UNIVERSITY OF CALIFORNIA, SAN DIEGO

Molecular Mechanisms of Ligand-Dependent Repression by the Estrogen Receptor and Derepression by Inflammatory Signaling

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

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2010

The Dissertation of Eliot Michael Bourk is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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TABLE OF CONTENTS

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LIST OF FIGURES

VITA

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ABSTRACT OF THE DISSERTATION

Molecular Mechanisms of Ligand-Dependent Repression by the Estrogen Receptor and Derepression by Inflammatory Signaling

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Eliot Michael Bourk

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2010

Professor Michael G. Rosenfeld, Chair

Uncovering the linkage between inflammatory signaling pathways and transcriptional regulatory programs is key to understanding the role of inflammation in homeostasis and disease. An intriguing aspect of this connection has emerged with the identification of a cohort of genes that exhibit direct negative regulation by liganded ERα that can be reactivated by specific inflammatory signals. We found that ERα-dependent repression of the *BMP7* gene, which is overexpressed in up to 70% of

primary breast tumors and is reactivated by inflammatory signaling, utilizes an LXXLL motif-containing orphan nuclear receptor, SHP, as a corepressor to recruit the NCoR/TAB2 complex to the *BMP7* promoter. In response to inflammatory signals, transient association of the ubiquitin ligases TRAF6 and Ubc13 with the TAB2-containing NCoR complex on the *BMP7* promoter catalyzes K63-linked polyubiquitination of TAB2, permitting promoter-specific recruitment of MEKK1 and licensing clearance of the NCoR complex. We have identified similar inflammation-induced reactivation of additional ERα-repressed genes, including *BAK1*, *NCOA3*, and *ZNF217*, which also exhibit TRAF6-dependent derepression. We suggest that this TAB2/TRAF6/MEKK1 corepressor clearance pathway regulates specific gene expression programs in response to diverse stimuli and in other cell types, including the hematopoietic and nervous systems, identifying a novel pathway for selective gene activation by inflammatory signals.

INTRODUCTION

Precise control of gene expression is essential for proper differentiation and function of cells, and dysregulation of gene expression is a common feature of many diseases, including cancer. Transcriptional regulation is achieved by a complex network of interactions between genetic and epigenetic elements, transcription factor and cofactor availability and competition, and receptor-mediated signal transduction pathways.

Nuclear steroid hormone receptors serve both as transcription factors and as ligand-dependent switches for coordinating patterns of gene expression in response to hormonal cues, and drive the growth of certain hormone-dependent cancers, including those of the breast, ovary, and prostate. The activity of nuclear receptors is modulated not only by their chemical ligands but also by signal transduction pathways, enabling an additional layer of regulatory control over their transcriptional output.

A growing area of interest in many fields of disease research is the contribution of inflammation to disease development and progression. Leukocytes such as macrophages and lymphocytes infiltrate tumors and secrete pro-inflammatory cytokines, including interleukin-1β (IL-1β), that contribute to invasion and metastasis. Interfering with the signaling pathways downstream of these inflammatory mediators may prove to be an effective strategy for preventing tumor progression, but first it is necessary to elucidate the molecular events downstream of inflammatory signals that contribute to aberrant gene expression.

In the following chapters I present evidence of novel corepressor-mediated strategies by which the estrogen receptor α (ERα) represses transcription of a subset of its target genes. I also describe a molecular pathway by which inflammatory signals, through a ubiquitin-dependent signaling cascade, lead to the reactivation of ERα-repressed genes, but not ERβ-repressed genes. Finally, I identify other genes that are coordinately regulated as part of the ERα-repression program and show that their reactivation by inflammatory signaling depends on a similar mechanism.

Some of the genes represented in this program, including BMP7, NCOA3, and ZNF217, are associated with invasion, metastasis, and survival of tumor cells, and are frequently overexpressed and amplified in breast cancer. Reactivation of these genes from ERα-mediated repression *in vivo* by tumor-infiltrating leukocytes may contribute to their increased expression in cancer.

The signal transduction pathway downstream of IL-1β is similar to those activated by other plasma membrane receptors such as Receptor Activator of NF-kB (RANK), p75 neurotrophin receptor, Toll-like receptor 4 (TLR4), and the T and B cell antigen receptors, suggesting that a similar transcriptional derepression program may operate in other cell types that express these receptors.

Chapter 1:

Macrophage/Cancer Cell Interactions Mediate Hormone Resistance by a Nuclear Receptor Derepression Pathway

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SUMMARY

Defining the precise molecular strategies that coordinate patterns of transcriptional responses to specific signals is central for understanding normal development and homeostasis as well as the pathogenesis of hormone-dependent cancers. Here we report specific prostate cancer cell/macrophage interactions that mediate a switch in function of selective androgen receptor antagonists/modulators (SARMs) from repression to activation in vivo. This is based on an evolutionarily conserved receptor N-terminal L/HX$_7$LL motif, selectively present in sex steroid receptors, that causes recruitment of TAB2 as a component of an N-CoR corepressor complex. TAB2 acts as a sensor for inflammatory signals by serving as a molecular beacon for recruitment of MEKK1, which in turn mediates dismissal of the N-CoR/HDAC complex and permits derepression of androgen and estrogen receptor target genes. Surprisingly, this conserved sensor strategy may have arisen to mediate reversal of sex steroid-dependent repression of a limited cohort of target genes in response to inflammatory signals, linking inflammatory and nuclear receptor ligand responses to essential reproductive functions.

INTRODUCTION

The pattern of transcriptional response to the multiple signaling factors impacting each cell reflects, in part, an ability to integrate these inputs into a coordinated program of gene activation and repression. The actions of nuclear receptors have provided an ideal model in which to investigate this question, as androgen and estrogen receptors can bind both to agonists, such as dihydrotestosterone (DHT) and 17-β-estradiol (E_2), or to selective androgen and estrogen receptor modulators (SARMs, SERMs), which act as antagonists or as weak agonists in a context-dependent fashion.

Androgens, acting via androgen receptor (AR), are essential for normal growth and function of the prostate gland and in all animal models of prostate carcinogenesis. While androgen ablation is a standard treatment for prostate cancer (Feldman and Feldman, 2001; reviewed in Debes and Tindall, 2004), there is an invariant progression from androgen-dependent to androgen-independent growth, even though high levels of AR generally persist (Feldman and Feldman, 2001; Chen et al., 2004; reviewed in Debes and Tindall, 2004). Resistance to antiandrogen treatment has been postulated to reflect diverse mechanisms such as changes of AR expression and functions (silencing of the AR gene, mutations in the AR sequence, and increased levels of AR), alterations of levels of nuclear receptor cofactors, activation of growth factor or kinase pathways, and decreased expression of tumor suppressors or increased expression of antiapoptotic genes (Feldman and Feldman, 2001; Chen et al., 2004; reviewed in Debes and Tindall, 2004); but each of

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these could only account for a subset of resistance events. Analogous to other members of the nuclear receptor superfamily, AR actions require AF-2 transcription-activation function and an extended N-terminal domain with a strong AF-1 function (Onate et al., 1998; Bevan et al., 1999). Agonists and antagonists induce different conformations of helix 12, with antagonists blocking AF-2 function by preventing formation of an effective ''charge clamp'' for the LXXLL interaction motif (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998).

