Université de Montréal

Study of nuclear receptor dynamics by BRET

David Cotnoir-White

Biologie moléculaire, Faculté de médecine

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Résumé

Les récepteurs nucléaires (RN) sont des facteurs de transcription ligand dépendants qui contrôlent une grande variété de processus biologiques de la physiologie humaine, ce qui a fait d'eux des cibles pharmacologiques privilégiées pour de nombreuses maladies. L'un de ces récepteurs, le récepteur de l'œstrogène alpha (ERα), peut activer la prolifération cellulaire dans certaines sections de l'épithélium mammaire tandis qu'un autre, le récepteur de l'acide rétinoïque alpha (RARα), peut provoquer un arrêt de la croissance et la différenciation cellulaire. La signalisation de ces deux récepteurs peut être altérée dans le cancer du sein, contribuant à la tumorigénèse mammaire. L'activité d'ERα peut être bloquée par les anti-oestrogènes (AE) pour inhiber la prolifération des cellules tumorales mammaires. Par contre, l'activation des voies de RARα avec des rétinoïdes dans un contexte clinique a rencontré peu de succès. Ceci pourrait résulter du manque de spécificité des ligands testés pour RARα et/ou de leur activité seulement dans certains sous-types de tumeurs mammaires.

Puisque les récepteurs nucléaires forment des homo- et hétéro-dimères, nous avons cherché à développer de nouveaux essais pharmacologiques pour étudier l'activité de complexes dimériques spécifiques, leur dynamique d'association et la structure quaternaire des récepteurs des œstrogènes. Nous décrivons ici une nouvelle technique FRET, surnommée BRET avec renforcement de fluorescence par transferts combinés (BRETFect), qui permet de détecter la formation de complexes de récepteurs nucléaires ternaires. Le BRETFect peut suivre l'activation des hétérodimères ERα-ERβ et met en évidence un mécanisme allostérique d'activation que chaque récepteur exerce sur son partenaire de dimérisation. L'utilisation de BRETFect en combinaison avec le PCA nous a

permis d'observer la formation de multimères d'ERα fonctionnels dans des cellules vivantes pour la première fois. La formation de multimères est favorisée par les AE induisant la dégradation du récepteur des oestrogènes, ce qui pourrait contribuer à leurs propriétés spécifiques.

Ces essais de BRET apportent une nette amélioration par rapport aux tests de vecteurs rapporteur luciférase classique, en fournissant des informations spécifiques aux récepteurs en temps réel sans aucune interférence par d'autres processus tels que la transcription et de la traduction. L'utilisation de ces tests nous a permis de caractériser les propriétés de modulation de l'activité des récepteurs nucléaires d'une nouvelle classe de molécules hybrides qui peuvent à la fois lier ER ou RAR et inhiber les HDACs, conduisant au développement de nouvelles molécules prometteuses bifonctionnelles telles que la molécule hybride RAR-agoniste/HDACi TTNN-HA.

Mots clés : Récepteur nucléaire, pharmacologie, FRET, BRET, estrogène, acide rétinoïque.

Abstract

Nuclear receptors (NRs) are ligand-dependent transcription factors that control a wide variety of biological processes in human physiology, which has made them preferred pharmacological targets for many diseases. One such receptor, the estrogen receptor alpha (ERα), can activate cell proliferation in some sections of the mammary epithelium while another, the retinoic acid receptor alpha (RARα), can cause growth arrest and cellular differentiation. Signalling by these receptors can be altered in breast cancer, contributing to tumorigenesis. ERα can be blocked by antiestrogens (AEs) in the clinical setting to inhibit tumor cell proliferation. However, attempts to activate the RARα pathway with retinoids have not proven beneficial in clinical trials. This may result from the lack of specificity of the tested ligands for RARa and/or from their activity only in a subset of breast tumors.

Since nuclear receptors form homo- and heterodimers, we sought to develop novel pharmacological assays to study the activity of specific receptor-dimer complexes, their dynamics and quaternary structure. We report here a new FRET technique dubbed BRET with fluorescence enhanced by combined transfers (BRETFect) that can detect the formation of ternary nuclear receptor complexes. BRETFect can monitor the activation of ERα-ERβ heterodimers and highlights an allosteric mechanism of activation that each receptor exerts on its dimer partner. Use of BRETFect in combination with PCA-BRET has allowed us to observe the formation of functional ERα multimers in live cells for the first time. The formation of multimers is favored by AEs which induce receptor degradation, and may underlie their specific properties.

These assays are a net improvement over the classical luciferase-reporter experiment as they deliver real-time receptor specific information with no interference by other process such as transcription and translation. Using these BRET assays we developed a new class of NR hybrid ligands that can modulate ER or RAR activity and inhibit HDACs. This has allowed for the development of promising new bifunctional molecules such as the RAR-agonist/HDACi hybrid molecule TTNN-HA.

In conclusion, the work presented here brings new insight in NR dynamics and quaternary structure and offers novel tools to study their mechanism of action or design new modulators of NR activity such as hybrid AE-HDACis and Retinoid-HDACis.

Key words: Nuclear receptors, pharmacology, FRET, BRET, estrogen, retinoic acid.

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List of Abbreviation

AF-1/2	Activation function ½
AI	Aromatase Inhibitor
ATP	Adenine triphosphate
BRET	Bioluminescence Resonance Energy Transfer
CoA	Coactivator
CoR	Corepressor
CRABP	Cellular retinoic acid binding protein
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
E3	Estriol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen Response Element
eRNA	enhancer RNA
ESC	Embryonic Stem Cells
FACS	Fluorescence activated cell sorting
FRET	Förster Resonance Energy Transfer
GR	Glucocorticoïd receptor
HAT	Histone acetyl transferase

HDAC	Histone deacetylase
HER2	Human EGF receptor 2
HMEC	Human mammary epithelial cell
HMT	Histone methyl transferase
HRE	Hormone Response Element
IPA	Ingenuity Pathway Analysis
KDM	Lysine specific demethylase
LXR	liver X receptor
MAPK	Mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NCOA	Nuclear coactivator
NCOR	Nuclear corepressor
PCA	Protein complementation assay
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PSC	Pluripotent Stem Cells
PXR	pregnane X receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROR	RA receptor-related orphan receptor
RTK	Receptor Tyrosine Kinase
RXR	Retinoid X receptor
SAHA	Suberoylanilide hydroxamic acid

SAR	Structure Activity Relationship
SERD	Selective Estrogen Receptor Down-regulator
SERM	Selective Estrogen Receptor Modulator
SHR	Steroid Hormone Receptor
SMRT	Silencing mediator of RAR and TR
SRC	Steroid coreceptor
TF	Transcription Factor
TR	Thyroid hormone receptor
TSA	Trichostatin A
VDR	Vitamin D receptor

Acknowledgements

When I was fired from my Master's I was told I "didn't have it". That was hard to accept. I couldn't imagine who would take me on for PhD at that time. I don't remember how I came across Dr. Mader's lab page while browsing around for a future but there was something about nuclear receptors mechanism that seemed irresistible (it still is!). I was very lucky that Sylvie called me in for an interview and then doubly so to be offered a spot in her lab. The work reported here is not what I started out doing here; nothing on GPR30 or artificial transcription factor projects… Sylvie always stayed positive even when these projects crashed and/or burned and it was good to have that attitude guiding me. It's important for a student to feel his supervisor thinks "he's got it" and I never doubted that with Sylvie. I am also grateful to have been giving some liberties with my projects to just "try some things out" which led to a good deal of the stuff that is reported here.

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Special thanks go out to Mohammed for all his work in developing our lab's BRET assays with me.

My father (Dr. White) warned me when I got to university "Don't do a PhD!" It was great advice and I relay it to every young student that comes through our lab. Yet, even though I disobeyed him, he remained supportive and shared quite a bit of insight on how to navigate grad school bureaucracies and other PhD candidate traps. Thank you dad.

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CHAPTER 1: INTRODUCTION

Receptors

Health and disease of the human body are regulated by an intricate balance of signalling within and between the tissues. The overwhelming majority of signalling is regulated by families of receptors that respond in most cases to the presence or absence of their ligand [1-3], a molecular trigger whose binding to the receptor causes a shift in their structural conformations that allows for the receptors activity to be modulated. Receptors can be divided into two categories [4-6] dependent on their localization, i.e. as membrane receptors and intracellular receptors. Membrane receptors can be further split into three classes: G protein coupled receptor [7], receptor tyrosine kinase [8] and Cys-loop (ionotropic) receptor [9].

Membrane receptors

Cys-loop receptors, also known as ligand-gated ion channels or ionotropic receptors, are a class of receptors mostly associated with the nervous system. They allow the exchange of ions such as Na+, Ca2+, K+ and Cl- between extracellular and intracellular space when bound by neurotransmitters to generate action potentials which will be further propagated by voltage-gated ion channels or modulate cell biology [10]. Ligand binding will induce a shift in conformation, which will allow the free flow of the ions specific to the receptor along their gradient [11]. Ionotropic receptors are the target of most anaesthetics and certain psychotropic drugs [12-14].

Receptors tyrosine kinase (RTKs) are membrane receptors characterized by an extracellular ligand-binding/chaperone domain, a single transmembrane domain and an intracellular kinase domain [15-18]. Notable examples of the RTKs are the vascular endothelial growth factor receptors (VEGFR1, 2 and 3) [19], ephrin receptors and the erbb family of receptors – erbb1 or epidermal growth factor receptor (EGFR), erbb2 or heregulin receptor 2 (HER2), erbb3 and 4 [20]. The ligands to these receptors are usually small peptides circulating in the extracellular space that cause receptor dimerization when binding to their target [21-23]. The dimerization of the RTKs brings their intracellular domain into close proximity and leads to reciprocal phosphorylation on tyrosine residues of receptor monomers, which become activated [24]. The apo-ligand-binding domain of the RTKs is inhibitory to that process [25] as extracellular domain deletion or specific point mutation of the receptor are often constitutively active, suggesting this domain acts as a chaperone to the receptors [26, 27]. After activation and autophosphorylation the modified tyrosine residues act as binding sites for adapter proteins which contain the src-homology 2 (SH2) domain [28]. In turn the adapter proteins, which often have their own tyrosine kinase domain, can recruit and activate effector proteins to engage changes in plethora of cellular activities such as cell morphology, cell cycle control, metabolism etc… [29]. Several RTKs are disregulated in cancer. For instance, HER2 is amplified in 25-30% of breast cancers and EGFR in many cancers such as breast and colorectal carcinomas [30-32]. Targeting RTKs for drug development has led to novel cancer treatments such as the HER2 inhibitor herceptin (Trastuzumab) and various VEGFR inhibitors to treat renal cell carcinomas and various metastatic diseases [33]. The efficiency of these treatments is often limited by the inbuilt

redundancy of the signalling network they are part of and the often ubiquitous expression of some of the target receptors, as with the EGFR [34].

G protein-coupled receptors (GPCRs), also known as seven transmembrane (7TM) domains receptor, are a family of approximately 800 genes [7] characterized by their signalling through the heterotrimeric G-proteins to engage rapid signalling cascades of events through calcium release, cyclic-AMP production [35], kinase activation[36], etc…[37]. GPCRs are classically activated by binding of extracellular ligands [38, 39] which can range greatly in size and nature from a small sugar molecule for the taste receptors TAS1R2/3 [40] to multi-kilodalton proteins such as Wnt1 for the Frizzled receptor, which feeds into many cell proliferation and differentiation pathways [41]. While GPCRs have been shown to be active as monomers *in vitro*, *in vivo* dimeric signalling is a major component of signalling regulation and specificity. As previously mentioned, taste receptor TAS1R3 will be activated in the presence of sucrose when dimerized with TAS1R2 [42], while dimerization with TAS1R1 will cause activation in the presence of L-glutamate [40]. Adding to the layer of signalling regulation, different ligands binding to the same GPCR may stabilize different conformations of the receptor, which may in turn activate a sub-selection of signalling pathways [43]. This emerging area of research is referred to as ligand-biased signalling, which we will cover later on. It is acknowledged that over 50% of today's approved pharmaceuticals target GPCRs yet their targeting in cancers has only recently started [44-46]. One recent breakthrough has been the selective targeting and inhibition of the entothelin receptor A (ET_aR), which has protumoral activity in gastroenteric tract cells [47]. Conversely, the activation of

receptors with pro-apoptotic signalling such as 2AR could also provide novel treatment options for certain malignancies [48].

Intracellular receptors

Intracellular receptors are ligand-dependent transcription factors made up of the nuclear receptor super-family and its lone cousin the aryl-hydrocarbon receptor (AHR). The AHR is a basic helix-loop-helix transcription factor that is activated by planar aromatic hydrocarbons [49] such as natural plant flavonoids and phenols [50]. Upon binding to these ligands, the AHR will activate the transcription of specific target genes by recruiting cofactors to the promoter and enhancer regions of these genes [51, 52] (in a mechanism that highly resembles that of type 1 nuclear receptor which we will cover later) such as the P450 enzymes which metabolize xenobiotics [53]. The AHR also regulates a plethora of other pathways in the absence of xenobiotics such as cell growth, proliferation, differentiation and death [54-56]. Interestingly the AHR has recently been identified as a key player in hematopoietic stem cell expansion, making its targeting an active area of research to develop novel ways to perform stem cell transplants [55].

Nuclear receptors, which this thesis focuses on, form a family of 48 genes in humans encoding for over two hundred transcription factors when accounting for different isoforms [57, 58]. While some of these are orphan-receptors, meaning that no ligand have been assigned to them to date, they are overwhelmingly considered druggable targets [59]. In absence of their ligand, nuclear receptors are kept inactive by

chaperone [60] or co-repressor proteins [61]. Once bound by their ligands, they shed these proteins. Nuclear receptors homo- or heterodimerize with partner receptors and bind key regulatory sites in the genome to modulate transcriptional activity [62]. Nuclear receptors influence many (if not all) signalling pathways such as placental development[63], metabolism [64] and circadian rhythms [65, 66]. Most receptors with known ligands already have FDA-approved targeted therapies [67] or molecules in clinical trials [68], yet as we will see the scope of the targeting strategies that have been used to date are limited as they focus on individual receptors while those receptors actually act in concert with other NR partners in dimeric complexes.

Nuclear receptor signaling

Nuclear receptor superfamily

Nuclear receptors are a hallmark of metazoans and are not present in lower eukaryotes [69, 70]. While the human genome harbors 48 nuclear receptor genes, other species will have evolved fewer, such as Drosophila melanogaster with only 18 genes [71], or many more like Caenorhabditis elegans that hosts an impressive 283 nuclear receptor-encoding genes [72]. It is theorized that nuclear receptors first evolved as “energy sensors” that could sense the availability of nutrients by binding free fatty acid derivatives in multi-cell organisms to modulate their growth [69, 73]. Through duplication of the genes and mutations the family acquired the ability to sense different molecules, either exogenous or endogenous, to integrate physiological and environmental cues in multicellular organisms.

The first nuclear receptor was identified by Elwood Jensen in the 1950s when he showed using a radiolabeled ligand that only estrogen-responsive tissues could bind and concentrate estrogen in cells [74]. Before this breakthrough, it was believed that steroid hormones acted as nutrients that delivered energy to specific tissues where they could be metabolized [75]. The discovery of a protein that bound hormones and translated this signal into physiological changes in target tissues eventually led to the identification of other hormone receptors such as androgen receptor (AR) [76, 77] and the progesterone receptor (PGR) [78, 79]. Pierre Chambon's and Ron Evan's group were the first to clone and sequence NR - ERα [80] and GR [81] respectively and subsequently all NRs were characterized before genome sequecing by homology cloning.

Nuclear receptor domain organization

Nuclear receptors are defined by a modular structure composed of a ligand-independent activation function (AF-1) [82], a zinc finger type DNA-binding domain (DBD) [83], and overlapping ligand-binding domain (LBD) and a ligand-dependent activation (AF-2) (Fig. 1A) [84, 85]. Notable exceptions to this rule are DAX1 and SHP, which constitute the NR0 sub-family of receptors; these receptors do not have AF1 domains or DBDs [86, 87].

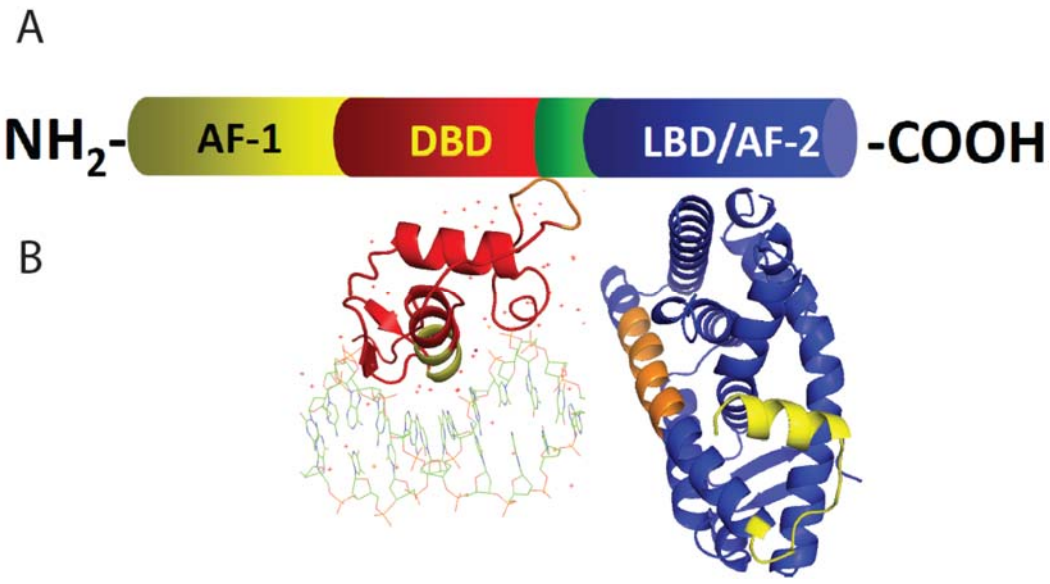

Figure 1. Nuclear receptor structure.

(A) Schematic view of the modular organization nuclear receptors with AF-1 (yellow), DBD (red), hinge (green) and LBD/AF-2 (blue) domains. (B) ERα DBD and LBD crystal structures. The DBD (red) is drawn bound to an ERE half-site with the P-box in yellow and D-box in orange (adapted from structure 1HCQ). The E2- bound LBD (blue) is drawn with helix 12 in yellow and the helix 11 dimerizing interface in orange (adapted from structure 1ERE).

DNA binding domain

The DNA-binding domain is the most conserved domain within the nuclear receptor super-family. All nuclear receptor DBDs possess two treble-clef (or C4-type) zinc finger motifs that allow for direct DNA interactions [88]. While both zinc fingers make stabilizing contacts, the specificity of DNA binding is determined by the amino acids of the P-box at position 2-6 in the first zinc finger (Fig. 1B). These amino acids make contact with 4 adjacent base pairs that form the core of the nuclear receptor response motif. The

response element is complemented by two flanking base pairs – one on each side – that make less crucial contacts with the DBD [89]. The P-box sequence diverges between nuclear receptors and thus contributes for diversification of possible genomic sites of action for the receptors [90-92]. The DNA binding domain also contains a dimerization interface called the D-box (Fig. 1B) which, with other interfaces in the LBD, allows nuclear receptor to dimerize – a behavior typical of nuclear receptors [93]. Thus a typical nuclear receptor response element (RE) is composed of two motifs bound by one nuclear receptor each [94-97]. The organization of the receptor dimers determines the REs that can be bound, with variable spacing and orientation of the motifs [98-101]

The C-terminal part of the DBD is linked to a hinge region. While this domain was long thought to merely serve a string to tie the DBD to the LBD it has recently been shown that it is a critical hub that relays signal from LBD to DBD and can integrate signals coming from the two dimeric receptors to allow intra-dimeric allosteric control [102-104].

Ligand binding domain and AF2 transcriptional activation function

The ligand-binding domain, which follows the hinge region, is structurally conserved within the NR super-family. It is formed of a specific 12 alpha helix bundle structure also called alpha-helical sandwich, which contains a hydrophobic ligand-binding pocket [84]. Much of the surface-exposed amino acids are conserved between nuclear receptors and play roles in receptor dimerization and cofactor recruitment [93, 105, 106] (Fig. 1B). The amino acids of the LBD core, whose side chains and backbones are buried

inside the LBD structure, form the lining of the ligand binding pocket [107, 108]. These amino acids define the shape, size and polarity of the ligand binding pocket, resulting in ligand binding specificity [109]. The binding typically occurs via hydrophobic water exclusion, aromatic stacking, and van der Waals forces [110]. Endogenous ligand binding to the receptor is usually selective ; for example endogenous estrogens come in three varieties: estrone (E1), estradiol (E2) and estriol (E3) that are all high affinity agonists of ER and ERMiller. Moreover the ERα ligand-binding pocket can also bind 27-hydroxycholesterol in the low micromolar range [111, 112]. 27-hydroxycholesterol (27HC) induces a different conformation of the LBD that is less conducive to activation, so that in absence of estrogens 27HC will cause partial activation of estrogenic signalling while in the presence of estrogens high levels of 27HC will inhibit full activation of the receptor [113]. 27HC is thus considered to be a partial agonist or an endogenous selective estrogen receptor modulator (SERM) [114, 115]. Other receptors such as the xenobiotic sensing receptors CAR and PXR [116, 117], on the other hand appear to bind with more promiscuity to allow the detection of a greater variety of molecules.

Ligand binding induces conformational changes in the LBD. These changes modulate the affinity of the receptor for partner proteins, which allows the receptor to switch from inactive to active state [118]. For instance, while in the inactive state, the steroid receptors interact with the chaperone protein Hsp90, which stabilizes the receptors and limits their DNA binding and cofactor recruitment [119-122]. This interaction is required because of the inherent instability of the LBD in the apo-state. When the receptor binds a ligand the conformational changes induced stabilize the LBD, cause the release of Hsp90 and expose a surface that can interact with cofactors.

The LBD overalps with the C-terminal AF2 transcriptional activation region, which mediates cofactor recruitment [123, 124]. The core of the AF-2 domain is contained in the terminal helix (H12) of the LBD, whcih acts as a lid to the ligand-binding pocket and stabilizes the ligand [125-127]. Upon ligand-binding, helix H12 folds onto the to create with residues from helices 3-5 a coactivator binding groove in which a coactivator motif (often corresponding to the consensus LXXLL) can bind to form a receptor-coactivator complex [128-130]. A ligand-bound nuclear receptor homo or heterodimer contains two binding grooves, and may thus accommodate two motifs per receptor dimer. In addition, most coactivators contain more than one coactivator-motif increasing the avidity of receptor-coactivator interaction [131]. NR antagonists disrupt AF-2 folding and prevent cofactor interactions [132, 133] [134-138].

While AF-2 domains have a conserved helical ternary structure they have some level of sequential diversity. This primary structure diversity allows different receptors to recruit different cofactors and to achieve different intramolecular conformations [139, 140]. The two main classes of coactivator motifs are the leucine and phenylalanine repeats – defined by the LXXLL and FXXLF sequences respectively. Receptors may bind both of them, like in the case of the glucocorticoid receptor (GR), or only one of them, like ER to the LXXLL motif and the androgen receptor (AR) to the FXXLF motif [140]. Moreover, AF-2, along with H3 and H11, determines the capacity of the receptor to recruit, in the absence of ligand, corepressors through the aliphatic repeat corepressor nuclear receptor box motif (CoRNRBox) defined as (I/L)XX(V/I)I [139]. The AF-2 domain can also mediate intramolecular interactions between the C-terminal LBD and the N-terminal AF-1 domain as is observed for ERα [141] and AR [142]. The ERα and β do not bind corepressors in the absence of ligand and rely on a variety of mechanisms such as

nuclear exclusion, chaperone protein binding and AF-1 to AF-2 looping [143] to exclude binding of the coactivator to the AF-2 function and help keep the receptor in an inactive state.

AF1 transcriptional activation function

The AF-1 domain is a loosely unstructured domain at the N-terminus of the receptor [144] which is missing or incomplete in some receptors such as CAR [145] but can also comprise most of the receptor itself as in the case of Nur77 [146]. The diversity of sequence and structure imparts a high variety of function to the AF-1 region. When isolated from the rest of the receptor, it functions as a ligand-independent activation-function. Within the receptor, it may interact with cofactors in the absence of agonist and even in the presence of antagonist [144, 147]. Some receptors, such as Nur77, are highly dependent on AF-1 activity as they do not mediate cofactor recruitment through the AF-2 domain and may require a dimeric NR partner (such as RXR) to mediate AF-2 contact with the coactivator [146, 148]. As described above the AF-1 domain can bind the AF-2 domain and often does so through coactivator like motifs encoded in its sequence – this conformation is observed in the receptors apo-state[143]. When the receptor is activated, the AF1 domain is released and can interact with cofactors that contain a conserved glutamine rich region [149]. This alternate method of cofactor recruitment allows NRs to interact with a wide range protein. For example, while the AR does not strongly bind the LXXLL motifs of SRC-1 through its AF-2domain it can stably interact with SRC-1's glutamine rich region and recruit SRC-1 to the chromatin, yet AR can also recruit cofactors such as RNF14 solely through the AF2-domain binding a FXXLF motif of the cofactor [150].

Nuclear receptor 3D structure

Because of their modular nature and their domains' small size many crystallographic and solution structures have been acquired for nuclear receptors in complex with DNA RE, cofactors, ligands and/or dimerization partners. Recently full length receptor dimers bound to DNA have also been solved for RXR heterodimers [151-153].

The DNA binding domain structure of GR was first solved in 1991 and confirmed its helical zinc-finger motifs. Further studies that co-crystallized NR DBD with their response-element highlighted the contacts made between the NR P-box and DNA and how those defined the selectivity of receptors for their respective elements [154, 155].

The ligand binding domains of RXR, RAR and TR were also crystalized soon after and revealed the LBD's helical bundle structure [156-158]. The addition of coactivator peptide to ligand bound receptor generate crystal structure that showed how the AF-2 domain folds down between helices 3 and 12 and is held in place in a charge clamp (amino acids E380, D538, D545, and R363 in of ERα) (Fig. 3). In this conformation helices 3, 4, and 12 create a coactivator binding groove where the LXXLL or FXXLF peptide lays down [159, 160]. In the case of the estrogen receptors, helix twelve also makes important contacts with asp351 that stabilizes it in its activated conformation [161]. Corepressor peptide were also crystalized with the RARa that define the structural basis for corepressor recruitment in the case of apo-RAR receptor but not for apo-RXR or apo-ER complexes [162-165].

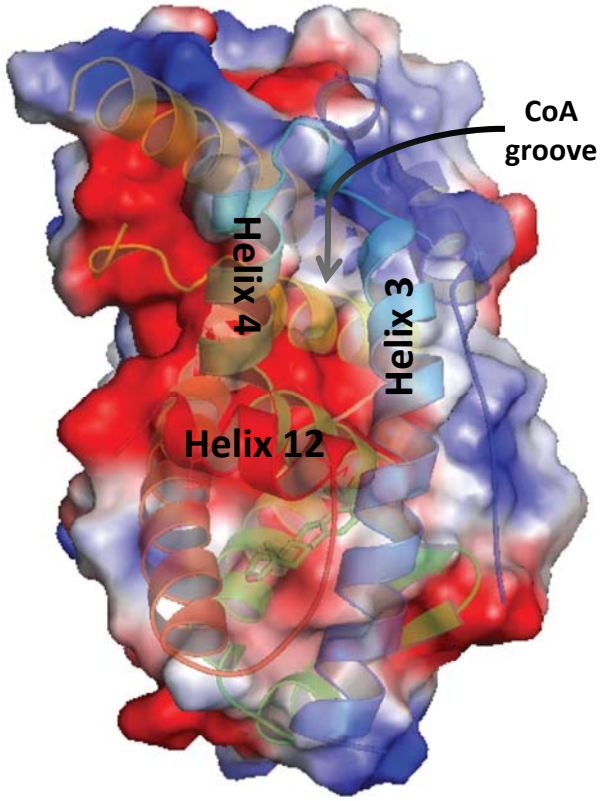

Figure 2. ERα's LBD with coactivator binding groove and lining helices identified. Electrostatic surface of ERα's LBD with helical structure overlaid. The negative (red) and positive (blue) charges of the charge clamp are apparent around helices 4 and 12 (negative) and 3 (positive).

The full structure of AF-1 and much of the C-terminal of the LBD – which can stretch more than 50 amino acids past helix 12 – have not been solved and it is believed that they are naturally disordered in the absence of larger macro-complexes [166, 167]. The main function of these domains is to act as a scaffold for cofactors to bind the nuclear receptor and recruti them to nuclear receptor binding sites to modulate transcription [168, 169]. Importantly, they contain sequences that are subject to post-translational

modifications such as acetylation, phosphorylation etc. that can alter receptor activity or turnover [170].

Nuclear receptor interactome

Nuclear receptors coordinate the assembly of large transcription complexes at their sites of action, directly or indirectly recruiting a plethora of different cofactors and mediator proteins to transduce regulation of target genes [171-173], which can in some instances be located many kilobases away from the response-element [174]. The three most characterized interactions are the NR-NR dimerization, the AF-1 and AF-2 cofactor recruitment.

NR dimerization

The dimerization of nuclear receptors happens in part through contacts of the helices 11 of both receptors in their LBDs and the DBD D-box. This H11 to H11 contact is kept together by a leucine zipper-like structure between the two receptors [106, 175]. Mutations in the leucine residues in H11 with large polar amino acids have been shown to destabilize or prevent dimerization [141], correlating with loss of transcriptional activity in dimerization dead mutants. This is of course due in part to lack of DNA binding of most monomeric receptors [176] but the contribution of regrouping two AF-2 and AF-1 domains in a single complex also increases the affinity of the cofactors for the nuclear receptor complex [177].

Dimerization of the receptors is also completed via contacts between the two DBDs and these interactions are stabilized by the binding of the DBDs to their response element [176]. The response elements are organized in two half-sites, each one with a sequence recognized by one of the dimerization partner, and a nucleotide spacer that separates the two half-sites. The spacer length is specific to the receptor pairing, as RXR can bind to RE with any spacer length between 1 and 5 nucleotides but RAR-RXR heterodimers prefer 5 nucleotide spacers while RXR-VDR pairs prefer 3 nucleotides [89, 98, 100, 178].

Moreover, the orientation of the half-sites to one another is crucial for dimer recognition [179]. Half-sites can have translational symmetry and be called direct repeats; these REs have both half-sites on the same DNA strand separated by 1 to 5 nucleotides. Half-sites can also be positioned with reflectional symmetry; if the sites are 5'-3' to 3'-5' the RE is considered an inverted repeat, if the orientation is 3'-5' to 5'-3' the RE is an everted repeat. The required orientation of the RE half-sites is dictated by the identity of the NR dimer targeting the site [180].

Through literature mining, Amoutzias et al. [181] catalogued 88 certifiable NR dimers – meaning directly observed in two separate publications – and another 101 probable dimers that were characterized in a single publication between NRs of various phylogenetic commonalities.

The association of two receptors *in vivo* will however be limited by the co-expression of these receptors, as in most tissues only about half of all receptors are expressed. Moreover, the activity of the receptor dimer complex may be conditional on the presence of the ligand to one or both of the receptors [182].

Furthermore, some reports indicate that NRs may attain higher order complex through tetramerization, or dimer of dimers. While the tetrameric conformation has only been observed in vitro and for homotetramers of RXR [183] and TR [184], one could speculate that *in vivo*, in the presence of different NRs, heterotetramers could form to add another layer of possible NR-NR interactions beyond dimers. Crystal structures have been generated of the RXR LBD tetramers in a repressed state showing the AF-2 domains of a dimer being exchanged with AF-2 domains of a second dimer binding and with the C terminal part of the four H11 helices coming together with stabilizing hydrophobic and aromatic interactions [185].

Cofactor recruitment

NR receptors primarily – but not exclusively – interact with cofactors via their N-terminal AF-1 and C-terminal AF-2 domains. In the absence of agonists, or in some cases of activating post-translational modifications, the receptor cannot interact with co-activators and for a large subset of NRs, corepressors (NCoR and SMRT) are recruited to enforce a repressive activity on the target genes [61, 139, 165] (Fig 2). Alternatively, some corepressors such as LCoR [186] and RIP140 [187] contain LXXLL motifs and can bind to activated receptors – in this cases ligand activation of a receptor would lead to repression of transcription. Corepressors serve as platforms for the assembly of histone-modifying enzymes that chemically modify histone tails to make the chromatin environment less favorable to transcription [188]. Examples of these enzymes are the histone deacetylases (HDACs), the histone methyltransferases (HMTs) and the histone demethylases.

HDACs remove acetyl groups from lysine residue side-chains in the N-terminal tail of core histone proteins: H2A, H2B, H3 and H4 (one report has identified a site of acetylation on histone H1) [189]. The removal of acetylation marks from lysine side chains restores their positive charge and allows stronger interactions between histones and DNA which in turn contracts chromatin and prohibits the binding of the transcriptional machinery and gene expression [190]. HDACs bind the corepressors' repression domains (RDs) and also require the activity of the corepressors' deacetylases activating domain (DAD) to properly function [191].

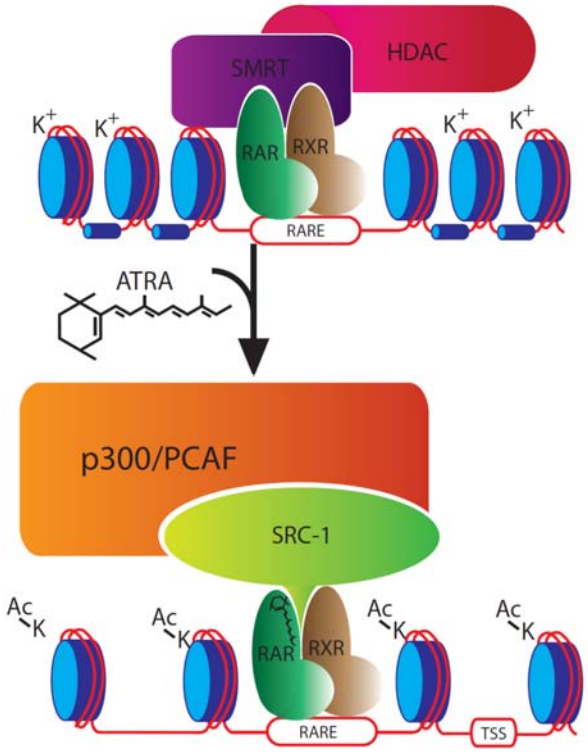

Figure 3. Example of nuclear receptor activation on the chromatin.

A retinoic acid receptor(RAR)/retinoic acid X receptor(RXR) heterodimer recruits the corepressor SMRT (NCoR2) which in turns recruits HDAC complex to remove acetyl groups from histone tails. Binding of all-trans retinoic acid (ATRA), the RAR ligand, causes release of SMRT and recruitment of the coactivator SRC-1 (NCoA1), acetylation of histone tails and assembly of the p300/PCAF complex. A transcription start site (TSS) is then revealed to allow for transcription.

HMTs play a more nuanced role in genomic regulation. While histone deacetylation is considered a purely repressive mark – note that this only refers to cis-repression; the mark can repress the chromatin at the site of a repressor complex binding site and lead to trans-activation of a distal promoter [192] [193] – histone-tail methylation can be an activating or repressing mark [194]. Methylation takes place on lysine or arginine side-chains and modulates not so much the histone-DNA contacts but instead leaves marks that can be recognized by other transcription factors. Methylation of histone-tail residues further differentiates itself from acetylation as it can occur several times on the same residues; up to three methylation marks can be left on a lysine residues while only one acetylation mark can be fitted on. The level of methylation of the residues will affect what proteins are recruited to the chromatin [195, 196]. Generally, the methylation of H3K4 and H3K36 are associated with activation while H3K9 and H3K27 methylation is associated with repression.

While histone methylation was once thought to be permanent because of their long half-lives, several proteins with *in vivo* histone demethylase activity or lysine-specific demethylase (KDM) have been identified [197, 198]. As with HMT, histone demethylases can act as coactivators or corepressors depending on which methylation mark they recognize and remove from histone-tail residues [199]. The JARID family of histone demethylase specifically removes H3K4 methylation marks to silence active promoters, and as such they are considered corepressors [200, 201]. Removal of H3K36 leads to promoter inactivation yet H3K36 methylation is also a multidisciplinary mark recognized by other transcription and replication factors outside of the promoter area and can have

effects on splicing and elongation of transcripts as well as replication of origin firing [202-205]. Thus, disruption of H3K36-trimethyl marks outside of promoters may not directly lead to repression of expression.

Together the work of HDACs, repressive HMTs and KDMs recruited by corepressor complex will reshape the chromatin to an inactive state to prevent transcription. To maintain the active repression exerted by the nuclear receptor complexes, other transcription factors like heterochromatin protein 1 (HP1) will recognize the closed chromatin conformation, bind it and stabilize a repressed state that will be maintained even in the absence of NR action [205, 206].

To reverse the repression imposed on the chromatin, nuclear receptors must recruit coactivators to remove repressive marks and add activating ones. For a subset of receptors that do not interact with corepressors in their inactive states, such as the estrogen-receptor, a basal interaction can be observed with coactivator in the absence of ligand because the cofactor binding groove is not obstructed by a corepressor and AF-1 domain is available [207, 208]. As such, overexpression of coactivators, such as SRC-1, 2 and 3, has been shown to activate the transcription of ER target genes in the absence of estrogen [209, 210]. Alternatively, coactivators can be recruited to ligandless NRs following post-translational modification of the receptor; many kinase cascades can target the receptor leading to phosphorylation of residues in the LBD, which alters the conformation of the receptor to increase its affinity for coactivators [211]. Examples for ERs?

One major mechanistic difference between nuclear receptor corepressors and coactivators is that corepressors do not possess intrinsic enzymatic activity and rely on the recruitment of HDACs, whereas coactivators interacting with NRs can possess histone acetyltransferase (HAT) activity [212, 213], while serving as scaffolds for the recruitment of other cofactors with enzymatic activity such as HMTs [214] (Fig. 2). The action of cofactivators with HAT activity, such as SRCs, is rapid and causes acetylation on numerous histone tail residues across all core histones. This weakens association of DNA with histones and increases the repositioning or shuffling of the histone along the DNA, exposing TF binding sites that were hidden before [215, 216]. Moreover, acetylated histone can be specifically recognized by tandem bromodomains present in a variety of chromatin remodelling proteins, propagating the activation signal along the chromatin [217, 218]. Thus, recruitment of the p160/p300 compelx by the estrogen receptor to upstream promoter regions in an estrogen target gene will cause acetylation of neighboring histones on many residues, including the histone 4 tail residues K5, K6, K12 and K16 [219] which will be recognized by the general coactivator CBP bromodomain. The binding of CBP will propagate the acetylation marks to the promoter of the target gene, making it mnore accessible to transcription factors and the transcription machinery.

HATs are divided into two classes. Class A represents the nuclear HATs with genomic action and class B the cytoplasmic, novel histone-modifying HATs [220, 221] [222]. While some HATs share properties of class A and B, generally nuclear receptors

recruit class A HATs. HATs can be further subdivided into different phylogenetic families: the Gcn5-related N-acetyltransferases (GNATs) family [223], the MOZ-Ybf2-Sas2-Tip6 (MYST) family [224] and the "orphan-class" family which groups together such proteins as p300 and the SRCs that show intrinsic HAT activity but do not possess the consensus domain found in both GNAT and MYST HATs [225].

Early activation events by NRs usually relies on the SRCs and other AF1- and AF2-interacting domain cofactors of the orphan-class family [223]. The interaction between the NR and the SRCs is transient but allows for the recruitment of other HATs to the chromatin such as p300-CBP associated factor (PCAF) of the GNAT family [226, 227]. Since not all HATs share the same acetylation specificity, the regulation of HAT composition of transactivation complexes allows for another level of transcriptional control.

Genomic action of nuclear receptors

Nuclear receptors are known to activate G-proteins and kinase cascades in rapid, non-genomic effects but the best characterized function they hold is to regulate genomic pathways [228]. Nuclear receptors act as powerful switches to determine cell-fate, proliferation, energy consumption etc. The mechanisms used to regulate the gene expression required to oversee these functions separate nuclear receptors into two classes.

Class I Nuclear Receptors

Class I nuclear receptors are best exemplified by the NR3 subfamily that encompasses the steroid hormone receptors (SHRs) of which ER and AR are members [5]. Class I receptors are thought to be kept inactive in the absence of ligands as monomers in association with the chaperone protein HSP90, which usually plays a role in protein folding by facilitating the condensation of the hydrophobic core of nascent proteins in the cytoplasm's hydrophilic environment [229]. Since unliganded SHRs have exposed hydrophobic surfaces (ligand-binding pocket and cotactivator binding groove), they require stabilization to prevent misfolding and degradation. Thus, inhibition of HSP90 will lead to inhibition of SHR signalling for most receptors [230] [231]. Furthermore, HSP90 is often overexpressed in cancers either to generally handle the increased protein synthesis or to allow the proper function and expression of several oncogenic kinases such as AKT and the EGF-family of RTKs [232-234]. HSP90 inhibitors have thus been developed to treat certain forms of cancers and may in some cases result in SHR inhibition [235-238].

Once released from chaperones, the receptors are free to dimerize with other activated receptors. While homodimerization is accepted as the norm for steroid receptors [239], heterodimer formation has also been reported [240, 241]. While in theory both receptors should have bound their ligands, reports have been published of heterodimers between two receptors following treatments with a selective ligand to one of the partners [242]. BE MORE SPECIFIC

After ligand binding and release from chaperone proteins the class I receptors are shuttled to the nucleus by the nuclear import machinery [243-245]. This is achieved by exposing nuclear localisation signals that were concealed by the chaperone protein binding and it ensures that once active the nuclear receptors remain nuclear [246, 247]. This can be easily visualized with fluorescence tagged receptors that display a diffuse distribution in absence of treatment and concentrate in the nucleus in the presence of their ligands. Interestingly, while the estrogen receptors have initially been reported to follow this mechanism like other SHR, in breast cancer cells they are mostly nuclear in the absence of ligand, but can in certain situations be observed in the cytoplasm [248-250].

When receptors are localized to the nucleus they are able to bind to their response elements (RE) or interact with other transcription factors already localized to the chromatin. In the case of direct DNA binding, the REs recruit receptor dimers via interactions with each units of the dimer. The receptor can also be tethered to the chromatin through other TFs bound to their own response elements [251, 252]. Liganded receptors can recruit cofactors to modulate the transcription of the targeted genes. It is still unclear whether receptors can recruit coactivators in the absence of DNA binding.

Class II Nuclear Receptors

Nuclear receptors of the Class II variety are exemplified by the RXR dimer complexes. While class I receptors mostly homodimerize after ligand activation, class II receptors generally heterodimerize – often with RXR [253, 254] – and are bound to their

RE before ligand binding [255]. Following synthesis, the receptors are shuttled directly to the nucleus [256]. In the nucleus, they will dimerize with the available partners and bind to REs with motif sequences and spatial configurations compatible with the receptor heterodimer. All this can be achieved in the absence of ligand, although some reports show that ligand presence can alter the dimerization partner preferences of RXR (Giner et al, Manuscript in preparation)[257].

In their inactive states many class II complexes will recruit corepressors such as NCoR1 and SMRT [258, 259] through binding of the corepressor CoRNRbox motif to the NR AF-2 domain [260]. This will allow the formation of a repressor complex on the chromatin and propagate an active inhibition of transcription on the target gene. This negative regulation is dependent on many factors such as the availability of the corepressor, the expression of competing receptors and the absence/presence of their ligands in the cells.

The identity of the receptors interacting with RXR will influence the permissiveness of the receptor heterodimer complex: in a permissive complex, binding of a ligand to either of the two partners activates the complex and exchanges the corepressor for a coactivator, while in a non-permissive complex only one receptor – and its ligand – is responsible determining the activity of the receptor [164, 261-263]. Note that the permissiveness of a complex is also determined by cellular context as the TR-RXR dimer in thyroid cells is non-permissive; only thyroid hormone binding to TR will activate the complex, RXR is a "silent partner" and binding of its ligand 9cis-retinoic acid will not influence the complex activity [262, 264]. Yet in pituitary cells TR-RXR is a permissive complex and the RXR ligand

9 cis-retinoic acid will activate transcription. This variability may be due in part by the balance of corepressor and coactivator expression in different cell types. [262] Once class II receptor dimers are activated, they recruit cofactors and modulate transcription in the same fashion as class I receptors would [265].

Other smaller groups of receptors can be classified as class III, characterized by class II-like receptors that only homodimerize and bind direct repeats, and class IV receptors which act as monomers [5].

Chromatin action

Nuclear receptors have been observed to bind all over the genome but their activity is mostly concentrated at the promoter and enhancers of target genes [261]. Of these two regions, enhancers are often considered the most crucial site of action [174, 266]. Enhancers are chromatin regions located at variable distances from the TSS that act as distal landing platforms for transcription factors. Enhancers are characterized by specific chromatin modifications such as H3K4mono-methyl [267].

Nuclear receptors are in charge of activating large genomic programs that define cell fate, identity and function. Receptors such as the RARs are important triggers for embryonic stem cells (ESCs) differentiation into pluripotent stem cells (PSCs) [268, 269] and can further direct the neuronal differentiation of PSCs [270]. Yet, in those cases the

same RAR will be modulating a different set of target genes due to the different set of available enhancers it will be targeted to [271]. Enhancers themselves are rendered accessible by pioneering factors, transcription factors that bind to intergenic regions and lay down specific histone modification such as histone 3 lysine 4 monomethylation [272]. The sum of these modified regions forms a super-enhancer which determines cellular identity [273-275].

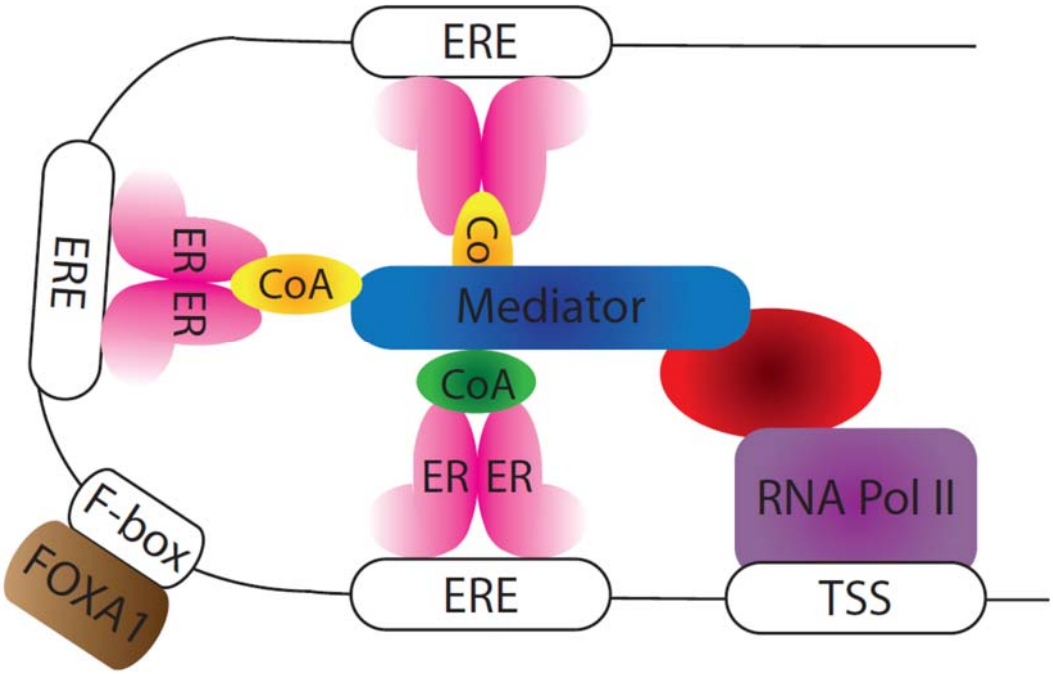

Figure 4. The estrogen receptor acting at an enhancer and looping chromatin to promoter.

The estrogen receptor (ER) bound to its response element (ERE) recruits coactivators (CoA) and mediator complex that links the receptors to the transcription initation complex and RNA polymerase II (RNA pol II) at the transcription start site (TSS).

The enhancer's mechanism of action is still not completely understood but its actions have been studied extensively in the case of nuclear receptor control. Upon activation the enhancer regions will often loop and become physically associated through

protein-protein interaction with the transcriptional start site (TSS) of the gene the enhancer regulates [276, 277] (Fig. 2). This allows for the existence of cell-specific enhancers for genes expressed in different cell types. Note that one sub-region of the enhancer can also be looped to another part of the enhancer and not necessarily to the TSS directly, as shown in a previous study from our laboratory on the GREB1 gene , which contains multiple high affinity estrogen response elements in its upstream regulatory sequences [174]. How looping is coordinated and how contacts between two distal sites are initially made is not completely worked out but the presence of active transcription complexes on the enhancer is emerging as an important step for enhancer activity. Indeed, recent studies using genomic run-on sequencing (GRO-seq) and chromatin immunoprecipitation (ChIP) have shown how RNA polymerase II is recruited to and actively transcribes the enhancer sequence bidirectionally to produce enhancer RNA (eRNA). eRNA levels are correlated with mRNA levels of the enhancer's target genes and eRNA knockdown by siRNA correlated with decreased mRNA levels of the target gene [278-281]. The mechanism behind this is not yet understood but the eRNA may act as a scaffold for the assembly of TFs and may bridge the TF binding sites in the enhancer and promoter to loop the chromatin [282].

Gene ontology analysis for nuclear receptor target genes often indicates that large cellular programs such as proliferation [283], metabolism [284] or apoptosis [285] are regulated. It is thus hardly surprising that dysregulation of NRs can bring about catastrophic consequences such as cancer, aberrant development or a plethora of other pathologies [286-288].

Nuclear receptors and cancer

Nuclear receptors contribute to organogenesis by balancing cell growth and differentiation. The estrogen receptor alpha (ERα) signalling is essential for the growth and differentiation of many tissues [289-292]. In the mammary gland, ERα action is required to create an architecturally sound and functional gland [293-295]. On the other hand, the retinoic acid receptor works as a brake to ERα – the accelerator – to slow down the growth of the mammary gland during development to the gland [296]. If ERα signalling were to go unchecked by RAR it could lead to runaway proliferation and oncogenesis driven by oestrogens and ERα [297-299]. Table 1 shows a brief overview of how nuclear receptors can affect breast tumor biology.

Nuclear receptors	Tumor subtypes (Expression)			Action	Therapy
	Luminal	HER2+	Triple-Negative		
ERα	**+**	**-**	**-**	Proliferation	SERM/SERD
ERβ	**+/-**	**-**	**-**	Antiproliferative	
ERRα	**+/-**	**+**	**+**	Proliferation	
ERRβ	**-**	**-**	**-**	Antiproliferative	
ERRγ	**+**	**-**	**-**	Proliferation	Tamoxifen
GR	**+**	**+/-**	**+/-**	Subtype and context-dependent	Dexamethasone/ Mifepristone
MCR	**+**	**+/-**	**+/-**	Subtype and context-dependent	Corticosteroids
PGR	**+**	**-**	**-**		Levonorgestrel/ Mifepristone
AR	**+/-**	**+/-**	**+/-**	Proliferation	SARM
RARs	**+**	**+/-**	**+/-**	Antiproliferative	Retinoids
PPARs	**+**	**+**	**+**	Proliferation	Glitazones (agonists)
LXRs	**+**	**+**	**+**	Antiproliferative	
VDR	**+**	**-**	**-**	Antiproliferative	Calcitrol
Nur77	**+**	**+**	**+**	Context dependent	
Nurr1	**+**	**+**	**+**	Proliferation	

Table 1. Sampling of various nuclear receptor expression and action in breast cancer. [300-314]

Breast cancer and the estrogen receptor-alpha

ERα expression or overexpression is observed in a majority (two thirds) of breast tumors. Mammary gland tumors that express the estrogen receptor are defined as luminal because normal epithelial cells expressing ERα are found in the lumen of ducts and lobules in the mammary gland [315]. These luminal breast tumors are subdivided in two types, A and B by gene signatures identified from unsupervised hierarchical clustering of tumors based on their gene expression levels (microarray analyses). Luminal A tumors are generally HER2-negative, p53 wt and poorly proliferative, while luminal B tumors have increased expression of cell cycle related genes and can overexpress the membrane RTK HER2 [316]. Moreover, ERα expression is positively correlated with progesterone receptor (PR) expression since PR is a primary target gene of ERα [317, 318]. Note however that other ER target genes may be more tightly correlated with ER expression, at least at the mRNA level. Other sybtypes of breast cancers are classified as HER2+, a luminal-like subtype that overexpress HER2 but not ERα expression, and triple negative, which are composed of cells that resemble the basal layer of the duct and lobules in that they do not express ERα, PR or HER2 [319, 320]. This latter tumor type is the most aggressive, being often associated with genomic instability, and does not benefit from targeted therapies yet.

Antiestrogens

Since ERα controls the expression of genes affecting processes invovled in cancer progression, such as proliferation and apoptosis inhibition, its inhibition by various means has led to the first targeted therapies in breast cancer treatment [321] to replace ovarectomies which were initially used to reduce estrogen levels.

The compounds known as antiestrogens (AE) are competitive inhibtors for the ligand binding pocket of ERα that interfere with the folding of the AF2 domain, preventing the receptor from achieving a fully active conformation [322]. AEs are divided into two classes: the selective estrogen receptor modulators (SERMs) [323], previously known as partial agonists, and the selective estrogen receptor down-regulators (SERDs), which are also referred to as full-antiestrogens [324].

SERMs are a class of molecules that exhibit gene- and tissue-specific effects [325]. Tamoxifen is the prototypical SERM; it is used as a breast cancer therapy because of its antagonism on the ERα in the mammary gland but it can partially activate the receptor in other tissues such as bone or uterus [326]. While SERMs like tamoxifen disrupt the folding of H12 to prevent the proper formation of the coactivator binding cleft, they do allow receptor dimerization and DNA binding [327]. In the presence of SERMs, cofactors can be recruited to the receptors through the AF1-domain, which restricts the group of coactivators that can interact with the receptor and therefore the set of genes that can modulated [147, 328]. Additionally, in mammary epithelial cells, tamoxifen-bound receptors can preferentially recruit corepressors, leading to reverse-agonist activity on ERα target genes [329, 330]. On the other hand, SERMs act as activators of estrogen

programs in bone and uterine tissue [331, 332]. While the agonism of SERMs is desirable in bones where ER signalling is physiologically required for maintaining bone density, agonism in the uterus is associated with endometrial cell proliferation and is a risk factor for endometrial cancer[333]. Thus, SERMs can be developed with different properties to treat different conditions related to estrogen signalling such as Raloxifene or Bazedoxifene, which do not exhibit agonist activity in the endometrium but are agonists in bone, with marketed application for the treatment of post-menopausal osteoporosis [334]. 27-hydroxycholesterol has been identified as an endogenous SERM which can bind and regulate ERα in a mostly agonistic capacity but with different efficacy than estrogens [112].

Unfortunately, SERM-based therapies for breast cancer often fail due to acquired resistance [335], which is not necessarily due to loss of ER exrpression. SERDs, which completely shut down ERα signalling, possibly due to induction of its degradation [336] may be used as second line treatment [337]. Both types of antiestrogens act via different mechanisms. Whereas SERMs stabilize the H12 in ERα AF2-domain in an alternative position, SERDs possess a bulky side-chain that prevents refolding of H12 on the LBD [338]. This destabilization of H12 correlates with an increased insolubility of the receptor and its inactivation [138]. Furthermore our laboratory has observed that the receptor is heavily SUMOylated in the presence of SERDs and that this modification is to some extent responsible for disabling ERα signalling [338]. The SERD-bound receptor is also ubiquitinated [339] which leads to its degradation but this down-regulation of the receptor, from which SERDs get their name, may not be essential for antiestrogenic

activity as inhibition of degradation does not abrogate the “full-antiestrogenicity” of SERDs in MCF7 cells? [340] and full antiestrogenicity can be observed in HepG2 cells, which do not exhibit an increased turn-over rate of the receptor in the presence of SERDs. More research is required to identify the links between insolubility, SUMOylation and ubiquitination and their contribution to the antiestrogenic potential of SERDs. Of note, while tamoxifen or fulvestrant best represent the notion of SERMs and SERDs, respectively, other molecules such as raloxifen and bazedoxifen show some attributes of both classes of antiestrogens [138].

Aromatase inhibitors

Another approach to block activation ERα is to suppress the production of estrogens [341-343]. Estrogens are synthesized by the aromatase enzyme (CYP19A1) through oxidation of androgens [344]. The aromatase enzyme is part of the cytochrome P450 superfamily of monooxygenase and is expressed in ovaries and peripheral tissues including adipose tissue [345]. In postmenauposal women, estrogen production comes from aromatase action in peripheral tissues and aromatase expression is often increased in the micro-environment of luminal breast tumors leading to increased proliferation of the cancer cells [346]. Aromatase inhibitors (AIs), such as letrozole, can decrease estrogen levels and inhibit ERα signalling in the tumor [347], but will also block estrogen action in bones [348], brain [349] and the cardiovascular system [350]. While AI therapy leads to longer disease free survival than tamoxifen therapy, it does not improve overall

survival much [351, 352] and AIs do present some adverse side-effects not found with SERMs, such as bone mineral density reduction.

Histone deacetylases inhibitors

Other classes of compounds that do not target specifically the estrogen receptors can also show surprisingly efficacious ERα down-regulation. Histone deacetylase inhibitors (HDACis) are molecules that inhibit the catalytic removal of acetyl moieties from lysine residues by HDACs by blocking their catalytic sites [353]. HDACis can non-selectively inhibit all HDACs, as is the case for trichostatin-A (TSA) [354] or suberoylanilide hydroxamic acid (SAHA) [355], or they can selectively target an HDAC subclass or a specific HDAC, like entinostat [356], acting on class I HDACs, or tubacin, targeting HDAC6 [357]. Interestingly non-selective HDACis cause a rapid shutdown of ERα signalling and subsequent degradation of the receptor and reduction of its expression through inhibition of its gene promoter [358, 359]. These effects may be mediated by different HDACs but HDAC3 inhibition was shown to be specific in abrogating estrogen signalling in breast cancer cells by destabilising ERα mRNA [360]. Unfortunately, HDACis have not shown efficacy on the treatment of solid tumors, which may be due to poor pharmacokinetic properties, in particular the short half life of the first FDA-approved compounds such as SAHA [361]. Further development of HDAC-specific molecules with better pharmacokinetics may improve HDACi therapies for breast cancer [362, 363]. Entinostat is now in clinical trials in combination with the SERD fulvestrant [364] or with

the AI exemeststane (Aromasin) [365] to treat advanced estrogen receptor-positive breast cancer.

Experimental approaches to nuclear receptor study

Much of the molecular biology describing nuclear receptor dynamics comes from the early days following their discovery. Thus the technology used to write the books on nuclear receptor was limited largely to ex-vivo approaches for DNA binding studies and cofactor recruitment. For example, one of the early clones of the estrogen receptor, HE0, used to investigate its mechanism contained a critical mutation substituting Gly400 for a valine residue [366]. This mutation caused a destabilisation of the LBD and went unnoticed for years. While the LBD was destabilized, ligand binding did bring about enough reordering that the receptor could work almost normally. Yet, data collected and published concerning the apo-state receptor was erroneous and may have contaminated the knowledge pool of the research community.

Initial studies of NR complexes often used liquid chromatography and size exclusion columns to isolate different complexes that bound radio-labeled ligands [367, 368]. While the technique itself has the advantages of providing some amount of quantification to the interactions observed over what GST-pulldown and IP can yield, it is strictly an in vitro analysis and the interactions measured are very dependent on the buffer conditions – salt concentration, buffering agent and pH.

As the pharmacopeia of the receptors grow each year, novel methods have been implemented to characterize NR dynamics in live cells. One such method which has gained in popularity this last decade is the Förster Resonance Energy Transfer (FRET) [369].

FRET

FRET is based on the electronic transfer of energy from a donor chromophore to an acceptor chromophore [370]. When an electron of the donor is excited to a higher state it will relax to its ground state and release a photon but if an acceptor is present in its vicinity and has an equivalent excited state for its electrons the energy of the donor can be transferred to the acceptor by dipole-dipole coupling. When the acceptor's excited electron relaxes it will land on a different ground state than it would have in the donor and the acceptor thus emits a photon of longer wavelength than donor (red-shifted emission). FRET is then measured by a ratio of the red-shifted emission of the acceptor over the emission of the donor. The transfer of energy is dependent on the proximity of the donor-acceptor pair as efficiency decreases exponentially as the distance between the two increases; generally 10nm is considered to be the longest distance where FRET can happen (Fig 3). Moreover, the absorption and spectrum of the acceptor must overlap with the emission spectrum of the donor; this allows for the overlapping excited state integrals to transfer energy from the donor to the acceptor. Finally, since the transfer is done through dipole-dipole coupling, the dipoles of the donor and acceptor must be

aligned; the efficiency of the transfer is a function of the sinus of the relative angle between the two partners [371].

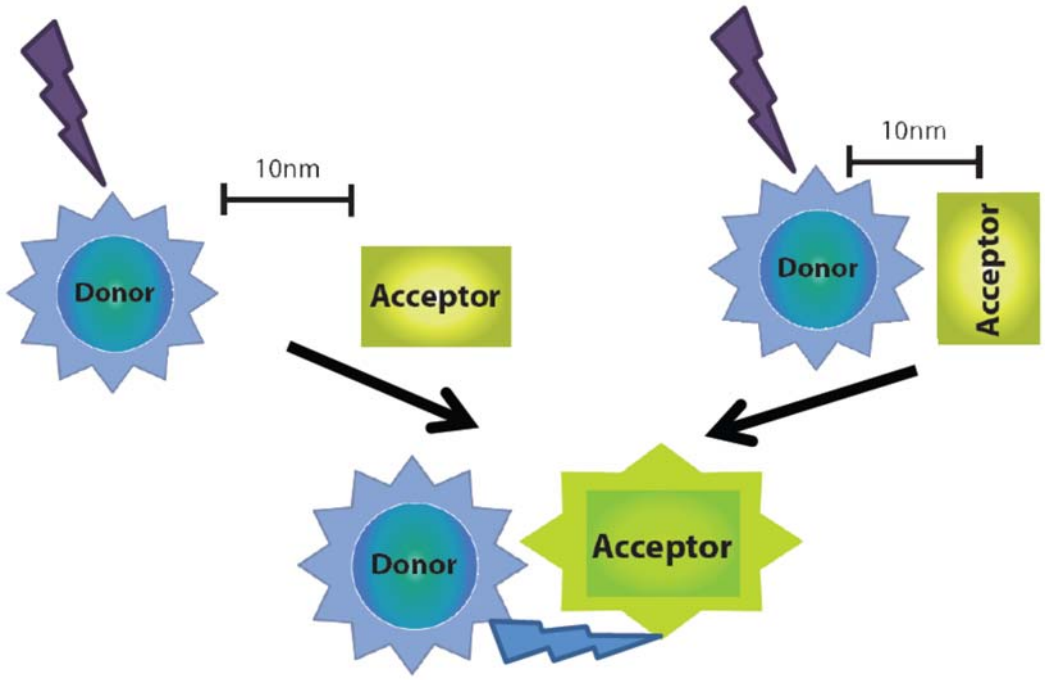

Figure 5. Basic principle of the Förster Resonance Energy Transfer (FRET).

A Fluorescent FRET Donor absorbs short wavelength energy (purple) to reemit longer wavelength light (blue). If an Acceptor is close enough (less than 10 nm) and in the proper orientation, energy can be transferred and reemitted at an even longer wavelength (green).

In this way FRET allows for the monitoring of changes in bimolecular-complex conformation. By labelling a protein with a donor chromophore such as a fluorescent protein (FP) or a luciferase (Luc) and a second protein with another red-shifted fluorophore it is possible to monitor the binding of one protein to the other or a change in their relative alignment [372] [373]. This has been applied to nuclear receptors such as the estrogen receptors α/β [242], retinoic acid receptor [374] and androgen receptor

[142]. In this manner the receptor dimerization and cofactor recruitment has been studied in live cells and has proven helpful in deciphering the behavior of nuclear receptors in their normal environment.

Since the light used to excite the donor may also excite the acceptor and create a contaminating signal, enzymatic bioluminescent donors were developed using the Renilla Reniformis luciferase protein Rluc giving rise to Bioluminescence Resonance Energy Transfer (BRET). The substrate of Rluc, coelenterazine, is added to the system and emits light when metabolized by the Rluc donor and will not directly excite the acceptor without FRET taking place. Mutant variants of Rluc and chemical modification to coelenterazine allows for a several donor emission spectrum.

FRET donor and acceptor pairings can also be combined to monitor a ternary complex. If the acceptor protein can also be a donor to a secondary acceptor one can monitor three proteins in a complex where energy is transferred sequentially from the donor to the first acceptor and finally to the second acceptor. This method is called triple-FRET or three color-FRET and is mostly performed through microscopes. Using a Rluc donor and combining it with two fluorescent protein acceptor to act as subsequent acceptors, a variant referred to as serial BRET-FRET (SRET), simplifies the method and allows it to be done with a simple luminescence reader. Unfortunately this method has only been validated in GPCR and G-protein complexes.

Objectives

Current molecular approaches used in nuclear receptor functional studies or in ligand screening strategies include luciferase and FRET assays, which have complementary strengths and limitations. Luciferase assays rely on indirect detection of NR activity, and require prolonged incubation with the ligand to yield sufficient accumulation of the reporter gene product. As such, they are susceptible to multiple levels of regulation beyond nuclear receptor activation/inhibition, such as protein turnover. Luciferase assays also rely on response elements that can be shared between numerous receptors, leading to increased "off-target" hits.

In opposition to luciferase, FRET readout takes place as soon as the receptor is activated, in the order of minutes after ligand addition rather than hours. The assay also takes place in live cells, which selects for ligands that have good cell permeability. However, FRET measures the interaction of two partners at a time while nuclear receptors work in ternary complexes that usually encompass two dimerization partners and at least one cofactor. Furthermore, the recent reports of nuclear receptor tetramers forming *in vitro* cannot be certifiably verified *in vivo* with existing methods.

The goal of this first section was to design assays that could interrogate complexes formed by three partners. Being able to monitor ternary complex formation will allow the characterization of specific nuclear receptor heterodimer mechanism and the interplay between two different cofactors – be it a coactivator and corepressor – binding

to the same receptor complex. Beyond the opportunity to study the promising pharmacological avenue of heterodimer selective ligands and ligand synergy, ternary complex monitoring could unlock insights into the basic biology of nuclear receptors such as the formation of tetramers or higher-order complexes in live cells.

CHAPTER 2: MONITORING TERNARY COMPLEX FORMATION IN LIVE CELLS BY BRETFECT

David Cotnoir-White, Etienne Gagnon, Michel Bouvier and Sylvie Mader.

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As first author I developed the BRETFect assay (biophysical design, recombinant protein construction and proof of concept experiments), performed and analyzed all experiments and produced all figures. I co-wrote the manuscript with Sylvie Mader. Étienne Ganon and Michel Bouvier contributed to experimental design and figure presentation.

Monitoring ternary complex formation in live cells by BRETFect

David Cotnoir-White[1], Etienne Gagnon[1,2], Michel Bouvier[1,3] and Sylvie Mader[1,3,4]

[1]Institute for Research In Immunology and Cancer, [2]Department of Medicine, [3]Department of Biochemistry, [4]Centre de Recherche du Centre Hospitalier Universitaire de Montréal, Université de Montréal, Montréal, Canada.

Correspondence should be addressed to SM

Detection of multimeric complexes in live cells is important for systems biology and pharmacological applications, but current methods are limited by low sensitivity and/or signal cross-contamination. Here, we introduce BRET with Fluorescence Enhancement by Combined Energy Transfer (BRETFect), based on energy transfer from luciferase to both intermediate and terminal acceptors as well as between the acceptors, to monitor ternary complex formation in live cells. We validated BRETFect for the robust detection of coactivator recruitment by estrogen receptor dimers without spectral unmixing and for the identification of ligands activating ERα/β heterodimers.

Fluorescence/Bioluminescence Resonance Energy Transfer (FRET/ BRET) both detect protein-protein interactions in real-time in intact cells [375, 376]. FRET allows reliable monitoring of sequential transfer between three fluorophores in a three-color or triple-FRET assay [377, 378], but suffers from problems due to photobleaching and contaminating cross-excitation. Sequential Resonance Energy Transfer (SRET), which combines BRET between an initial donor (ID) and intermediate acceptor (IA) and resonance energy transfer between the IA and a terminal acceptor (TA) [379], is based

on a similar principle but is handicapped by a low signal output and high signal cross-contamination (see below).

Our aim was to develop a BRET assay that reliably monitors ternary complexes without the need for spectral unmixing and with robust signal output to enable the dissection of ternary protein complexes and applications to high-throughput screens. One of the limitations of SRET is the need to transfer energy sequentially from the ID (RLucII activated with Coelenterazine 400a for SRET^2) to the IA (uvGFP) and subsequently from the IA to the TA (eYFP) [379]; this limits the choice of ID and TA to prevent direct transfer from ID to TA, which would bypass the IA. However, we reasoned that the contribution of an IA can be detected provided that it uses a part of the emission spectrum of the ID that only marginally contributes to excitation of the TA, and can re-emit it to efficiently excite the TA in combination with the ID. This conceptual change should result in increased signal output and greater flexibility in the choice of the emission/excitation wavelengths for the different reporter proteins. We chose the mTFP1 [380] and Venus fluorescent proteins as intermediate acceptor (IA) and terminal acceptor (TA) because of the high quantum yield of mTFP1 (0.85) and the minimal overlap in emission spectra between the two fluorophores (**Supplementary Fig. 1**). The donor molecule remained RLucII but replacement of coelenterazine 400a by coelenterazine H improved emission 30-fold (**Supplementary Fig. 2**), while shifting the emission peak from 420 nm to 485 nm (**Supplementary Fig. 1**) [381]. This drastically changes the pattern of energy transfer: here the energy emitted by RLucII is expected to be transferred directly to both IA (mTFP1) and TA (Venus) (**Fig. 1a**). Importantly, the energy emitted from RLucII in the shorter wavelengths of the spectrum (400 to 460 nm), which are not favorable for TA excitation, can be efficiently transferred to the IA mTFP1, which will emit at 492 nm, a highly

favorable emission wavelength for Venus excitation (**Supplementary Fig 1 and Supplementary Table 1**). This energy combines with that from RLucII, enhancing the total energy transferred to the TA (Venus). This assay is termed "BRET with Fluorescence Enhancement by Combined Energy Transfer (BRETFect)", as it monitors an amplification of the BRET signal in a donor+IA+TA condition versus the donor+TA and donor+IA control conditions.

As a proof of concept, we monitored recruitment of coactivators by homo- and heterodimers of nuclear receptors ERα and ERβ, which dimerize and recruit coactivators to stimulate target gene transcription when bound to agonists [88, 382-384]. Two ER molecules were tagged with RLucII and with mTFP1, while a coactivator motif peptide (CoApep) was tagged with Venus (**Fig. 1a**). Corresponding expression vectors were cotransfected into HEK293 cells. Spectral analyses of the various complexes validated the combined energy transfer between ID, IA and TA in presence of the agonist 17β-estradiol (E2) (**Fig. 1b**). A red shift in the emission peak in "ERα-RLucII+ERα-mTFP1" versus "ERα-RLucII alone" indicated that energy was efficiently transferred between ID and IA. A potentialization of the TA emission could be observed when ERα-mTFP1 was added to ERα-RLucII+CoApep-Venus complex (**Fig. 1b**). The differential emission plot representing the emission of the ternary complex (ID-IA-TA) minus that of the binary complex between ERα-RLucII and CoApep-Venus (ID-TA) (**Fig. 1c**) illustrates the significant signal drop centered at 464 nm, due to energy absorption by mTFP1, and the corresponding increase in signal with a maximum at 528 nm, compatible with energy transfer from mTFP1 to Venus (**Supplementary Table 1**). These results confirm the predicted pattern of energy transfer and amplification in BRETFect.

We then monitored BRET ratios at 550 nm vs 485 nm for the binary and ternary complexes in the presence of E2 or of the antiestrogen 4-hydroxytamoxifen (OHT), the latter being permissive for ER dimer formation but repressing coactivator recruitment [132, 138]. In the control ERα-RLucII+CoApep-Venus condition, treatment with E2 produced a robust BRET signal, reflecting complex formation, while OHT decreased basal CoApep recruitment (**Fig. 1d**). Cotransfecting ERα-mTFP1 significantly increased BRET levels in the presence of E2 or the absence of treatment (**Fig. 1d**). Transfer between ERα-RLucII and ERα-mTFP1 in the absence of CoApep-Venus produced only small BRET signals under all treatment conditions (**Fig. 1d**), as the larger part of the emission spectrum of mTFP1 is not detected in the 550 nm channel. Thus, the BRET amplification observed in the ternary condition is due to energy transfer from ERα-mTFP1 to CoApep-Venus and not to emission of ERα-mTFP1 at 550 nm. Interaction between ERα-mTFP1 and CoApep-Venus could be verified by FRET after direct excitation of mTFP1 (**Supplementary Fig 3**). The differential BRET activity observed between the three partner condition and the sum of those observed with the acceptors separately in control transfections (Delta BRET, **Fig. 1e**) provides evidence for efficient coactivator recruitment to ERα dimers assembled in the presence or absence of E2, but not in the presence of OHT. Non ERα-dimerizing proteins fused to mTFP1 failed to amplify BRET, and a non ERα-interacting mutated peptide yielded only residual BRET signal (**Supplementary Fig. 4**). The BRET levels in the ternary condition were dependent on the concentration of CoApep-Venus and were saturable under all treatments, as expected for a specific interaction (**Supplementary Fig. 5a&c**). Signal amplification increased with the concentration of ERα-mTFP1, but not of untagged ERα (**Supplementary Fig. 5b&d**). Together, our results indicate that the IA yields an increase in BRET signal that is dependent on its presence in a ternary complex.

As a comparison, we performed similar measurements using SRET parameters (Coel400 instead of CoelH and measurements at 530/400 nm). Direct excitation of the TA by the ID was observed in the SRET set-up, indicating combined rather than serial transfer (**Supplementary Fig. 6**). Calculating a differential signal between the ternary complex and binary complexes with separate acceptors (delta SRET) resulted in signals that were much smaller than in the above-described BRETFect assay, or even negative as with OHT (**Fig. 2**). Thus, BRETFect can be applied to the study of complexes that cannot be adequately analyzed using SRET.

Development of dimer-selective nuclear receptor ligands is an important pharmacological opportunity [385, 386] that necessitates robust assays for the activity of specific homo- and heterodimers. Using BRETFect assays with different combinations of ERα and ERβ fused to RLucII or mTFP1, which were validated for the recruitment of CoApep-Venus in the presence of E2 but not of OHT (**Supplementary Fig. 6**), we tested whether the ERα-specific agonist propylpyrazole triol (PPT) or the ERβ-selective agonist diarylpropionitrile (DPN) [242, 387, 388] can lead to heterodimer activation. PPT as expected did not activate ERβ homodimers **(Fig. 3a)** but was active on ERα homodimers (**Supplementary Fig. 7**). Further, the addition of ERα-mTFP1 to ERβ-RLucII led to a PPT-stimulated coactivator recruitment **(Fig. 3a),** indicating activation of the ERα-ERβ heterodimer. Interestingly, PPT has less potency for the ERα-ERβ heterodimer than the ERα homodimer (EC50 28 vs 0.5 nM, **Supplementary Table 2**), suggesting differential allosteric effects between homo/heterodimeric partners. Similarly, DPN, which activated preferentially ERβ over ERα homodimers with a 20-fold difference in EC50s, had an intermediate EC50 for ERα-ERβ heterodimers **(Supplementary Table 2).** Assays performed with the SRC1 receptor-interacting-domain (RID), which contains multiple

CoApep-like interacting motifs, confirmed that PPT and DPN promoted recruitment of the entire RID to the heterodimer with intermediate potency compared to homodimers (**Fig. 3c-d, Supplementary Table 3**). Further, titration curves indicate that while the coactivator peptide recruitment capacity was reduced by half in the heterodimer with PPT, which binds only ERα, compared to E2 or DPN, which bind both heterodimeric partners, this was not observed for the RID, consistent with the reported stoichiometry of two coactivator peptides but only one RID per dimer (**Supplementary Fig. 8**) [177]. Finally, to verify the usefulness of BRETFect as a screening assay for heterodimer specificity in live cells, we tested the robustness of the ERα-ERβ heterodimer activity assay. A Z-factor of 0.528 was obtained (**Supplementary Fig. 9**), supporting the applicability of this assay to high-throughput screening.

Our study demonstrates that BRETFect is a powerful tool to monitor ligand-dependent recruitment of coactivators to specific nuclear receptor homo- or heterodimers in live cells. BRETFect assays generate robust signals compatible with high-throughput screens without the need for spectral unmixing and can be developed for a variety of donor and acceptor proteins. BRETFect monitors trimer formation but the same constructs can also be used to detect each dimeric interaction in the trimer. BRETFect should be generally applicable to reveal and characterize a wide variety of ternary complexes as illustrated here for ER dimer-coactivator complexes.

METHODS

Methods and any associated references are available in the online version of the paper

ACKNOWLEDGMENTS:

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AUTHOR CONTRIBUTION:

D.C.W. developed the BRETFect assay, performed all experiments and prepared all figures under supervision from S.M. E.G. and M.B. contributed to experimental design and figure presentation. D.C.W. and S.M. wrote the manuscript with input from E.G. and M.B.

COMPETING FINANCIAL INTERESTS:

The authors declare no competing financial interest.

FIGURE LEGENDS

Figure 1. BRETFect monitors ternary complex formation in live cells. (**a**) Description of ID (ERα-RLucII), IA (ERα-mTFP1) and TA (CoApep-Venus) fusion proteins and schematized energy transfer in the presence of two or three partners. (**b**) Spectral analysis of BRETFect reveals energy transfer from ERα-RLucII to CoApep-Venus through ERα-mTFP1. (**c**) Subtraction of emission in the presence of ID and TA [Em(ID-TA)] from ternary complex emission [Em(ID-IA-TA)] across the wavelength spectrum. (**d**) Net BRET signals (550/485 nm) for the binary and ternary complexes after treatment with ligands (1 μM). (**e**) BRETFect signals (Delta BRET ID+IA+TA-[(ID+IA)+(ID+TA)] indicate that OHT suppresses coactivator motif recruitment to ERα dimers. Graphs are representative of 3 biological replicates and error bars represent the SEM from 3 technical replicates. * $p < 0.01$, Bonferroni-test post-hoc ANOVA.

Figure 2: Comparison of net trimer signals in the BRETFect and SRET setups. Net trimer signals represent the delta BRET for BRETFect assays and delta SRET for SRET assays in **Supplementary Fig. 6**. Displayed graphs were prepared from one representative biological replicate with 4 technical replicates; error bars represent the standard deviation.

Figure 3: Activation of ERα-ERβ heterodimers by selective ligands. (**a**-**d**) HEK293 cells were transfected with indicated receptors fused to RLucII or mTFP1 and with CoApep-Venus (**a-b**) or SRC1-RID-Venus (**c-d**). Displayed graphs were prepared from 3 biological replicates and error bars represent the SEM from 3 technical replicates.

ONLINE METHODS (Note: all protocols will be made available on ProtocolExchange)

Cell lines, Plasmids and Reagents

HEK293 cells (Sigma-Aldrich, Oakville, Ontario, Canada) were grown in DMEM with 10% fetal bovine serum (Wisent Inc., St-Bruno, Quebec, Canada). Polyethyleneimine (25 kDa molecular mass, linear or branched forms) was obtained from Sigma-Aldrich. Coelenterazine H and Coelenterazine 400a were obtained from NanoLight Technology (Pinetop, AZ). 17-beta-Estradiol (E2) and 4-hydroxytamoxifen (OHT) were purchased from Sigma-Aldrich; RU58668 (RU58), ICI182,780 (ICI182) and raloxifene (Ral) were purchased from Tocris Cookson Ltd (Minneapolis, MN). pcDNA-RLucII vectors are described in [381]. pCMV-Venus and pCMV-mTFP1 were generated by amplification of the relevant fluorophore genes and replacement of the eGFP in the peGFP-N1 vector (PerkinElmer Corp., Wellesley, MA). ERα/β and Nur77 cDNAs were cloned into the above-described vectors by PCR amplification of coding sequence and digestion of 5' and 3' ends with appropriate restriction enzymes. The coactivator construct was generated by inserting oligonucleotides coding for a repeat of the first NCOA2 LXXLL motif (WT sequence: GAT CTA ACC ATG AAG CAT AAA ATT TTG CAC AGA CTC TTG CAG GAC AGC AGT CTC GAG ATG AAG CAT AAA ATT TTG CAC AGA CTC TTG CAG GAC AGC AGT CTC GAG, non-interacting mutant sequence: GAT CTA ACC ATG AAG CAT AAA ATT GCG CAC AGA GCC GCG CAG GAC AGC AGT CTC GAG ATG AAG CAT AAA ATT GCG CAC AGA GCC GCG CAG GAC AGC AGT CTC GAG) and a GR-derived NLS sequence (sequence: GAT CGA GCC CAC TCC ACA CCT CCA AAA AAC AAA CGA AAC GTT CGA GAT CCC AAG GAT CGA GCC CAC TCC ACA CCT CCA AAA AAC AAA CGA AAC GTT CGA GAT CCC AAG) into pCMV-Venus. The ERα(L507R) mutant was created by overlapping primer site-directed mutagenesis. The SRC1-RID was

cloned from NCOA1 cDNA with primers flanking the third and fifth LXXLL motif in 5' and 3' respectively (positions 2155 to 2517 in NCBI Reference Sequence NM_003743 – oligo sequence: atg tac tct caa acc agt cac aaa and TGA GGG GCT ACC CTC CTG).

HEK293 Cell Transfection

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) in 175 cm^2 culture flasks. Two days before experiments, HEK293 cells were harvested and switched to phenol red-free DMEM containing 10% charcoal-stripped serum. HEK293 cells were transfected via PEI with 1.66 µg of DNA, 1.66 µg PEI-branched and 5 µg PEI-linear per 10^6 cells. DNA mixes and PEI dilutions were prepared in a total volume of 75 µl PBS separately before mixing. After 10 min incubation, HEK293 cell suspensions ($1.25x10^6$/ml) were added directly to the DNA-PEI transfection mixes (850 µl of cells per 150 µl of transfection mix) and 100 µl of DNA-PEI-cell suspensions was aliquoted per well in 96-well white-bottom culture plates or 1 ml per well in 12-well culture plates and grown for 48 h.