Investigation of active repression of gene expression by unliganded nuclear receptors has led to the identification of the nuclear receptor corepressors, N-CoR (Hörlein et al., 1995) and SMRT (Chen and Evans, 1995; Sande and Privalsky, 1996), which contain multiple repressor domains that could transfer their active repression function, recruiting histone deacetylases (HDACs). Based on genetic analyses, N-CoR proves to be required for the inhibitory function of estrogen receptor (ER) antagonists (SERMs) (Jackson et al., 1997; Smith et al., 1997; Lavinsky et al., 1998; reviewed in Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). N-CoR has been found as a component of many complexes, including TBL1/TBLR1/HDAC3/GPS2 (Guenther et al., 2000; Li et al., 2000; Wen et al., 2000; Zhang et al., 2002; Yoon et al., 2003), TAB2/HDAC3 (Baek et al., 2002), Sin3/HDAC1, 2 complexes (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), complexes containing SWI/SNF-related proteins (Underhill et al., 2000), and multiple other complexes (reviewed in Jepsen and Rosenfeld, 2002). Sequences referred to as the CoRNR box (Hu and Lazar, 1999) or alternatively as LXX I/H I XXX I/L motifs (Nagy et al., 1999; Perissi et al., 1999), appear to bind in the hydrophobic pocket that is occupied by the coactivator LXXLL helical motifs upon binding of ligand.

Recently, it has been recognized that there is an increased monocyte/macrophage infiltration in the adipose tissue of obese individuals and that these macrophages may be a major localized source of the inflammatory cytokines that are linked to insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Among their phenotypic actions, proinflammatory signals can derepress genes regulated by the p50 homodimer of NF-κB DNA binding factors by dismissal of the N-CoR complex (Baek et al., 2002), influencing the behavior of prostate cancer cells in vitro (Kim et al., 2005). Based on analysis of *IKKβ* gene-deleted mice, interruption of the IKK/NF-κB pathway attenuates inflammation-associated tumors (Greten et al., 2004). It is thus of particular interest to determine whether a macrophage/prostate cancer cell interaction occurs if it is a common event in prostate cancer and whether this serves to elicit inflammatory signals capable of impacting the therapeutic effectiveness of SARMs. These compounds typically function initially as antagonists in vivo but almost invariably become ineffective over a period of time (Feldman and Feldman, 2001; Chen et al., 2004; reviewed in Debes and Tindall, 2004).

In this manuscript, we establish that specific interactions between prostate cancer cells and macrophages appear to occur in most prostate cancers examined and convert SARMs to function as agonists by activating a functional program of specific proinflammatory signals that cause dismissal of the N-CoR holocorepressor complex from AR. This derepression effect requires TAB2 (Takaesu et al., 2000), recruited to steroid hormone receptors through a specific, evolutionarily conserved, N-terminal L/HX_7LL motif found in sex steroid receptors but not in other nuclear receptors. This motif serves as a discriminatory molecular beacon for specific proinflammatory cytokine signals, modulating a coordinated program of gene derepression. One evolutionary basis for this signal processing module appears to be the importance of inflammatory signal-dependent derepression of a cohort of sex steroid-repressed genes in reproductive biology.

RESULTS

Macrophage/Prostate Cancer Cell Interaction Causes Resistance to SARMs

To examine potential direct interactions between macrophages and prostate cell lines, we initially used RWPE1-transformed prostate cells and fluorescein-labeled THP-1 monocytes/macrophages and found a specific RWPE1/macrophage interaction (Figure 1A). Pretreatment of RWPE1 cells with TNFα, LPS, or IL-1 induced the expression of *VCAM-1* (data not shown) and enhanced the engagement of macrophages (Figures 1A and 1B). We found that these interactions were blocked by addition of a specific VCAM-1 monoclonal IgG (Figures 1A and 1B). These data raised the possibility that such interactions might also operate in vivo in prostate cancer. We therefore examined tissue arrays containing patient-matched sections with both normal and cancer-containing regions of prostates, stained for CD68, a specific marker of macrophages. This revealed that virtually 100% of the tumor samples exhibited macrophage infiltration as well as stromal interactions with macrophages (Figure 1C). There was much less interaction between macrophages and histologically ''normal'' cellular areas in the resected tumors (Figures 1C and 1D).

Therefore, we considered that macrophage/prostate cancer cell interactions might represent a possible in vivo mechanism involved in SARM resistance in prostate cancer. We explored whether the direct cell-to-cell interactions that we observed between macrophages and prostate cancer cells might cause changes in the activity of SARMs based on macrophage activation and release of proinflammatory cytokines. Nuclear microinjection studies were performed in RWPE1 cells pretreated with TNFα, where indicated, using a *prostate-specific antigen* (*PSA*) promoter-controlled reporter. Following microinjection of the *PSA* reporter, THP-1 cells were added to the prostate cells for 6 hr and then either DHT or bicalutamide was added for 6 hr. We observed the expected activity of the *PSA* reporter whether or not the cells had been pretreated with TNFα (Figure 1E), with a robust increase in activity after DHT, but not bicalutamide (Figure 1E). Addition of macrophages to untreated RWPE-1 cells clearly increased the basal activity, although less than TNFα-pretreated cells, due probably to lesser adhesion of macrophages to prostate cells (Figure 1A). In TNFα-pretreated