Bioluminescence Resonance Energy Transfer Assays

Unless otherwise indicated, BRET assay transfection mixes contained 150 ng of RLuc-tagged donor and 0 to 1.5 µg of Venus-tagged acceptor for titrations or 1.5 µg for single point experiments. 48 h after transfection, cells were washed with PBS and treated with specified ligands or vehicle (0.1% DMSO) in 100 µl PBS for 40 min at 37°C. Coelenterazine H was added to a final concentration of 5 µM, and readings were immediately collected on a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), with sequential integration of signals detected with BP485 nm (Renilla luciferase emission) and LP550 nm (Venus emission) filters. BRET ratios displayed for titration curves are the stimulated

Venus emission (exc 485 nm, em 550 nm) acquired with a FlexStation 3 Microplate Reader (Molecular Devices) divided by Luciferase emission as recorded by the Mithras LB 940 in the 485 nm channel ([Venus]/Luc). Net BRET values (BRET ratios for fused proteins minus BRET ratios with fused Luciferase but unfused FP) are represented as a function of Log_{10}(Fluorescent protein/Luc), where levels of the fluorescent proteins are measured after direct stimulation at appropriate wavelengths. Emission spectrum experiments (Fig. 1a) were performed on a Synergy Neo microplate reader (Biotek, Winooski VT, USA) using 2 nm intervals from 400 to 600 nm and Relative Light Units (RLU) are calculated as a fraction of the maximal value recorded in each condition (arbitrarily set at 1.00). All graphs were built using GraphPad Prism 5.00 which analyzed titration data using non-linear regression curve fit with variable slope (log(agonist) vs. response) and one point comparison using ANOVA and the Bonferroni post-hoc test to determine confidence intervals.

SRET, BRETFect and FRET assays

Unless otherwise indicated, BRETFect and SRET assay transfection mixes contained 100 ng of LucII tagged donor, with or without 400 ng of mTFP1-tagged IA and/or 1 µg of Venus-tagged acceptor (total DNA concentration kept constant with pcDNA3.1-Hygro). For spectral analysis (Fig. 1a), cells were transfected with 250 ng ERα-LucII, 500 ng untagged ERα for ID+TA condition or 500 ng ERα-mTFP1 for ID+IA+TA condition and 750 ng CoApep-Venus for ID+TA and ID+IA+TA. Cells were plated at a higher concentration (500 000 cells per well instead of 100,000) to optimize signal output for spectral analysis. For BRETFect titration (Supplementary Fig. 5), levels of ERα-mTFP1 transfected remained constant at 400 ng while transfected amounts of CoApep-Venus varied from 0 to 1 µg

(Supplementary Fig. 5a) and levels of CoApep-Venus remained constant at 1 µg while transfected amounts of ERα-mTFP1 or unfused ERα varied from 0 to 400 ng (Supplementary Fig. 5b). Cell treatment and acquisition was performed as for BRET assays. Readings were collected using 485 nm (Donor) and LP550 nm (Acceptor) filters for BRETFect and 400 nm (Donor) and 530 nm (Acceptor) filters for SRET on the Mithras LB 940. LP550 filters were used instead of 530 nm to limit bleed-in from mTFP1 emission in BRETFect assays but use of 530 nm filter yielded similar delta BRET signals. The mTFP1 emission captured in the 485 nm-RLuc filter more than compensated for the quenching of Luc emission (data not shown). BRETFect signals correspond to the delta BRET measures calculated by subtraction from the three-partner BRET ratios of the sum of those obtained with either of the mTFP1 or the Venus partners. Net SRET was calculated as described in [379] and delta SRET was calculated as for delta BRET using Net SRET measurements. FRET assays were performed on transfected cells before addition of Coelenterazine using the FlexStation 3 Microplate Reader with excitation of mTFP1 at 420 nm, and measuring emission of mTFP1 at 495 nm and of Venus at 550 nm. FRET ratios were calculated as (em550nm)/(em495nm).

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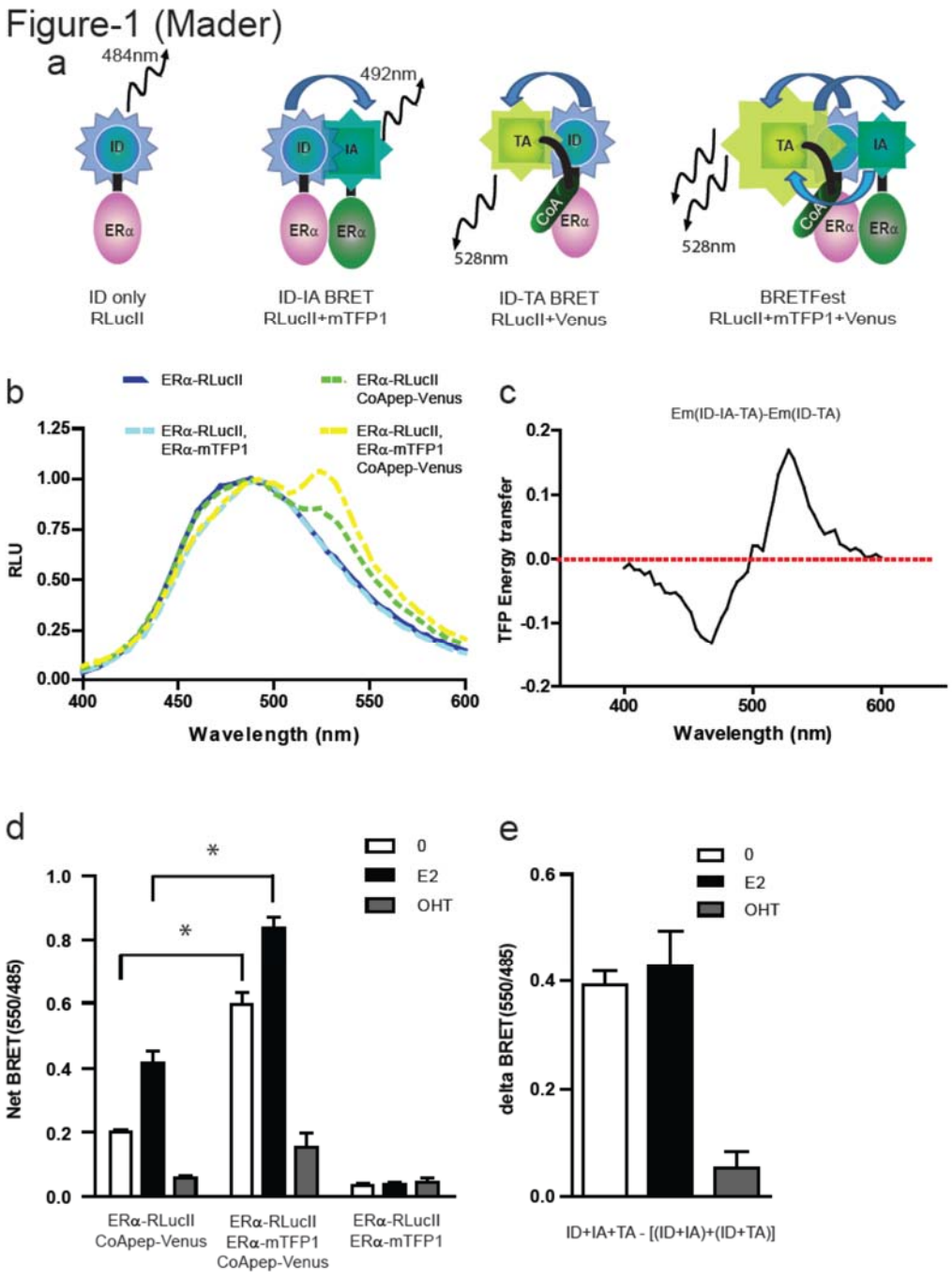


Figure 1. BRETFect monitors ternary complex formation in live cells.

Figure-2 (Mader)

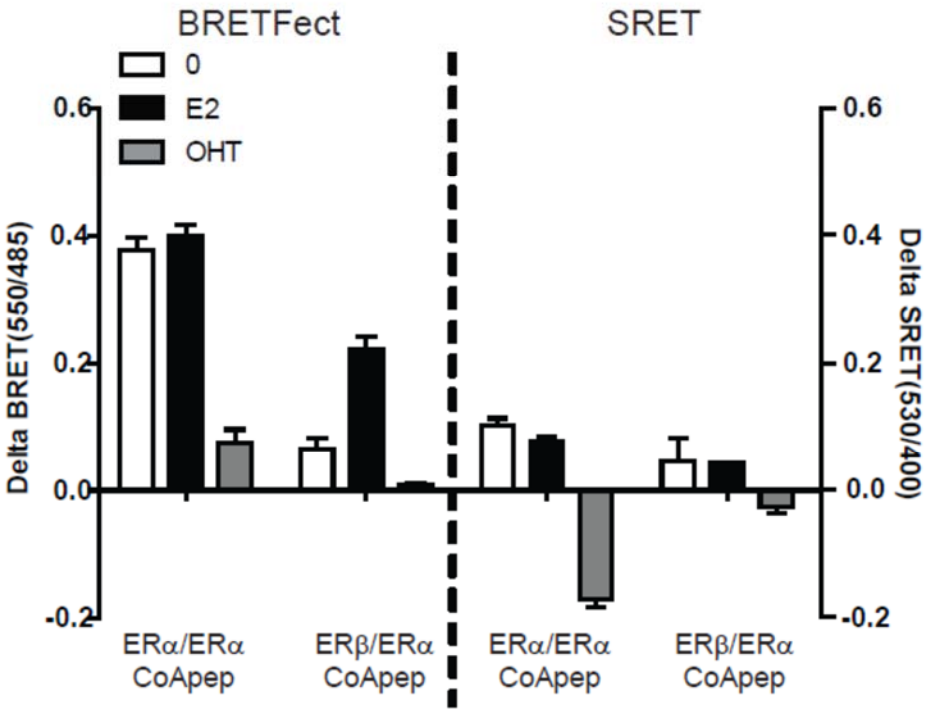


Figure 2. Comparison of net trimer signals in the BRETFect and SRET setups.

Figure-3 (Mader)

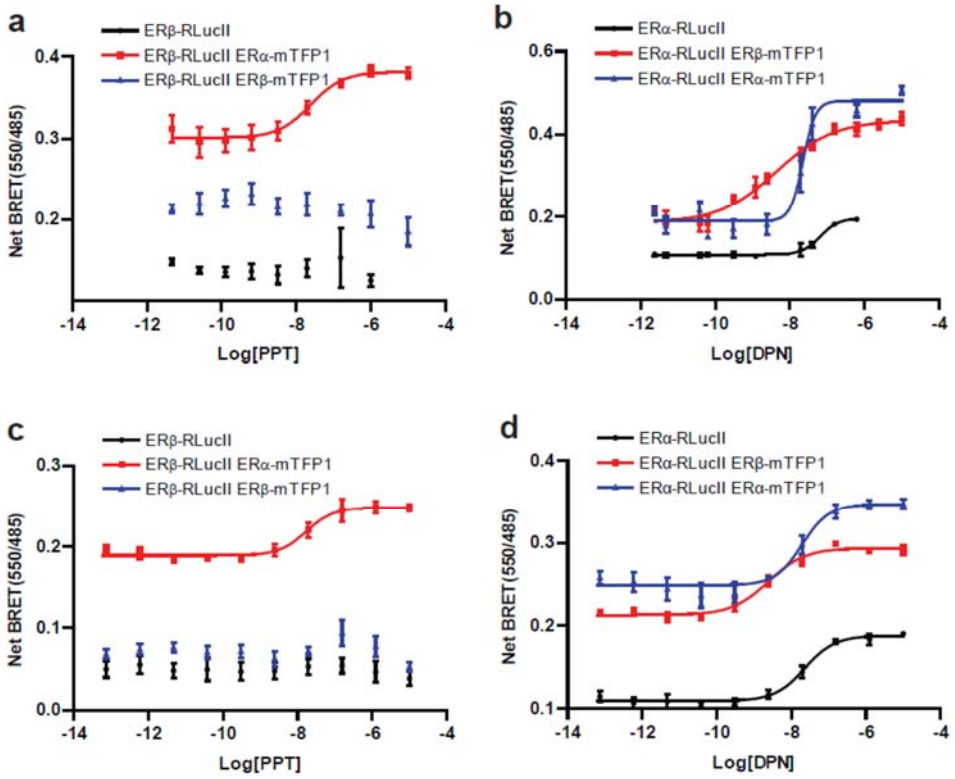
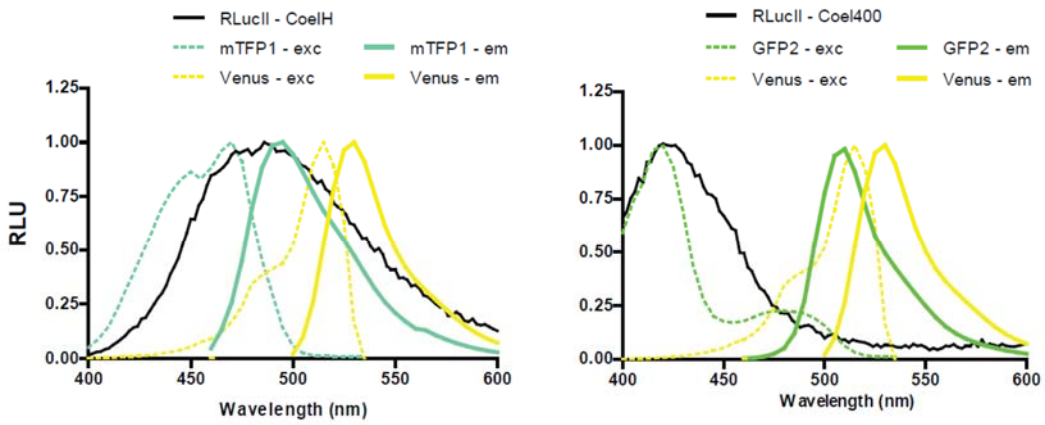


Figure 3. Activation of ERα-ERβ heterodimers by selective ligands.

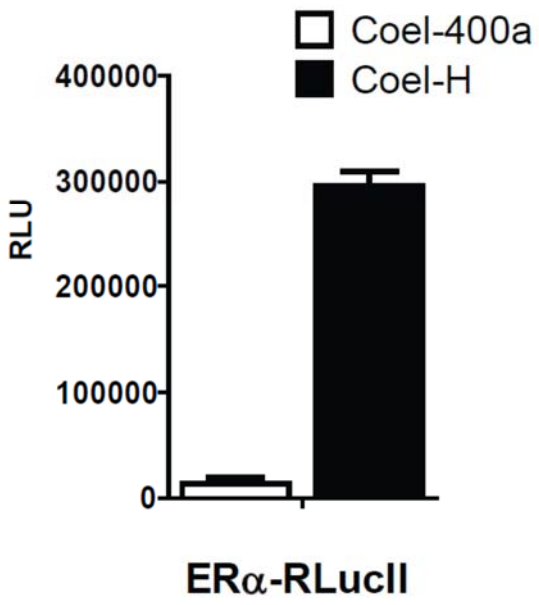
Supplementary Figure-1 (Mader)



Supplementary figure 1. Absorption and emission spectra of different donors and acceptors used in BRETFect and SRET. Cells were transfected with free RLucII, mTFP1, GFP2 or Venus and fluorescence was measured from 460 to 600 nm with excitation at 400 to 535 nm with 5 nm increments. Luciferase emission was acquired with a monochromatic luminescence reader (400 nm to 600 nm with 2 nm increments) after incubation of cells with coelenterazine H (CoelH) or coelenterazine-400a (Coel400). Relative light units (RLU) are calculated as a fraction of the maximal value recorded in each condition which is set at 1.00.

Supplementary Figure 1. Absorption and emission spectra of different donors and acceptors used in BRETFect and SRET.

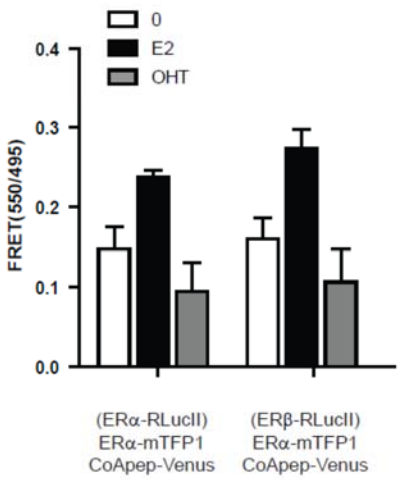
Supplementary Figure-2 (Mader)



Supplementary figure 2. Use of coelenterazine H instead of coelenterazine 400 greatly increases signal output. HEK293 cells were transfected with expression vectors encoding ERα-RLucII and unfused mTFP1 as a transfection control. Cells were treated with CoelH or Coel400 and luminescence was measured at 485 nm for CoelH and 400 nm for Coel400. Data was normalized over control free mTFP1 fluorescence to give relative light units (RLU).

Supplementary Figure 2. Use of coelenterazine H instead of coelenterazine 400 greatly increases signal output.

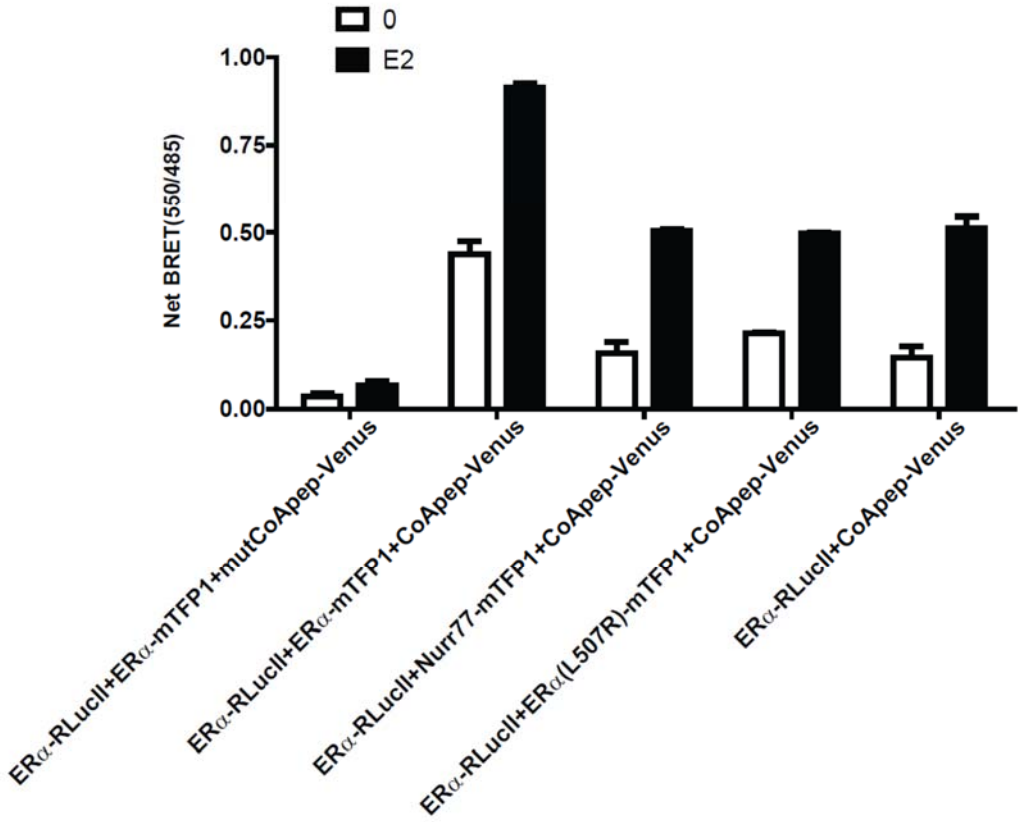
Supplementary Figure-3 (Mader)



Supplementary figure 3. FRET measurement of ERα and ERβ recruitment of CoApep-Venus. Cells prepared as in Fig. 2 but measurements were taken in a fluorometer by stimulating at 425 and detecting at 495 and 550 nm.

Supplementary Figure 3. **FRET measurement of ERα and ERβ recruitment of CoApep-Venus.**

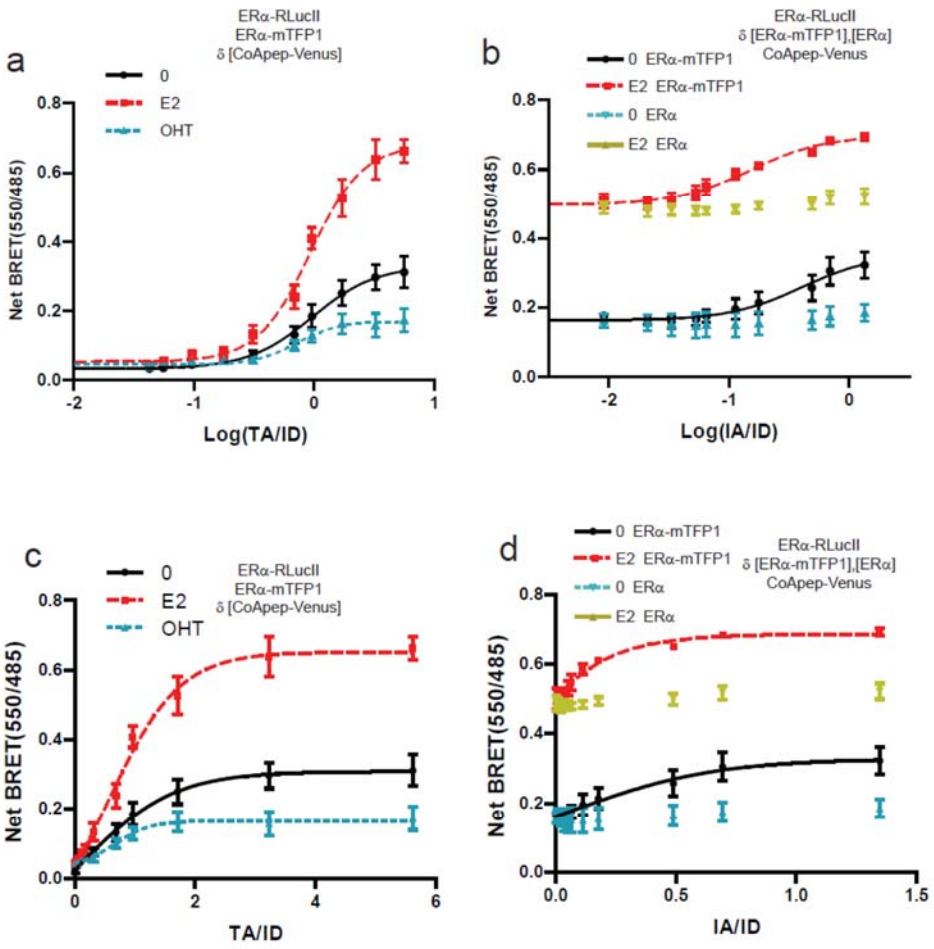
Supplementary Figure-4 (Mader)



Supplementary figure 4. Specific interactors are required to achieve BRETFect. Experiment was carried out as in Fig. 1b but TA was switched to a mutant of the CoApep with the LXXLL motif mutated to AXXAA that cannot interact with ERα or IA was substituted for the non-interacting partners Nur77-mTFP1 or ERα(L507R).

Supplementary Figure 4. **Specific interactors are required to achieve BRETFect**

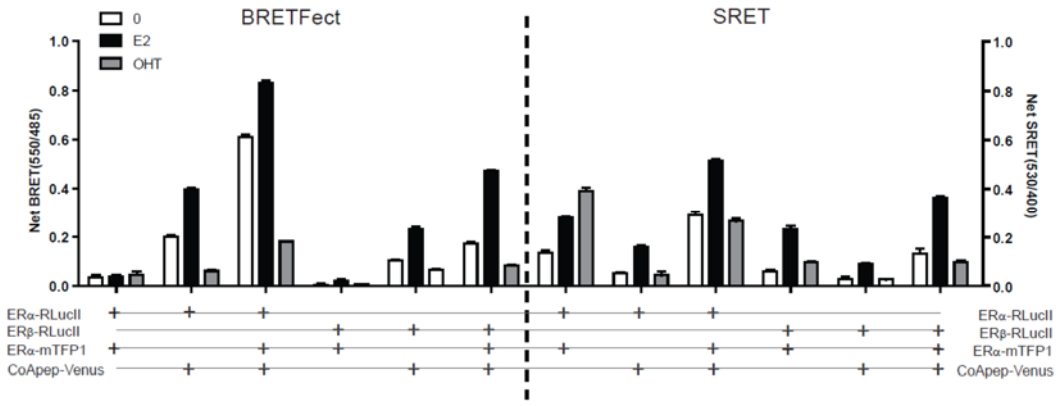
Supplementary Figure-5 (Mader)



Supplementary figure 5. (a) ERα dimer interaction with CoApep-Venus is concentration-dependent and saturable. (b) BRETFect amplification is dependent on ERα-mTFP1 concentration. (c-d) Linear displays of BRETFect titration curves.Graphs are representative of 3 biological replicates and error bars represent the SEM from 3 technical replicates.

Supplementary Figure 5. **ERα dimer interaction with CoApep-Venus is concentration-dependent and saturable.**

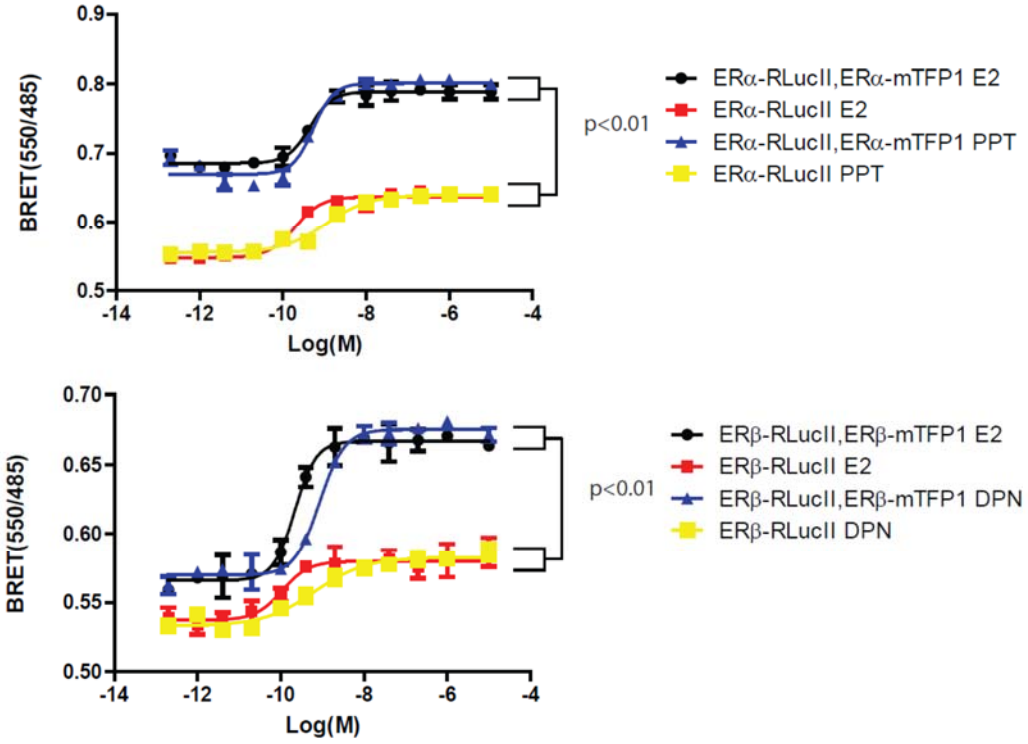
Supplementary Figure-6 (Mader)



Supplementary figure 6. BRETFect assays detect ERα-ERβ heterodimer activation. (a) BRETFect and SRET measurement of ERα-ERβ heterodimer recruitment of coactivator motif. Cells were transfected with specified constructs and were treated with ligands (1 μM) before measurement of BRET (550/485 nm) or SRET (530/400 nm) upon stimulation with coelenterazine H or coelenterazine 400, respectively.

Supplementary Figure 6. BRETFect assays detect ERα-ERβ heterodimer activation.

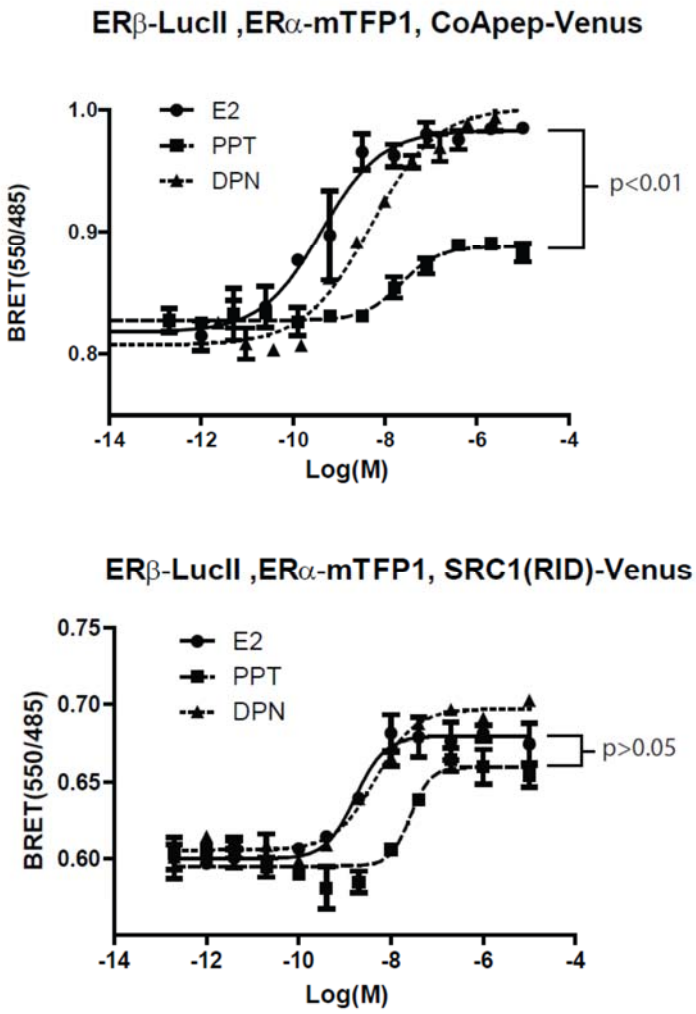
Supplementary Figure-7 (Mader)



Supplementary figure 7. Comparison of CoApep recruitment by ERα-ERα or ERβ-ERβ homodimers in the presence of E2 and PPT or DPN, respectively. The experiment was carried out as in Fig. 3a-d in the trimeric or dimeric conditions to illustrate the impact of ligand titration on signal gain in the trimeric condition. P-value obtained through Student's t-test.

Supplementary Figure 7. Comparison of CoApep recruitment by ERα-ERα or ERβ-ERβ homodimers in the presence of E2 and PPT or DPN, respectively.

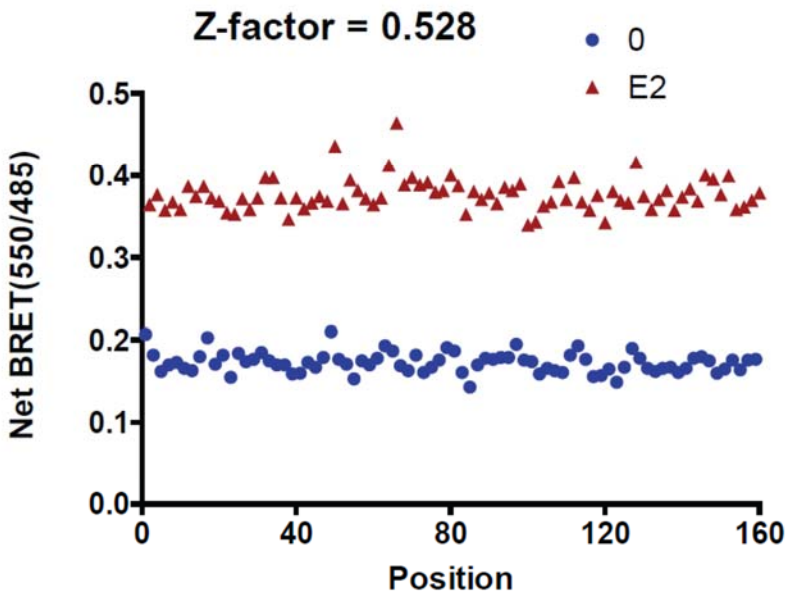
Supplementary Figure-8 (Mader)



Supplementary figure 8. Comparison of CoApep or SRC1-RID recruitment by ERα-ERβ heterodimers in the presence of E2, PPT or DPN. Experiment was carried out as in Fig. 3a-d in the trimeric condition. BRET ratios are displayed for ERβ-ERα heterodimer recruitment of CoApep-Venus or SRC1-RID-Venus in the presence of varying concentrations of each ligand. While PPT reaches only 50% of the BRETmax observed with E2 or PPT for CoApep recruitment, it is not significantly weaker for SRC1-RID recruitment. P-value obtained through Student's t-test.

Supplementary Figure 8. **Comparison of CoApep or SRC1-RID recruitment by ERα-ERβ heterodimers in the presence of E2, PPT or DPN.**

Supplementary Figure-9 (Mader)



Supplementary figure 9. BRETFect assays are excellent assays for high-throughput screening of heterodimer activity. Cells transfected with ERβ-RLucII, ERα-mTFP1 and CoApep-Venus were seeded in two 96 well plates and alternate rows were treated with vehicle (0) or estradiol (E2). BRET ratios from individual wells are plotted on the graph against the position of the wells. The Z-factor was calculated as described in Zhang et al 1999 [18]. With a Z-factor higher than 0.5, the BRETFect assay qualifies as an excellent assay.

Supplementary Figure 9. BRETFect assays are excellent assays for high-throughput screening of heterodimer activity.

Supplementary Table 1: Excitation and emission wavelengths of ID, IA and TA used in this study.

Role	Protein-tag	Exc(nm)	Em(nm)
ID	RLucII coel 400		420
	RLucII coelH		484
IA	mTFP1	462	492
TA	Venus	515	528

Supplementary Table 2: Allosteric effects of heterodimeric partners for recruitment of the coactivator motif peptide revealed by BRETFect titration curves with receptor-selective ligands. EC50s are taken from displayed curves of Figure 3a-b. Note that titration curves using inverted tagging of LucII and mTFP1 for the heterodimers yielded similar results. Data are the average of three biological replicates.

Ligands	EC50 (nM) (95% confidence interval)		
	ERα-ERα	ERα-ERβ	ERβ-ERβ
E2	**0.341** (0.255 to 0.481)	**0.419** (0.214 to 0.822)	**0.469** (0.222 to 0.831)
PPT	**0.547** (0.421 to 2.22)	**28.4** (10.3 to 38.6)	**n/a**
DPN	**20.1** (15.2 to 40.4)	**5.11** (2.76 to 11.8)	**1.02** (0.196 to 2.12)

Supplementary Table 3: Allosteric effects of heterodimeric partners for recruitment of SRC1-RID revealed by BRETFect titration curves with receptor-selective ligands. EC50s are taken from displayed curves of Figure 3c-d. Note that titration curves using inverted tagging of LucII and mTFP1 for the heterodimers yielded similar results. Data are the average of three biological replicates.

Ligands	EC50 (nM) (95% confidence interval)		
	ERα-ERα	ERα-ERβ	ERβ-ERβ
E2	**0.404** (0.268 to 0.784)	**0.503** (0.109 to 2.65)	**0.218** (0.166 to 0.286)
PPT	**0.968** (0.729 to 1.18)	**25.1** (7.43 to 84.7)	**n/a**
DPN	**69.7** (35.1 to 447)	**17.6** (4.12 to 22.3)	**0.757** (0.318 to 1.80)

CHAPTER 3: LIGAND-SPECIFIC MODULATION OF ESTROGEN RECEPTOR CONFORMATION AND MULTIMERIZATION IN LIVE CELLS

David Cotnoir-White, Mohammed El Ezzy, Martine Bail, Michel Bouvier and Sylvie Mader priorities sad Tengo eert

Manuscript prepared for PNAS (Awaiting publication of previous chapter for submission)

As first author I developed the sliced-BRET nuclear receptor assay performed and analyzed experiments (All except for SUMO and UBI assays of **figure S7B** carried out by Mohammed El Ezzy) and produced figures. Martine Bail reproduced some experiments with N-terminal fusion of PCA tags. I co-wrote the manuscript with Sylvie Mader. Michel Bouvier contributed to experimental design and figure presentation.

Ligand-specific modulation of estrogen receptor conformation and multimerization in live cells

Cotnoir-White D[1], El Ezzy M[1], Bail M[1], Bouvier M[1] and Mader S[1].

[1]Institut de recherche en immunologie et en cancérologie (IRIC), Université de Montréal, Montréal, Québec, Canada.

SUMMARY:

While both selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs) inhibit recruitment of coactivators via the ligand binding domain of estrogen receptors (ERs), SERDs have additional suppressive effects that have been linked to the induction of ubiquitination and SUMOylation of the receptor. Using assays combining BRET and PCA (sliced-BRET), we show here that SERDs and SERMs both recruit the AF1-interaction domain of SRC coactivators but not their LXXLL motifs in live cells, in spite of inducing different conformations of ERα dimers. However, SERDs favor assembly of multimers (at least three subunits) of ERα via determinants additional to the dimerization interface. Multimeric complexes can be detected with ERa and/or ERβ and are competent for coactivator recruitment in the presence of agonists, ruling out non-functional aggregates. Enhanced multimerization in the presence of SERDs correlated with SUMOylation and ubiquitination in antiestrogen derivatives with side chains of variable lengths.

INTRODUCTION:

Nuclear receptors are a class of ligand dependent transcription factors that control the expression of genes necessary to several processes including cell proliferation, metabolism and fate-determination. Generally, receptors bind their specific DNA response elements as homodimers or as heterodimers with a second nuclear receptor and recruit cofactors to regulate gene transcription upon ligand binding. Activation of steroid hormone receptors upon ligand binding is thought, based on biochemical and immuno-localization experiments, to involve release from inactive complexes with heat-shock chaperones, shuttling of the receptor to the nucleus, homodimerization, DNA binding, and recruitment of transcriptional cofactors [1]. Use of fluorescence-based techniques for detection of receptor complex formation has validated the role of ligand in co-activator recruitment. Variations are observed among steroid receptors in subcellular localization in the absence of ligand. For instance, unliganded GR, MR and AR are predominantly in the cytoplasm in the absence of ligand while ER and ERR are located mainly in the nucleus in breast cancer cell lines and in transfected cells [2-5]. However, ligand-induced dimerization has been reported for estrogen receptors in FRET experiments as well as MR, GR and AR [6], while *in vitro* studies have reported a constitutive dimerization of estrogen receptors [7].

The impact of antagonists on receptor conformation has been extensively studied by biophysical approaches but their effects on receptor conformation and dimerization in solution remain less clear. Early assays described SERDs as dimerization stabilizers [8] or

inhibitors [9] and SERMs as dimerization enhancers while in vivo BRET assays show enhanced association between ERα units with SERDs relative to SERM [10]. Discrepancies between *in vitro* and *in vivo* studies may be due to the presence of coactivators in cell-based studies, since coactivator peptides selectively stabilized agonist-bound receptors *in vitro* [11].

Interestingly, using fluorescence anisotropy Margeat et al described the formation of higher-order structures of ERs that suggested the formation of a DNA-bound tetramer [12]. The organization of nuclear receptor in tetramers had also been observed *in vitro* for RXR when the receptor was inactive in the absence of ligand and response element [13, 14]. Such oligomerization could complicate interpreation of receptor-receptor interactions in cell-based assays such as BRET and FRET. However, the possibility of forming estrogen receptor oligomers and the role of different agonists/antagonists in modulating these higher order interactions have not been characterized in cell-based systems.

In the present study we have observed the dynamic formation of ERα oligomers in a ligand-modulated manner. The receptor appears to be at least partially constitutively dimeric in transfected HEK293 cells, but its ligand binding domain is conformationally flexible. Both SERMs and SERDs preventing coactivator recruitment via AF2, but were permissive for recruitment of coactivators via AF1. However, we observe that SERDs selectively stabilize higher order complexes involving at least three receptors. Formation of these complexes in the presence of SERDs preceded induction of ubiquitination and sumoylation of the receptor. Receptor mutants that display increased transcriptional

activation properties in the presence of SERDs also displayed reduced oligomer formation. Together, these results suggest that the conformational effects induced by SERDs *in vivo* lead to facilitated oligomerization, recruitment of ubiquitin or SUMO conjugating enzymes, modulated recruitment of cofactors and altered transcriptional activation properties.

RESULTS :

Luc-PCA and sliced BRET detect distinct conformations of ER in the presence of SERMs and SERDs.

While previous studies including our own have observed that treatment of cells with either agonists or antagonists induces BRET between two ER fusion proteins [6, 10], the interpretation of these observations should be subject to caution as BRET is a proximity assay that can be sensitive to conformational changes and higher order oligomerization. To further examine the role of ligand in receptor dimerization, we used a luciferase (Luc) complementation assay wherein the Luc molecule is split into two fragments F1 and F2 at amino acid 110 [15]. Luc PCA was favored over the previously-described YFP PCA because complementation of Luc, but not YFP, fragments is reversible [15]. These fragments were fused either at the N-terminus or C-terminus of ER, reconstituting Luc activity upon interaction of molecules fused with each Luc fragment, provided the resulting conformation is compatible with fragment complementation (Fig. 1A). Luc PCA is thus more sensitive to conformation than BRET, where transfer can take place between

two molecules within a 100 Å radius. However, the Luc PCA signal reflects strictly dimeric interactions that lead to Luc reconstitution.

As observed in BRET, E2 increased the basal signal for ER□-ER□ interaction in the PCA assay with C-terminally fused fragments. The effect of E2 was however much more pronounced in the PCA assay (about 12-fold, Fig. 1B). This increase was also observed in ERα-ERβ heterodimers (not shown). Note that the specificity of both the basal and induced signal is demonstrated by the lack of luminescence when ER is paired with either RXR,RAR or Nur77 (Fig.1B). In addition, introduction of a mutation that prevents dimerization, L507R [16], also abolished the signal. On the other hand, fusing the Luc fragments N-terminally to ER□□led to a signal that was constitutive (Fig. 1B). Titration curves confirmed that this observation is independent from the ratio of the two fusion proteins (Fig. 1CD), suggesting that under the conditions of our assay ERα can dimerize in the absence of ligand. This was confirmed using the G400V mutation (HE0), which destabilizes dimerization in the absence of ligand [17] and accordingly suppressed the basal Luc PCA signal while maintaining similar signals as the wt receptor in the presence of E2 and ICI182,780 treatment (Fig. S1). Note also that incubation with the HSP90 inhibitor geldanamycin did not lead to specific modulation of signal in the absence of ligand either with N-term or C-term fusions (Fig. S2). Together, these results thus indicate that that the PCA assay with C-terminal tag fusion detects estradiol-induced conformational differences in the ligand binding domain rather than effects on ER□ dimerization. Note that this is compatible with *in vitro* results [17], although it is possible that interaction is favoured by high receptor concentrations or cell-context dependent.

With both N- and C-terminal fusions, SERMs induced complementation to levels comparable to those with E2 (Fig. 1EF). By contrast, SERDs ICI164,384, ICI182,780 and RU58,668 were markedly less efficient at inducing Luc complementation with C-terminal tags (Fig. 1E), while they had the opposite effect on Luc reconstitution with N-terminal fusions (Fig. 1F). Titration of ER□-F1Luc by ER□-F2Luc indicates different capacity but similar affinity for receptor dimerization in the presence of E2 and ICI182,780 (Fig.1CD). Titration experiments with N-terminal fusions on the other hand confirmed a lack of difference in capacity or affinity under vehicle, E2 or SERM treatment. Together, these results strongly suggest that SERDs induce a conformation of the receptor that differs from those in the presence of E2 or SERMs.

Both SERDs and SERMs block AF2 activity but are permissive for coactivator recrtuiment via the AF1 domain.

Both SERMs and SERDs are expected to block coactivator recruitment via the AF2 interface, although their differential impact on coactivator interaction with the AF1 interface has not been characterized. To assess coactivator domain recruitment by the receptor under its different ligand-modulated conformations, we combined our PCA assay with a BRET assay in which energy can be transferred from the ER□-F1/2Luc dimers to a YFP tagged coactivator peptide derived from GRIP1 (sliced BRET, see methods) and compared results to a standard BRET assay using ER□ fused to full R-Luc (Fig. 2A, B). In both assays, a strong BRET signal was observed in the presence of E2, but not SERM or SERD treatment (Fig. 2C, D). The dimerization-deficient mutant L507R was not able to recruit the peptide in either assay, providing a negative control for these experiments

(Fig. S3). Notably, the signal in the absence of added ligand was proportionally stronger in the sliced-BRET assay, which monitors specifically coactivator recruitment by the fraction of receptors in the appropriate conformation for Luc complementation. Titration curves with either the synthetic coactivator peptide construct or with the NCOA1 NR interaction domain indicate that a BRET signal was detected at lower coactivator concentrations in the presence vs absence of E2, in keeping with the more than 10-fold higher proportion of reconstituted Luc molecules. In addition, the capacity for synthetic peptide binding was significantly lower in the absence vs the presence of E2. With the NCOA1 domain, high concentrations of the coactivator domain were however capable of compensating for the weaker interaction, as indicated by the higher cooperativity of interaction (increased slope of the curve for the unliganded ERα). Note that we verified that peptide recruitment parallels transcriptional activity using mutant L536A, which has increased basal activity [10] (Fig. 3A), and mutant D351L, which has undetectable activity in the absence of ligand and reduced activity in the presence of agonists. (Fig. S4). Finally, both SERMs and SERDs abrogated recruitment of the coactivator peptide or the NCoA1 domain.

The cell-specific activity of SERMs has been attributed to the recruitment of coactivators by the AF1 region, which interacts with the LBD in the absence of ligand but is freed by agonist/antagonist binding [18]. However, it is unclear whether the full antiestrogenicity of SERDs results from lack of capacity to recruit coactivators via AF1 or is due to other properties such as induced ubiquitination and SUMOylation. As expected, YFP-tagged AF1-interacting domain was recruited in the presence but not in the absence of E2. Also compatible with the previously reported interaction of AF1 with AF2 in the absence of E2 resulting in lower availability of both regions for coactivator recruitment, we observed

that deletion of AF1 increased the capacity for coactivator recruitment in the absence of hormone (Fig. S4A). Both OHT and ICI182,780 led to the recruitment of the AF1-interacting domain to an intermediate capacity compared to E2. Yet, only ICI182,780 treatment increased SUMOylation and ubiquitination of ERα dimers in a conformation dependent manner while OHT behaved like E2 (Fig. S4B).

Together, our results indicate that SERMs and SERDs are both permissive for recruitment of the AF1-interacting domain but not of the coactivator peptide. This suggests that the different properties of SERMs and SERDs result from differences in other functional properties.

SERDs facilitate formation of higher order receptor multimers.

In contrast with the Luc PCA assay with tags fused at the C-terminus of ER (Fig. 1), a regular BRET assay with two ER fusion proteins (Fig. 3A) resulted in signals that were higher in the presence of full antiestrogens than in the presence of E2 or SERMs (Fig. 3B), similar to results obtained with N-terminal fusions in the Luc PCA assay but with a larger relative increase in signal for SERDs. These results are compatible with the expected greater tolerance for conformational effects in BRET vs PCA with C-terminal fusions, but the reason for the increased signal with full AEs is unclear. As SERDs have been described to induce ER aggregation and/or resistance to extraction from cells in the absence of detergent [10], we investigated whether the increase in the BRET signal may result from interaction between more than two ER molecules; indeed, transfer from one Luc molecule to several YFP molecules would be expected to result in stronger BRET signals. To test for receptor multimerization, we used a sliced-BRET assay with LucF1, LucF2, and

YFP fused c-terminally to ER (Fig. 3C), thereby detecting formation of complexes containing at least three ER molecules. Of note, receptor tetramers have been observed *in vitro* for the RXR family of nuclear receptors in the absence of ligand [13, 14, 19, 20], although they have not been documented in live cells.

In this assay, specific basal interaction was detected as evidenced by comparison with non-specific controls L507R or RARα (Fig. 3D). Treatment with E2 only modestly induced the sliced BRET signal, and OHT had little impact (Fig. 3F). Strikingly however, ICI182,780 induced a marked increase in sliced BRET signal (Fig. 3D) in spite of a lower input signal from Luc complementation for the SERDs, reflecting an increased capacity and apparent affinity in the trimer signal. Further, the mixed SERM-SERD Ral induced trimer assembly more efficiently than SERMs or E2 but less than ICI182,780 (Fig. S6A). Finally, a similar increased signal in the presence of ICI182,780 was also observed in a sliced BRET assay with N-terminal Luc fragment fusions, indicating that this effect is independent on the modulation of the Luc PCA signal by this antiestrogen (Fig. S6B).

Full antiestrogenicity has been linked with the length of the SERD side chain, which in the case of the ICI164,384 molecule was observed to interact with a hydrophobic pocket in the coactivator binding groove[21]. We have generated derivatives of ICI164,384 with different side chain lengths and shown that lengths of 15-19 atoms are associated with the maximal degree of antiestrogenicity in a luciferase assay in HepG2 and T47D cells [22]. Therefore, we tested the impact of side chain length on receptor multimerization with antiestrogens containing side chains with amides at different positions (RMS757 and 759 vs RMS760 and 762) and different overall side chain lengths (shorter in RMS757 vs 759 and in RMS760 vs 762). Sliced BRET assays indicate that ligands with short side chains

(RMS757 and RMS760), which behave as SERMs, did not lead to an increased signal in sliced BRET, while ligands with longer side chains, which behave like SERDs, led to a higher capacity signal like observed with ICI182,780. This correlated for both ligand pairs with the capacity to induce SUMOylation and ubiquitination as observed in a BRET assay between ERα-Luc and SUMO- or Ubi-YFP (Fig. S7).

Together, these results indicate that ERα can form multimers containing at least three monomers and suggest that full antiestrogens and to a lower extent Ral facilitate multimerization *in vivo*.

Multimerization correlates with resistance to detergent solubilization but is dependent on dimerization and preserves receptor functionality.

The increased signal in the presence of full antiestrogen reflects a specific mutlimeric state as demonstrated by the increased resistance of this complex to incubation with increasing concentrations of NP40 (Fig. 4A). On the other hand, mutation L536A, which increased transcriptional activity in the presence of ICI182,780 [10](Fig. S4B), suppressed the increase in sliced BRET induced by this ligand (Fig. 8A) and the differential resistance to increasing concentrations of detergent (Fig. 4B). Similar effects on the suppression of the ICI182,780 selective effect were also observed with other mutants in helix 12 leading to increased activity in the presence of ICI182,780 (L539A, L540A, data not show).

To further characterize the multimeric complexes revealed by sliced BRET, we investigated whether trimerization depends on the dimerization interface, which was

shown to be crucial for coactivator recruitment as well as ubiquitination/SUMOylation. No interaction was detected when using the L507R mutant in sliced BRET. This is not simply explained by the lack of Luc reconstitution with this mutant as a lack of signal was also observed in BRET with this mutant in the presence of ICI as well as when using ERα and RARα fusions in the presence of both ligands. However, when using an L507R-YFP fusion together with WT ERα fused to LucF1 and LucF2, trimer formation was essentially abolished in the absence of ligand or the presence of E2, but was still significant in the presence of ICI182,780 (Fig S8B). This suggests that trimerization in the presence of SERDs requires dimerization but that addition of a third molecule is not dependent on the integrity of the dimerization interface. Further, mutation D351L led to markedly reduced trimerization in the presence of all ligands (Fig 8C) in spite of its lack of impact on dimerization (Fig. S8D), again indicating that determinants of dimerization and trimerization can be dissociated. Finally, mutant L536A increased the Luc PCA dimeric signal but abolished the differential trimerization signal in the presence of ICI182,780 vs E2, correlating with its increased agonist activity in the presence of full AEs.

To further investigate whether multimerization corresponds to aggregates of non-functional receptors or whether receptor functionality is preserved in the multimers, we employed BRETFect assays, a novel technique to monitor ternary complex formation based on detection of increased transmission of energy from Luc to a YFP-linked terminal acceptor due to relay by a TFP-linked intermediate acceptor (Cotnoir-White et al., in preparation). We combined this assay with the Luc-PCA assay to detect complexes composed of four molecules, including three ERα and either a coactivator peptide or a SUMO tag (Fig. 5A). In this context, binding of ERα-mTFP1 to an ERα-F1/2Luc dimer will amplify the BRET signal observed by the binding of CoApep-Venus to ERα dimers.

Amplification of signal was observed in the presence or absence of E2, but was significantly smaller with OHT and null with ICI182,780. Thus, trimers formed in the presence of E2 were functional for recruitment of the coactivator peptide (Fig. 5B). On the other hand, the SUMOylation marks observed under SERM treatment are enriched in ICI-induced trimers versus Ve, E2 and OHT conditions, further distinguishing multimers formed after agonist or SERD binding (Fig. 5C). No significant amplification was observed when pairing the ERα-mTFP1/F1/F2Luc constructs with Ubiquitin-YFP (data not shown), possibly due to a non-permissive relative positioning of the tags.

Together these results demonstrate that the ligand-specific functional properties of estrogen receptors are preserved in the multimers.

DISCUSSION :

Novel BRET and PCA assay monitoring ternary complex conformation provide evidence that the estrogen receptor can undergo ligand-induced multimerization in live cells, a property shared with the androgen receptor. This conformation had been observed *in vitro* for RXR and THR, and while THR and RXR tetrameric conformation exist in the unliganded state it appears that steroid-receptors assemble as multimers after ligand activation. Moreover, antagonists such as SERMs can induce multimerization of ERα similar to agonists while SERDs seem to favor the multimerization mechanisms, which may be linked to insolubility of the receptor, and to the post-translational modifications that lead to its degradation. Of note, a crystal structure of ERα LBD complexed to estradiol reported a crystal-packing event resulting in displacement of H12, which extends away from the body of the LBD and binds to the surface of a neighbouring molecule. This leads to formation of a dimer of dimers, stabilized by intermolecular disulfide bonds between two monomers in different dimers and tetramer formation with di-sulfur bridges involving Cys 530 at the end of H11 [23]. This is achieved without significant alteration in the overall fold of the ligand binding domain. SERDs, by blocking the refolding of H12 on top of the LBD to accommodate coactivator-binding, may allow for interactions between helices 12 within a multimer and disulfide bridge formation between neighboring Cys530. Such critical misfolding of the receptors may then be recognized by E3 ligases to target the receptors for degradation. As in the case of THR and RXR, our model for ER multimerization involves a distinct interface surface than the ones used for dimerization but still requires dimerization to occur to allow for higher order structures to form.

The transcription factor p53 is known to require tetramerization to function properly. P53 oligomerization allows proper DNA binding and tetra-p53 is recognized specifically by cofactors when activated. Interestingly many cofactors, such as SRC-1, contain more than two LXXLL motifs so they could in theory selectively recognize a multimer over a dimer. Moreover, the formation of higher molecular weight complexes may limit the receptors' flexibility and allow for stable orientation of the receptors N- and C-termini. This orientation may be critical for proper binding of SRC-1 to both activation functions and transcriptional activity. Tetramerization may also increase DNA binding affinity through avidity effect as a tetramer would have a much lower kd to an ERE than a dimer. This increase in affinity may also facilitate recognition of a greater array of non-consensus ERE. This effect is observed in p53 DNA binding, a proper coordination of all DBD being required for the recognition of imperfect p53RE. In turn, this mechanism ensures proper control of p53's genomic program. Since most binding sites for ERα discovered by ChIP-chip analysis are not consensus EREs, a similar coordination between multiple DBDs in the ER multimer may be required to bind these sites in the genome. In addition, the presence of more than one dimer in the same complex could be a way to bridge two distal response elements, resulting in chromatin looping. Whether active-state multimerization is present in other nuclear receptor sub-family remains to be explored, but good candidates for testing would be phylogenetically related receptors of the NR5 subfamily, of which SF-1 has already been shown to heterodimerize with AR.

MATERIALS AND METHODS

Plasmids and Reagents

Polyethyleneimine-linear (PEI-lin) and Polyethyleneimine-branched (PEI-branched) (25 kDa molecular mass, linear or branched form), was obtained from from Sigma (Sigma, Oakville, Ontario, Canada). Coelenterazine H and Coelenterazine 400a were obtained from NanoLight Technology (Pinetop, AZ). Estradiol (E2) and 4-hydroxytamoxifen (OHT) were purchased from Sigma (Sigma, Oakville, Ontario, Canada); Ru58668 (Ru58), Fulvestrant (ICI182) and raloxifen (Ral) were purchased from Tocris Cookson Ltd. (Ellisville, MO). Ru39411 (Ru39) was a kind gift from (???). ICI164,384 (ICI164) was synthesized by Rodrigo Mendoza-Sanchez at McGill University. MG132 was purchased from EMD Biosciences (La Jolla, CA). pSG5-ERα was a kind gift from Professor P. Chambon. pcDNA-F1LucII/F2LucII-Hygro and pCMV-rLuc vectors were kind gifts from Professor M. Bouvier. pCMV-Topaz(YFP) and pCMV-mTFP1 were generated by amplification of the releveant gene and ligation in the peGFP-N1 vector (PerkinElmer Corp., Wellesley, MA) instead of eGFP. Mutations at positions 351, 507, 536 and 540 were introduced by site-directed mutagenesis using PCR amplification of the ERα cDNA (the sequence of oligonucleotides used for mutagenesis is available upon request). Expression plasmids for ERα mutants were generated by PCR amplification and ligation into the appropriate vector. Clones for each mutant were characterized by sequencing. To create pcDNA-ERα-F1LucII, pcDNA-ERα-F2LucII-Hygro and peYFP-N1-ERα and mutant derivatives (D351L, L507R, L536A and L540A), the coding sequences of the receptors without the stop codons were amplified by PCR from the corresponding pSG5

expression vectors. The amplified cDNA fragments were then subcloned into the *Not*I (using *Bsp120*I to digest the ERα 5' end) and *Cla*I sites of pcDNA-F1LucII/F2LucII-Hygro vectors (ref to Michnick methods) or *EcoR*I and *BamH*I for the pCMV-Topaz or pCMV-mTFP1 vector.

Cell Culture

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Two days before experiments, HEK293 cells were switched to phenol red-free DMEM containing 10% charcoal-stripped serum.

Luciferase Reporter Assays

For transcription reporter assays, mixes of DNA containing 500ng of pMX2-EREx3-TATA-Luc (see Lupien et al 2007 for details) 250ng of pSG5-ERα (0ng for control transfection), 100ng of peGFP-N1 for transfection control and 800ng pcDNA3.1-Hygro to complete to 1,65ug DNA per mix in 75ul of PBS were prepared. Transfections were performed as reported for BRET assays. The following day treatment was performed by adding 50ul of phenol red-free DMEM containing 3X compound to the cells for a final concentration of E2 (10nM), OHT (1000 nM), ICI182,780 (1000 nM) or vehicle (0,1% DMSO). 24 hours post-treatment, medium was removed from cells and lysis buffer was added (100mM Tris pH 8.00, 0.5% NP40) and left on cells for 20 minutes before addition of luciferin solution and luminescence was collected on a Mithras LB 940. Statistical analysis was performed using Student's t test analysis.

Bioluminescence Resonance Energy Transfer Assays

For BRET assays, mixes of DNA containing 75ng of pcDNA3.1-ERα-F1LucII vector, 75ng of pcDNA3.1-ERα-F2LucII vector, from 0 to 1,5 ug of pCMV-ERα-Topaz vector and pcDNA3.1-Hygro to complete to 1,65ug DNA per mix in 75ul of PBS were prepared. PEI solutions containing 1,65ug of PEI-branched and 5ug of PEI-lin in 75ul of PBS were added dropwise to the DNA mix, vortexed briefly and incubated 13 minutes at room temperature. HEK293 were harvested in phenol red-free DMEM containing 10% charcoal-stripped serum and diluted to 1.2 million cells/ml. 850ul of HEK293 suspension was added to the DNA/PEI transfection mix after the incubation period. The cell and transfection mixture was mixed gently and aliquoted to a white flat-bottom 96 well plate at 100ul/well. Cells were grown 48h in 96 well plates and the medium was removed and replaced with PBS containing E2 (10nM), OHT (1000 nM), Ru39411 (1000 nM), Ral (1000 nM), ICI164,384 (1000 nM), ICI182,780 (1000 nM), Ru58668 (1000 nM) or vehicle (DMSO). Cells were incubated for 40 minutes at 37°C. Coelenterazine H was added to a final concentration of 5 µM, and readings were immediately collected on a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), with sequential integration of signals detected at 485 nm (*Renilla* luciferase emission) and 530 nm (YFP emission). The BRET ratio was defined as described in REF. ERα-YFP to PCA-LucII ratios were calculated for each transfection condition ERα-YFP expression vector in the presence of a fixed amount of the ERα-F1/F2LucII vector as the total YFP signal measured by direct YFP stimulation [YFP] minus the basal signal from cells transfected with only ERα-rLuc [YFP_0] divided by the PCA-Luc signal [Luc] in the cotransfected cells. Expression ratio were presented as Log10([YFP]/[Luc]). Statistical analysis was performed using Student's t test analysis.

BRETFest assays

For BRET assays, mixes of DNA containing 75ng of pcDNA3.1-ERα-F1LucII vector, 75ng of pcDNA3.1-ERα-F2LucII vector, 750ng of pCMV-CoApep-Topaz (0ng for control transfection), from 0 to 750ng of pCMV-ERα-mTFP1 vector and pcDNA3.1-Hygro to complete to 1,65ug DNA per mix in 75ul of PBS were prepared. Transfections, treatment and acquisition were performed as reported for BRET assays. BRETFest effect is defined as all increases in BRET ratio (530/480) that cannot be attributed to mTFP1 fluorescence bleeding into the 530nm channel as observed in the no YFP control and therefore is the product of mTFP1 to Topaz FRET.

Figure Legends

Figure 1. Different tools can monitor the conformation of ERα dimers under different treatment. (A) Schematic representation of luciferase complementation assay. (B) ERα-F1LucII forms dimers with luciferase activity with ERα and ERβ-F2LucII but not RXRγ, RARα or Nur77-F2LucII. Transient transfection of ERα-F1LucII and ERα, ERβ, RXRg, RARα or Nur77-F2LucII. Cells were then treated for 40 minutes with 10nM E2 and 100nM 9cis-RA or 100nM AM580 in the case of RXRg and RARa transfection repectively, luciferase activity was quantified, and relative luciferase activity was calculated by setting the value of 1 to untreated cells. (C-F) SERMs affect ERα dimer conformation in comparable fashion to E2 while SERDs elicit a different conformation. (C, D) Transient transfection of stable amount of (C)ERα-F1LucII and increasing amount of ERα-F2LucII or (D) F1LucII-ERα and increasing amount of F2LucII-ERα in HEK293 were performed. Transient transfection of (E) ERα-F1LucII and ERα-F2LucII or (F) F1LucII-ERα and F2LucII-ERα in HEK293 were performed. Cells were then treated for 40 minutes with 1 uM of SERD (OHT, Ru39411, Ral, ICI164,384, ICI182,780 or Ru58668) or 10nM E2, luciferase activity was quantified, and relative luciferase activity was calculated by setting the value of 1 to untreated cells.

Figure 2. SERMs and SERDs prevent recruitment of a coactivator peptide to ERα in the presence of E2 but allow for the binding of additional ERα partners. (A,C) schematic of BRET1 (A) and sliced-BRET (C) coactivator recruitment experiment. Transient transfection of (B) ERα-rLuc and the CoApep-YFP or (D) ERα-F1LucII, ERα-F2LucII and the CoApep-YFP was performed. Cells were treated as specified previously for in Fig.1C. (C-

D) Transient transfection of stable amount of ERα-F1LucII, ERα-F2LucII and increasing amount of (E) CoApep-YFP or (F) NCoA1-YFP in HEK293 were performed. Cells were then treated for 40 minutes with E2 (▪) (10nM), ICI182,780 (Δ) (1000nM), OHT (◊) (1000nM) or vehicle (•) (DMSO). BRET ratios were calculated as described in *Materials and Methods*.

Figure 3. SERDs form different higher order ERα oligomers than pure SERMs and E2. (A,D) schematic of BRET1 (A) and sliced-BRET (D) oligomerization assay. Transient transfection of stable amount of (B-C) ERα-rLuc or (E-F) ERα-F1LucII, ERα-F2LucII and (B-E) stable or (C-F) increasing amount of ERα-YFP in HEK293 were performed. (B-E)Cells were treated as in Fig.1C. (C-F) Cells were treated as in Fig.2E. BRET ratios were calculated as described in *Materials and Methods*.

Figure 4. SERD induced oligomers are biochemically different from E2 induced counterparts. (A) Transient transfection of ERα-F1LucII, ERα-F2LucII and ERα-YFP was performed. Cells were then treated for 20 minutes with 10nM E2 or 1 uM of SERDs (ICI182,780) before being partially lysed with increasing concentration of NP40 for 20 minutes before BRET acquisition. BRET ratios were calculated as described in *Materials and Methods*.

Figure 5. Novel BRETFect experiments reveal the formation of heterotetramer composed of three ERα subunits and a coactivator peptide. (A) Schematic of the

BRETFect assay; ERα-trimer coactivator-recruitment. (B-C)Transient transfection of ERα-F1LucII, ERα-F2LucII (B) with CoApep-YFP or (C) without CoApep-YFP and increasing amount of ERα-mTFP1 was performed. Cell treatment was performed as in Fig.2E. BRET ratios were calculated as described in *Materials and Methods*.

Supplementary Figure 1. The G400V mutant (HE0) fails to dimerize in absence of ligand. Experiment performed as in Fig. 1C with the ERα(G400V)-F1/2LucII constructs.

Supplementary Figure 2. Treatment with geldanamycin does not prevent dimerization or recruitment of cofactors to ERα. Cells were transfected with indicated constructs and treated for 1 or 6 hours with geldanamycin. Vehicle, E2, ICI182 or OHT treatments were then applied for 40 minutes before BRET ratio were measured.

Supplementary Figure 3. Dimerization mutant ERα(L507R) does not properly recruit coactivator motifs. Experiment was carried out as in Fig. 2C with ERα(WT)-RLuc and ERα(L507R)-RLuc.

Supplementary Figure 4. Receptor affinity for the LXXLL coactivator motif reflects transactivation potential. (A) Transient transfection and treatment were performed as in Fig. 2EF but with L536A and D351L mutant receptors. BRET ratios were calculated as described in *Materials and Methods*. (B) HEK293 cells were transfected ERα, ERα(L536A)

or ERα(D351L) vector and an EREx3-TATA-Luc reporter vector and treated with E2, ICI182,780 or OHT 24 hours after transfection. Cells were lysed and luciferase activity was assayed 24hours post-treatment. Luminescence levels are normalized to GFP transfection control levels of each transfection.

Supplementary Figure 5. AF1 function is not disrupted by SERDs but SUMoylation and ubiquitination is increased. (A) Cells were transfected with the AF-1 interacting domain (AF1-ID) derived from SRC1 and the indicated ERα constructs then treated for 40 minutes with vehicle, E2, ICI182,780 or OHT before BRET ratios were measured. (B) Cells were transfected with the indicated constrcuts and treated with 0, E2, ICI or OHT with MG132 for the ubiquitin assay or without for the SUMOylation assay. BRET ratios were measured after 40 minutes of treatment.

Supplementary Figure 6. High oligomerization is a correlates to SERD activity of AEs. Experiments carried out as in Fig. 3F with addition of raloxifen (Ral) treatment (A) or use of F1/2LucII-ERα constructs (B) instead of ERα-F1/2LucII.

Supplementary Figure 7. Increased oligomerization potential of molecules correlate with their ability to induce sumoylation and ubiquitination of the receptor. (A) Transient transfection performed as in Fig.4F. Cells were then treated for 40 minutes with 10nM E2, 1 uM of ICI182,780 or 10uM of RMS molecules reported in Hilmi et al. 2012. (B) Transient transfection of stable amount of rLuc-ERα and stable amounts of SUMO-YFP

or Ubiquitin-YFP in HEK293 were performed. Cells were then treated for 2 hours for sumoylation assay or 6 hours for ubiquitination assay with 10nM E2, 1 uM of ICI182,780 or 10uM of RMS molecules.

Supplementary Figure 8. ERα oligomerization state reflects proper activity of the receptor and SERD-susceptibility. Transient transfection and treatments performed as in Fig.3F but with (A) L536A, (B) WT and L507R or (C) D351L receptors as specified on graphs. (D) Dimerization assay carried out with receptor WT and mutants as in Fig. 1B. to GFP transfection control levels of each transfection.

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Figure 1

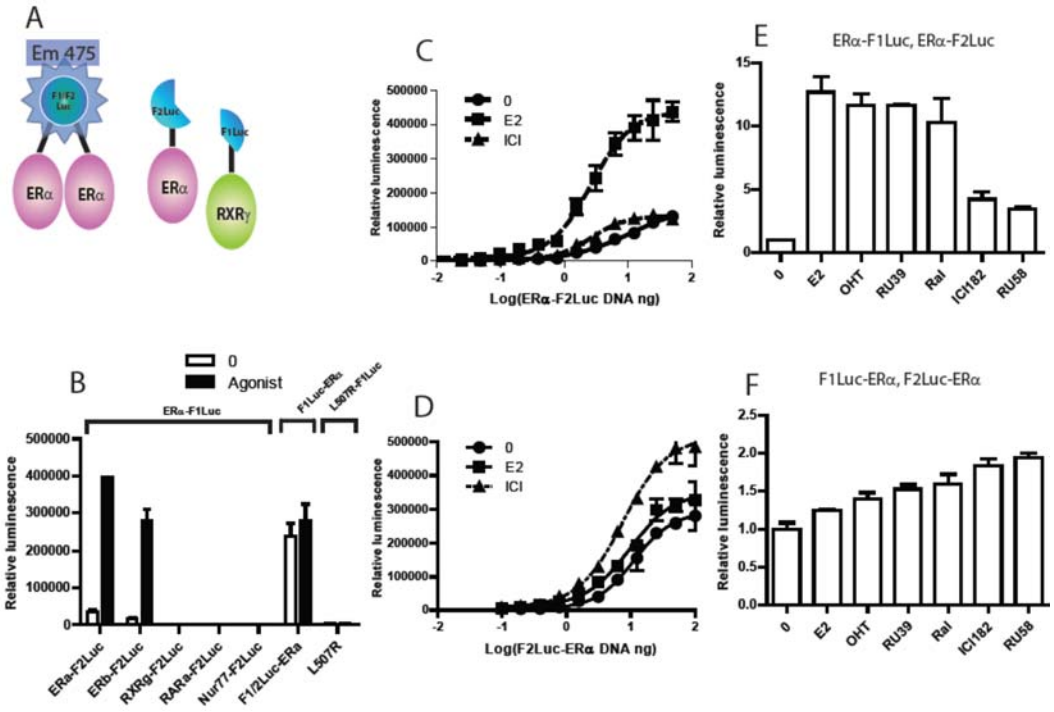


Figure 1. Different tools can monitor the conformation of ERα dimers under different treatment.

Figure 2

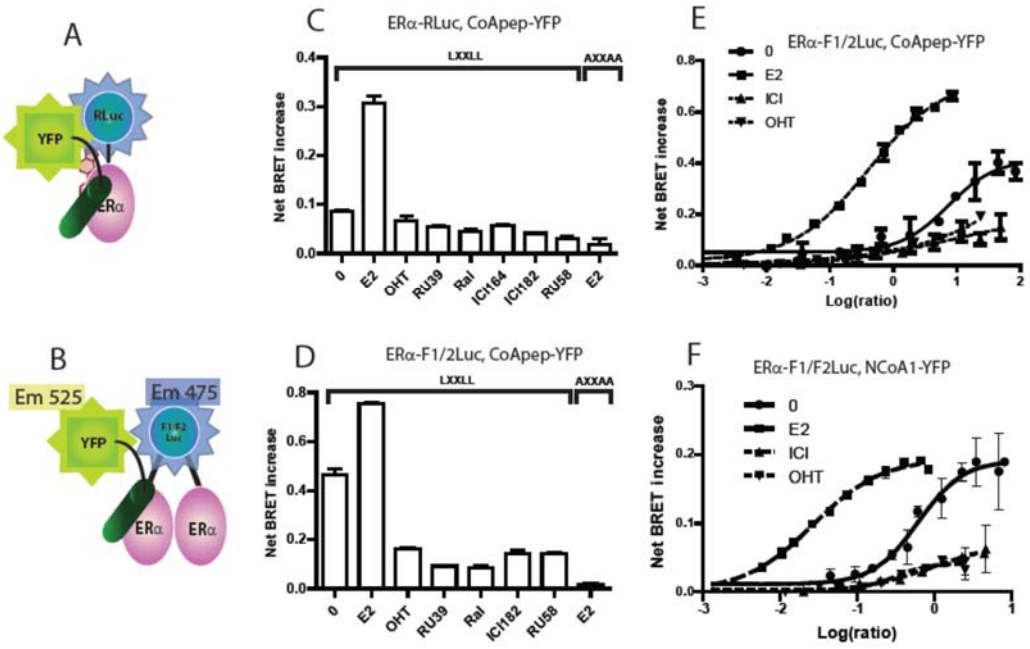


Figure 2. SERMs and SERDs prevent recruitment of a coactivator peptide to ERα in the presence of E2 but allow for the binding of additional ERα partners.

Figure 3

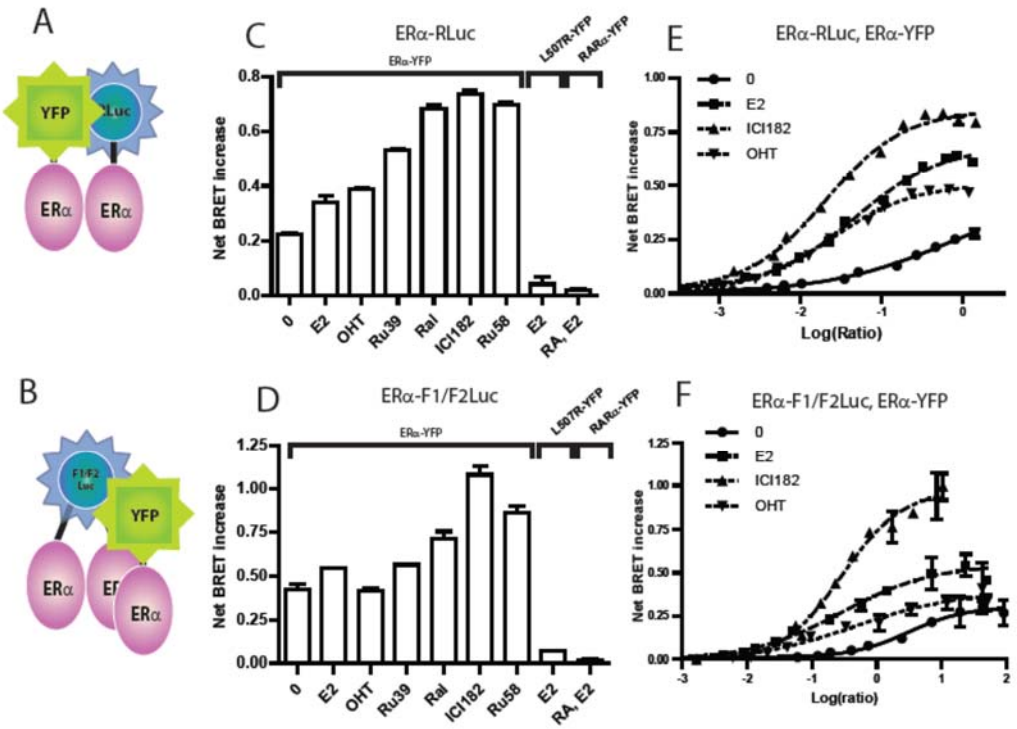


Figure 3. SERDs form different higher order ERα oligomers than pure SERMs and E2.

Figure 4

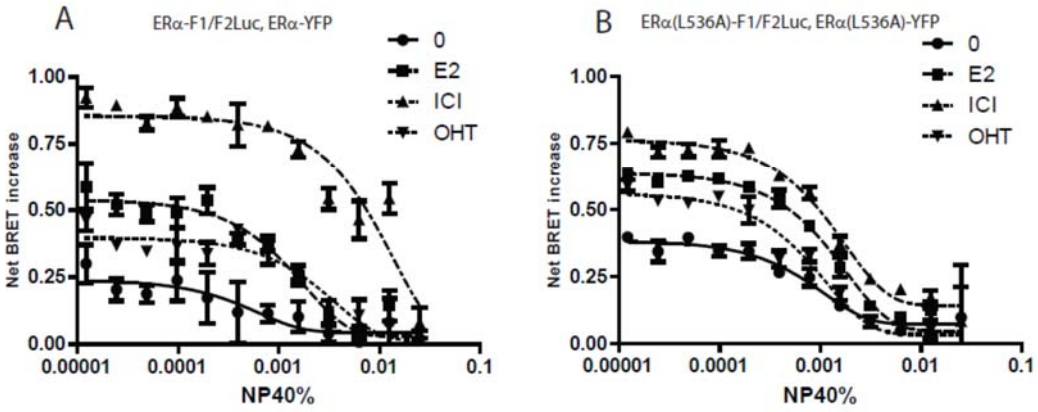


Figure 4. SERD induced oligomers are biochemically different from E2 induced counterparts.

Figure 5

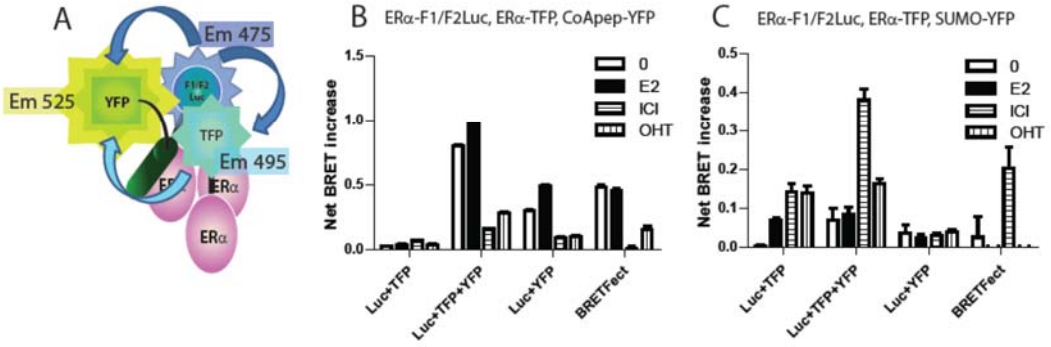
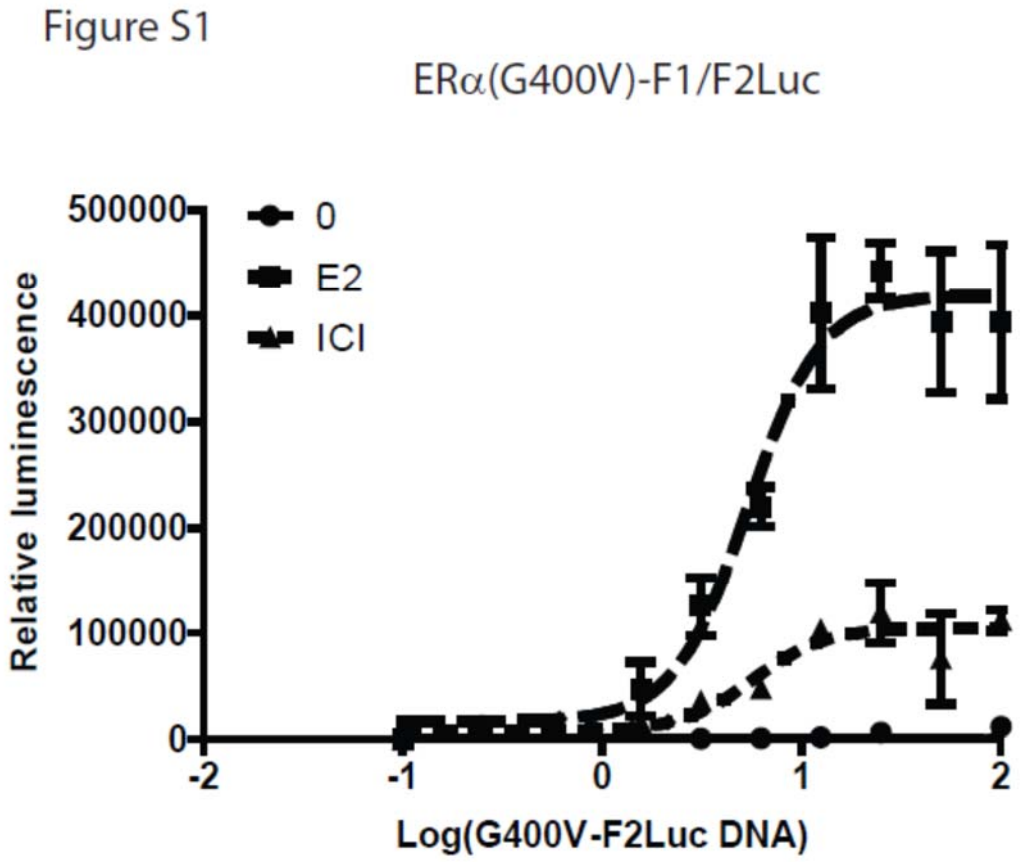
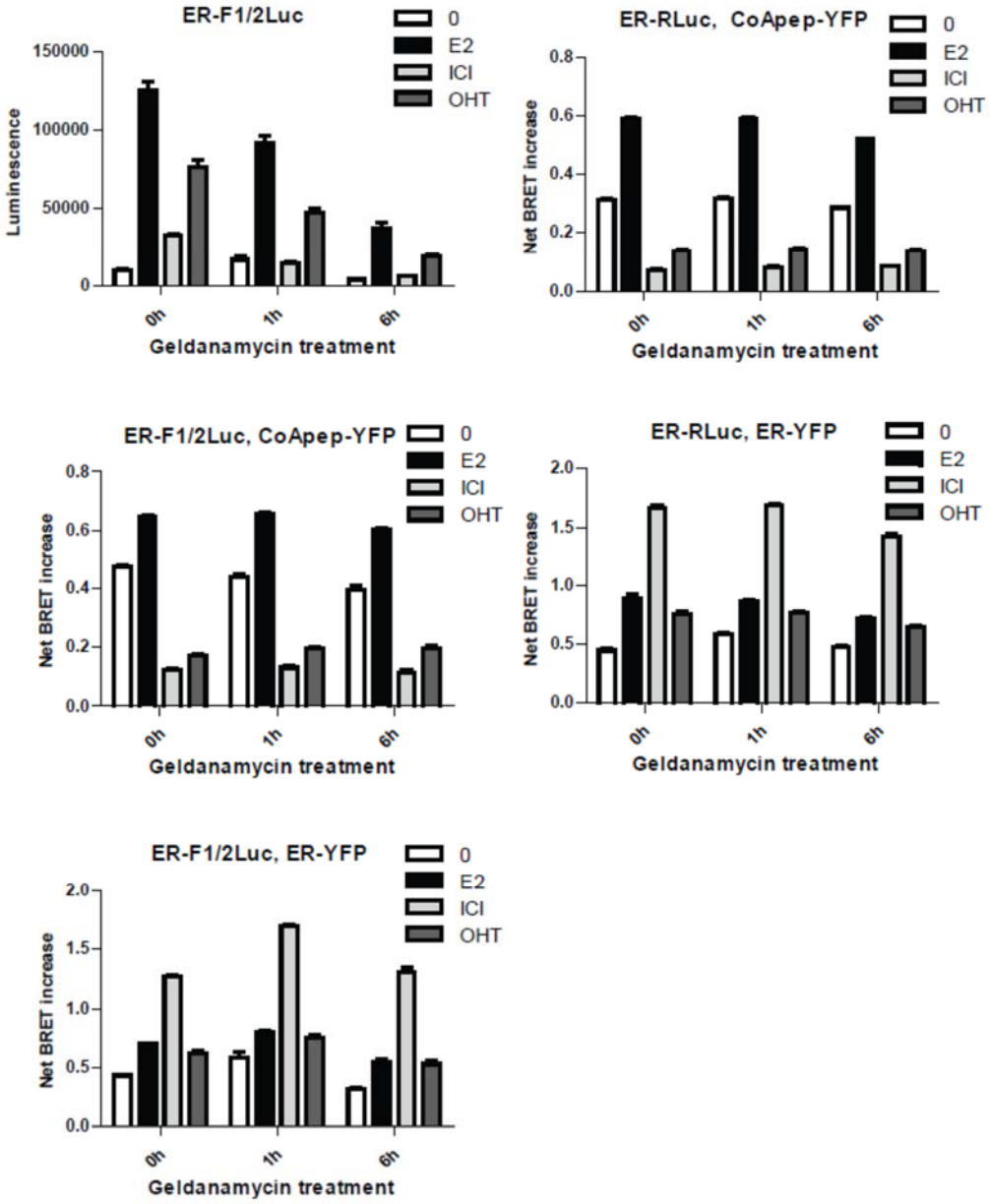


Figure 5. BRETFect experiments reveal the formation of heterotetramers composed of three ERα subunits and a coactivator peptide.