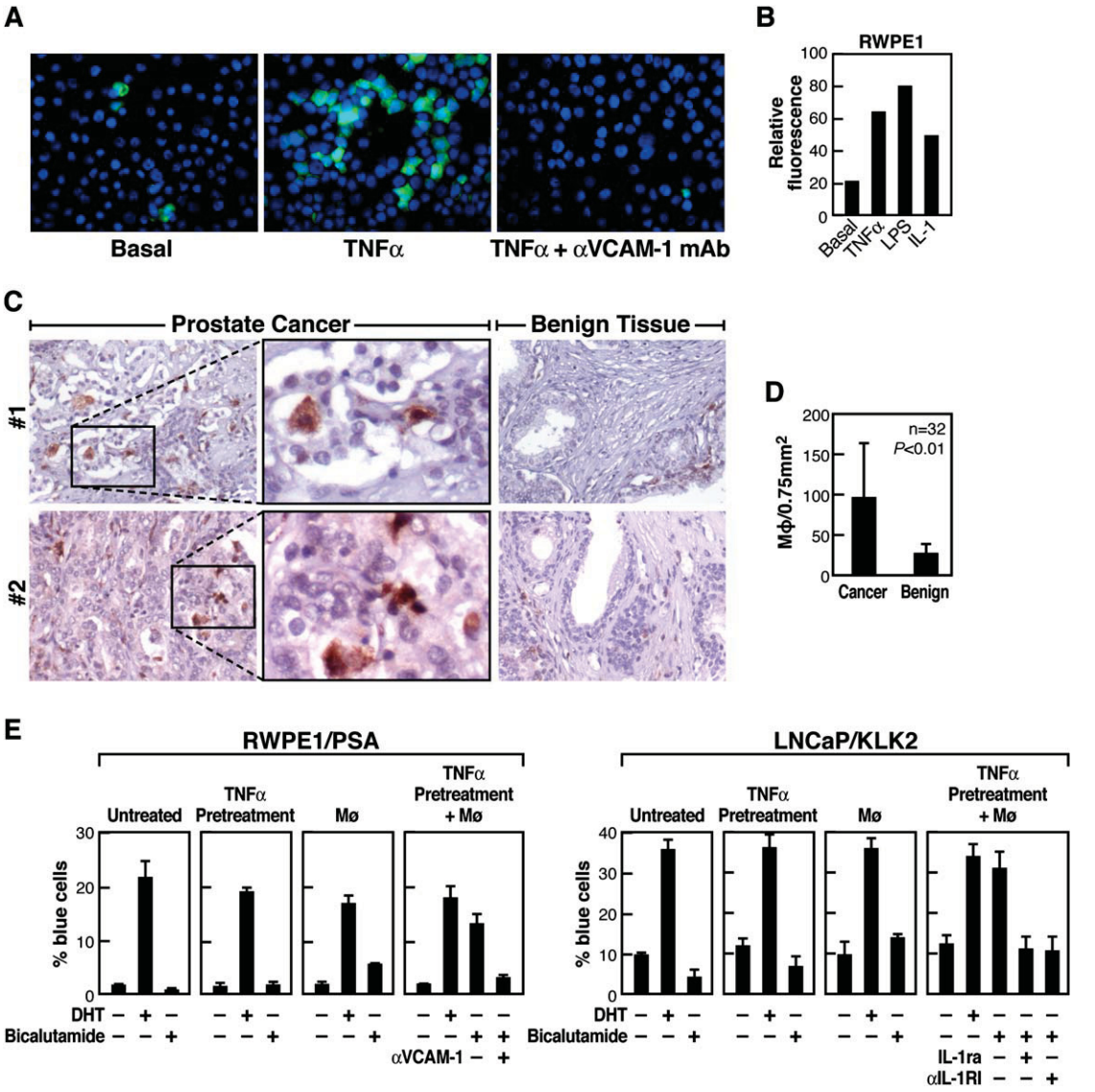

Figure 1. Macrophage-Prostate Cell Interaction Converts an AR Antagonist to an Agonist

(A) Interaction between fluorescein-labeled THP-1 monocyte/macrophages (green) and RWPE1 transformed prostate cells upon stimulations. DAPI staining (blue) indicates the cell nuclei.

(B) Quantitative analysis of relative fluorescence that represents the amount of macrophages engaged to prostate cells in (A). One representative of three independent experiments is shown.

(C) Tissue microarrays (A302) from prostate cancer patients were stained with macrophage-specific marker CD68. Representatives of cancer tissues and patient-matched pathologically benign tissues are shown. CD68+ cells appear in dark red. Higher magnification shows a specific macrophage-cancer cell interaction.

(D) Quantitative evaluation of infiltrating macrophages in prostate tissues of 32 patients. P value was calculated by Student's t test.

(E) Effects of macrophage/prostate cell interaction on AR antagonist. RWPE1 and LNCaP cells were single-cell microinjected with lacZ reporters driven by the *PSA* and *KLK2* promoters, respectively. After being incubated with THP-1 monocyte/macrophages and neutralizing antibodies against human VCAM-1 (αVCAM-1) where indicated, RWPE1 or LNCaP cells were treated with ligands and/or blocking reagents (IL-1ra: human IL-1 receptor antagonist; αIL-1RI: neutralizing antibodies against human IL-1 receptor type I). Data are represented as mean ± SEM.

RWPE1 cells cocultured with macrophages, there was an agonist-like induction of reporter activity in the presence of bicalutamide, which was reversed by the addition of VCAM-1 antibody prior to macrophage addition (Figure 1E). We observed similar effects using the promoter of another AR target gene, *KLK2,* in LNCaP cells (Figure 1E). To further test the role of IL-1β, one of the major cytokines secreted by macrophages that derepresses p50-dependent genes (e.g., *KAI1*), we blocked IL-1 signaling by adding IL-1 receptor antagonist (IL-1ra) or neutralizing antibodies to type I IL-1

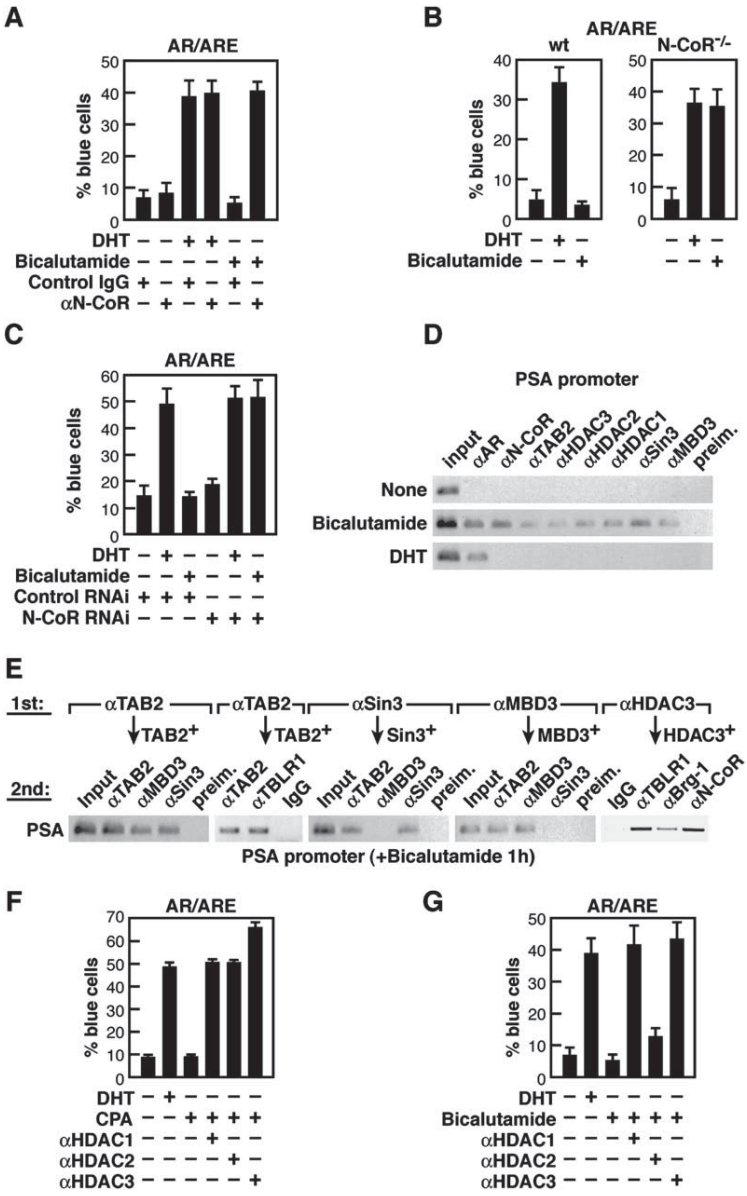

Figure 2. N-CoR Corepressors in AR Antagonist Actions

(A) Single-cell nuclear microinjection of αN-CoR IgG largely relieved the bicalutamide-dependent repression of an ARE-lacZ reporter.

(B) In N-CoR$^{-/-}$ MEFs, bicalutamide acted as an agonist.

(C) Microinjection of N-CoR siRNA resulted in a switch from repression to activation.

(D) Chromatin immunoprecipitation (ChIP) assay of AR occupancy on the *PSA* promoter in response to hormone treatment in LNCaP cells (bicalutamide: 10 μM; DHT: 100 nM for 1 hr).

(E) Serial 2-step ChIP assay to determine whether the different corepressor complexes are assembled on the same promoter. Soluble chromatin from LNCaP cells (treated for 1 hr with bicalutamide or CPA for samples using αTBLR1, αBrg-1, and αN-CoR in the 2nd ChIP) was first immunoprecipitated with indicated antibodies (1st IP) and reimmunoprecipitated with antibodies as shown (2nd IP).

(F) Microinjection of IgGs against HDAC1, HDAC2, or HDAC3 fully relieved the repression by CPA bound AR.

(G) In response to bicalutamide, HDAC1 and HDAC3, though not HDAC2, were required for repression. Data are represented as mean ± SEM.

receptor (α-IL-1RI) (Figure S1) into the medium after microinjection of reporters. Under those conditions, the macrophage-induced agonistic activity of bicalutamide was efficiently abrogated (Figure 1E), suggesting an important role of IL-1 signaling in resistance to SARMs.

IL-1β Converts Androgen Antagonists to Function as Agonists

Because IL-1β blocks the activity of corepressors including N-CoR (Baek et al., 2002), we then conducted mechanistic studies to investigate the role of N-CoR and other repressors in SARM-dependent transcriptional repression. Single-cell nuclear microinjection of specific α-N-CoR IgG abolished the repression of a reporter under control of an AR response element in the presence of bicalutamide (Figure 2A). AR antagonists behaved as agonists in MEFs from *N-CoR*$^{-/-}$ mice, stimulating reporter gene expression (Figure 2B). These results were validated using siRNAs against N-CoR (Perissi et al., 2004), which reversed bicalutamide-dependent repression function (Figure 2C), consistent with the finding that failure of N-CoR recruitment occurs in aggressive tumors with AR overexpression and antagonist resistance (Chen et al., 2004). To a lesser extent, SMRT was also required for SARM-dependent repression (data not shown).

We observed the presence of individual components of many distinct N-CoR complexes on the *PSA* promoter (Cleutjens et al., 1996) in bicalutamide-treated cells using chromatin immunoprecipitation (ChIP) assay (Figure 2D), but it was unclear whether these complexes are independently

recruited as distinct N-CoR complexes or combinatorially recruited as a holocomplex (Shang et al., 2002). Sequential ChIP assay of the *PSA* promoter revealed that many components that mark the independently isolated N-CoR complex, including the TAB2/HDAC3-, the TBL1/TBLR1-, the Sin3-, and the Brg1-containing complexes, were apparently corecruited in bicalutamide-dependent repression (Figure 2E). In contrast, an MBD3-containing complex (Zhang et al., 1999) may not be simultaneously assembled with other components, such as the Sin3 complex, on the *PSA* promoter (Figure 2E).

Injection of specific purified IgGs against HDAC1, 2, or 3 showed that blocking the actions of any of these HDACs relieved cyproterone (CPA)-dependent repression, while HDAC1 and 3, but not HDAC2, were required for bicalutamide-mediated repression (Figures 2F and 2G). Because HDACs that lack functional HDAC activity failed to rescue the knockdown effects of each HDAC siRNA in the presence of bicalutamide or CPA (Figures S2A, S2B, and S2C) and functional HDAC2 and HDAC3 failed to compensate for the loss of HDAC1 activity (Figure S2D), distinct HDAC enzymatic activities are suggested to be combinatorially required for maintenance of the antagonistic effects of SARMs on AR.

Recombinant IL-1β ''switched'' bicalutamide or CPA to function as an agonist, and this switch could be overcome by overexpression of N-CoR (Figure 3A). Upon stimulation by IL-1β, there was a progressive and complete dismissal of all components of the N-CoR-containing complex recruited by SARM bound AR for the *PSA* promoter assessed using ChIP assay (Figure 3B). N-CoR was detected in both nuclear and cytoplasmic locations in either nontreated or SARM-treated LNCaP prostate cancer cells but became more preferentially localized to cytoplasm after IL-1β treatment (Figure 3C). Consistent with the observation that TAB2 is a substrate for MEKK1, potentially causing the exposure of a nuclear export signal (Baek et al., 2002), IL-1β-dependent loss of the N-CoR complex from AR was accompanied by a transient recruitment of MEKK1 (Figure 3B). Intriguingly, MEKK1 remained transiently associated with the promoter after dismissal of TAB2, which may reflect subsequent MEKK1/coactivator interactions, as MEKK1/Tip60 interactions can be detected by coimmunoprecipitation analysis (Figure S3A). Injection of either αTAB2 or αMEKK1 IgG abolished the activation of an androgen-dependent reporter in the presence of IL-1β and SARM (Figure 3D). Similarly, IL-1β-dependent agonistic activity of SARMs was abolished using MEFs either from *MEKK1* gene-deleted mice (Figure 3E) or from *TAB2* gene-deleted mice (Figure 3F), supporting the model that MEKK1 recruitment to TAB2 is required for the removal of N-CoR complexes and derepression of AR target genes. In *TAB2*$^{-/-}$ MEFs, the SARM bicalutamide acted as an antagonist even with IL-1β treatment unless TAB2 was reexpressed or N-CoR/SMRT expression was abrogated by microinjection of specific siRNAs (Figure 3G). In *MEKK1*$^{-/-}$ MEFs, we rescued SARM bound AR activation by IL-1β by expression of wild-type MEKK1 or a MEKK1 protein with a point mutation to block its internal ubiquitin ligase activity (C478A) (Lu et al., 2002). MEKK1 harboring a mutation blocking its protein kinase activity (D1369A) failed to function in AR derepression (Figure 3H). An RNA-profiling experiment revealed that almost all DHT-stimulated gene transcripts recorded were also induced by bicalutamide in the presence of IL-1β, suggesting that this strategy regulates most or all AR-dependent genes (Figure 4A, Table S1).

Molecular Mechanisms of Steroid Hormone Receptor-Specific Derepression by IL-1β

Analogous to events on SARM bound AR, IL-1β-dependent conversion of SERMs (4-hydroxytamoxifen, 4-OHT) to activators of estrogen receptor-α (ERα) also failed to occur in cells null for either *MEKK1* or *TAB2* (Figures 4B and 4C). Similarly, antagonists of progesterone receptor (PR) were also switched to agonists in response to IL-1β, and both MEKK1 and TAB2 were required for agonistic actions of PR antagonists (Figure 4D). In contrast, even in the same LNCaP cells, retinoic acid receptor-α (RARα)-dependent repression was not altered by IL-1β either in the absence of ligands or even with binding of the RARα antagonist LG815 (Figure 4E). Moreover, RARα-dependent activation or repression (mediated by N-CoR) was not altered by disrupting TAB2 or MEKK1 functions using IgGs or dominant negative mutant counterparts (Figure 4F). ChIP assays revealed that the N-CoR holocorepressor complex, including TBLR1, mSin3A/B-containing complexes, and HDAC3, was present on the *RARβ* promoter (Figure 4G). On this RARα target, TAB2 recruitment could not be detected in either the presence or absence of antagonists. This is in contrast to the presence of TAB2 in the N-CoR holorepressor complex on the *PSA* promoter in response to SARMs (Figure 4G and data not shown).

The receptor specificity of the IL-1β response is consistent with the hypothesis that IL-1β-dependent dismissal of N-CoR holocorepressor complexes on AR, ER, and PR requires the presence of a component of the N-CoR complex, TAB2, that is not successfully recruited to unliganded or to antagonist bound RXR/RAR complexes, raising the question of why TAB2 is recruited to some nuclear receptors but not to others. One striking difference between sex hormone receptors and retinoic acid and thyroid hormone receptors is the relative importance of the N terminus in gene activation events and the presence of distinct activation domains (AF1) in the N terminus of ER and AR (Alen et al., 1999; Bevan et al., 1999; Tremblay et al., 1999). Indeed, the N-terminal domain of ERα is > 180 aa, and that of AR is > 530 aa, compared to a relatively short (<90 aa) RARα N terminus.