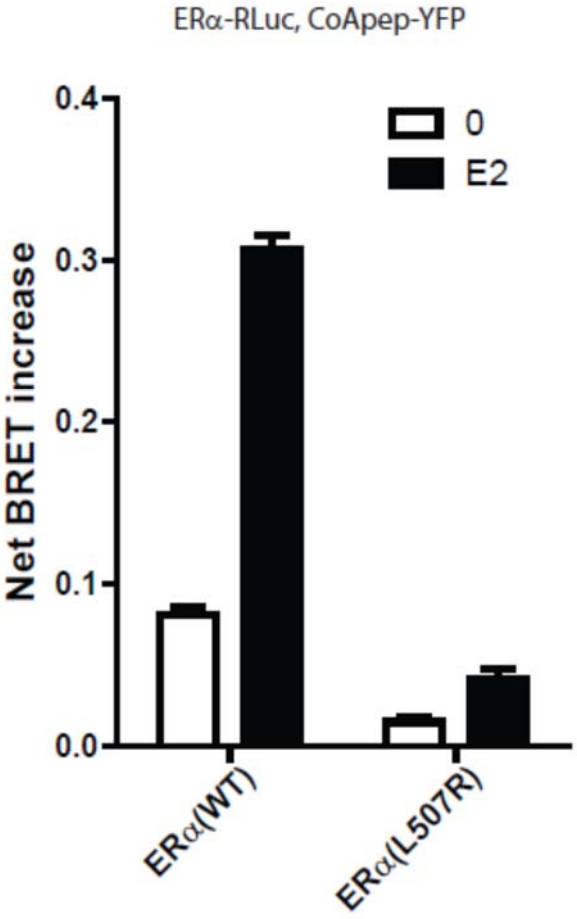


Supplementary Figure 1. The G400V mutant (HE0) fails to dimerize in absence of ligand.



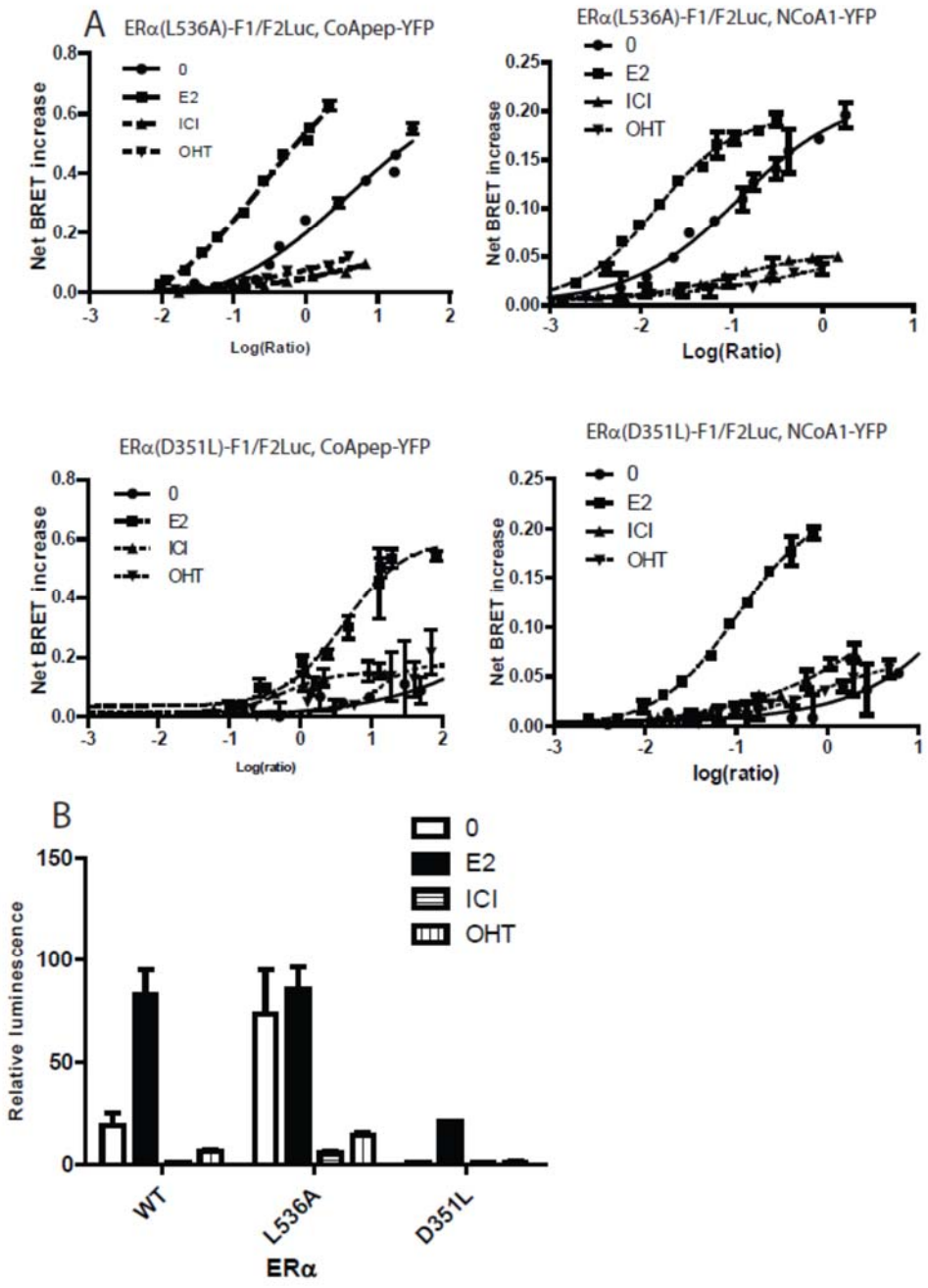
Supplementary Figure 2. Treatment with geldanamycin does not prevent dimerization or recruitment of cofactors to ERα.

Figure S3

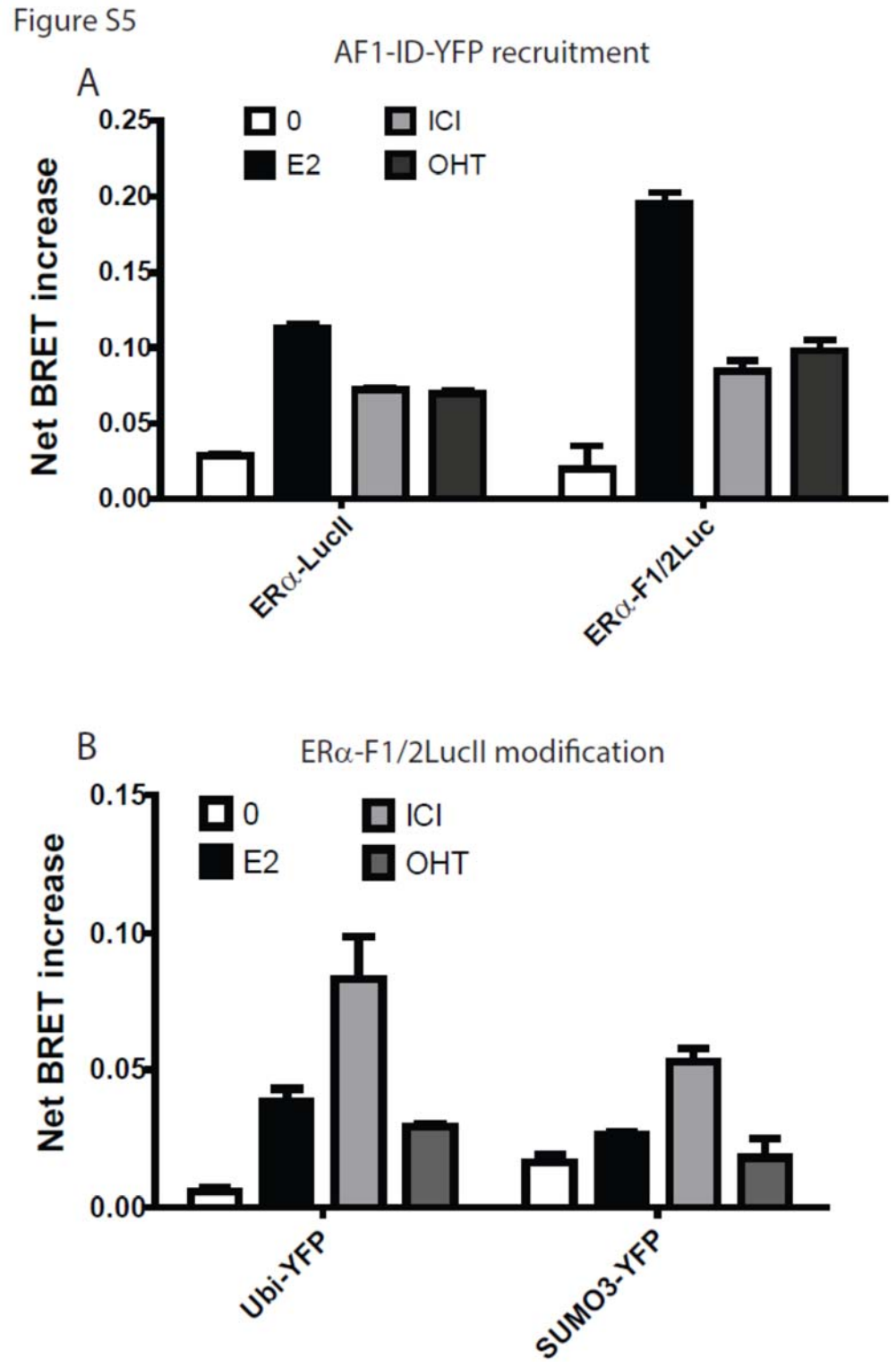


Supplementary Figure 3. Dimerization mutant ERα(L507R) does not efficiently recruit coactivator motifs.

Figure S4

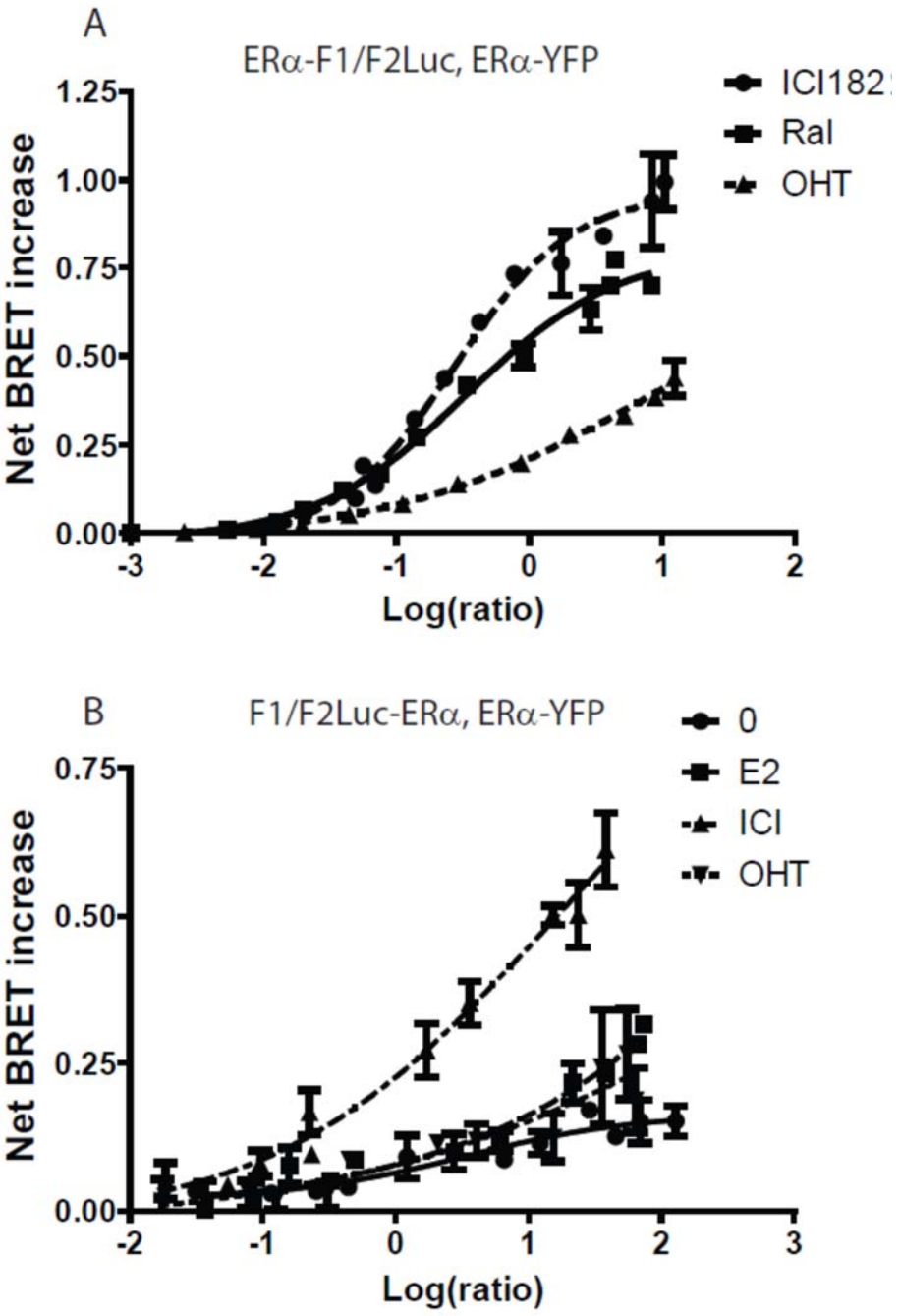


Supplementary Figure 4. Receptor affinity for the LXXLL coactivator motif reflects transactivation potential.



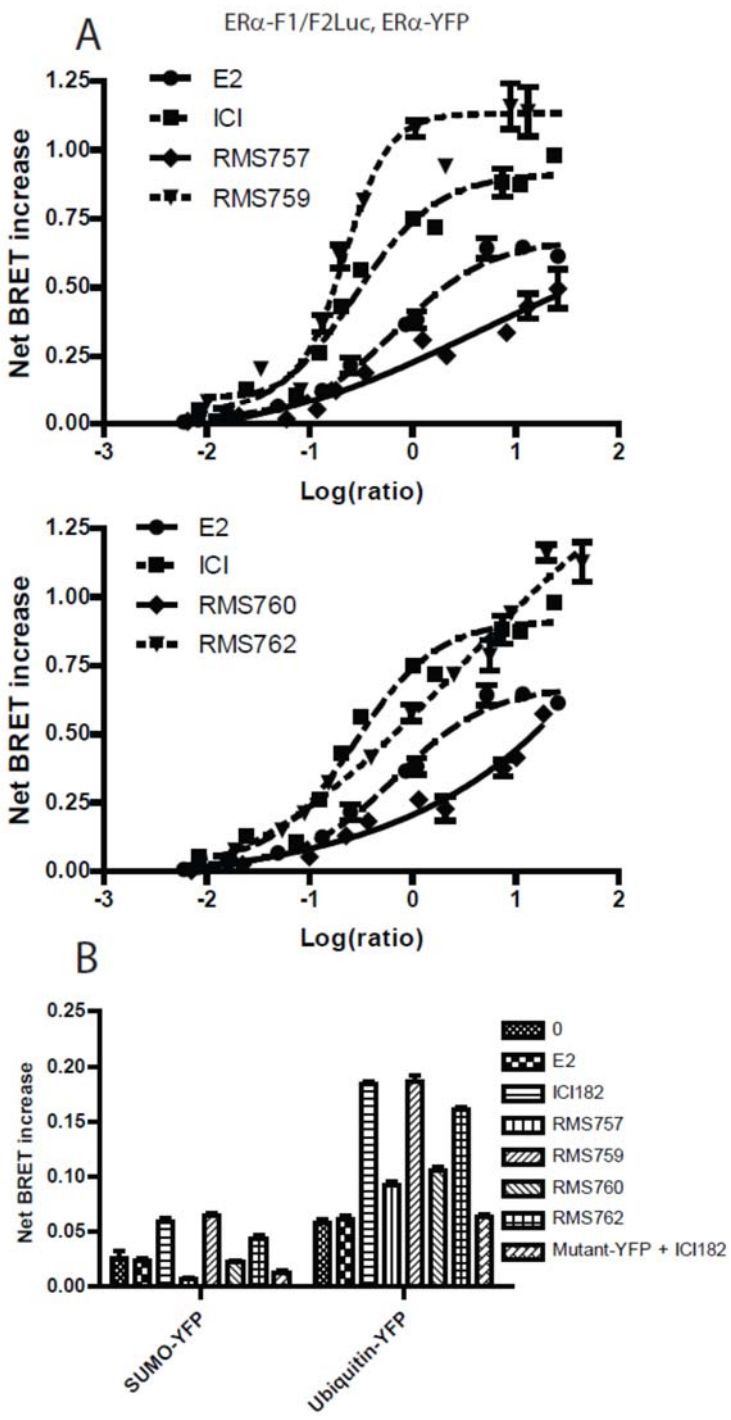
Supplementary Figure 5. AF1 function is not disrupted by SERDs but SUMoylation and ubiquitination is increased.

Figure S6



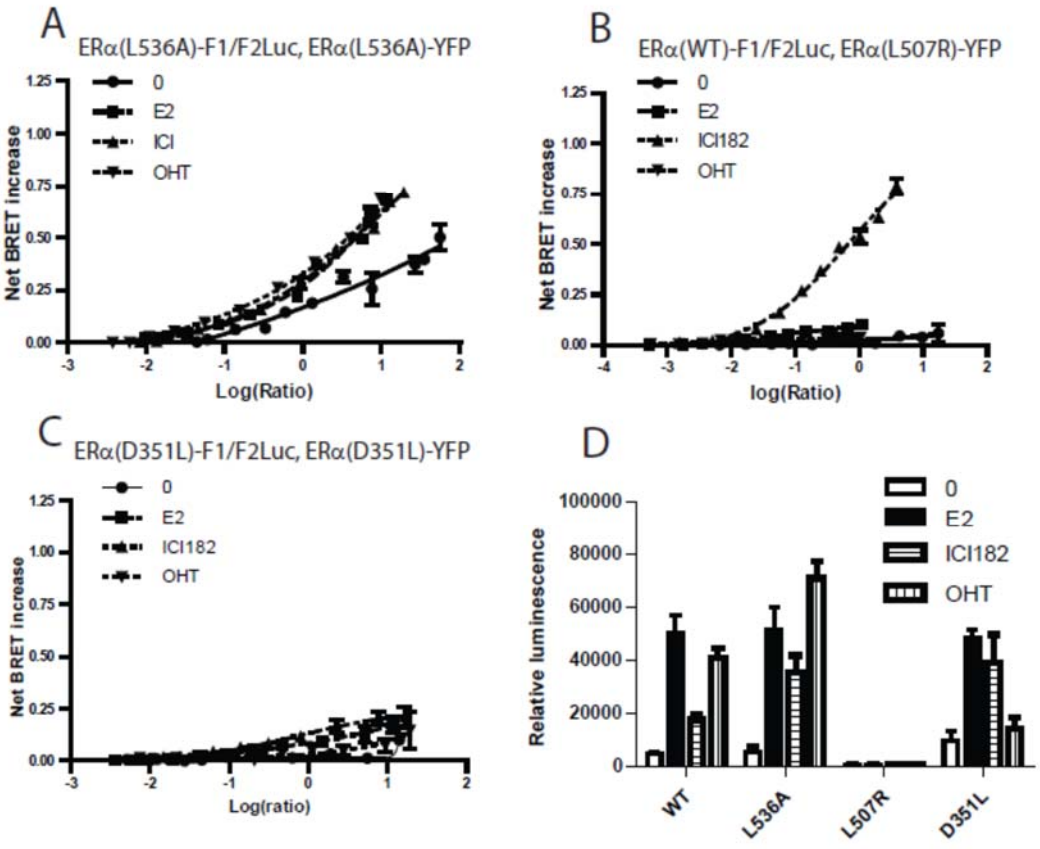
Supplementary Figure 6. High oligomerization correlates with SERD activity of AEs.

Figure S7



Supplementary Figure 7. Increased oligomerization potential of molecules correlates with their ability to induce sumoylation and ubiquitination of the receptor.

Figure S8



Supplementary Figure 8. ERα oligomerization state reflects activity of the agonist-bound receptor and transcriptional repression by SERDs.

CHAPTER 4: INVESTIGATION OF SHR MULTIMERIZATION CAPACITY AND HETEROMULTIMER PROPERTIES.

David Cotnoir-White, Sylvie Mader.

Results reported in this section are not ready for publication but I thought it important to include them as they confirm in some ways the findings of chapter 3 even though I cannot part an encompassing conclusion from them yet. All experiments were carried out by the author of this thesis.

INVESTIGATION OF SHR HOMO AND HETERO-MULTIMERIZATION PROPERTIES.

Following our discoveries on the multimerization of ERα and ERβ we decided to test if this quaternary structure extended to the other nuclear receptors of subfamily 3. Additionally, since SHR form not only homodimers but also heterodimers, the resulting asssays would enable to probe for interactions involving different combinations of receptors. For instance, the glucocorticoid receptor (GR) has been reported to form heterodimers with the mineralocorticoid receptor (MCR) [1] and the androgen receptor (AR) [2]. While contacts via the receptor DBDs have an impact on the DNA binding specificity of the resulting dimers [3], the dimerization interface in the LBD is crucial for the stability of these dimers in solution. Analysis of the conservation of the dimer interface in helix H11 (Fig.1A) of six SHRs: ERα, ERβ, ERRα, GR, MCR and AR indicates two groups of homology, NR3A and B (ERα, ERβ and ERRα) and NR3C (GR, MCR and AR) (Fig.1BC), correlating with the DBD sequence homology and DNA binding specificity of these receptors (Table 1 of Cotnoir-White et al 2011 [4]). Sequence conservation of the interacting part of helix H11 was perfect within NR3C subgroup (Fig.1B), which explains their propensity to dimerize. These receptors all share similar recognition motifs, suggesting that multiple dimeric species can assemble on DNA. However, the greater divergence observed in the rest of the LBD results in different ligand and/or cofactors specificity for each receptor.

Multimerization of AR

First we tested the ability of AR to engage in multimerization as observed with ERα and ERβ. Recombinant AR-F1 and F2lucII proteins behaved similarly as ERα-F1/2lucII, displaying a basal level of luminescence that was increased by treatment with the agonist dihydrotestosterone (DHT) or the antagonist bicalutamide (Casodex, CDX), but not of Dexamethasone (Dex) (Fig. 2AB). This basal interaction, also observed in ERα, suggests that AR can dimerize in a partially constitutive manner under the conditions of the assay, the increase in luminescence observed being either the result of an increased affinity of dimerization or of a change in the conformation of AR AF2 domain following ligand binding. In the presence of DHT, the AR-F1/2LucII dimers also recruit an additional AR-YFP, but not ER-YFP (Fig.2C), suggesting a specificity to the multimeric interactions.

Contrary to ERα, AR does not undergo multimerization in the vehicle condition, which may be due to AR's retention to the cytoplasm in the absence of hormonal treatment. Titration curves with AR-YFP indicates competition with the AR-F1lucII and AR-F2lucII, preventing luciferase complementation by displacing compatible PCA partners (Fig.2D). Of interest, the IC50s observed in the presence of DHT was 3-fold higher than in its absence (Fig.2E), compatible a need to compete away three molecules within a tetramer compared to one in a dimer, assuming that it is possible for any two complimentary luc fragments within multimers to recombine regardless whether they are part of the same dimer.

GR and AR heterodimerize

GR-F1/2LucII PCA gave similar results to AR (Fig.2A) and could form heterodimers with AR-F1/2LucII (Fig.2B). Recombination only increased when the ligand to the F2LucII tagged receptor was added. As reported previously, GR homodimers could equally recruit a LXXLL or FXXLF motifs but AR homodimers could only efficiently recruit the FXXLF motif (Fig.2C). Surprisingly the AR-GR heterodimer could only recruit the FXXLF motif, suggesting that allosteric control between the two partners could alter the cofactor selectivity of GR in a heterodimer (Fig.2C). Moreover, DHT or DEX treatment alone did not reach the maximal capacity of FXXLF-YFP recruitment, which required addition of both ligands (Fig.2C). GR homodimers did not display any ability to multimerize with AR-YFP but the GR-AR heterodimer could in the presence of both DHT and DEX multimerize with AR (Fig.2D). The requirement for both ligands again highlights the allosteric inhibition of apo-GR on AR to prevent the recruitment of interacting proteins as it does for FXXLF motifs.

Heterodimerization within NR3A and B groups.

ERα and ERβ have very high sequence homology in the H11 region (Fig.1C), which explains their propensity for hetero-dimerization [5], whereas ERRα shows more divergence with the estrogen receptors. However, as ERRs are reported to heterodimerize with ERα [6], we investigated whether ERβ and ERRα could heterodimerize.

The ERRα homodimers did produce a large increase in luminescence that as expected was not modulated by 17β-estradiol (E2) (Fig.3A). On the other hand, luminescence was

increased by E2 treatment for ERα and β homodimers along with ERα-ERβ heterodimers. As predicted, an ERβ-ERRα heterodimer could be formed as well as the ERα-ERRα heterodimers (Fig.3A). The luminescence was not regulated by E2 in this case even if the tags were inverted between receptors (data not shown). The receptor dimers showed similar restrictions on multimerization as did AR and GR; ERβ homodimers did not multimerize with ERRα-YFP and vice-versa but ERβ-ERRα heterodimer did multimerize with ERβ or ERRα molecules (Fg. 3B). The recruitment of ERRα-YFP to the heterodimer did not appear influenced by any treatment but ERβ interactions seemed to be modulated under 4-hydroxytamoxifen (OHT) and ICI182,780 (ICI) treatment (This was also observed for ERRα-ERα heterodimer multimerization with ERα-YFP, not shown). The patterns of interaction of homodimers versus heterodimer multimerization point towards different interfaces for dimerization and multimerization; interestingly, the multimerization interface appears more restrictive than the dimerization interface. The possibility for more SHR heterodimers and heteromultimers is apparent and will be investigated in the future. These associations maybe what marks certain areas of the genome as NR hotspots or super-enhancers [7] integrating the inputs of several genomic programs to modulate gene expression.

Material and Methods.

Plasmids and reagents

The FXXLF-YFP construct was cloned from oligos encoding RNF14 sequence between positions 450 and 461 inserted in place of the LXXLL motif in the CoApep-YFP construct described in Chapter 4. AR-YFP, ERβ-YFP and ERRα-YFP were cloned by PCR amplification

of cDNAs and ligation in the YFP variant of peGFP-N1 vector described in Chapter 4. AR, GR, ERβ and ERRα were cloned into the PCA system in pcDNA3.1-F1/2LucII vectors as described in Chapter 4.

Structural and sequence analysis.

ERα LBD structure (pdb 1ERE) was acquired from pdb.org and images were generated using PyMOL (DeLano Scientific LLC). Sequence alignment was performed using the multiple sequence alignment online tool CLustal Omega [8, 9].

PCA-Luc and PCA-BRET assays

Experiments were carried out as described in Chapter 4.

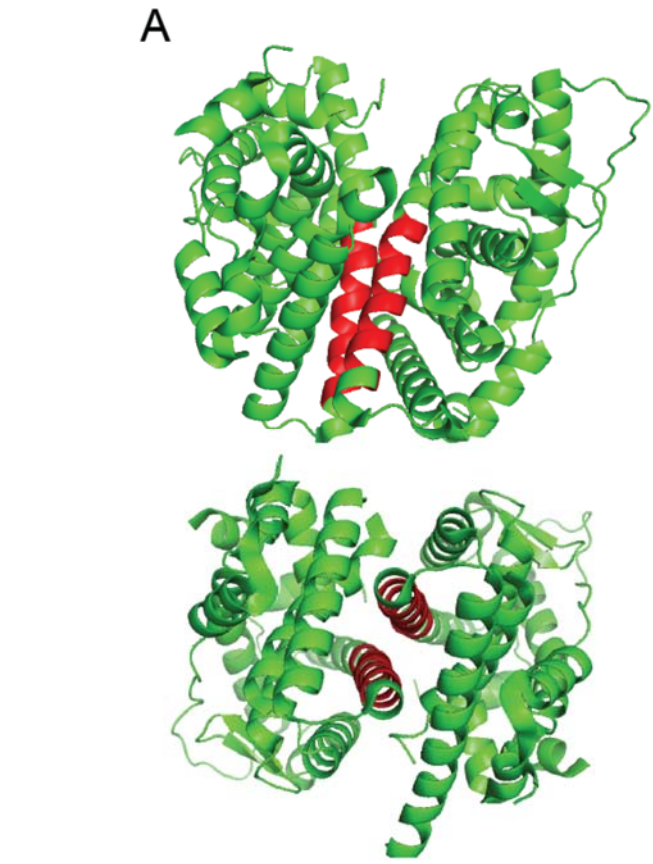


Figure 1. Heterodimers are predicted to form within NR3 nuclear receptor subgroups.

(A) ERα LBD dimer from PDB 1ERE with H11 dimer contacts highlighted in red. (B) Alignment of NR3C receptors (AR, GR and MCR) (C) Alignment of ERα and homologous sequences of ERβ and ERRα. Amino acids were color coded according to polarity and charge.

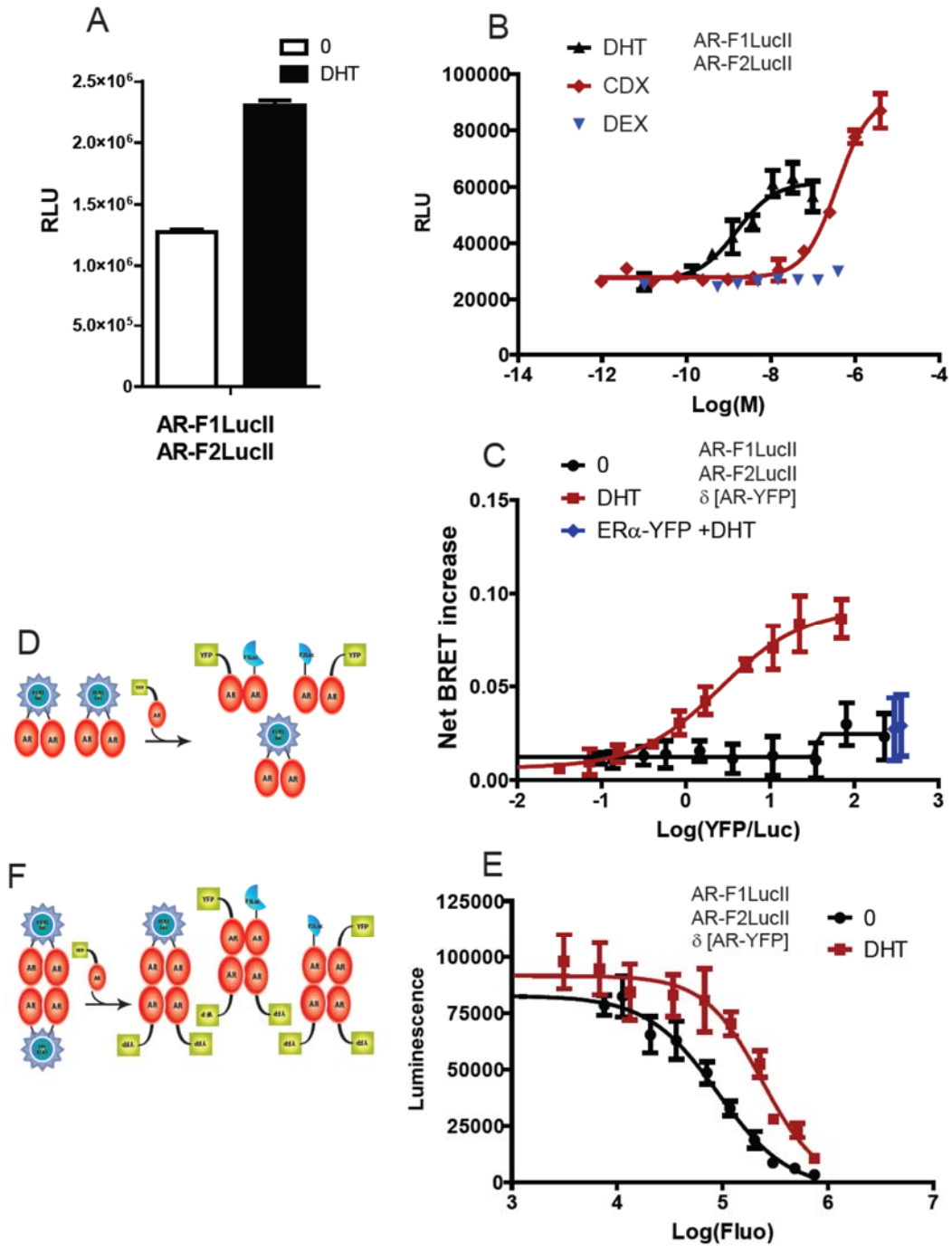


Figure 2. The Androgen Receptor tetramerizes.

(A-B) Basal and ligand-modulated AR interactions in a PCA-luciferase assay. (B) Dihydrotestosterone (DHT) and bicalutamide (CDX), but not dexamethasone (DEX) cause

concentration-dependent complementation of luc fragments. (C) Titration of AR-F1/F2 LucII dimers with increasing amounts of AR-YFP suggests a specific DHT dependent multimerization of AR. (D-F) AR-YFP competes more efficiently with AR-F1LucII and AR-F2LucII in unliganded vs liganded complexes.

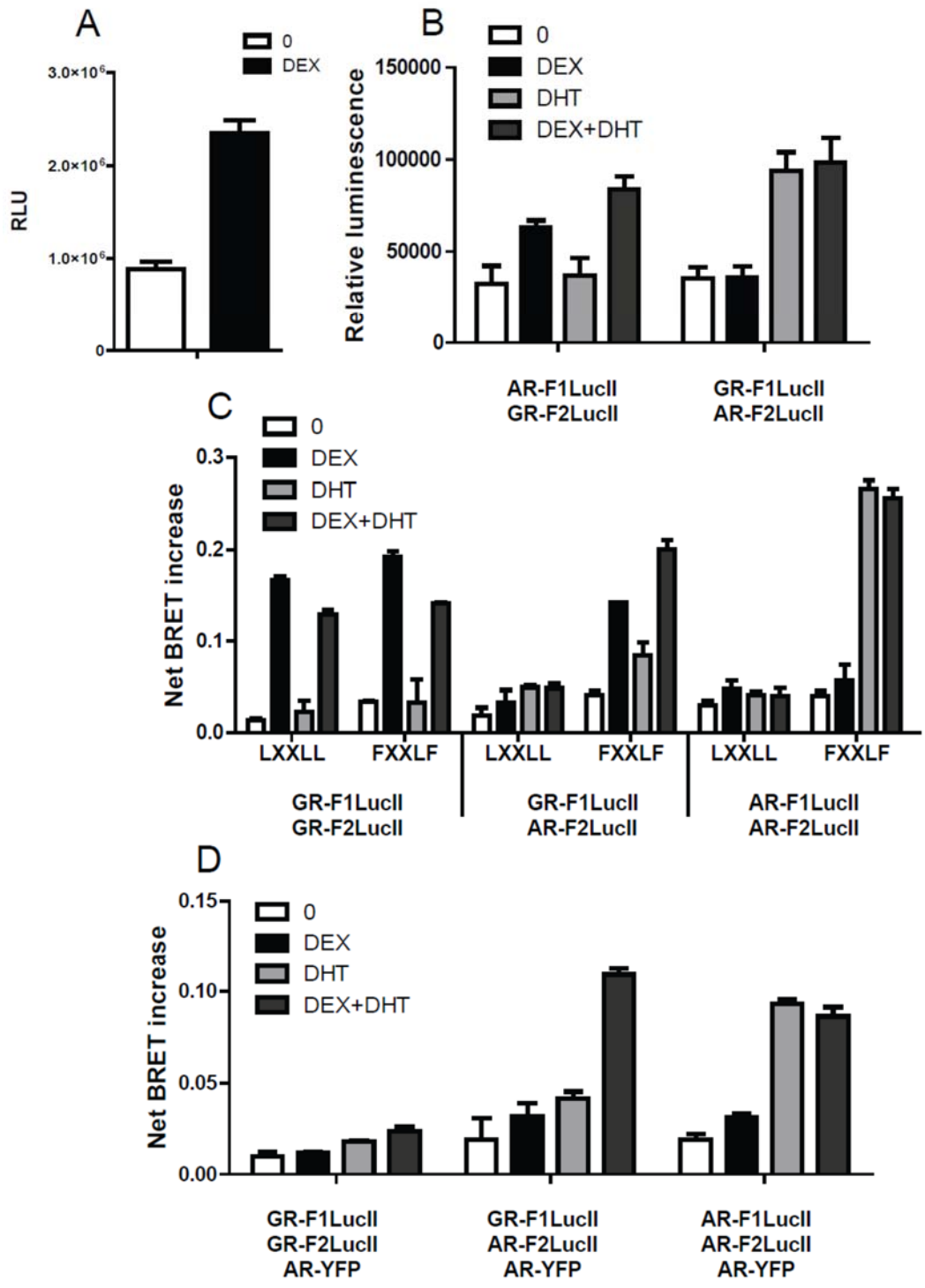


Figure 3. Androgen and Glucocorticoid Receptor hetero-multimerization and selective coactivator recruitment.

(A-B) PCA-Luc assays of GR and AR indicate hetero- as well as homodimerization; note that the conformation required for recombination of luc fragments is achieved by treatment with ligands of the F2-tagged receptor. (C) Selectivity of coactivator motif recrtuiment for receptor hetero- and homo-dimers. GR efficiently recruits LXXLL and FXXLF motifs while AR homodimers and GR-AR heterodimers are selective for FXXLF-YFP motifs. (D) Heteromultimerization between GR and AR. AR can be recruited by AR-GR heterodimers as well as AR homodimers when both receptors are liganded.

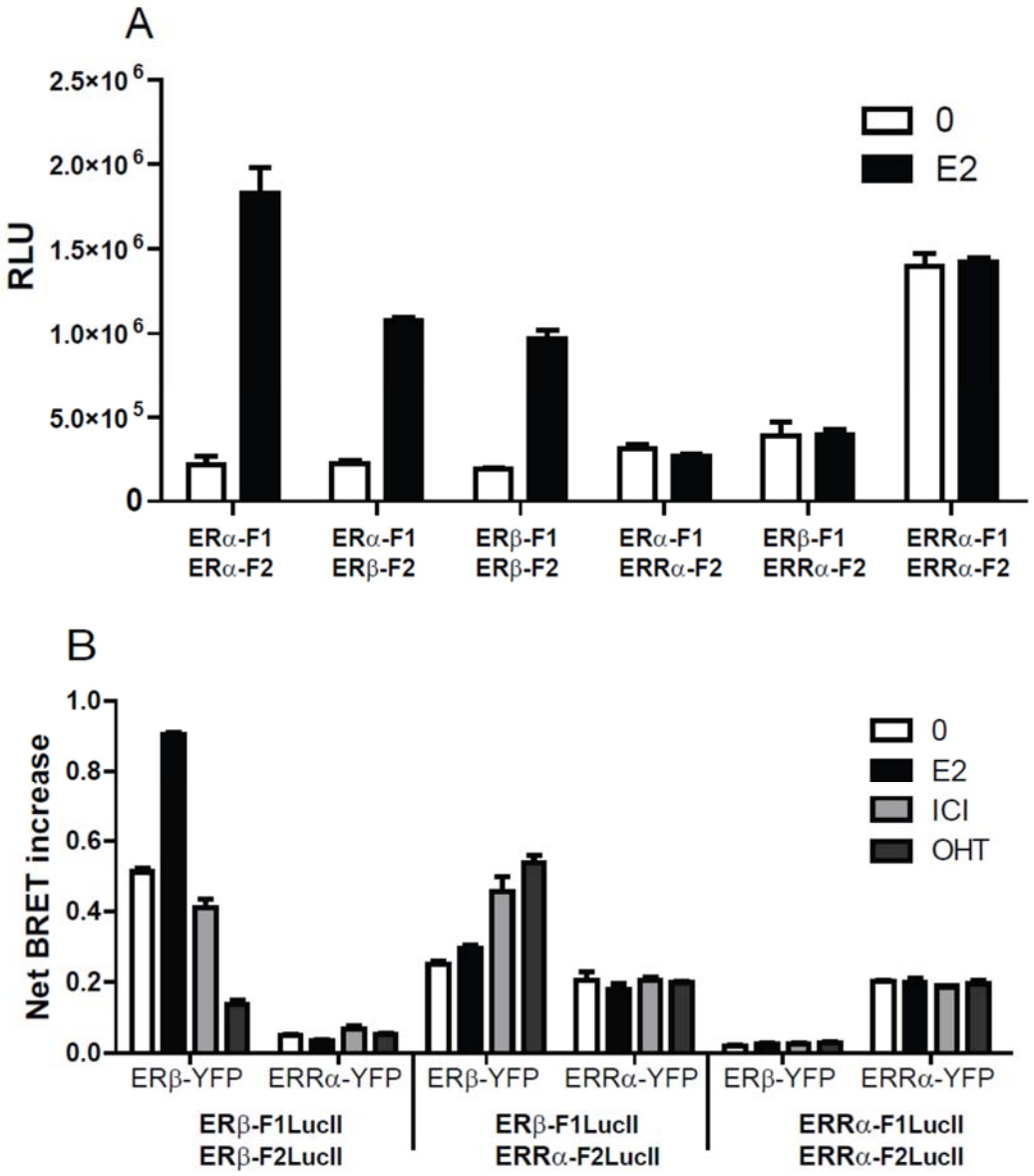


Figure 4. Promiscuity of interactions between SHRs.