We therefore examined the possibility that the N termini of AR, ER, or PR harbored the key for inclusion of TAB2 into the N-CoR holocorepressor complex. Consistent with this model, we find that TAB2 can interact with the N terminus of either AR (aa 1–512) or ERα (aa 1-173) in coimmunoprecipitation assays (Figure 5A). Further, with removal of the AR N terminus (aa 2-500), in comparison to androgen holoreceptor, there was no change in the recruitment of receptors themselves and other components of the N-CoR holocomplex, including HDAC3, TBLR1, and mSin3A/B in

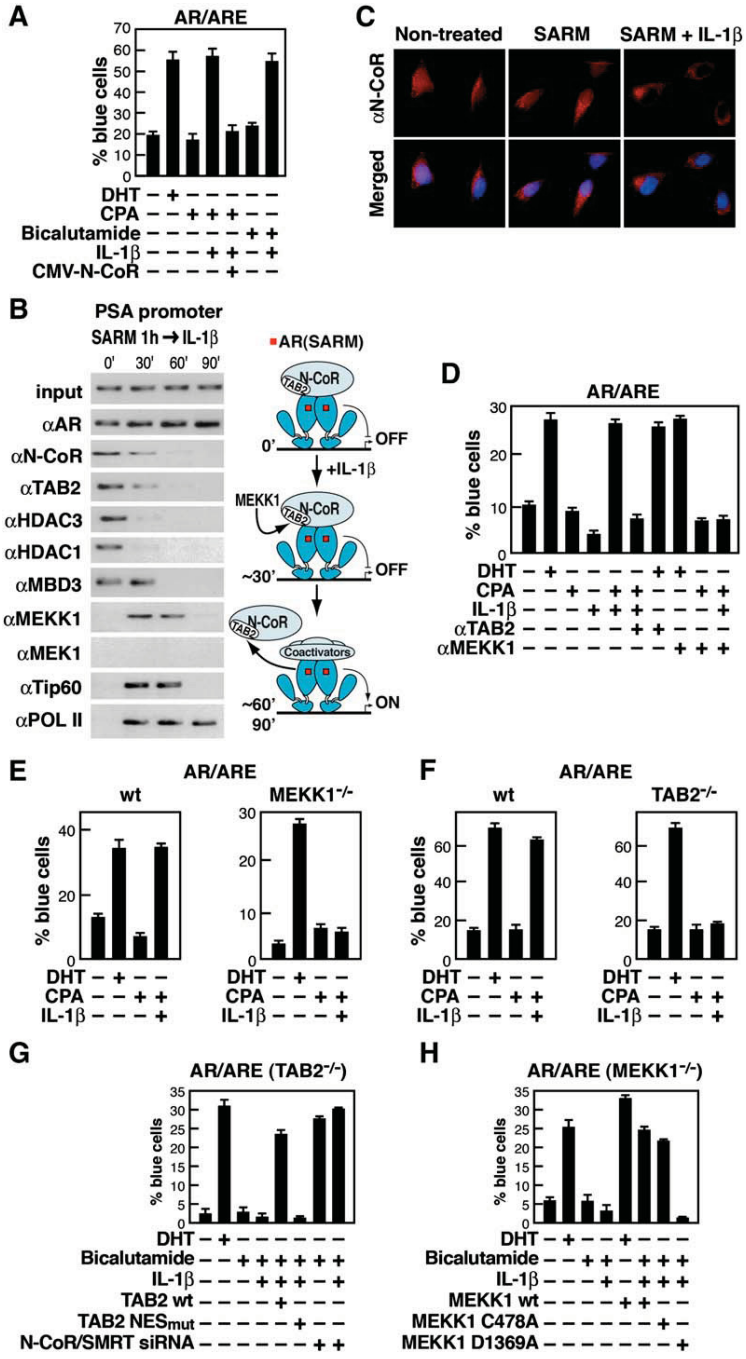

Figure 3. IL-1β Converts AR Antagonists to Agonists

(A) Pretreatment of cells with IL-1β abolished CPA or bicalutamide-mediated repression of an ARE-containing reporter, and microinjection of CMV-N-CoR expression plasmid restored the antagonist function.

(B) ChIP assay on the *PSA* promoter in LNCaP cells in the presence of CPA and IL-1β. LNCaP cells were pretreated with CPA for 1 hr, and dismissal of the corepressor complex was assessed at indicated times after IL-1β treatment.

(C) LNCaP cells were maintained in charcoal-stripped medium and exposed to CPA in the absence or presence of IL-1β. Cells were stained with αN-CoR IgG and visualized using deconvolution microscopy. Merged images with nuclear DAPI staining (blue) are shown.

(D) Microinjection of αTAB2 and αMEKK1 IgGs into Rat-1 cells with AR and ARE-LacZ reporter in the presence or absence of DHT or CPA.

(E) A reporter under the control of ARE was microinjected into either *MEKK1*$^{-/-}$ or wild-type (wt) MEFs, and the effects of treatment of DHT, CPA, or IL-1β were tested in the presence of AR.

(F) In *TAB2*$^{-/-}$ MEFs, IL-1β-dependent androgen-like activity of CPA was abolished.

(G) Role of TAB2 in bicalutamide-dependent repression of AR function. Using *TAB2*$^{-/-}$ MEFs, an AR-dependent reporter gene is not activated by bicalutamide/IL-1β cotreatment unless wt TAB2 is expressed or N-CoR/SMRT are depleted by 48 hr of specific siRNAs.

(H) In *MEKK1*$^{-/-}$ MEFs, bicalutamide acts as an antagonist upon bicalutamide/IL-1β cotreatment unless wt MEKK1 or a C478A mutant is added. Data are represented as mean ± SEM.

response to SARMs, but there was a loss of ability to recruit TAB2 (Figure 5B, and data not shown). A similar result was found with ERα, wherein the presence of the N terminus (aa 1-170) is required for recruitment of TAB2 but not for other components of the N-CoR holocomplex in response to SERMs (data not shown). Finally, with replacement of (or addition to) the RARα N terminus (aa 1-60) with that of either the AR (aa 1-512) or ERα (aa 1-173), the RARα fusion proteins (AR-N'/RAR, ER-N'/RAR) can now exhibit MEKK1- and TAB2-dependent activation with RARα antagonist or in the absence of ligands in response to IL-1β (Figure 5C and data not shown). Further, a serial two-step ChIP assay confirmed that N-CoR and TBLR1 components were present on both RARα and the AR-N'/RARα fusion receptor and that TAB2 could be recruited only to the fusion receptor (Figure 5C and data not shown), suggesting that the steroid hormone receptor N termini are required for TAB2 recruitment to the N-CoR complex during repression. Retinoic