(A) ER-ERR interaction by PCA-luc. ERα, ERβ and ERRα can form dimers in PCA-luc assays.

(B) ERβ-ERRα heterodimers can interact with additional ERβ or ERRα molecules to form heteromultimers.

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CHAPTER 5: ANTIESTROGEN AND HISTONE DEACETYLASE INHIBITOR MOLECULAR HYBRIDS.

Rodrigo Mendoza-Sanchez, David Cotnoir-White, Justyna Kulpa, Isabel Jutras, Sylvie Mader and James L. Gleason

Manuscript prepared for Journal of Medicinal Chemistry

We are presently in discussion to have me included as a co-first author on this paper. My contribution consisted in screening the newly synthesized molecules for antiestrogenic activity using the BRET assays that I had perfected (The assays were required because the potential HDACi activity of the molecules prevented us from relying on classical luciferase-reporter assays.) I would then screen promising molecules were for HDACi activity by WB. I tested the molecules that displayed good activity (either AE, HDACi or both) in growth assays on MCF-7 and MDA-MB-231. Justyna Kulpa provided complimentary luciferase assays and Isabelle Jutras performed enzymatic HDACi assays. All chemistry and in vitro assays were performed by Rodrigo Mendoza-Sanchez under the supervision of James L. Gleason.

Author's note: While only 4 hybrids are presented in this paper we tested over 50 molecules altogether over the course of five years. Our biggest obstacle was preventing molecules from *gaining* estrogenicity when adding HDAC-inhibiting chemical groups. The molecules reported were chosen for the paper because intellectual protection was already taken out on them. We now have better molecules with IC50 on HDAC and ER activities in the mid-nanomolar range (100-300nM) we are just not ready to publicize them yet...

Antiestrogen and histone deacetylase inhibitor molecular hybrids

Rodrigo Mendoza-Sanchez[†], David Cotnoir-White[‡], Justyna Kulpa[‡], Isabel Jutras[‡], Sylvie Mader[‡*], James L. Gleason[†,*]

[†] Department of Chemistry, Otto Maass Building, McGill University, 801 Sherbrooke Street West, Montreal, Québec, Canada, H3A 0B8

[‡] Institute for Research in Immunology and Cancer, Pavillon Marcelle-Coutu, Université de Montréal, 2950 chemin de Polytechnique, Montréal, Québec, Canada, H3T 1J4

TITLE RUNNING HEAD. Antiestrogen and histone deacetylase inhibitors molecular hybrids.

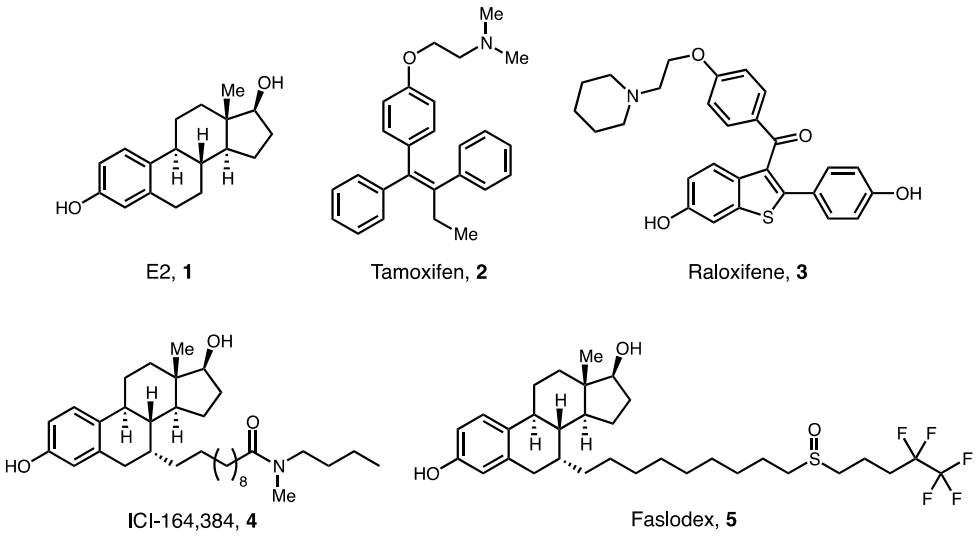
* to whom correspondence should be addressed

INTRODUCTION

Breast cancer is the most common form of neoplasia in women in the Western world and second cause of mortality after lung cancer.[1] Estrogens, mainly 17β-estradiol (E_2, **1**), play a crucial role in the development of female secondary sexual characteristics including normal breast growth.[2] E_2 signaling is mediated by intracellular estrogen receptors (ERα and ERβ), members of the nuclear receptor superfamily that regulate gene expression through binding to DNA response elements associated within target genes.[3] While ERα is expressed at low levels in normal tissues it is overexpressed in about 67% of hormone dependant tumors, which represent 75% of breast cancers.[4] Therefore, E_2 stimulation of ERs is a major contributor of mammary tumorigenesis and is thus an important target in the treatment of breast cancer.[5]

Two classes of competitive inhibitors of the ER have been developed for treatment of breast cancer, selective estrogen receptor modulators (SERMs),[6] as exemplified by Tamoxifen (**2**)[7] and Raloxifene (**3**),[8] and full antiestrogens,[9] as exemplified by ICI-164,384 (**4**)[9b] and Faslodex (fulvestrant, ICI-182-780, **5**).[10] SERMs display partial agonist activity in a tissue- and gene-specific manner.[11] For instance, tamoxifen is an antagonist in breast cancer cells but has estrogenic effects on the uterus and bone mass, while raloxifene has less uterotrophic activity but retains agonist activity in bone.[12] Full antiestrogens, like Faslodex, are antagonists in all tissues. Faslodex induces ubiquitination and degradation of ER via the proteasome pathway.[13] In addition, induction of SUMOylation of the receptor also contributes to full antiestrogenicity.[13c]

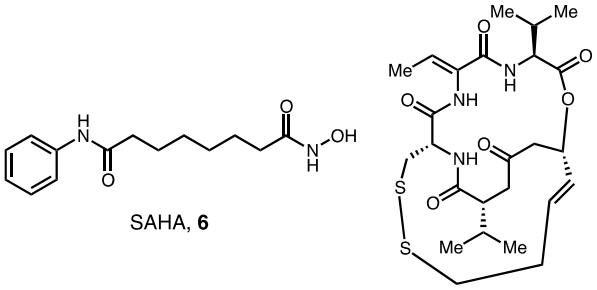
SERMs are used as first-line therapy with about 50% response rate overall (21-33% in patients with metastatic disease).[14] Full antiestrogens are used mainly in second line after progression on prior hormonal therapy with an objective response rate evaluated at 7.4 % in the EFECT trial.[15] Thus there is considerable room for improvement in reversal of resistance to hormone therapy.



.Figure 1. Selected ER ligands

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) regulate the acetylation pattern of histones and non-histone proteins.[16] In normal cells HDACs and HATs play an important role as transcriptional regulators by controlling compaction of DNA in the nucleosome and an aberrant expression of these enzymes has been observed in different cancer cell lines, including breast cancer.[17] In recent years, histone deacetylase inhibitors (HDACi's) have emerged as potential therapeutic agents for the treatment of cancer.[18] Preclinical studies of HDACi's have indicated roles in the regulation of differentiation, gene expression, cell cycle arrest, inhibition of

proliferation and the promotion of apoptosis in a variety of cancer cell lines, including breast cancer cells.[19] Currently, SAHA (**6**) and romidepsin (**7**) are approved by the FDA for cutaneous T-cell lymphoma.[20] In addition, a variety of novel HDACi's have been investigated and reached clinical trials.[21] Current efforts aim at increasing stability, dosage and reducing dose-dependent side effects, which include thrombocytopenia, diarrhea, nausea, and fatigue.[22]



. Figure 2. Selected HDACi's

HDACi's have shown promise in association with other anti-cancer agents to improve treatment of solid tumors and also to combat development of resistance to these agents.[23] Importantly, while SAHA did not meet RECIST (Response Evaluation Criteria in Solid Tumours) criteria for adequate response as a single agent in the treatment of metastatic breast cancer, the presence of objective response (disease stabilization in 29% patients) suggested that they could be considered in combination with other drugs.[24] Tamoxifen was found to cooperate with different HDACi's for suppression of ER+ breast cancer cell proliferation.[25] A separate study showed that the

combination of SAHA and Faslodex resulted in a more potent effect to regulate the expression of cell cycle proteins, to induce downregulation of ERα, and to decrease the transcription of ERα targets in MCF-7 cells than the treatment of Faslodex alone.[26] The above observations spurred the initiation of a phase II study of SAHA (400 mg daily for 3 out of 4 weeks) with tamoxifen (20 mg daily) in 43 patients with ER-positive tumors progressing on endocrine therapy indicated a 19% objective response rate and a 40% clinical benefit rate (response or stable disease >24 weeks).[27]

Although combination therapy is the traditional method for taking advantage of synergistic or additive effects between two drugs,[28] our group and others have explored the concept of hybrid drugs, agents that combine two activities in a single molecule.[29] In particular, given the propensity of HDACi's to synergize with other chemotherapies, there are several reported HDACi based hybrids, including those with combined HDACi activity and EGFR/Her2, PI3K, and Abl/PDBFRb/c-Kit kinases.[30] Our group pioneered the incorporation of HDACi pharmacophore into a nuclear receptor ligand. We developed triciferol, a hybrid molecule which possessed agonism for the vitamin D receptor and HDACi activity and which show enhanced cytostatic and cytotoxic activity against a number of cancer cell lines.[31] This molecule was followed with a wide variety of hybrids based on both secosteroidal and non-secosteroidal cores, showcasing the versatility of structures with analogous SAR.[32] Very recently, Oyelere et al. showed that it was possible to prepare HDACi hybrids with either ER agonist or antagonist activity, the latter using the tamoxifen backbone.[33] Herein we describe several bi-functional antiestrogen/HDACi hybrids based on the ICI-164384 and Faslodex structures as potential treatments for breast cancer.

RESULTS AND DISCUSSION

Design and synthesis. The structure of the estradiol-based full antiestrogens appeared ideal for development of hybrid molecules as their structural features seemed well suited to merging with those of HDACi. On the one hand, full antiestrogens possess a long alkyl chain attached to the core of E_2 in either the 7α or 11β positions.[9b] In the structure of ICI-164,386 (**4**) the side chain attached at position 7α in the E_2 core is an eleven carbon aliphatic chain terminating in a tertiary amide. This side chain disrupts normal folding of helix 12 of the ER over the ligand binding pocket which is required for the binding of transcriptional co-activators through LXXLL motifs.[34] On the other hand, structural studies on HDACi bound to HDACs have revealed a canonical pharmacophore comprised of three distinct motifs:[35] a) a zinc binding group (ZBG) required for chelation to the zinc ion in the active site, b) a hydrophobic linker that occupies the hydrophobic tunnel in the enzyme, and c) a bulky, generally aromatic, cap group that interacts with the surface of the enzyme. In designing hybrids, we envisioned the E_2 core functioning as both a binding motif for ERα and a cap group for HDACi function. The side chain of ICI-164,382 could serve both as an antiestrogenic structural element as well as that of the linker in an HDACi. Finally, the tertiary amide, which does not engage in interaction with the ER ligand binding domain, might be replaced with ZBGs, such as a hydroxamic acid, N-butyl-hydroxamate or *ortho*-amino-anilide.

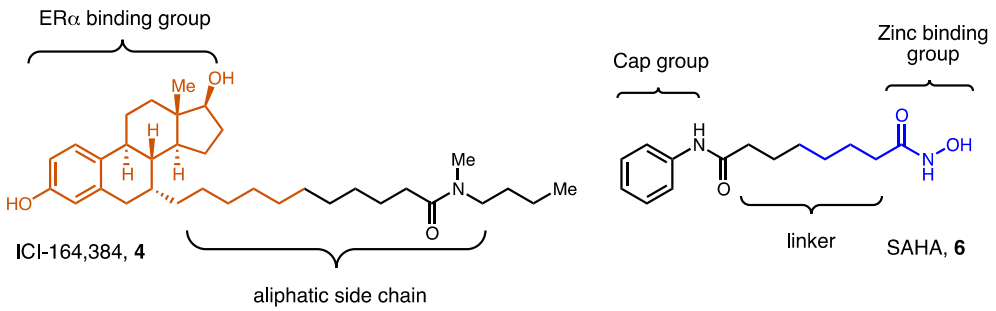
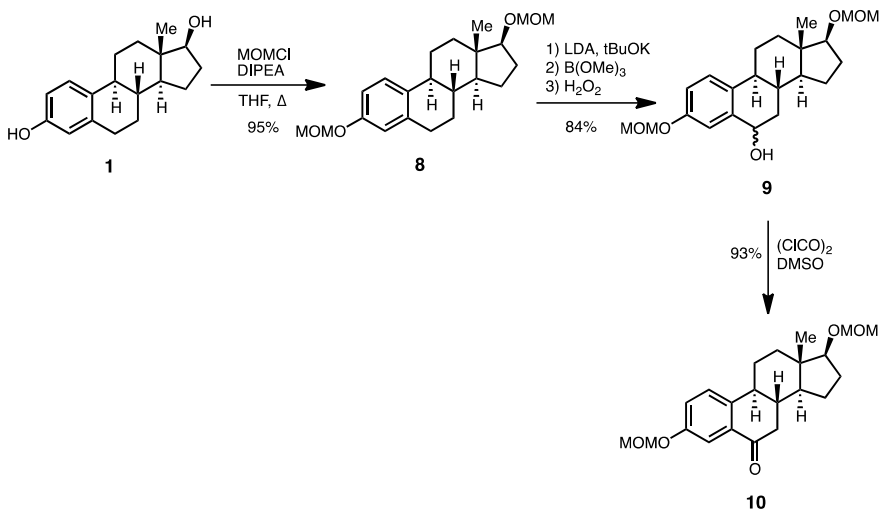


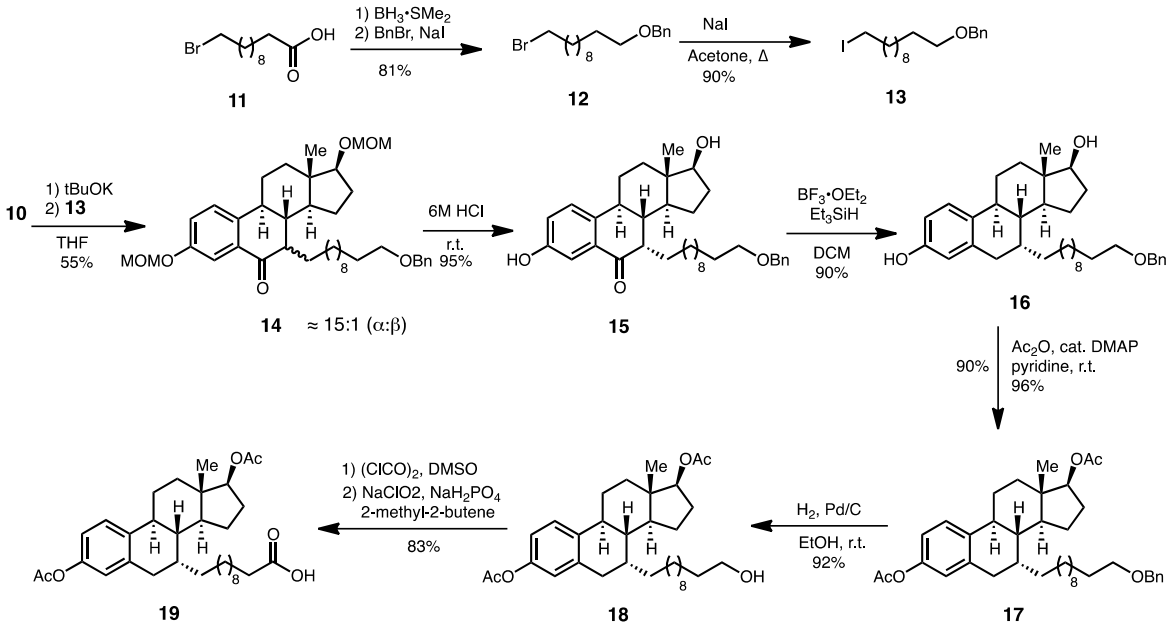
Figure 3. Pharmacophores of full antiestrogen ICI-164384 and SAHA

The synthesis of our first three hybrids was modeled on the work of Zhu et. al.[36] using carboxylic acid **19** as a common intermediate. E_2 was protected at both hydroxyl groups with MOM ethers, which allowed the subsequent benzylic oxidation at C6. This capricious reaction was best achieved under strong basic conditions using LDA and ^{t}BuOK to effect benzylic lithiation, followed by trapping with trimethyl borate and subsequent oxidation in the presence of H_2O_2. Swern oxidation of the resulting alcohol afforded ketone **10** which could be alkylated at C7 by treatment with ^{t}BuOK and alkyl iodide **13** to give **14** as a 15:1mixture of α to β isomers. Treatment of **14** with concentrated acid served to remove the MOM groups as well as epimerize any remaining 7β isomer, affording **15** in 95% yield. Reductive deoxygenation of the benzylic ketone was carried out with Et_3SiH and $BH_3 \bullet OEt_2$ and followed by reprotection of the diol to their corresponding acetates to afford **17**. Finally, removal the benzyl group on the side chain using palladium on carbon as a catalyst followed by oxidation of the resulting primary alcohol to the carboxylic acid in a two-step fashion (Swern protocol followed by Pinnick oxidations) completed the synthesis of carboxylic acid **19** in 34% yield over 7 steps from ketone **10**.

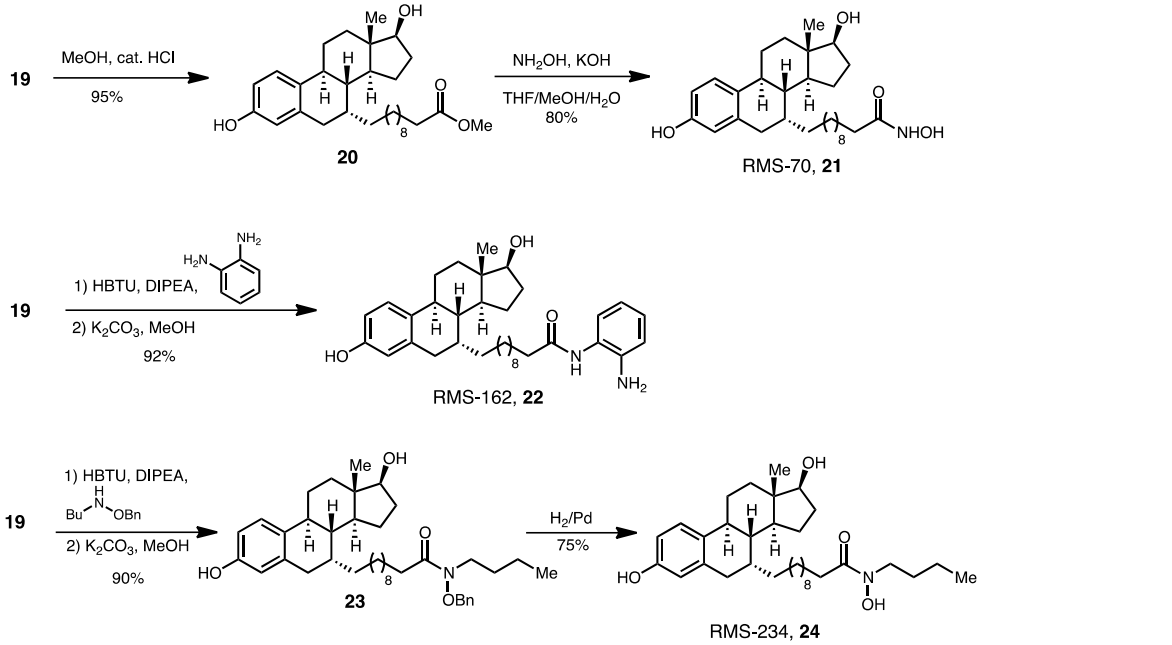


Scheme 1. Synthesis of ketone 10

Carboxylic acid **19** served as a common intermediate for hybrids RMS-70 (**21)**, RMS-162 (**22)** and RMS-234 (**24)**. Treatment of **19** with acidic methanol simultaneously cleaved the acetyl groups and esterified the carboxylic acid, and the resulting methyl ester **20** was directly transformed to the corresponding hydroxamic acid upon treatment with hydroxylamine under basic conditions to give RMS-70 (**21**) in 80% yield. Alternatively, carboxylic acid **19** was coupled with 1,2-diaminobenzene using HBTU/DIPEA conditions followed by treatment with K_2CO_3 in methanol to cleave the acetyl groups to provide RMS-162 (**21**) in 92% yield. Finally, **19** was coupled with N-butyl-O-benzylhydroxylamine using HBTU/DIPEA conditions followed by sequential deprotection of the acetyl and benzyl groups with K_2CO_3/methanol H_2/Pd-C to give RMS-234 (**24**) in 67% yield over two steps.



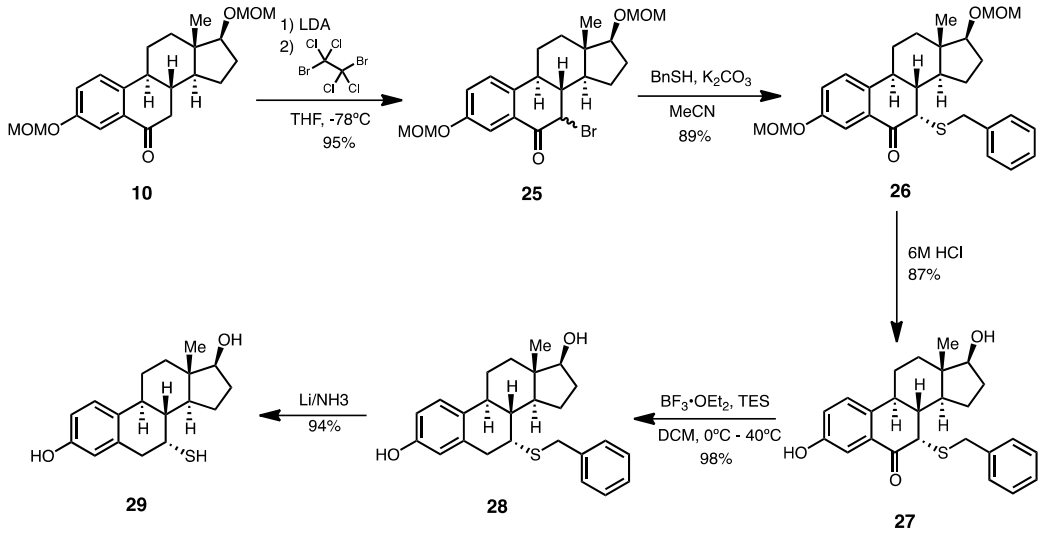
. Scheme 2. Synthetic sequence for carboxylic acid 19



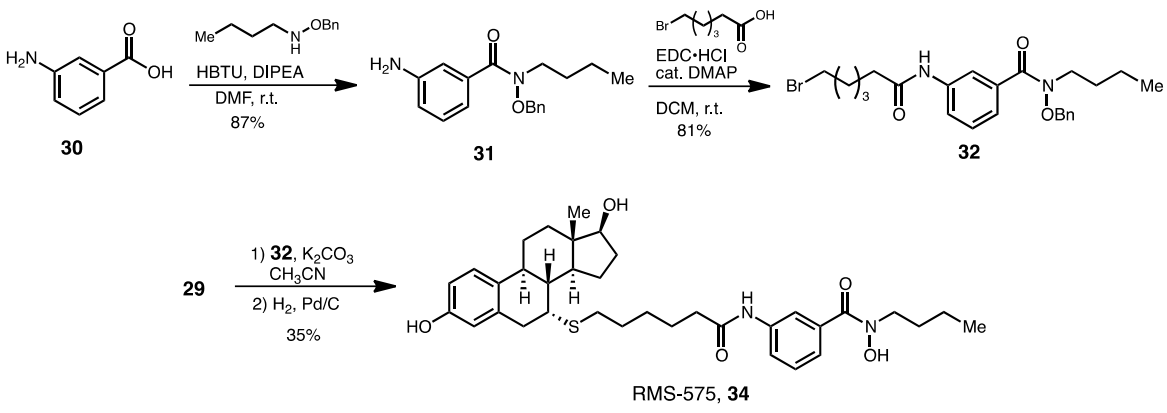
Scheme 3. Synthesis of hybrids RMS-70 (21), RMS-162 (22) and RMS-234

(24)

A fourth hybrid, RMS-575, was synthesized via a modification of a strategy previously reported previously by Napolitano and Komm.[37] 7α-Mercapto-E_2 (**29**) was synthesized in a five step protocol involving initial bromination of ketone **10** with LDA and 1,2-dibromotetrachloroethane followed by nucleophilic displacement of the bromide with benzyl mercaptan to give **26** as a single stereoisomer. Subsequent deprotection of the MOM groups and reduction of the keto group was achieved as above. Finally, deprotection of the benzyl group afforded thiol **29** in 68% yield over 5 steps. Thiol **29** was efficiently coupled with alkyl halide **32**, prepared in 2 steps from 3-aminobenzoic acid acid (**30**), followed by debenzylaton to afford hybrid RMS-575 (**34)** in 35% yield over 2 steps.



Scheme 4. Synthesis of thiol **29** from ketone **10**



Scheme 5. Synthesis of RMS-575 (34)

Antiestrogenic profile of hybrids. The antiestrogenic activity of our hybrids was initially assessed using both a bioluminescence resonance energy transfer (BRET) assay[38] in transiently transfected HEK293 cells. In this assay, luciferase is fused to ER and green fluorescent protein (GFP) is fused to a LXXLL-coactivator peptide.[39] In the presence of E_2 or other ER agonists, the coactivator peptide associates with the ER ligand binding domain, resulting in energy transfer from activated luciferase to GFP, while antagonists prevent coactivator association induced by agonists and thus reduce the BRET signal. Our four hybrids RMS-70 (**21**), RMS-162 (**22**), RMS-234 (**24**) and RMS-575 (**34**) showed suppression of BRET signal induced by E2 below basal levels when used at 1.0 μM, consistent with antiestrogenic activity and similar to controls 4-OHT and ICI-164,384 (Fig. 4 and data not shown). For the luciferase reporter assay, transcriptional activity of ERα was measured using an ERE3-TATA-Luciferase construct[40] in T47D-KBLuc cells (ATCC® CRL2865™).[41] While none of the hybrids were as potent as either ICI-164,384 or 4-OHT, all four hybrids displayed some antiestrogenic activity (**Table 1**), with RMS-575 (**34**) and RMS-70 (**21**) having IC_{50} values in the submicromolar range.

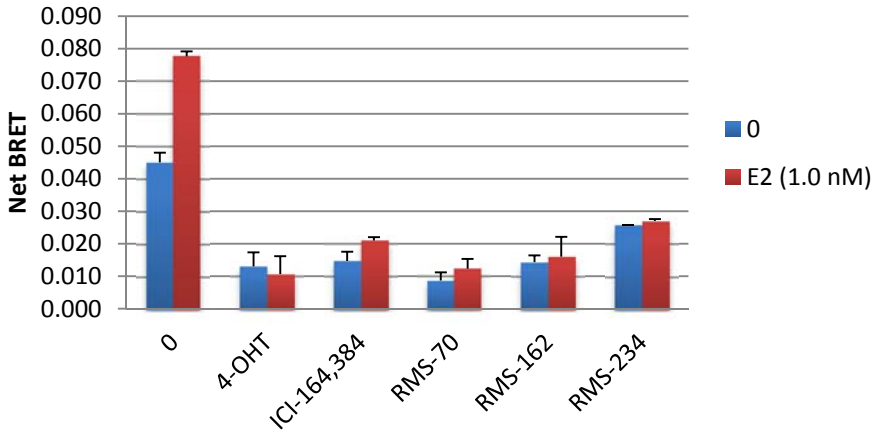


Figure 4. Antiestrogenic activity of hybrids in a BRET assay for agonist-induced coactivator recruitment.

	Luciferase transactivation (IC_{50} in μM)
ICI-164,384	0.05
4-OHT	0.01
RMS-70	0.44
RMS-162	4.07
RMS-234	2.10
RMS-575	0.11

Table 1. IC50s of hybrids in luciferase assays

HDAC inhibition profile of hybrids. The HDAC inhibitory activity of our hybrids was tested using a standard competition assay with a fluorescent substrate against purified human recombinant HDAC3 and HDAC6.[42] Although displaying weaker potency than either SAHA or entinostat, all four hybrids displayed inhibitory activity against HDAC3, a class I HDAC, with RMS-70 (**21**) being the most potent (0.95 μM). Against Class IIb HDAC6, only RMS-70 (**21**) had a potency within one order of magnitude of SAHA, whereas the remainder hybrids were significantly less potent as displayed in **Table 2**.

The difference between the HDAC3 and HDAC6 results can be rationalized considering the known SAR available for these enzymes. HDAC6, a class II HDAC, has a narrow hydrophobic pocket that connects the protein surface with the active site. This pocket can accommodate simple hydroxamic acids and thiols. In contrast, HDAC3, a class I HDAC, has a larger active site that includes a secondary pocket adjacent to the catalytic site, which is capable of accommodating larger groups. *O*-aminoanilides contain a large group in the ZBG moiety, which is more suitable to fit within the secondary pocket making this class of inhibitors generally more selective towards Class I HDACs, such as HDAC3. These observations could explain the higher affinity of *o*-aminoanilide RMS-162 (**22**) for HDAC3. The larger HDAC3 site can apparently accommodate the N-butyl group in RMS-575 (**34**)

	Enzymatic assay (IC_{50} in μM)	
	HDAC3	HDAC6
SAHA	0.17	0.35
Entinostat	0.31	N.D.
RMS-70	0.96	1.15
RMS-162	3.18	>50.0
RMS-234	>5.0	>50.0
RMS-575	1.55	43.7

Table 2. In vitro HDACi activity of hybrids using purified HDAC3 and HDAC6.

Antiproliferation studies. The studies above established that the four hybrids possessed both antiestrogenic and HDACi activity. We subsequently assessed the antiproliferative effects of the four hybrids in MCF-7 cells in the presence and absence of E_2. In the absence of E_2, RMS-70 (**21**), RMS-162 (**22**) and RMS-234 (**24**) did not induce cell growth while in contrast, partial estrogenic activity was observed for RMS-575 with an EC_{50} value of 0.03 μM. All four hybrids were able to inhibit E_2 induced proliferation of MCF-7 cells, with RMS-234 (**24**) and RMS-575 (**34**) possessing IC_{50} values of 0.84 and 0.34 μM respectively. These antiestrogenic effects were similar, but not significantly superior, to those of SAHA, entinostat, ICI-164,384 and 4-OHT. Interestingly, RMS-575 (**34**) displayed the highest potency of all four hybrids, even though it displayed estrogenic activity at lower concentrations.

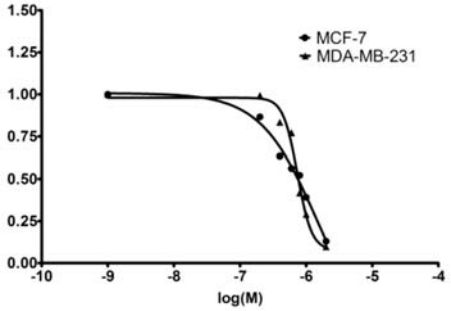
	Estrogenic (EC_{50} in μM)	Antiestrogenic (IC_{50} in μM)
ICI-164,384	> 5.0	0.93
4-OHT	> 5.0	0.15
SAHA	> 5.0	0.32
Entinostat	> 20.0	0.35
RMS-70	> 5.0	3.85
RMS-162	> 20.0	9.11
RMS-234	> 20.0	0.84
RMS-575	0.03	0.34

Table 3. Antiproliferative activity of hybrids in MCF7 cells.

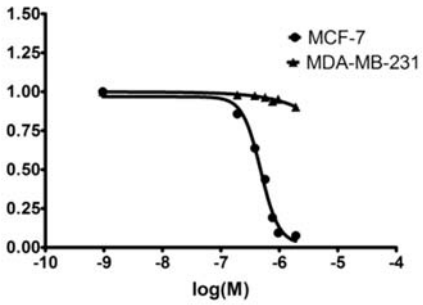
We further examined the antiproliferative effect of RMS-575 in ER– MDA-MB-231 cells. This cell line is sensitive to SAHA while but not to antiestrogens including Faslodex. Intriguingly, MDA-MB-231 cells were sensitive to RMS-575 (Figure 5 and 6) with an IC50 which was within one order of magnitude of that of SAHA, even though it is a significantly weaker HDACi. These results again underscore the bifunctional nature of the hybrids.

Docking studies. In order to study the binding modes of our hybrids, we performed molecular docking studies using Glide (Glide 5.0, Shrodinger LLC), which we previously demonstrated can aid understanding of SAR in antiestrogens, and FORECASTER which has recently been parameterized for modelling HDAC. The crystal structure of ICI-164,384 bound to ERβ (PDB code 1HJ1) provided a wealth of knowledge of interactions of full antiestrogens. In this crystal structure, H12 is disordered and the long side chain interacts with the coactivator binding groove (CBG). We docked RMS-70 (**21**), RMS-162 (**22**) and RMS-234 (**24**) in rigid mode in E_2, Faslodex, and ICI-164,384.

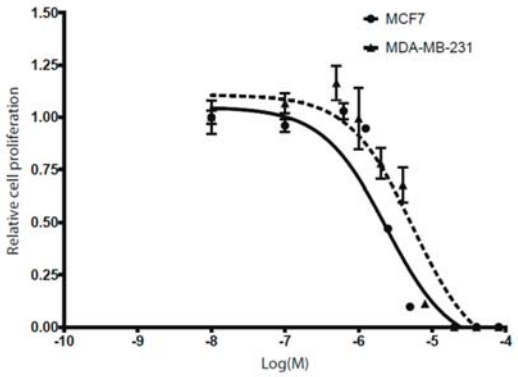
a) SAHA



b) Faslodex



c) RMS-575



. Figure 5. Comparison of antiproliferative effects of SAHA, Faslodex and RMS-575 on MCF-7 and MDA-MB-231 breast cancer cells.

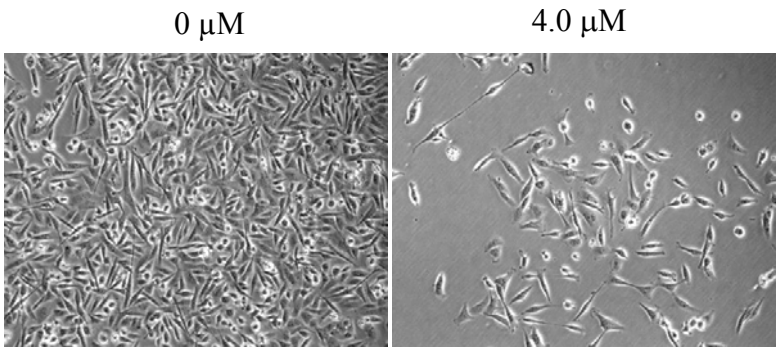


Figure 6. MDA-MB-231 cells treated with RMS-575

ICI-164,384 was self-docked and resulted in similar conformation to the one observed in the original crystal structure with the side chain occupying the CBG along the surface of the protein. A visual inspection of the poses showed a relationship between the polarity of the ZBG's and the conformation of the side chain. Side chains isosteric to that of ICI-164,384 are long enough to reach the highly hydrophobic CBG. When the side chain was functionalized with a hydroxamic acid, the side chain did not interact with the surface defined as the CBG. However, less polar o-amino anilides and N-butyl hydroxamates were able to interact with the CBG. The conformations of the side chains of hybrids RMS-162 (**22**, yellow) and RMS-234 (**24**, violet) were analogous to that of ICI-164,384 (green) where the side chain interacted with the CBG. However, the side chain in RMS-70 (**21**, orange) protruded along the surface of the protein without interacting with the CBG. This observation is illustrated in **Figure 7** that shows the superimposed poses predicted for three hybrids with ICI-164,384 (green) using Forecaster

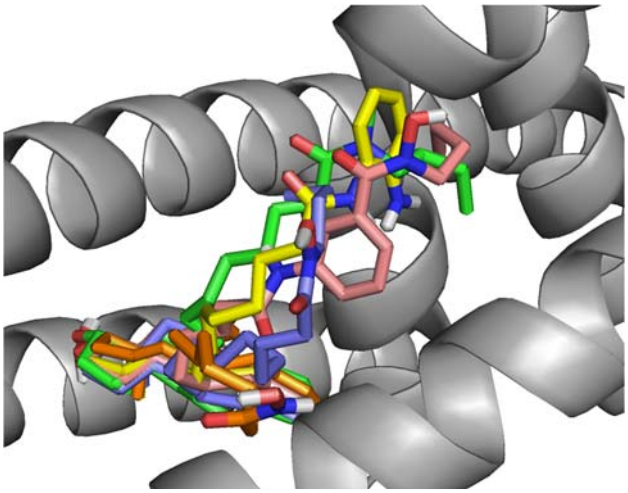


Figure 7. **Docking overlay of RMS-162 (**22**, yellow), RMS-234 (**24**, violet) and RMS-70 (**21**, orange) with ICI-164,384 (green)**

Conclusions. Our results demonstrate that it is feasible to synthesize hybrid molecules that have inhibitory activity against both HDACs and estrogen receptor. Starting from the ICI164,384 backbone, modification of the side chain by inclusion of a zinc binding group resulted in gain of HDACi activity while retaining antiestrogenic activity. Given the use of HDACi's in combination with antiestrogens in breast cancer treatment, these molecules may constitute new leads for combined therapies with simplified pharmacokinetic studies and administration schedules. Future studies will aim at improving affinity for each target and testing efficacy in preclinical models.

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CHAPTER 6: A HYBRID RETINOIC ACID RECEPTOR AGONIST / HISTONE DEACETYLASE INHIBITOR AS AN ANTIPROLIFERATIVE AGENT IN BREAST CANCER

David Cotnoir-White, Bin Zhao, Shuo Xing, Rodrigo Mendoza-Sanchez, James L. Gleason and Sylvie Mader.

Manuscript in preparation for Journal of Biological Chemistry

As first author I developed and performed the RAR BRET assays to assess the retinoicity of the hybrid molecule. I also performed the western blot of acetylated proteins, qPCR of modulated genes and growth assays. Rodrigo Mendoza-Sanchez performed the *in vitro* HDAC assays and, together with Bin Zhao and Shuo Xing, the chemistry under the supervision of James L. Gleason.

Author's note: Contrary to our AE-HDACi hybrid project which ran concurrently, we only developed a dozen or so molecules and TTNN-HA was the fourth molecule to be tested. More recently we tested molecules that showed better RAR activation profiles and experiments are ongoing. The molecule has been licensed out to HaRo pharmaceuticals which carried out encouraging MTD tests and independently reproduced our growth assays results. We also proceeded with PK/PD experiments in which I took part (more specifically the formulation step to conceive an IV, IP and PO delivery solution).

A Hybrid Retinoic Acid Receptor Agonist / Histone Deacetylase Inhibitor as an Antiproliferative Agent in Breast Cancer

David Cotnoir-White[2], Bin Zhao[1], Shuo Xing[1], Rodrigo Mendoza-Sanchez[1], James L. Gleason[1]* and Sylvie Mader[2]*

[1]Department of Chemistry, McGill University, 801 Sherbrooke St. West, Montreal, QC Canada, H3A 2K6

[2]IRIC, University of Montreal, CP 6128 Succursale Centre Ville, Montrela. QC Canada, H3C 3J7.

*Corresponding author

Abstract: Both retinoids and histone deacetylase inhibitors (HDACi's) have anti-tumor properties in the clinic and have been shown to work cooperatively in combination in pre-clinical models. Here we show that the incorporation of histone deacetylase inhibitory (HDACi) activity into a retinoic acid receptor (RAR) agonist results in a molecule with improved antiproliferative activity. The hydroxamic acid of RAR agonist TTNN has HDACi activity while maintaining RAR agonist activity. This hybrid molecule inhibits the growth of breast cancer cell lines that are both sensitive (MCF-7, SkBr-3) and resistant (MDA-MB-231, MDA-MB-468) to retinoids and is more efficient in these cells than the combination of retinoids with SAHA. It is also less toxic than SAHA in normal and immortalized cell lines. This molecule has potential value for the treatment of breast cancer and other cancers in which RA and HDACis are antiproliferative.