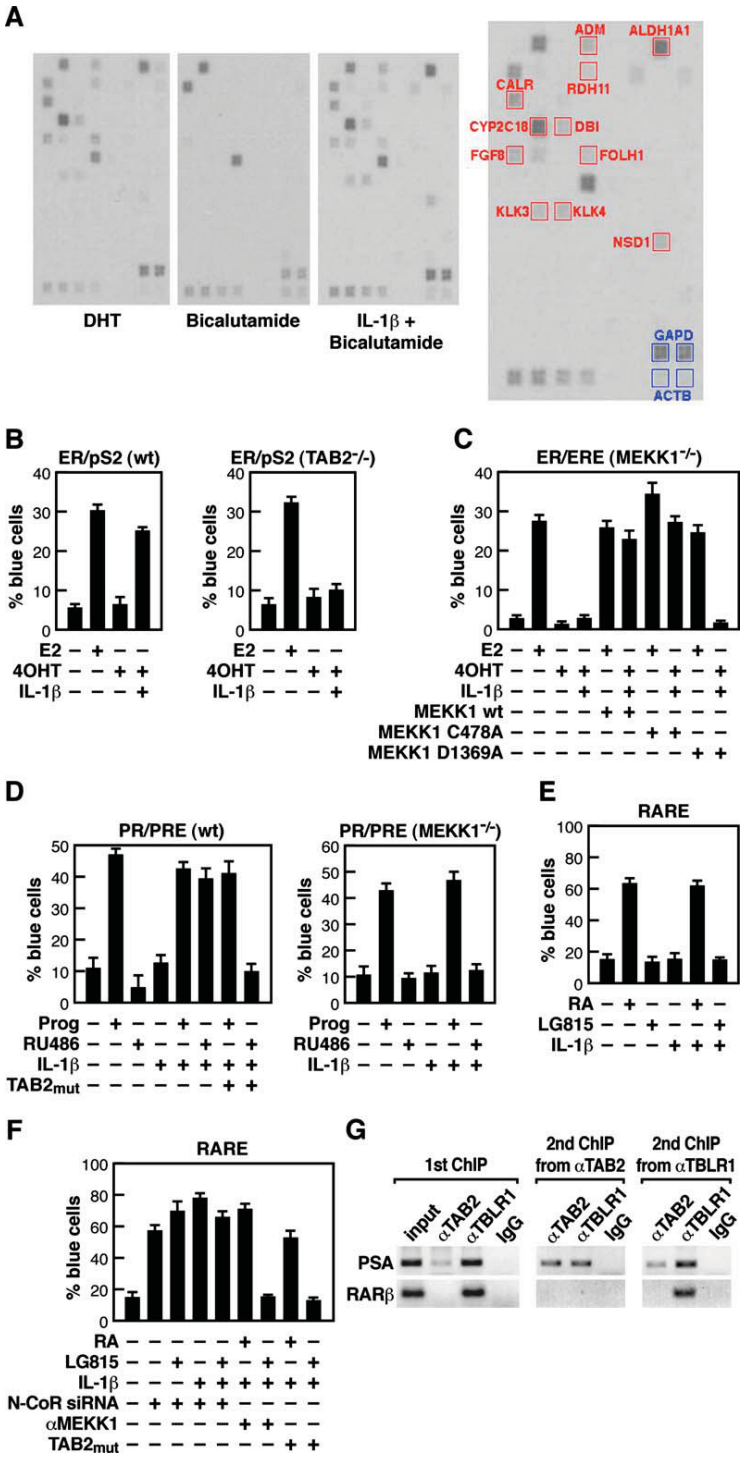

Figure 4. Steroid Hormone Receptor-Specific Derepression by IL-1β

(A) Expression-profiling of AR target genes using Superarray HS-031 in LNCaP cells treated with DHT, bicalutamide, and bicalutamide/IL-1β. Detectable upregulated genes upon normalization were marked in red. The list of the genes and quantitative expression levels are shown in Table S1.

(B) Effects of IL-1β on *pS2* promoter-dependent reporter in wt or *TAB2*$^{-/-}$ MEFs.

(C) The ability of MEKK1 expression vectors to rescue 4-OHT-mediated activation in response to IL-1β. Wt MEKK1, C478A MEKK1 (mutant of E3 ligase activity), or D1369A MEKK1 (mutant of kinase activity) was used to rescue IL-1β-dependent activation of the ERE promoter by 4-OHT in MEFs from *MEKK1*$^{-/-}$ mice.

(D) Effects of progesterone (Prog) and RU486 on a PRE-dependent reporter in response to IL-1β, using single-cell nuclear microinjection assays in wt and *MEKK1*$^{-/-}$ MEFs.

(E) IL-1β fails to activate unliganded or antagonist (LG815) bound RAR in Rat1 cells.

(F) Antibody against MEKK1 and a dominant-negative TAB2 (TAB2mut) show no effect on LG815 or RA actions in response to IL-1β. Data are represented as mean ± SEM.

(G) Two-step ChIP analysis of *RARβ* and *PSA* promoters in LNCaP cells, showing that *PSA* promoter recruits TBLR1 and TAB2 when cells are treated with CPA for 1 hr, while *RARβ* promoter only recruits TBLR1, though not TAB2, in cells cultured in charcoal-stripped medium.

acid stimulated the chimeric RARα, but neither E_2 nor DHT exerted activation effects (Figure 5C and data not shown).

To localize a potential interaction domain(s), HA-NLS-tagged fragments of ERα N terminus and endogenous TAB2 were tested by coimmunoprecipitation/Western blot analysis. These studies revealed that only aa 1-45 of the ERα N terminus permitted TAB2 binding (Figure 5D). Again, the initial 45 aa of ERα N terminus was sufficient to cause direct interactions with TAB2 using bacterially expressed ERα fragments. Further mapping revealed that this interaction

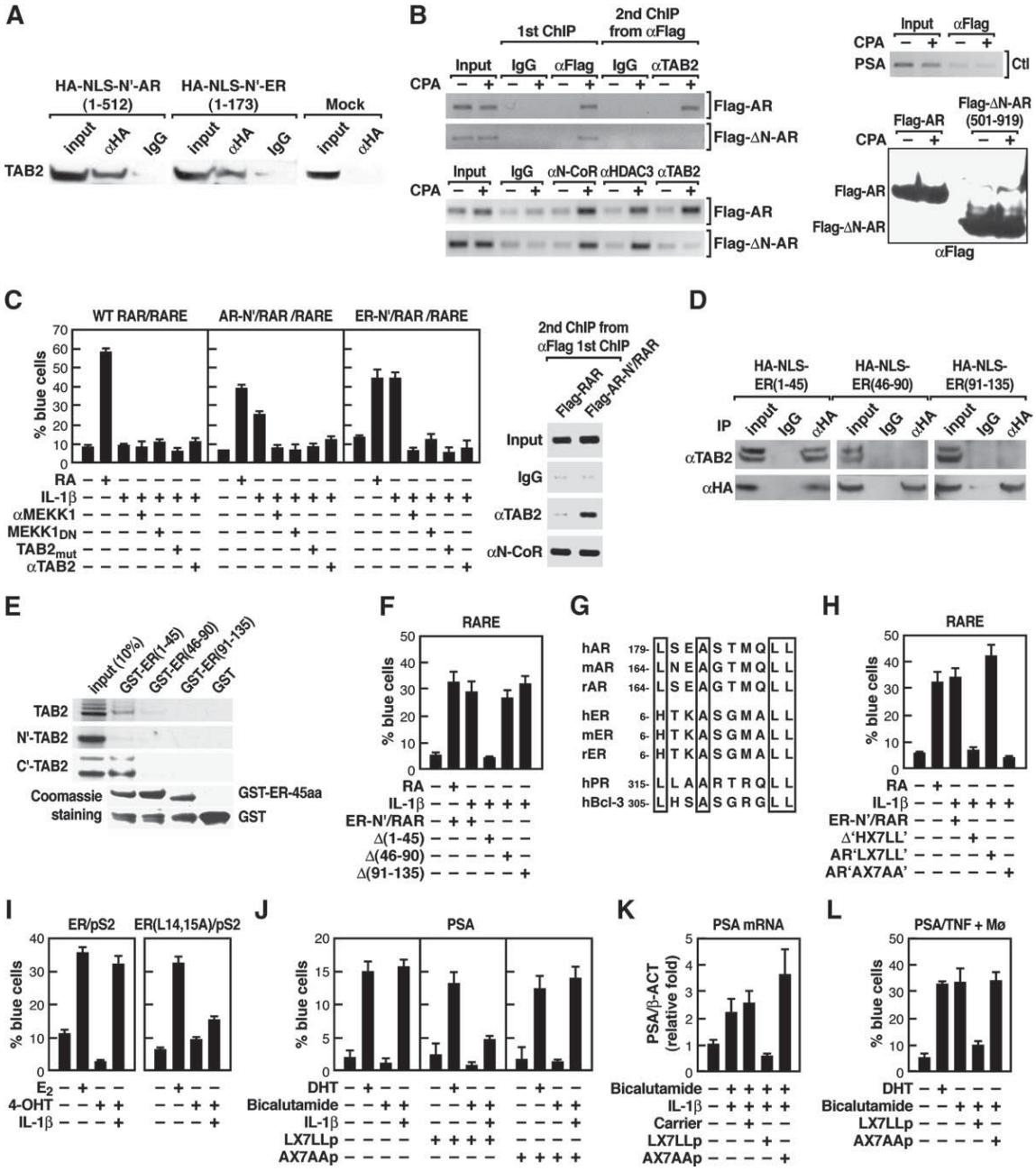

Figure 5. The Evolutionarily Conserved N-Terminal L/HX$_7$LL Motif of Steroid Hormone Receptors Is Crucial for TAB2 Recruitment and IL-1β-Induced Derepression