Introduction

All-trans retinoic acid (ATRA), the oxidized form of Vitamin A, is a potent antiproliferative and differentiating agent that acts as an agonist of the retinoic acid receptors (RARα, -β and -γ), members of the superfamily of ligand-regulated nuclear transcription factors. Its isomer 9-cis-RA acts as an agonist for both RARs and retinoic acid X receptors (RXRs). Due to the instability of ATRA and 9-cis-RA, many synthetic analogs have been developed with several possessing class and/or subtype selectivities. ATRA displays potent antiproliferative activity against a wide range of cancer cell lines and is used clinically in the treatment of acute promyelocytic leukemia where, in combination with anthracycline, it achieves a 90% remission rate. Retinoids can inhibit the growth of normal mammary epithelial cells and breast cancer cell lines by inducing G1 arrest and/or apoptosis.[1-6] However, although retinoids were shown to reduce second malignancies in the breast[7-9], their promise in breast cancer cell lines has not translated well to clinical treatment of breast cancer. This may be due to intrinsic and/or acquired resistance, which can readily be observed in vitro in breast cancer [10-13] and leukemic cell lines. While estrogen receptor (ER) positive (ER+ve) cells, such as MCF7, are sensitive to the anti-proliferative effects of ATRA, most ER negative (ER–ve) cells are not.[14] This may be due to induction of RAR expression by estrogens[15-17] and/or to other levels of cross-talk between the two receptors.[18-20] HER2 amplification, which occurs in 25% of breast tumors, has been reported to correlate with lack of ER expression and resistance to RA.[21]

Histone deactylase inhibitors (HDACi's) are a relatively new class of therapeutic agent which have shown promise in areas from cancer to HIV treatment. Histone

deactylases (HDACs), in conjunction with histone actyltransferases, control the acetylation state of nuclear and cytoplasmic histones as well as numerous other proteins (e.g. HSP90, p51, tubulin). HDACi's include trichostatin A, a natural product which is the prototype of the class, as well as suberoyl anilide hydroxamic acid (SAHA) which is approved for treatment of B-cell lymphoma. HDACi have shown promise in pre-clinical models of solid tumors including breast cancer. Our prior work showed that HDACi's repress transcription of ER[22] a therapeutic target for 2/3 of breast tumors. In addition, HDACi's down-regulate HER2, a proto-oncogene amplified in 25% breast tumors, both at the transcriptional level and through increased HER2 protein turnover, and sensitize HER2-amplified breast cancer cells such as SkBr3 to herceptin or chemotherapeutic drug treatment.[23,24]

Numerous studies have shown synergy using combinations of HDACi with ATRA and synthetic retinoids. Notably, APL resistance to ATRA is most commonly linked with an HDACi fusion which counteracts the transcriptional activation by ATRA. Studies *in vitro* have shown that resistance in APL may be overcome by coadministration of HDACi's and this protocol has had some limited clinical success. It has been observed that HDACi's synergize with RA to inhibit growth and induce apoptosis in breast cancer cells.[25] While both RA and the HDACi trichostatin A are anti-proliferative in MCF7 cells, comparison of patterns of gene expression upon treatment with either compound revealed only partial overlap in regulated genes, possibly explaining their cooperative action; e.g., while RA and TSA induce expression of CDKI p19,[26,27] TSA but not RA strongly suppressed

expression of cyclin CCND1. These findings suggest that association of HDACi's with retinoids may represent a potent combination for inhibition of tumor growth.

While combination therapy is a traditional, well established method for taking advantage of synergy between therapeutic agents, we have been investigating as an alternative, the development of hybrid molecules which combine affinity for two targets in a single molecule. We developed triciferol, a molecule that combines structural features of 1,25-dihydroxyvitamin D (1,25D) and TSA and that shows improved cytostatic and cytotoxic activity compared to 1,25D. Further, we showed that it was possible to prepare a wide variety of related hybrids based on the structural backbones of both 1,25D and its non-secosteroidal analogs. We have also previously shown that cleavable conjugates of retinoids are possible and can display enhanced antiproliferative activity in resistant leukemic cells compared to both individual and combination therapy. However, clinically useful cleavable conjugates are difficult to design as they should be sufficiently stable to reach the target cell but labile enough to be cleaved into their constituent drugs upon reaching the target. Given the synergy between retinoids and HDACi, we reasoned that a stable hybrid capable of acting as an RAR agonist and an HDACi would be of significant benefit.

Retinoic acid and its analogs bind to RARs through a combination of hydrogens bonds to the carboxylic acid and a series of hydrophobic contacts with the remainder of the molecule. Hydroxamic acid-based HDACi's such as TSA bind an active site zinc in a bidentate fashion, with the dienyl moiety spanning a hydrophobic tube which connects the active site to the surface. The aromatic group in TSA makes non-specific hydrophobic contacts with the surface of the enzyme. While a preliminary examination would suggest

that a simple hydroxamic acid of ATRA or another retinoid would form a functional hybrid, numerous carboxylic acid isosteres fail to act as RAR agonists. However, we hypothesized that a hydroxamic acid would enter into a similar series of hydrogen bond contacts as a carboxylic acid. Moreover, we chose to prepare a hybrid of a stable retinoid to avoid the potential for metabolic inactivation observed with ATRA. Advantageously, most stable retinoids use aromatic replacements for the labile polyene chain and the resulting aromatic hydroxamic acids are likely to be more acidic and more easily ionized in the RAR LBP, thus increasing their potential to mimic a carboxylic acid.

Results and Discussion

We prepared TTNN-HA (**3**), the hydroxamic acid of TTNN, a naphthoic acid-containing hybrid known to be selective for RARβ and -γ. Hybrid **3** was prepared by a modification of the procedure described by Dawson. We found that the Negishi cross-coupling to couple bromide X with bromide Y was inefficient. In our hands, a Suzuki cross-coupling was more effective as the reaction went to completion, simplifying purification. Although direct hydroxyamination of the coupling product could be carried out, we found the reaction to be inconsistent. However, the methyl ester could be easily and reliably transformed to the hydroxamic acid by ester hydrolysis, coupling with O-benzyl hydroxylamine and deprotection with H_2 on Pd/C.

The effects of hybrid **3** on RAR were initially assessed using a bioluminescence resonance energy transfer (BRET) assay. The BRET assay monitors agonist-dependent

recruitment of coactivators to RARs in transfected HEK293 cells, chosen because of their high degree of transfectability. Energy transfer between luciferase (fused to RARs) and eYFP (fused to the AF2-interacting domains of NCoA1/SRC-1 and NCoR1) occurs only when these moieties are juxtaposed by the RAR-NCoA1 or RAR-NCoR1 interactions, and can be detected by the emission of fluorescence. Assayed at 10 μM concentration, ATRA, TTNN and TTNN-HA were all shown to be agonists of RARβ/γ while HX600, an RXR-selective ligand, did not increase the BRET signal, and TTNN-HA showed no potency on RXR activation (**Figure S1**). Further dose-response comparison showed that TTNN-HA was in fact selective for RARβ/γ over RARα (NCoR1-IC50 = 210(β) and 290 nM(γ) versus 1620 nM(α), **Figure 2, Table 1**). Coactivator recruitment yielded similar results with TTNN and TTNN-HA being selective for RARβ/γ (**Table 2**).

We further examined the agonist activity of TTNN-HA through its effects on RAR target genes (**Figure 3**). In both MCF-7 (data not shown) and MDA-MB-231 cell lines, RT-qPCR analysis showed clear induction of RARB2 and DHRS3, two direct RAR target genes. Furthermore, genes that are reportedly regulated by HDACi's such as RARB1 and CyclinD1 follow similar regulation by TTNN-HA and SAHA in MDA-MB-231 [31].

We assessed the potential of TTNN-HA to act as an HDACi using both a fluorescence assay as well as by assessing its hyperacetylation effects on target proteins. Using a standard fluorescence assay, TTNN-HA was found to have IC50's of 5.0 μM and 148 nM against purified human HDAC3 and -6, respectively (**Table 3**). These HDACi activities compare favourably to that observed with vitamin D receptor agonist/HDACi hybrids as well as with SAHA which has reported IC50's of 6 and 4 nM, respectively, against HDAC3 and -6. We further assessed HDACi activity in MDA-MB-231 breast cancer

cells by measuring levels of acetylated histone H4 and tumor suppressor protein p53 by Western blotting. Although not as pronounced as effects observed with the highly potent HDACi SAHA, treatment with 10 μM TTNN-HA induced clear hyperacetylation of both targets over an 18-24 h time course (**Figure 4**). Interestingly, hyperacetylation of p53 causes its activation and the transcription of p53 target genes such as the pro-apoptotic genes PUMA, NOXA and BAX[31].

Taken together, the above data clearly showed that TTNN-HA possesses both RAR agonist and HDACi activity in a single compound. Given the known synergy between ATRA and HDACi, we assessed the effects of **3** on growth of several cancer cell lines including breast cancer cell lines (MCF-7, SkBr3, MDA-MB-231 and MDA-MB-468) as well as non-malignant cells (HMEC). Impressively, hybrid TTNN-HA was active against all four malignant cell lines, including normally ATRA-insensitive MDA-MB-231 and MDA-MB-468 (**Figure 5, Table 4**), with IC50's of 163 nM, 644 nM, 517 nM and 1086 against SkBr3, MCF-7, MDA-MB-468 and MDA-MB-231cell lines, respectively. TTNN-HA was also active against another ER-negative, HER2-negative, RA-insensitive cell line, BT-20 (data not shown). Importantly, TTNN-HA displayed only minimal effects on growth of normal HMEC cells compared to SAHA, which causes strong inhibition of HMEC growth, indicating a potentially useful therapeutic window. For comparison, in the MDA-MB-468 cell line, retinoids such as ATRA and TTNN had little to no effect, while treatment with SAHA induced a modest decrease in cell growth yet TTNN-HA caused complete arrest of proliferation. Moreover, co-treatment of TTNN-HA with the pan-RAR antagonist BMS493 increased proliferation of MCF-7 and MDA-MB-231 (**Figure S2**) while BMS493 alone had no effects in these cells, highlighting the importance of the retinoicity of TTNN-HA in inhibiting cancer cell growth. Our compound was also assayed by the NCI-60 DTP Human

Tumor Cell Line Screen[32] (**Table S1**) and revealed to be efficient at inhibiting cancer cell growth in a plethora of human cancer derived cell lines in ranges of mid-to high-nanomolar concentrations, which is below the tolerated dose of normal mammary epithelial cells (see above).

Material and methods

Reagents and plasmids

Retinoids were synthesized as described by authors BM, SX and RMS. TTNN was synthesized as reported by Dawson MI et al[33]. BMS493 and SAHA were purchased from Tocris Cookson Ltd (Minneapolis, MN). RARα, RARβ and RARγ were cloned from cDNA obtained from Pierre Chambon by incorporating in pRLuc-N1 (PerkinElmer Corp., Wellesley, MA). NCoA1-eYFP and NCoR1-eYFP were generated by cloning of SRC1 residues 625 to 699 and NCoR1 residues 2044 to 2278 in to peYFP-N1 vecotr described in Breton et al[34]. TRI reagent was purchased from Sigma-Aldrich.

Cell transfection and BRET assays

HEK293 cells were grown to confluence, trypsinized and plated at a density of 500k cells per well (12-well plates) in DMEM supplemented with 10% charcoal treated FBS (FBS-T). The following day cells were transfected with PEI using 0.1µg of RAR-RLuc vector and 1µg of NCoA1-eYFP vector (described previously) or NCoR1-eYFP vector. 48 hours post-

transfections cell were treated with retinoids for 2 hours before taking BRET measurement as described previously.

Reverse Transcription and Real-time PCR

MDA-MB-231 cells were grown in 5% FBS-T DMEM for 48 hours at 1M cells per 10 cm dish and treated for 16 hours with 1μM ATRA, TTNN, SAHA, TTNN and SAHA or TTNN-HA. DMSO was used as vehicle control (0). Cells were harvested in TRI reagent and RNA was extracted as specified by manufacturer. Total RNA (2 μg) was reverse transcribed using the RevertAid H first minus strand cDNA synthesis kit (MBI Fermentas, Burlington, Ontario, Canada) as recommended by the manufacturer. The reverse transcription product was diluted 10 times prior to real-time PCR. Each real-time PCR amplification reaction contained the reverse transcription dilution (2 μl), forward and reverse primers (150–300 nM), MgCl2 (3–4.5 mM according to primer pairs), dNTP (0.2 mM), SYBR Green (0.33×, Invitrogen, Burlington, Ontario, Canada), buffer for Jump Start Taq and Jump Start Taq (0.5 unit, Sigma) in a final volume of 20 μl. After denaturation at 95 °C for 7 min, samples went through a one-degree annealing temperature touchdown of 7 cycles starting from 60 °C (30 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C) followed by 40 cycles of amplification (30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C). A dissociation protocol followed the amplification program to characterize the amplified product(s). PCR was performed using a RotorGene 3000 (Corbett, Australia) and analyzed using expression levels of the p36b4 gene for normalization. For each set of primers, non-template control reactions were performed as a negative control. Each sample was

assayed in triplicate and each experiment was reproduced at least two times. A typical experiment is shown.

Western blotting

MDA-MB-231 were plated in DMEM 5% FBS-T at 600k cells per well in 6-well plates. The next day cells were treated with DMSO, TTNN (10µM), TTNN-HA (10µM) or SAHA(1µM) and the cells were collected in 95°C Laemmli buffer at various time points. Protein concentration of the extracts was analyzed by BioRad DC protein assay. 20µg of proteins per condition were loaded on an SDS-PA gel and transferred to a nitrocellulose membrane for blotting. Blotting was done using ABCAM primary antibodies EP356 (anti-p53) T3526 (anti-Tubulin) and AB1761 (anti-acH4).

Cell growth measurement

Cell were plated at 40k cells per well (6-well plate) in DMEM 5% FBS-T for all cell lines except HMEC that were plated in supplemented MEBM from Clonetics. Cells were treated every 48 hours and media was refreshed every 96 hours. After 10 days of treatment cells were harvested in 0.1N NaOH and growth was quantified by analyzing protein content of lysate with a Lowry assay.

NCI 60 screen

Information regarding NCI 60 screening can be found at http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

Figure Legends

Figure 1. Structure of hybrid molecule TTNN-HA and parental molecule TTNN. TTNN-HA was synthesized by hydrolysis of TTNNs methyl-ester followed by coupling with O-benzyl hydroxylamine and deprotection.

Figure 2. BRET assay reveal TTNN-HA's activation RARγ. Cells were transfected with RARγ-RLuc and NCoA1-YFP or NCoR1-YFP and treated with TTNN-HA from 10^{-10} to 10^{-5}M 48 hours after transfection. BRET measurements were made 40 minutes post-treatment.

Figure 3. TTNN-HA regulates retinoid and HDACi sensitive genes. MDA-MB-231 cells were treated for 24 hours with 1μM of indicated molecule. RNA was reverse transcribed and levels of retinoid target genes RARB2 and DHRS3 as well as HDACi sensitive genes CCND1 and RARB1 were assayed by real-time qPCR. mRNA levels were normalize to actin mRNA and presented as quantities relative to vehicke (0) treatment.

Figure 4. TTNN-HA has HDACi activity similar to SAHA. (A) MDA-MB-231 cells were treated with TTNN-HA for 8 hours at various concentrations. Proteins were harvested in Laemmli and separated by SDS-PAGE. Western blot was performed against acetylated p53 (Ac-p53) and acetylated tubulin (Ac-αTubulin) with total histone H3 as loading control. (**B**) MDA-MB-231 cells were treated with TTNN-HA (10μM) or SAHA (1μM) for 1 to 24 hours. Proteins were harvested in Laemmli and separated by SDS-PAGE. Western blot was performed against acetylated p53 (Ac-p53) and acetylated histone H4 (Ac-H4) with αTubulin as loading control.

Figure 5. TTNN-HA inhibits the growth of breast cancer cells but spares normal cells. Cells were grown for 10 days with treatments every two days starting at day 0. Cell content was harvested in 0.1N NaOH and protein content was assessed by Lowry assays. (**A**) Retinoid-sensitive SkBr-3 and MCF-7 and retinoid-insensitive MDA-MB-468 and MDA-MB-231 were treated with increasing concentration of TTNN-HA. (**B**) MDA-MB-231 were treated with ATRA, SAHA, ATRA and SAHA or TTNN-HA at increasing concentration for 10 days. (**C**) Light field microscopy of MDA-MB-231 growing for 120 hours with 0, 1 or 2 μM of TTNN-HA.

Figure S1. TTNN-HA selectively activates RARs. Cells were transfected with RARα-RLuc or RXRγ-Rluc and NCoA1-YFP. Cells were treated with retinoids 1μM ATRA, TTNN-HA or the rexinoid HX600 to verify receptor-type selectivity of TTNN-HA.

Figure S2. RAR antagonist BMS493 restores some growth to cells under TTNN-HA treatment. MCF-7 and MDA-MB-231 cells were grown for 10 days with treatments every two days starting at day 0 with TTNN-HA (1 and 2μM for MCF-7, 2 and 4μM for MDA-MB-231) and BMS493 (1μM). Cell content was harvested in 0.1N NaOH and protein content was assessed by Lowry assays. Growth is adjusted to vehicle condition.

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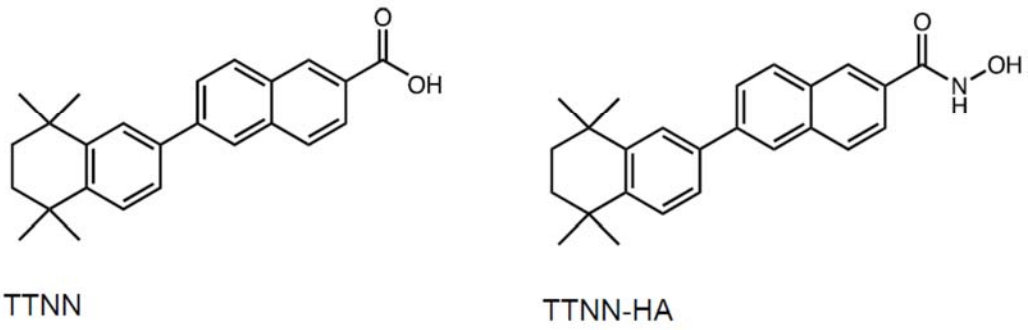
Figure 1



TTNN TTNN-HA

Figure 1. Structure of hybrid molecule TTNN-HA and parental molecule TTNN.

Figure 2

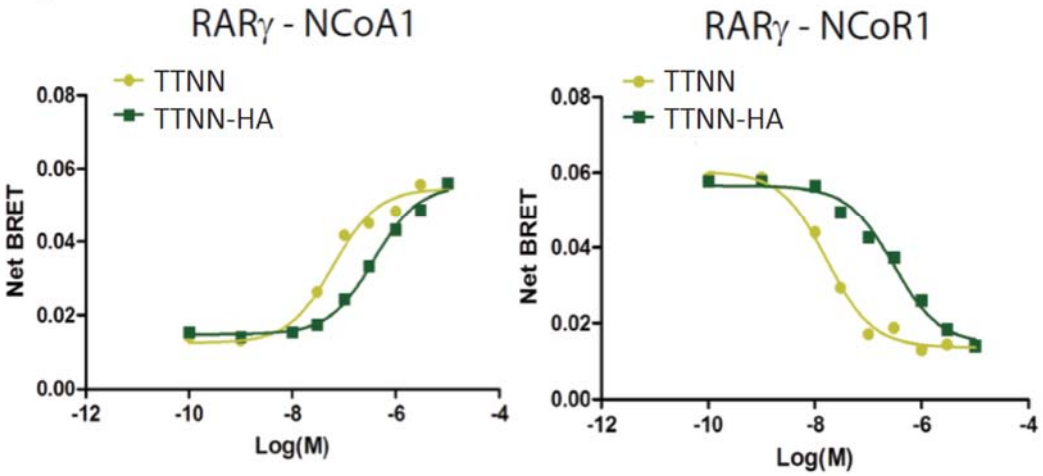


Figure 2. BRET assay reveal TTNN-HA's activation RARγ**.**

Figure 3

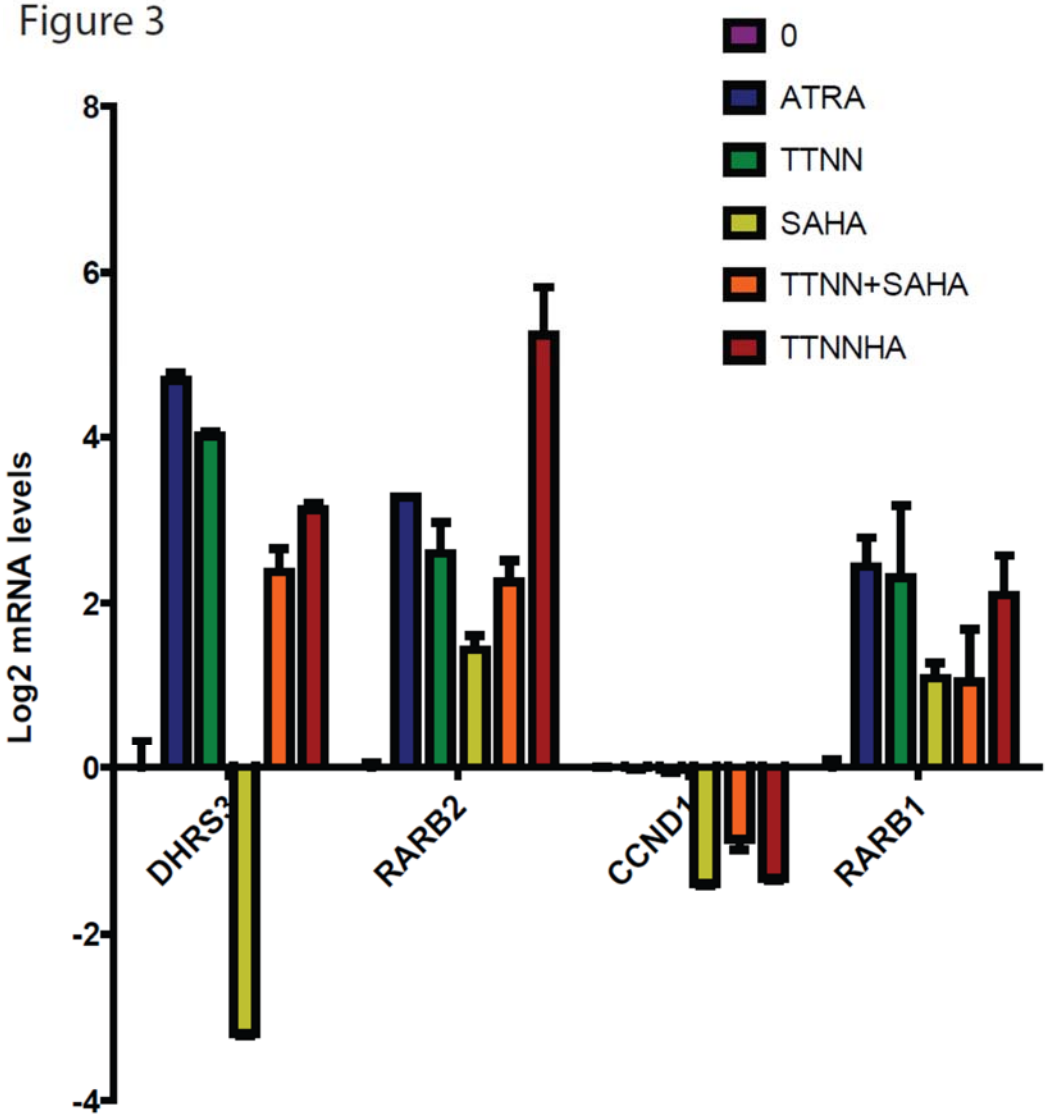


Figure 3. TTNN-HA regulates retinoid and HDACi sensitive genes.

Figure 4

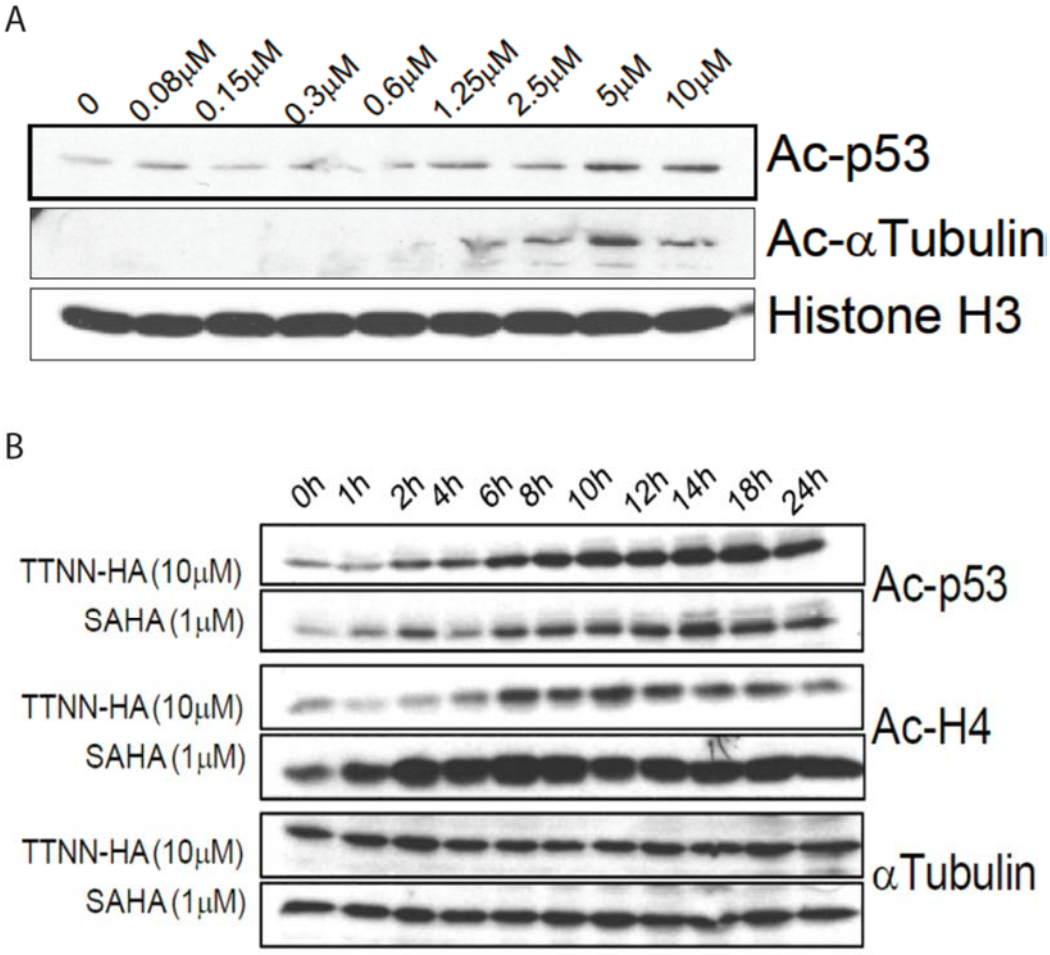


Figure 4. TTNN-HA has HDACi activity similar to SAHA.

Figure 5

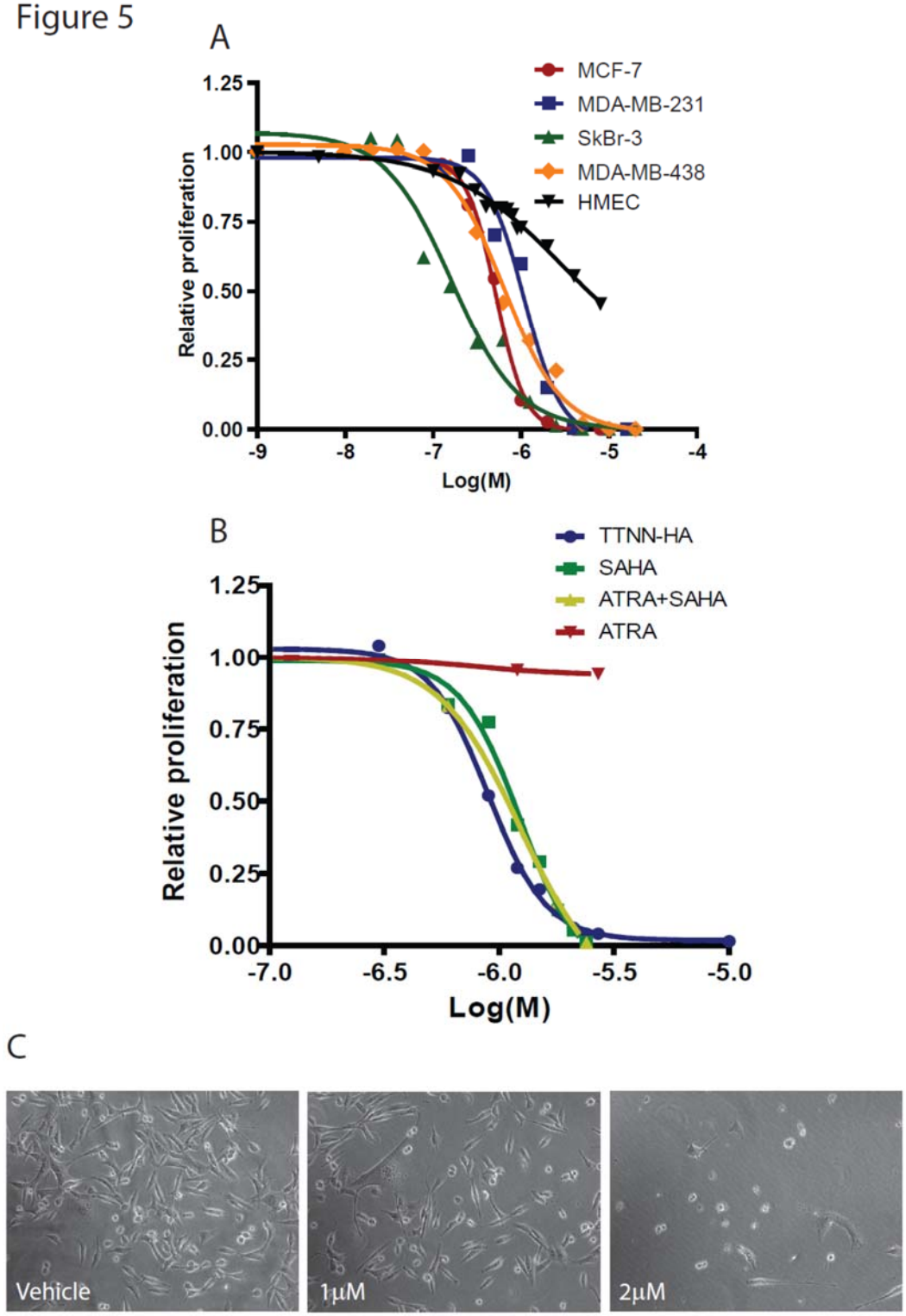


Figure 5. TTNN-HA inhibits the growth of breast cancer cells but spares normal cells.

Table 1. Efficiency of CoRNBOX motif release from RARs by TTNN and TTNN-HA

Ligands	**IC50 NCoR1 binding (nM)** (95% confidence interval)		
	RARα	RARβ	RARγ
TTNN	**123** (73.1 to 202)	**10** (5.07 to 20.3)	**16** (10.1 to 24.4)
TTNN-HA	**1620** (835 to 3580)	**210** (111 to 294)	**290** (165 to 532)

Table 2. Efficiency of NCoA1 domain recruitment to RARs by TTNN and TTNN-HA

Ligands	EC50 NCoA1 binding (nM) (95% confidence interval)		
	RARα	RARβ	RARγ
TTNN	**157** (90.2 to 273)	**28** (14.1 to 55.4)	**61.9** (33.0 to 116)
TTNN-HA	**1310** (819 to 2096)	**254** (181 to 355)	**377** (261 to 553)

Table 3. In vitro inhibition of HDAC3 and 6 by TTNN-HA

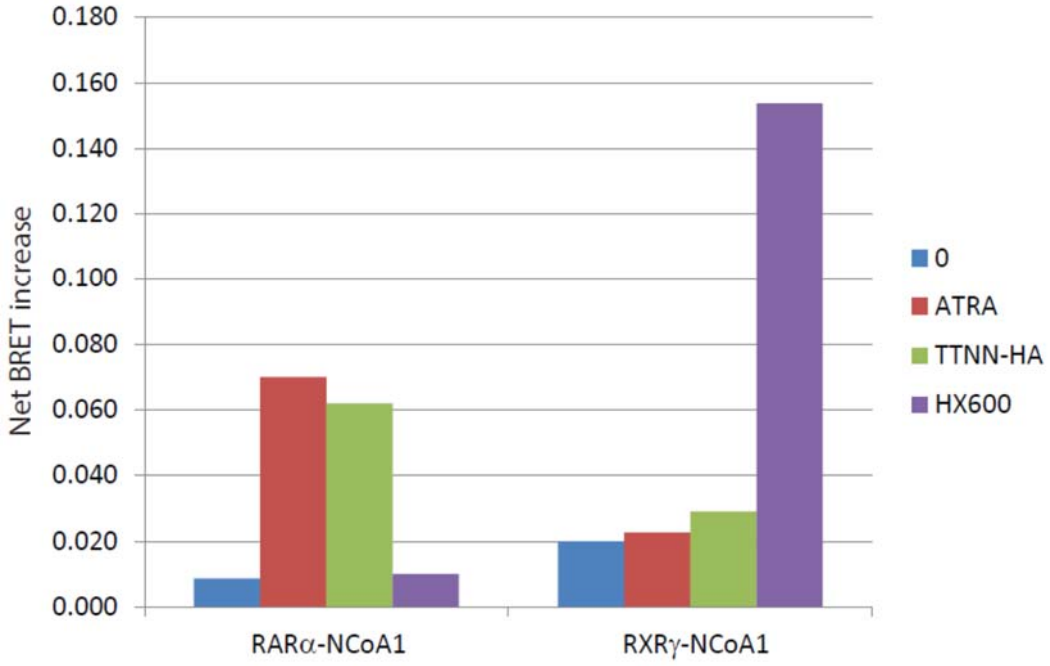
Enzyme	IC50 (μM) TTNN-HA
HDAC3	5
HDAC6	0.148

Table 4. Growth IC50 of TTNN, SAHA and TTNN-HA on various breast cancer cell lines and normal human mammary epithelial cells (HMEC). Cells were kept in growth media for 10 days with concentrations of 10 to 10 000nM of TTNN, SAHA and TTNN-HA. Protein content of cells was then measured to assess growth.

IC50 cell growth (nM)

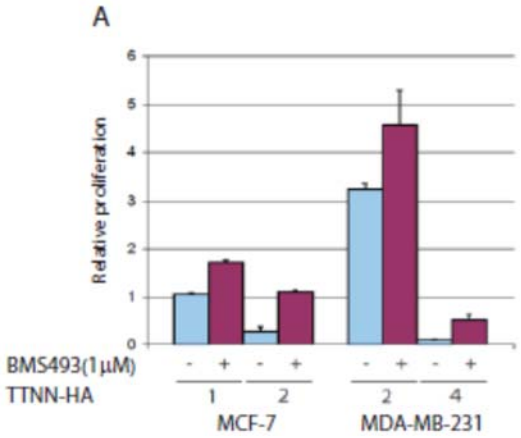
	TTNN	**SAHA**	**TTNN-HA**
SkBr-3	664	865	163
MCF-7	N/A	739	517
MDA-MB-468	N/A	N/A	644
MDA-MB-231	N/A	1540	1086
HMEC	~4000	54	6477

Figure S1



Supplementary Figure 1. TTNN-HA selectively activates RARs**.**

Figure S2



Supplementary Figure 2. RAR antagonist BMS493 restores some growth to cells under TTNN-HA treatment.

Table S1. Results of NCI 60 screen. Various cancer cell lines treated from 10^{-8} to 10^{-4}M of TTNN-HA for 48 hours growth inhibition and toxicity 50 concentrations are reported.

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC : D - 753858 / 1	Experiment ID : 1011RS52	Test Type : 08	Units : Molar
Report Date : February 28, 2011	Test Date : November 15, 2010	QNS :	MC :
COMI : TTNN-HA (96971)	Stain Reagent : SRB Dual-Pass Related	SSPL : 0Y1C	

			Log10 Concentration											
	Time		Mean Optical Densities					Percent Growth						
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.490	2.532	2.559	2.413	0.931	0.407	0.227	101	94	22	-17	-54	4.06E-7	3.63E-6	7.94E-5
HL-60(TB)	0.645	2.629	2.607	2.655	1.943	0.509	0.188	99	101	65	-21	-71	1.51E-6	5.70E-6	3.80E-5
K-562	0.187	1.482	1.504	1.498	0.825	0.114	0.075	102	101	49	-39	-60	9.66E-7	3.61E-6	3.30E-5
MOLT-4	0.448	2.066	2.127	2.127	0.847	0.266	0.188	104	104	25	-41	-58	4.78E-7	2.39E-6	3.46E-5
RPMI-8226	0.724	2.453	2.461	2.262	1.564	0.485	0.349	100	89	49	-33	-52	9.20E-7	3.93E-6	8.02E-5
SR	0.305	1.780	1.831	1.778	0.562	0.168	0.158	103	100	17	-45	-48	4.02E-7	1.90E-6	> 1.00E-4
Non-Small Cell Lung Cancer															
A549/ATCC	0.527	2.178	2.088	1.992	1.315	0.341	0.057	95	89	48	-35	-89	8.81E-7	3.76E-6	1.87E-5
EKVX	0.785	1.931	1.876	1.834	1.548	0.649	0.128	95	92	67	-17	-84	1.58E-6	6.22E-6	3.11E-5
HOP-62	0.428	0.933	0.924	0.987	0.654	0.082	0.062	98	111	45	-81	-86	8.34E-7	2.27E-6	5.68E-6
HOP-92	1.147	1.495	1.391	1.327	1.333	0.780	0.032	70	52	53	-32	-97	1.10E-6	4.22E-6	1.89E-5
NCI-H226	0.598	1.400	1.392	1.359	0.827	0.091	0.129	99	95	28	-85	-79	4.74E-7	1.78E-6	4.92E-6
NCI-H23	0.645	1.991	1.942	1.883	1.549	0.205	0.137	96	92	67	-68	-79	1.34E-6	3.13E-6	7.34E-6
NCI-H322M	0.615	1.583	1.628	1.594	1.180	0.852	0.116	105	101	58	24	-81	1.76E-6	1.70E-5	5.07E-5
NCI-H460	0.233	1.534	1.620	1.665	0.306	0.032	0.044	107	110	6	-86	-81	3.76E-7	1.15E-6	4.02E-6
NCI-H522	0.824	1.746	1.680	1.721	1.267	0.195	0.135	93	97	48	-76	-84	9.12E-7	2.43E-6	6.14E-6
Colon Cancer															
COLO 205	0.298	1.082	1.113	1.102	0.689	0.031	0.026	104	103	50	-90	-91	9.95E-7	2.28E-6	5.19E-6
HCC-2998	0.812	3.104	3.064	2.980	2.525	0.428	0.165	98	95	75	-47	-80	1.59E-6	4.10E-6	1.21E-5
HCT-116	0.209	1.564	1.619	1.605	0.649	0.005	0.006	104	103	32	-98	-97	5.64E-7	1.77E-6	4.29E-6
HCT-15	0.365	2.316	2.217	2.102	0.809	0.240	0.089	95	89	23	-34	-76	3.88E-7	2.51E-6	2.40E-5
HT29	0.197	1.162	1.198	1.183	0.555	0.045	0.118	104	102	37	-77	-40	6.33E-7	2.11E-6	.
KM12	0.499	2.226	2.268	2.264	1.456	0.094	0.066	102	102	55	-81	-87	1.09E-6	2.54E-6	5.90E-6
SW-620	0.220	1.080	1.075	1.095	0.668	0.052	0.049	99	102	52	-76	-78	1.04E-6	2.54E-6	6.23E-6
CNS Cancer															
SF-268	0.466	1.527	1.577	1.571	0.896	0.171	0.089	105	104	41	-63	-81	7.10E-7	2.46E-6	7.45E-6
SF-295	0.749	2.574	2.452	2.348	2.122	0.857	0.190	93	88	75	6	-75	2.31E-6	1.18E-5	4.94E-5
SF-539	0.951	2.293	2.161	2.044	1.735	0.798	0.066	90	81	58	-16	-93	1.30E-6	6.08E-6	2.76E-5
SNB-19	0.413	1.385	1.401	1.358	0.921	0.113	0.073	102	97	52	-73	-82	1.04E-6	2.62E-6	6.59E-6
SNB-75	0.679	1.189	1.125	1.058	1.009	0.325	0.060	87	74	65	-52	-91	1.34E-6	3.58E-6	9.57E-6
U251	0.340	1.503	1.417	1.430	0.778	0.043	0.039	93	94	38	-87	-89	6.02E-7	2.00E-6	5.02E-6
Melanoma															
LOX IMVI	0.246	1.872	1.861	1.773	0.638	0.060	0.051	99	94	24	-76	-79	4.25E-7	1.74E-6	5.54E-6
MALME-3M	0.727	1.439	1.374	1.414	1.307	0.671	0.188	91	96	81	-8	-74	2.25E-6	8.18E-6	4.33E-5
M14	0.375	1.423	1.410	1.445	0.932	0.203	0.028	99	102	53	-46	-93	1.07E-6	3.44E-6	1.23E-5
MDA-MB-435	0.488	1.901	1.836	1.819	1.447	0.203	0.095	95	94	68	-59	-81	1.39E-6	3.44E-6	8.56E-6
SK-MEL-2	0.943	1.761	1.862	1.837	1.824	1.039	0.094	112	109	108	12	-90	3.99E-6	1.30E-5	4.04E-5
SK-MEL-28	0.655	1.413	1.460	1.419	1.327	0.457	0.029	106	101	89	-30	-96	2.11E-6	5.57E-6	2.01E-5
SK-MEL-5	0.556	2.164	2.171	2.221	1.453	0.058	0.007	100	104	56	-90	-99	1.10E-6	2.42E-6	5.34E-6
UACC-257	0.779	1.785	1.666	1.645	1.661	0.888	0.030	88	86	88	11	-96	3.09E-6	1.26E-5	3.70E-5
UACC-62	0.730	1.982	1.989	1.978	1.218	0.073	0.050	101	100	39	-90	-93	6.57E-7	2.00E-6	4.90E-6
Ovarian Cancer															
IGROV1	0.386	1.601	1.665	1.708	1.142	0.262	0.143	105	109	62	-32	-63	1.35E-6	4.57E-6	3.80E-5
OVCAR-3	0.486	1.318	1.342	1.347	0.786	0.113	0.016	103	103	36	-77	-97	6.21E-7	2.09E-6	5.79E-6
OVCAR-4	0.383	0.927	0.919	0.890	0.532	0.141	0.013	99	93	27	-63	-97	4.54E-7	2.01E-6	7.15E-6
OVCAR-5	0.523	1.227	1.197	1.181	0.994	0.496	0.020	96	93	67	-5	-96	1.71E-6	8.45E-6	3.11E-5
OVCAR-8	0.407	1.683	1.678	1.643	0.825	0.112	0.066	100	97	33	-73	-84	5.38E-7	2.05E-6	6.10E-6
SK-OV-3	0.625	1.425	1.387	1.355	1.038	0.494	0.019	95	91	52	-21	-97	1.05E-6	5.14E-6	2.41E-5
Renal Cancer															
786-0	0.599	2.036	2.104	2.118	1.426	0.063	0.086	105	106	58	-90	-86	1.12E-6	2.46E-6	5.38E-6
A498	1.067	1.470	1.344	1.326	1.391	0.363	0.088	69	64	80	-66	-92	1.61E-6	3.54E-6	7.78E-6
ACHN	0.483	1.939	1.962	1.921	0.779	0.139	0.024	102	99	20	-71	-95	4.18E-7	1.67E-6	5.85E-6
CAKI-1	0.399	1.848	1.746	1.730	0.614	0.115	0.094	93	92	15	-71	-76	3.49E-7	1.49E-6	5.67E-6
RXF 393	0.622	1.126	1.140	1.108	0.891	0.117	0.112	103	96	53	-81	-82	1.06E-6	2.49E-6	5.86E-6
SN12C	0.463	1.674	1.688	1.668	1.044	0.098	0.078	101	99	48	-79	-83	9.12E-7	2.39E-6	5.91E-6
TK-10	0.666	1.253	1.236	1.296	0.940	0.181	0.021	97	107	47	-73	-97	8.78E-7	2.45E-6	6.43E-6
UO-31	0.624	1.620	1.509	1.544	1.052	0.199	0.093	89	92	43	-68	-85	7.19E-7	2.44E-6	6.87E-6
Prostate Cancer															
PC-3	0.368	1.455	1.450	1.420	0.997	0.293	0.027	100	97	58	-21	-93	1.26E-6	5.47E-6	2.56E-5
DU-145	0.342	1.219	1.261	1.272	0.723	0.165	0.003	105	106	43	-52	-99	7.86E-7	2.86E-6	9.58E-6
Breast Cancer															
MCF7	0.363	1.478	1.385	1.346	0.610	0.031	0.070	92	88	22	-92	-81	3.78E-7	1.57E-6	4.31E-6
MDA-MB-231/ATCC	0.550	1.156	1.104	1.088	0.813	0.247	0.077	91	89	43	-55	-86	7.12E-7	2.75E-6	8.86E-6
HS 578T	0.630	1.142	1.115	1.070	1.088	0.686	0.199	95	86	90	11	-68	3.18E-6	1.37E-5	5.86E-5
BT-549	0.788	1.550	1.607	1.582	1.342	0.166	0.050	107	104	73	-79	-94	1.41E-6	3.01E-6	6.44E-6
T-47D	0.684	1.591	1.560	1.459	1.044	0.227	0.196	97	85	40	-67	-71	5.94E-7	2.36E-6	6.95E-6
MDA-MB-468	0.595	0.992	0.971	0.986	0.826	0.072	0.112	95	99	58	-88	-81	1.14E-6	2.50E-6	5.50E-6

CHAPTER 7: DISCUSSION

On the quaternary structure of the estrogen receptors α and β

Estrogen receptor dimerization and subcellular localization

For the last 30 years type I nuclear receptors such as the estrogen and androgen receptors have been characterized as monomers in their inactive states and the biology and mechanism that surrounds them was adapted to that fact. Their interaction with DNA was regulated by the dimerization of the DBD upon activation which greatly increased their affinity for HRE's by avidity effects [81, 92, 161]. To prevent dimerization in the absence of ligand, the receptors were thought to be held inactive by heat-shock proteins [212]. This inactivation is also reinforced by segregation of the receptor to the cytoplasm with shuttling to the nucleus only possible when chaperone proteins are released [78, 223, 353, 354]. While this last step has been reported to be integral to estrogen receptor biology, ERα is rarely observed in the cytoplasm of cells [355]. A fraction of ERα is located at the cytoplasmic membrane where it can launch rapid signalling cascade through G-protein and mitogen activated protein kinase [229]. This may be the protein detected in the cytoplasm of past experiments. Moreover many previous demonstration of cytoplasmic ER relied on radiolabelled E2 binding in the cytoplasmic fraction of lysed cells as a proxy for ERα protein localization. This technique can lead to false positive signals as other proteins can bind E2 – albeit with lower affinity – and indicate cytoplasmic localization of steroid binding. Interestingly this caveat also applies to other experiments describing the protein complexes formed by ERα in cells.

Assessing estrogen receptor dimerization in live cells

Many recent reports on live cell behavior of NRs seemed to confirm previous work and established mechanisms [221, 352, 356]. The discrepancies with our work are in large part due to the widespread idea that FRET measures protein binding. This mindset ignores the other important parameter defining FRET efficiency, the orientation factor which varies as a function of sinθ (θ being the angle between donor and acceptor dipoles). Conformational changes in the partners that displace the chromophores and alter the relative angle between their dipoles may therefore lead to changes in FRET ratios. If said angle was close to 90 degrees in the inactive state, the FRET efficiency would be low and might be interpreted as evidence that the proteins do not interact in this state. Any changes in this angle after a stimulus would then be interpreted as binding of the two after activation. These misinterpretations of data may be quite common as many studies fail to run two sets of important experiments: a specificity of interaction control and an acceptor or donor titration experiment [213, 222, 357-360].

Controlling for specificity of interaction involves comparing the BRET ratios in the presence of the acceptor of interest and of an acceptor that should not interact with the donor [361, 362]. This non-interacting partner can be a non-specific partner or a mutant version of the acceptor of interest. In the case of ERα dimerization and cofactor recruitment we used both types of controls: the mutant ERαL507R and RARα for dimerization and the mutant AXXAA and CoRNBOX peptides for cofactor recruitment. The net BRET increase observed in non-specific acceptor conditions should be due solely to random collisions between donor and acceptor. Therefore the increase should be small and increase linearly as a function of acceptor concentration. Importantly, defining what the BRET ratio of non-specific transfer allows to determine if the BRET observed in the

"basal" state is significantly higher than random collision. In the case of ERα and ERβ dimerization, we notice a strong signal in the absence of any stimulation suggesting that a large fraction of the receptor is already dimerized. Cofactor recruitment also showed a sizable basal signal that could be diminished by AE treatment. Logically this basal cofactor recruitment could be attributed to the receptor fraction in the activate dimer form detected by dimerization BRET assays, as monomeric receptor are not expected to recruit coactivators. Indeed, the ERαL507R mutant failed to recruit the coactivator peptide in subsequent experiments.

The second experiment, the donor or acceptor titration, allows to derive two important components of the interaction: BRET50 and BRETmax. Classically the titration is done by increasing acceptor levels against stable donor expression. In this case the function plotted increases logarithmically and plateaus or reaches BRETmax at high acceptor to donor ratios when the interaction is saturated. Plotting this function on a linear ratio scale highlights the BRETmax reached by the assay, while plotting against a log scaled x axis or log10(Acceptor/Donor) highlights the ratio at which BRET value is half of the plateau, the BRET50 value. sigmoidal curve Note that the plateau is still present but is reached only at high x values. The BRET50 is a measure of relative affinity of the donor for the acceptor. To interpret BRET signal modulation as the formation of a complex, one should observe a change in BRET50 between two conditions and not simply a difference in plateaus. A change in plateau without a change in BRET50 is an indication either of conformational change that increases FRET efficiency between the donor and acceptor or of increased recruitment capacity (i.e. binding of additional acceptors to the donor.)

Impact of ligand on ER dimerization:

Without proper specificity and titration controls, previous research claims of observing ligand-induced dimerization of SHR in live cells by FRET fall short of being conclusive [352, 356]. Our experiments demonstrate that estrogen treatment does in fact lead to ERα binding additional receptor units. However, additional implications came from the data acquired. First, a sizable fraction of the receptor was already associated with other ERα units without ligand treatment in BRET experiments. Second, contrary to previous reports, ICI182,780 did not decrease the apparent dimerization of ERα. Rather, titration experiments revealed a higher affinity of ERα-RLuc for ERα-YFP and either an increased capacity of ERα binding or an alternative conformation. The PCA assays, performed with the same standards as the BRET assays, also indicate a constitutively dimerized receptor. These observations are consistent with the high basal activity of ERa on reporter vectors, which correlates with both receptor and coactivator levels. Thus, far from being "checked" by chaperones, the receptor appears to be in a free equilibrium between inactive dimers, active dimers and dimer-coactivator complexes. Binding of estrogen stabilizes the active dimer form, which has a high affinity for coactivators.

Allosteric effects of ER dimerization

The BRETFect also highlighted the allosteric controls one receptor exerts on his dimerization partner. While this mechanism has been suggested based on crystal structure, ours is the first observation of this phenomenon in live cells and indicates that receptor-specific ligands may not guarantee activity on only one receptor subtype. Indeed, PPT [363] was able to recruit coactivators to ERβ heterodimerized to ERα, albeit

at higher concentrations. This process is likely to affect other NR heterodimers, including RXR-containing complexes.

Our experiments confirm the ubiquitous nuclear localization of ERα and imply that ERα (and β) is constitutively dimeric. This is apparent in the amplification patterns in the ERα-mTFP1 titration (Chapter 2, fig. S5) and ligand titration of ER dimers (Chapter 2, fig. 3); if the receptor was overwhelmingly monomeric in the apo-state no amplification would be noticed in the vehicle condition (This conclusion was not included in the article manuscript to avoid a debate on dimerization dynamics). The use of protein complementation assay with *Renilla luciferase* fragments also demonstrated that the recombinant receptors were dimerized in their apo-state but that the binding of hormone or antiestrogen caused changes in LBD conformation that increased luciferase recombination when the Luc fragments were fused C-terminally but not when in the N-terminal position. This may be the effect that was registered by previous groups studying ER and AR by BRET and FRET [222, 352].

AR fusion proteins in our system were as expected cytoplasmic in the absence of androgens and shuttled to the nucleus after treatment with DHT or the anti-androgen bicalutamide (Casodex), but they appeared to be dimeric in presence and absence of ligands. Previous report have characterized nuclear shuttling of SHR as preceding their dimerization [356], but the dimerization assay was also reliant on receptor-receptor FRET, which may only be recording altered conformation of the receptors in the nucleus. Alternatively, the FRET ratio measured may have been an indication that the receptors were forming higher order complexes.

The effect of bicalutamide on the androgen receptor was unexpected as it is expected to inhibit dimerization [364, 365] yet our assays showed that AR dimerization was not decreased – and may have increased – in the presence of bicalutamide, and that while FXXLF peptide recruitment to the AF2 domain was inhibited, SRC1's AF1 interacting domain recruitment was increased. These assays have the potential for better characterization of anti-androgens activity. Recently, selective androgen receptor modulators (SARMs) development has increased for the treatment of a wide range of androgen signalling defects [366, 367], such as the lack of androgens in andropause [368] or overactive AR signalling in prostate hyperplasia [366]. The use of AR PCA-BRET to characterize novel SARMs could increase our understanding of androgen and AR SAR and help in the design of SARMs with optimal agonist/antagonist balance.

Moreover the trimerization/SUMOylation and ubiquitination assays developed for ERα are the best and, to our best knowledge, only direct SERD assays. Therefore it is possible that selective androgen down-regulator (SARDs) [366] operate in a similar fashion, with improper dimerization and multimerization of AR followed by post-translational modification leading to degredation. Our assays have detected AR multimerization in the presence of DHT and bicalutamide it would therefore be appropriate to test novel anti-androgens such as enzalutamide and the now defunct AZD3514 for multimerization and SUMOylation potential.

Assessing the impact of heat shock proteins on ER dimerization

Interestingly, HSP90 inhibition by geldanamycin treatment did not produce dramatic changes in receptor interactions. While ERα levels slowly and steadily declined after treatment with geldanamycin, there were no large spontaneous increases in BRET signal

in receptor-receptor or receptor-coactivator interactions in the absence of ligand treatment except for a small uptick in trimerization BRET. Studies of MCR have implicated HSP90 in keeping the receptor monomeric while it shuttles to the nucleus [356] so it may be that with ERα constitutively localized to the nucleus there are no constant interaction with nuclear HSP90 to prevent dimerization. In this scenario we could imagine apo-ERα being free to form dimers but requiring transient interactions with HSP90 to refold and stabilize the receptor when the LBD and AF2 domain's hydrophobic residues become exposed and misfold. In this scenario inhibition of HSP90 does not lead to increased dimerization in the absence of ligand but ERα protein will steadily be degraded as it cannot fold properly after translation or if it misfolds afterwards without hope of rescue. Yet HSP90 may have a greater role to play in ERα activity such as DNA binding or coactivator recruitment [213]. ChIP experiments – to verify ERα DNA positioning – and GRO-seq experiments – to monitor early activation of estrogen target gene – after HSP90 inhibition would contribute greatly to our understanding of the HSP90-ERα relationship and how it contrasts with other SHRs.

PCA-BRET for heterodimer studies

Our development of PCA systems for NR extends beyond the SHRs. Originally the SHR were put in PCA as a quality control for the NR PCA assays set up for RXR and RXR-partner receptors. Rexinoids have shown potential as therapeutic agents against neurodegenerative diseases such as Parkinson's and Alzheimer's disease or as a potentializer of rexinoid action. Unfortunately the ubiquity of RXRs in human tissues

causes undesired side effects. To side-step this problem we thought of designing a system that could identify heterodimer selective rexinoids; ligands that would only activate RXRγ-Nur77 dimers. While the original BRET1 system allowed for screens and counter-screens to easily separate pan-rexinoids and RXRγ-selective rexinoids the Nur77 activating potential of hits would not be resolved. Using a Nur77-F1LucII and F2LucII-RXRγ pairing and a YFP-tagged cofactor could discover both RXRγ and Nur77 ligands and allosteric modulator respectively – it is predicted that Nur77 LBD is unable to accommodate ligands and rely on control by RXR and post-translational modification to modulate transcriptional activity [133]. This setup works differently than the BRETFect dimer system discussed previously; BRETFect will give information concerning all luciferase-tagged receptor in any dimer confirmation (and the TFP-tagged receptor in FRET reading) the PCA system will only return information from the specific heterodimer of interest. In this fashion even if the dimeric conformation of interest constitutes a small fraction of our receptors its signal can be exclusively observed instead of being drowned out by other complexes. Heterodimer-selectivity of the hits can be assayed versus control dimers and receptors such as RXRγ-RAR and RXRα. The Nur77-RXRγ screen should proceed spring-summer 2014.

While initially tested with the LXXLL-YFP acceptor as a cofactor, the Nur77-RXRγ assay has been optimised for another cofactor: the AF1-ID-YFP cloned from the AF1-interacting glutamine rich domain of SRC-1. This factor appears to be particularly well suited to the assay because Nur77 interacts with cofactors solely through the AF-1 domain and this construct does not show recruitment to RXRγ in BRET1. We cannot determine whether

the AF1-ID construct is recruited to RXRγ or Nur77 in this setup but the recruitment is rexinoid-dependent (9cis-retinoic acid and bexarotene were validated as non-selective positive controls).

Nuclear receptor higher order complexes

Potential roles for multimerization in nuclear receptor action and biology

Nuclear receptor multimerization is not novel; as we stated, RXR and THR have both been described as forming tetramers in vitro. Yet their tetrameric form appears to be inhibitory to their action and tetrameric RXR and THR [168, 169] form a reserve of inactive receptor that can switch to active dimeric complex upon ligand biding. Our work shows that at least 5 of the 9 SHR form multimers, preferably when activated. AR showed the clearest ligand modulation of multimerization as energy transfer was insignificant in absence of agonist – or no greater than with the theoretically non-interacting ERα partnering. Moreover, the AR multimerization analysis reveals an equilibrium state of 4 units per multimer. In this manner, it may structurally mimic the THR and RXR tetramer that make the ternary contacts exclusively through their LBDs. An alternative mode of tetramerization could go through the alternative dimerization interface described for the glucocorticoid receptor [369] although in this case the complex would not be symmetrical and only one receptor of each dimer would make contact with the other dimer. This may be less likely as, unless allosteric effects prevent it, an extra surface would be available for multimerization on the receptor subunit not binding the second dimer leading to the addition of another dimer and so on… Yet, our experiments indicate a tetrameric form

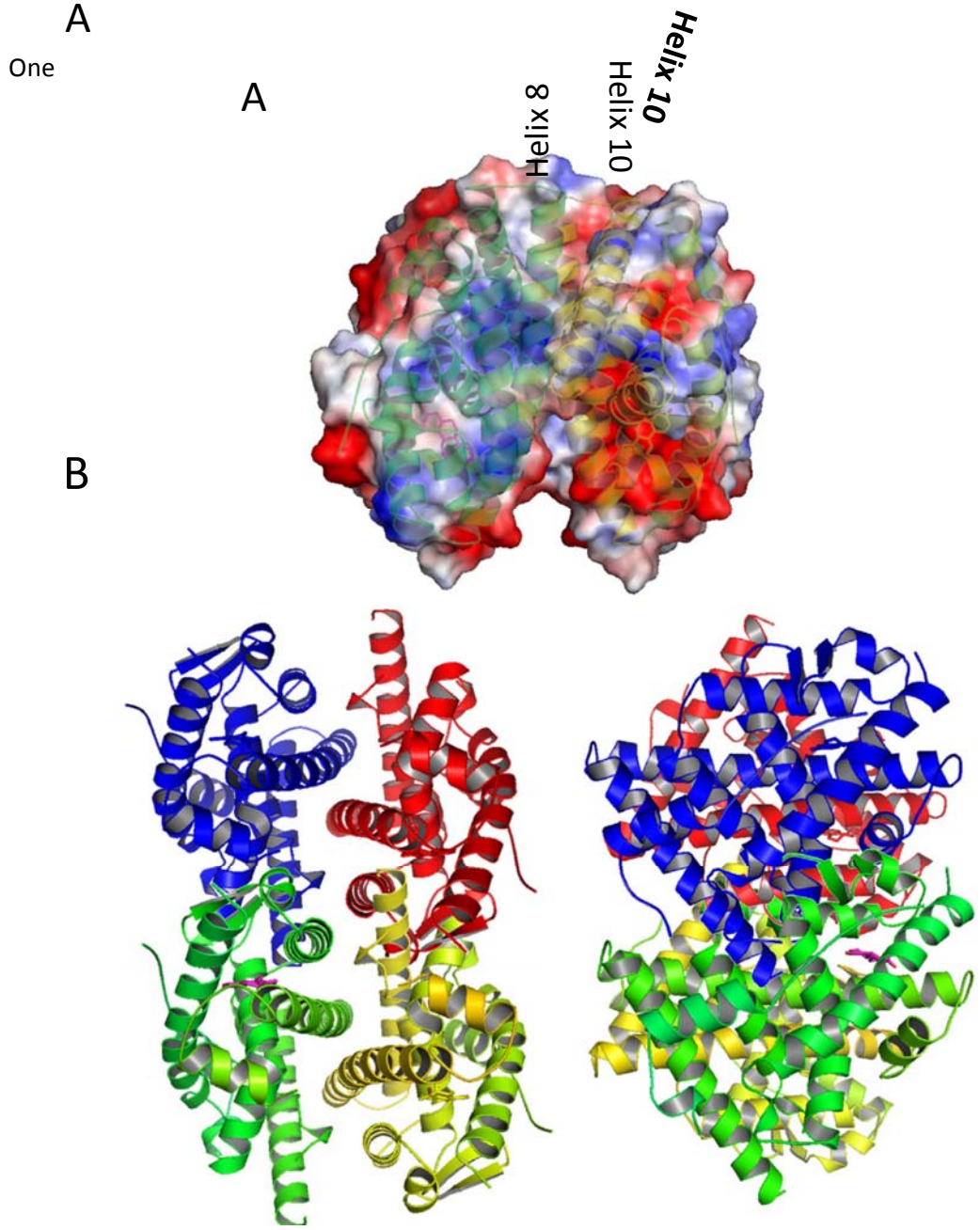
for AR – although the lack of energy transfer maybe due to physical constraints. Still, we cannot dismiss the possibility that tetramers may associate with more receptors; the answer in the receptor complex stoichiometry may reside in the function the receptor multimers serve.

The most informative SAR we obtained on multimerization came from the ERα mutant at residue 351 (D351L and D351K). While this residue was described as a stabilizer for helix H12 folding in the presence of estrogens and SERMs, to enable coactivator recruitment in the former [145] case and to modulate antiestrogenicity in the latter [370-372], its role does seem to extend to multimerization. Two hypotheses could explain this: aspartate 351 is part of the multimerization interface or positioning of helix H12 is a prerequisite for multimerization. Aspartate 351 being part of the multimerization interface would require an interface different than the ones previous proposed modeled after GR and THR/RXR as it does not sit along those described surfaces in crystal structures of ERα. Moreover, closing off the aspartate 351 area by binding of more receptor units would sterically hinder the access of coactivator motifs to the their binding groove. Still, these predictions are based on the ERα dimer crystal structure which may be much different from ERα as multimers. Conversely if aspartate 351's inability to stabilize helix H12 leads to multimerization it would fit the theory that ICI182,780 destabilization of H12 leads to anomalous multimers and insolubility of the receptor. In this fashion restoring H12 stability by mutations in leucine 536 and 539 would lead to the re-establishing proper multimerization which appears to agree with our data. A major difference we report from previous publications is that D351L and K [145] are not completely inactive and can

achieve 20-30% activity in the presence of E2 and OHT vis-à-vis the WT receptor, though in the absence of any ligand the mutants show no basal activity whatsoever. The complete lack of basal activity correlates well with the trimerization deficiency pattern observed by BRET under vehicle and tamoxifen treatment. It also correlates with the absence of coactivator motif recruitment by measured by BRET for vehicle and OHT condition but OHT transactivation does not require recruitment of coactivator to the LBD. Contrary to the D351L mutant, the L536A mutant displays increased activity in absence of ligand, increased trimerization signal and coactivator affinity in vehicle condition. This lets us theorize that 1) multimerization is not dependent on coactivators binding and bridging receptor dimers together, 2) multimerization is required for proper transactivation regardless of the receptor (AF1 and AF2 domain action included) 3) multimerization is required for recruitment of whole coactivator protein to target genes.

A

One



missing piece of this puzzle is how multimerization may affect SHR's chromatin interactions.

Figure 1. Possible multimerization interface and complexes of ERα.

(A) ERα multimer interface suggested from THR multimer studies. Helices 10 and 8 protrude the surface and could be the basis for multimerization. (B) Schematic of two ERα dimers into a multimer using the predicted THR tetramerization interface.

An inviting explanation of chromatin looping between HRE could be the direct linking of different dimers bound to HREs by multimerization. It has now been demonstrated that dimers of GATA3 [407] can bridge two distal elements without requiring additional cofactors. Such a mechanism maybe in action for SHRs as in the case of ERα's regulation GREB1 expression through an enhancer containing 4 verified EREs that under E2 treatment loop together and link the receptors to the transcriptional start site [174]. Interestingly the receptor does appear to be positioned at the HRE in the absence of E2 but they do not loop together; E2 treatments will (slightly) increase the detection of ERα on EREs but it is the linking of EREs that is most dramatically increased. We could even hypothesize that:

- Like their close cousins the estrogen-related receptors, ERα can easily bind EREs in the absence of hormone and thus can be detected by ChIP.
- Treatment with estrogens causes conformational changes to strengthen DNA binding which has been measured by ITC.
- Estrogen binding also stabilizes multimeric form of the receptor composed of two – or more – dimers biding their own EREs and looping the chromatin which is detected by chromatin conformation capture (3C).

Multi-ERE binding in this fashion could also stabilize DNA interactions by avidity effects as numerous DBDs will be brought in close proximity to several EREs. Avidity would increase the affinity of ERα for coactivators in the multimeric form which could also accommodate more LXXLL motifs (SRC1 has 7!) (Fig. 3).

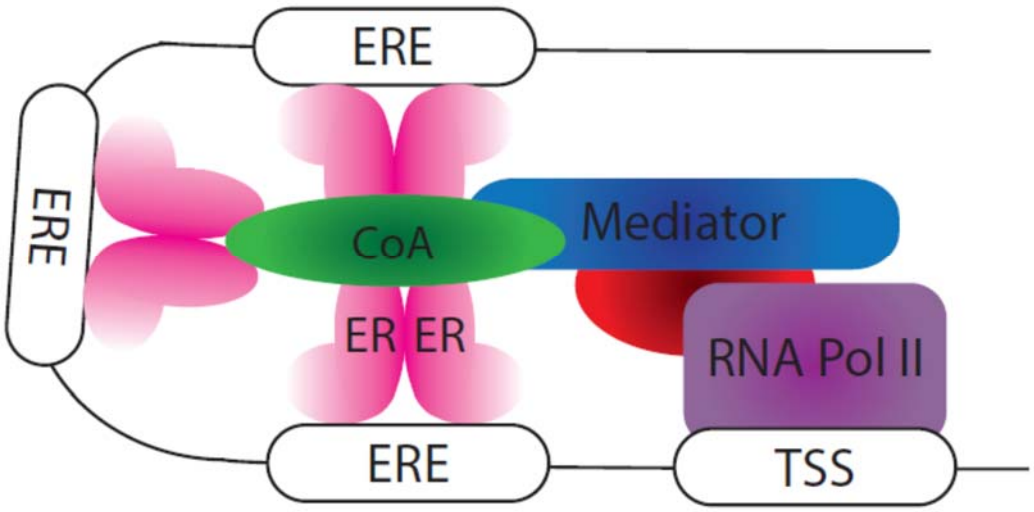


Figure 2. ER multimers looping chromatin and stabilizing a coactivator.

Experiments detecting ERR homomultimers and heteromultimers between ERRs and ERs indicate that multimers can be formed between different receptors of the same subfamily but appear to only form if the dimer studied contained the acceptor-tagged receptor; ERβ-ERRα heterodimers could recruit ERβ or ERRα but ERRα homodimers did not recruit ERβ and vice versa. This suggests that multimerization interface may only be compatible between ERs while the dimerization interface is more promiscuous and allows ERRα-ERβ pairing. The strong BRET signals of ERR-ERβ recruitment of ERRα and ERβ in the absence of treatments suggests that this complex is constitutive. This may be due to ERRs' constitutive activity which could allosterically activate ERs and cause formation of the heteromultimer. As multimerized receptors have thus far appeared more active, this potential ERR transactivation of ER could explain the AE resistance observed in a subset of ERRα overexpressing tumors [408]. As ERR expression increases and more ER-ERR heterodimers are formed that constitutively occupy ERE's and form multimers they activate estrogen target genes.

Nuclear receptor biology is complex, which is probably a prerequisite for their action; without networking complexity, 48 receptors could only operate 48 different genomic programs. To operate a tissue-, time- and context-specific program, nuclear receptors are required to interact with a plethora of cofactors and dimerization partner. It is now apparent that multimerization is a mechanism of nuclear receptors that adds an extra layer of possible regulation.

Hybrid HDACi-NR ligands

AE-HDACi molecules

The advantages of BRET assays over luciferase assays became clear in the development of AE-HDACi molecules; the action of HDACis alone – such as TSA or SAHA – can have impacts on luciferase reporter gene expression. In our case, which consisted of ERE-driven luciferase reporter, HDACi caused massive ER-independent increases in luciferase expression (data not shown). This made it impossible to assess if a molecule was estrogenic or had HDACi activity. The BRET assay was not affected by HDACi activity and allowed for a more quantitative measurement of AF2 and AF1 activity in the presence of hybrid molecules. Moreover, the PCA assay to measure receptor conformation provided a simple and efficient way of measuring the potency and cell entry kinetics of hybrid molecules. These assay should prove to be an improvement on luciferase reporter system for the screening and SAR of novel NR agonists and antagonists.

While we cannot report all molecules developed to simultaneously inhibit ERα and HDACs due to intellectual property issues, we would assert that it is absolutely possible to optimize antiestrogenicity of the hybrid while preserving a good HDAC IC50 through SAR of the hybrid side-chain. The side-chain length is the main factor dictating antiestrogenicity of the molecules as we previously reported. Yet the substitution of the terminal group with hydroxamic acid or aminobenzamide groups appeared to prevent SERD like activity and receptor multimerization and SUMOylation did not match those of their methyl derivatives. Moreover it was difficult to avoid partial agonism of the molecules for AF2 activity and LXXLL motif recruitment which may be due to the helix H12's inability to assume SERD [338] or classical SERM (OHT-like) position [132] with a large polar group at the end of the hybrid side-chain. It may be that only relatively small groups can accommodate the SERD side-chain binding pocket we identified in previous studies; large HDACi "warheads" cannot accomplish this. While we may not be able to generate SERD-HDACi hybrids, the HDACi action of the molecule may yet allow for receptor downregulation through ESR1 promoter silencing and receptor inactivation. So we may approach the problem from another direction and ask can we add HDACi activity to a SERM in the future.

Parallel studies on HDACi and AE combination have demonstrated synergy between the two in the inhibition of ER+ breast cancer cell lines [409]. Further studies to characterize the action of the two (SAHA and ICI182) and of our more potent hybrids on cell cycle by FACS revealed in part how they act on cells (Fig. 3). SAHA acts very quickly to

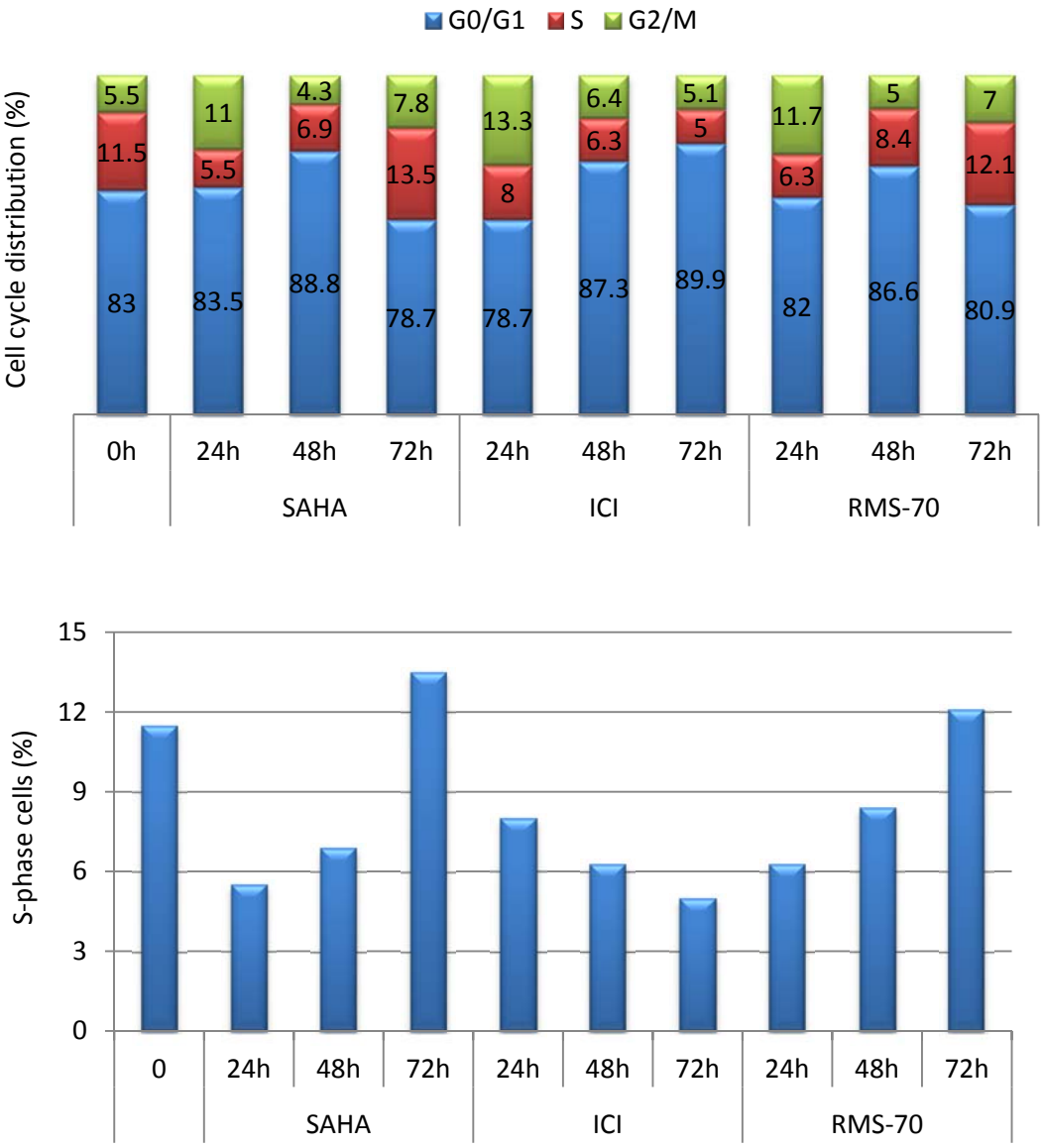


Figure 3. RMS-70 modulates cell cycle similarly to SAHA.

(A-B) MCF7 cells were treated with 1 µM SAHA, 1 µM ICI182,780 or 10 µM RMS-70 for 24 to 72 hours, fixed in cold ethanol and DNA was stained with propidium iodide. Cell cycle was analyzed by FACS.

stop growth and causes cell death but it is metabolize quickly and within 48 hours after treatment S-phase is repopulated as cancer cells start proliferating again. SERD activity takes longer to stop cell growth and does not cause much cell death but it lasts well beyond 72 hours as all cells appear to stay in G1/G0 phase. We can hypothesize that some of the synergy observed in combinatorial treatment is due to SERDs keeping the surviving cells from re-entering the cell cycle. When a second treatment of HDACi is given fewer cells are left to kill and the effects are amplified. Treatments with ICI will also downregulates anti-apoptotic genes such as Bcl-2 leaving cells more vulnerable to pro-apoptotic signals from HDACis. In our FACS assays hydroxamate-AE hybrid molecules showed profiles more akin to SAHA alone than ICI182 or combination treatment.

While AE have good half-lives in general [410, 411] (12 hours for tamoxifen and 40 **days** for ICI182), hydroxamic acid HDACis are quickly metabolised by glucuronidation of the hydroxamic group [412]. Thus addition of hydroxamate groups to AE can have the unwanted side effects of lowering the half-life of the antiestrogenic action of the molecule in favor of fast but short acting HDACi action. This hypothesis would explain the effect observed in FACS with our hybrids as they inhibit HDACs like SAHA but are quickly inactivated thus no long lasting antiestrogenic effect are seen. It is doubtful that a synergy on par with the SAHA-ICI182 pair will be observed in AE-hydroxamic acid derived hybrids. Fortunately, the alternative HDACi “warhead”, benzamides, show a dramatic increase in half-life compared to hydroxamates – 30 hours for entinostat [413]– thus these molecules hold more promise and should be prioritize.

We must note that contrary to some publications we have not observed re-expression of ESR1 following the treatment of ER-negative cells [414] with SAHA or hybrid compounds but the hybrids did have growth inhibitory potential in these cells similar to that of HDACis confirming their “hybridicity”. Moreover, while they may not act as great AE due to short half-lives the hybrid AE-HDACi molecules may supplant conventional HDACis by improving their pharmacokinetics. HDACis, and especially hydroxamic acid-types, have shown poor efficacy against solid-tumors which may be a result of poor tissue distribution, conversely, AE such as tamoxifen are quite efficacious in solid tumors. The combination of the two may hinder the antiestrogenic activity through metabolism but enhance HDACi efficacy by improving tissue distribution and penetrance compared to standard HDACi molecules.

Ret-HDACi molecules

Our retinoid-HDACi hybrid fared much better than the AE hybrid; it retained both retinoicity and HDAC inhibition in the same concentration range as the parent compounds. Moreover, TTNN-HA appears to display the HDACi-retinoid synergy observed in retinoid sensitive (SkBr-3) and insensitive (MDA-MB-486) breast cancer cell lines. Yet, TTNN-HA is less potent than pure HDACi such as SAHA (6-fold increase in IC50 against HDAC3 and 15-fold against HDAC6) but is still comparable in growth inhibitory potential in cancer cells to SAHA. Moreover, healthy tissue-derived HMEC and normal-like 184b1 cells showed more resilience to TTNN-HA treatment than cancer cells while SAHA was more cytotoxic to normal cells than cancer cells.

Retinoids and HDACi may be a better fit for a hybrid – compared to AE and HDACi – on the basis of their similar pharmacokinetic profiles with short half-lives. Thus, modification of the retinoid with a hydroxamate group does not change its half-life and interfere with its signalling dynamics. Yet one clear disadvantage of the added chemical group was a dramatic increase in EC50 of TTNN-HA to activate RAR recruitment of coactivators compared to the parent molecule TTNN. The retinoicity of TTNN-HA does appear to play an important part in the overall action of the hybrid as co-treatment of TTNN-HA with the pan-RAR antagonist BMS493 did increase cell proliferation compared to TTNN-HA treatment while BMS493 alone did not have an effect. This effect was not only observed in retinoid sensitive MCF-7 cells but also in retinoid insensitive MDA-MB-231 cells suggesting that some manner of retinoic acid signalling was restored in these cells after treatment with TTNN-HA. It would be of interest to use receptor specific antagonist in the future to identify which receptor subtypes are important for synergy with HDACi activity.

The detailed mechanism of the TTNN-HA growth inhibition remains mysterious as we do not have detailed genomic analysis of cells treated with the molecules. While we did consider performing microarray analysis the question of what to use as retinoid and HDACi parental controls remained unanswered. TTNN would probably be a suitable parental compound when used at levels that would activate RAR signalling similarly to TTNN-HA as it appears to retain the receptor subtype selectivity of TTNN. The HDACi parental control is harder to define. In our experiments we used SAHA as it was to date

the only FDA approved HDACi to treat cancer. Yet, SAHA, in the concentration range that inhibits proliferation, is not selective for any HDAC while TTNN-HA, in similar conditions, inhibits HDAC6 but not HDAC3. The parental control should display similar HDAC selectivity as TTNN-HA which would require a thorough characterization of TTNN-HA's HDACi activity. Alternatively, we could produce TTNN-HA derivatives with lower affinity for RARs. These molecules should not activate the RA pathways but could conserve the HDACi pharmacokinetics/dynamics of TTNN-HA.

The ret-HDACi synergy displayed by TTNN-HA may not work entirely at a genomics level if class II HDACs which act in the cytoplasm are the only enzymes significantly inhibited. Like the synergy between HER2 inhibition (by trastuzumab or lapitinib) and ATRA [415] and (*Roozendaal et al manuscript in preparation*), TTNN-HA combinatorial action may depend on genomic events being reinforced by post-translational event in the cytoplasm. In this case proteomic analysis would be required to elucidate the mechanism.

The wide array of subtype selective HDACis now available can be used to identify the HDAC(s) crucial to retinoid-HDACi synergy (few selective molecules were available at the time TTNN-HA was studied). This in turn would streamline the evaluation of new hybrids as they would only need to score a good IC50 against one enzyme. Moreover, subtype selective hybrids may incur fewer side-effects and show less cytotoxicity in normal tissue; it is noted that TTNN-HA proliferation IC50 in normal cells is similar to its IC50 on HDAC3.

Another factor that may explain ret-HDACi hybrids increased efficiency over AE-hybrids is the type of action exerted on the receptor: agonism versus antagonism. As an antagonist AE must exert their action on ERα constantly to counteract the proliferative effect of endogenous estrogens, as soon as it is metabolised the oncogenic signalling is restored and growth resumes – which may be why clinically effective AE have long half-lives and high affinities for ERα. Meanwhile, the retinoid activates a genomic program that is antiproliferative and that is not solely carried out by the retinoic acid receptors but also by their primary target genes, some of them transcription factors that also exert antiproliferative effects. In this fashion the NR ligand need only be present for a short period of time to activate the program, after being metabolized the effects will still be felt while the cells "wait". In this frame of mind the development of nuclear receptor ligand-HDACi hybrid should focus on agonist of NR that show growth inhibitory potential in cancer cells such as LXR and Nur77.

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