(A) Immunoprecipitation of extracts of 293 cells transfected (or mock-transfected cells as the control) with N terminus of AR (1-512 aa) or ERα (1-173 aa) (expressed as HA-tagged, NLS-containing fusion proteins) by αHA antibody. Specific TAB2 interaction with AR or ERα N terminus was detected by Western blot.

(B) Role of AR N terminus in TAB2 recruitment to SARM-bound AR on target genes (*PSA*). Expression vectors encoding Flag-tagged full-length AR or ΔN-AR (501-919) were transfected into LNCaP cells, and cells were treated with CPA for 1 hr if needed. Then a 2-step ChIP was performed, first using αFlag IgG and then αTAB2 IgG. Lower panels: CPA-treated or untreated LNCaP cells transfected with AR or ΔN-AR Flag-tagged vectors were examined by 2-step ChIP (first αFlag and then αcorepressors) on the *PSA* promoter for binding of N-CoR, HDAC3, and TAB2. Mock-transfected cells were used as the control for ChIP analysis (upper-right panel). Western blot confirmed equivalent expression of AR and ΔN-AR (lower-right panel).

(C) Single-cell nuclear microinjection assays on fusion proteins of N terminus of AR (aa 1-512) or ERα (aa 1-173) and ΔN-RARα (61-462), showing an IL-1β-induced, MEKK1- and TAB2-dependent activation of an RARα-dependent reporter in the absence of ligand. A 2-step ChIP showed specific cooccupancy of TAB2 with AR-N'/RARα fusion receptor but not with the wild-type RARα.

(D) Coimmunoprecipitation of endogenous TAB2 with HA-tagged different fragments of ERα N terminus.

was dependent upon the C terminus (aa 400-693) of TAB2, a region that also harbors the critical regulatory MEKK1 phosphorylation site (aa 419-423) and the NES site (aa 547-561) (Figure 5E). Finally, deletion of aa 1-45 of the ERα N-terminal sequence within the ERα-N'/RARα chimeric receptor caused loss of IL-1β response, while deletion of aa 46-90 or aa 91-135 failed to block IL-1β-induced activation (Figure 5F).

Within the TAB2 regulatory N-terminal sequences of AR and of ERα, we identified a sequence, L/HXXAXXXXLL (referred to as L/HX_7LL), conserved between species and between AR, ERα, and PR and also related to a sequence present in BCL3 (Figure 5G), which is recruited to the p50 site of *KAI1*, a site that also recruits TAB2 with the N-CoR holocomplex and that is derepressed in response to IL-1β (Baek et al., 2002). These residues are predicted to form a specific putative helical structure (Reid et al., 2002). To further test the hypothesis that this site is required or even sufficient to confer interactions with TAB2 and mediate IL-1β effects, we deleted the N-terminal 15 aa, including HX_7LL sequence from the ERα–N'/RARα fusion protein (ΔHX_7LL), finding that deletion of the sequence caused loss of its ability to transfer activation of RARα by IL-1β (Figure 5H). Replacement of the HX_7LL sequence of ERα with the corresponding LX_7LL sequence of AR, though not an AX_7AA mutant AR sequence, was sufficient to restore IL-1β-mediated activation of ERα–N'/RARα on an RARE or on the *RARβ* promoter (Figure 5H and data not shown). A mutant (L14,15A) of ERα holoreceptor with disrupted HX_7LL motif was found to lose response to IL-1β in the presence of antagonist, while the agonist-induced activation was fully intact (Figure 5I). As an independent confirmation, we transfected 293 cells with the tagged AR wild-type (LX_7LL) or mutant (AX_7AA) motif-containing RARα fusion receptors and tagged wild-type ERα or the ERα(L14,15A) mutant and performed a coimmunoprecipitation analysis, finding a decreased interaction of the mutant receptors with TAB2 (Figures S3B and S3C). Therefore, the ability to recruit TAB2 into the nuclear receptor-recruited N-CoR holocorepressor complex and to be derepressed by a proinflammatory cytokine are conferred by a specific, evolutionarily conserved sequence that is present in both AR and ERα.

To test whether competition for TAB2 binding to the L/HX_7LL motif might actually be sufficient to block IL-1β activation of androgen target genes by SARMs, we used the single-cell nuclear microinjection assay to introduce a 14mer synthetic peptide encompassing either the AR LX_7LL motif or the same sequence with L→A substitutions (AX_7AA) into cells expressing *PSA* promoter-dependent reporters. We found that the AR N-terminal LX_7LL peptide specifically blocked IL-1β-mediated activation function of bicalutamide, while the mutated AX_7AA sequence failed to inhibit induction (Figure 5J). Moreover, the antagonist/IL-1β-mediated induction of the endogenous AR gene target *PSA* was prevented by microinjection of LX_7LL but not AX_7AA peptide (Figure 5K). Therefore, this sequence is required and sufficient to mediate TAB2 recruitment and IL-1β-mediated derepression. To test whether the L/HX_7LL motif is also crucial for macrophage-induced derepression in prostate cells, we performed nuclear microinjection of the AR-N' LX_7LL 14mer peptide into RWPE1 cells pretreated with TNFα to induce VCAM-1 and interaction with THP-1 macrophages as described in Figure 1. Intriguingly, the LX_7LL-containing peptide, though not the AX_7AA mutant, blocked the macrophage-induced agonistic ''switch'' of bicalutamide (Figure 5L), implying a potential therapeutic approach to treating hormone resistance induced by macrophage/cancer cell interaction in prostate cancer.

We finally explored the mechanisms by which MEKK1 induces TAB2/N-CoR dismissal from the target gene promoters in response to proinflammatory signals via the suggested MEKK1-dependent phosphorylation of TAB2 (Baek et al., 2002). We performed coimmunoprecipitation of endogenous TAB2, N-CoR, and HA-tagged AR N terminus (1-512) after overexpression of MEKK1, observing that activation of MEKK1 kinase activity inhibited TAB2 interactions with the AR N terminus while enhancing its interactions with N-CoR, consistent with its function in removal of the N-CoR holocorepressor complex in derepression (Figure S3D).

Potential Physiological Roles of N-CoR/TAB2-Dependent Derepression

While these observations have produced initial evidence for an unexpected mechanism underlying some aspects of

(E) GST pull-down assays using different fragments of ERα N terminus fused to GST and in vitro translated full-length, N-terminal (aa 1-399) or C-terminal (aa 400-693) TAB2. The Coomassie blue staining of the purified GST fusion proteins showed similar amounts used in the assays.

(F) Deletion of the 1-45 aa of ERα N terminus, though not other fragments, abrogated the IL-1β -mediated derepression of the ERα-N'/RARα fusion receptor in the single-cell microinjection assays using an RARα-dependent reporter.

(G) Alignment of L/HX_7LL motif in AR, ERα, PR, and Bcl-3 of different species.

(H) Specific deletion and replacement of the L/HX_7LL motifs or mutant (AX_7AA) of ERα and AR in the ERα-N'/RARα fusion receptor in the single-cell microinjection assays using an RARα-dependent reporter.

(I) Single-cell microinjection assays using *pS2* promoter-controlled reporter and ERα expression plasmids in Hela cells. Mutations of L/HX_7LL motif of ERα (L14,15A) restored 4-OHT-dependent repression in the presence of IL-1β.

(J) In single-cell microinjection assays using *PSA* promoter-controlled reporter in RWPE1 prostate cells, synthetic 14mer peptide harboring wt LX_7LL motif but not the mutant AX_7AA abolished the IL-1β-mediated conversion of antagonist to agonist.

(K) *PSA* mRNA expression measured by real-time quantitative RT-PCR after single-cell nuclear microinjection of wt LX_7LL or mutant AX_7AA peptide in RWPE1 prostate cells. Values are relative ratios to basal expression and normalized to *β-actin* levels.

(L) Macrophage/prostate cell interaction was set up as described in Figure 1. By single-cell nuclear microinjection, the 14mer wt LX_7LL and mutant AX_7AA peptides were coinjected into RWPE1 prostate cells with a reporter driven by the *PSA* promoter, followed by the addition of ligands to the medium. Data are represented as mean ± SEM.

SARM/SERM resistance in prostate and breast cancers, they simultaneously raise a perplexing issue. Given the evolutionary conservation of the L/HX_7LL motif in specific nuclear receptors as well as the MEKK1/TAB2 effectors of the proinflammatory pathways, it is reasonable to conclude that there must be a physiologically important function that underlies this conserved response system. While many possible scenarios can be imagined, initial data led us to investigate the putative negative regulation of specific gene expression by E_2. Indeed, a number of genes are reported, directly or indirectly, to be downregulated by estrogens (Frasor et al., 2003; Cicatiello et al., 2004, Lin et al., 2004). One such gene that is negatively regulated in response to E_2 is *BMP7,* and this would be predicted to exert key biological roles in several tissues. It has been suggested that E_2 bound ERα might be recruited to the *BMP7* promoter and inhibit *BMP7* expression by direct mechanisms (Kusumegi et al., 2004; Lin et al., 2004). In fact, we found that the E_2-dependent repression of *BMP7* was relieved in response to IL-1β, following the same logic as the SARM/SERM derepression pathway (Figure 6A).

To better understand the extent to which the evolutionarily conserved mechanism that underlies resistance to SARM/SERM regulates derepression of agonist bound sex steroid receptors, we compared a genome-wide analysis of promoter occupancy by ERα using a human 20 k array designed for a new assay of genome-wide location analysis, the ChIP-DSL-Chip method (I.G.-B., Y.-S. Kwon, M.G.R., X.-D. Fu, unpublished data), and previous analyses (Laganiere et al., 2005) to a list of genes reported to be downregulated in MCF7 cells in response to estrogen. Remarkably, only 17 of these downregulated genes actually exhibit promoter occupancy by ERα, suggesting that the direct negative regulation by E_2 may actually be limited to a rather discrete set of genes. We therefore tested these candidates and verified that the transcripts of eight genes, including *ABCG2* and *BCL3*, were downregulated by E_2, while the positive target *pS2* was induced in MCF7 cells (Figure 6A and data not shown). Importantly, addition of IL-1β reversed E_2-dependent repression of these genes (Figure 6A), suggesting that the macrophage/IL-1β-mediated TAB2 derepression mechanism could underlie physiological events. To test the role of the N-CoR complex in E_2-dependent repression, we evaluated the activity of the *BMP7* or *ABCG2* proximal promoter-controlled luciferase reporter in wt and *N-CoR*$^{-/-}$ MEFs. E_2-induced repression of reporters was fully abolished in *N-CoR* knockout cells, revealing genetically the essential role of N-CoR in this event (Figure 6B). Using the *BMP7*-luciferase reporter in 293 cells, we confirmed that in the presence of ERα, E_2 decreased the reporter activity, whereas combination of E_2 and IL-1β restored the reporter activity to the baseline (Figure 6C). A dominant-negative mutant of TAB2, or *TAB2* siRNA, abrogated derepression of the *BMP7* promoter-dependent reporter, suggesting an essential role of TAB2 in this regulation. In contrast to wild-type ERα, an ERα construct harboring point mutations (E203A/G204A) which prevent its DNA binding-dependent activities but not trans activities (Jakacka et al., 2001) fully abrogated the E_2-mediated repression of the reporter, suggesting that the DNA binding ability of ERα is crucial for regulation of *BMP7* expression (Figure 6C). We noted that the promoters of *BMP7, ABCG2,* and *BCL3* each harbor putative half-ER binding sites. Consistent with these observations, ChIP analysis in MCF7 cells revealed that ERα, N-CoR, and TAB2 recruitment were enriched on the gene-regulatory regions of *BMP7, ABCG2,* and *BCL3* upon E_2 treatment and that N-CoR/TAB2 were released in response to E_2/IL-1β, although the relative amount of occupancy varied slightly on different promoters in response to E_2 and IL-1β (Figure 6D). Quantitative ChIP analysis of *BMP7* promoter by real-time PCR confirmed a robust enrichment of ERα, N-CoR, and TAB2 triggered by E_2 and a complete dismissal of N-CoR and TAB2 by addition of IL-1β (Figure 6E). These data provide a physiological explanation for evolutionary conservation of the N-CoR/TAB2/L/HX_7LL-dependent mechanism that underlies the ''switch'' of SARM function in response to IL-1β. This is particularly relevant in light of the fact that a key aspect of reproductive regulation, blastocyst implantation, involves a local induction of BMP7 in response to inflammatory signals (Monroe et al., 2000; Paria et al., 2001). The transporter ABCG2 plays a role in the placenta to protect the developing fetus from xenobiotic stimuli during gestation (Suzuki et al., 2003), and BCL3 is linked to the regulation of the p50/52 homodimer-dependent subset of NF-κB targets. This pathway is, of course, likely to serve other biological functions, perhaps as a release of inhibition by unliganded receptor under specific protein kinase regulation, exerting roles in the immune and reproductive systems.

DISCUSSION

A Macrophage/Prostate Cancer Cell Signaling Pathway Causes Resistance to SARMs

The studies reported in this manuscript have provided initial evidence that interactions between macrophages and prostate cancer cells serve to mediate specific aspects of tumor behavior and responses to AR antagonists based on an evolutionarily conserved sensor system. Thus, in addition to clear roles of macrophages in atherosclerosis (reviewed in Li and Glass, 2002), presumptive roles in diabetes mellitus, and correlation with vascularization of tumors (reviewed in Coussens and Werb, 2002), macrophages appear capable of serving as an important aspect of SARM resistance in prostate cancer. Here we have found that macrophage/prostate cancer interactions appear almost universal in clinical tumor samples, which could implicate macrophage-produced cytokines as a virtually ubiquitous signal in dictating prostate cancer cell responses. These observations are consistent with reports of monocytic/macrophage infiltration of prostate and breast cancers, particularly within surrounding stroma (Leek et al., 1996; Shimura et al., 2000). Prostate cancer cells are reported to be capable of producing chemoattractants, e.g., GM-CSF, which might be the initial step for macrophage recruitment (Chung et al., 1999). We have provided evidence that this macrophage/prostate cancer cell interaction, ultimately mediated via VCAM-1-dependent

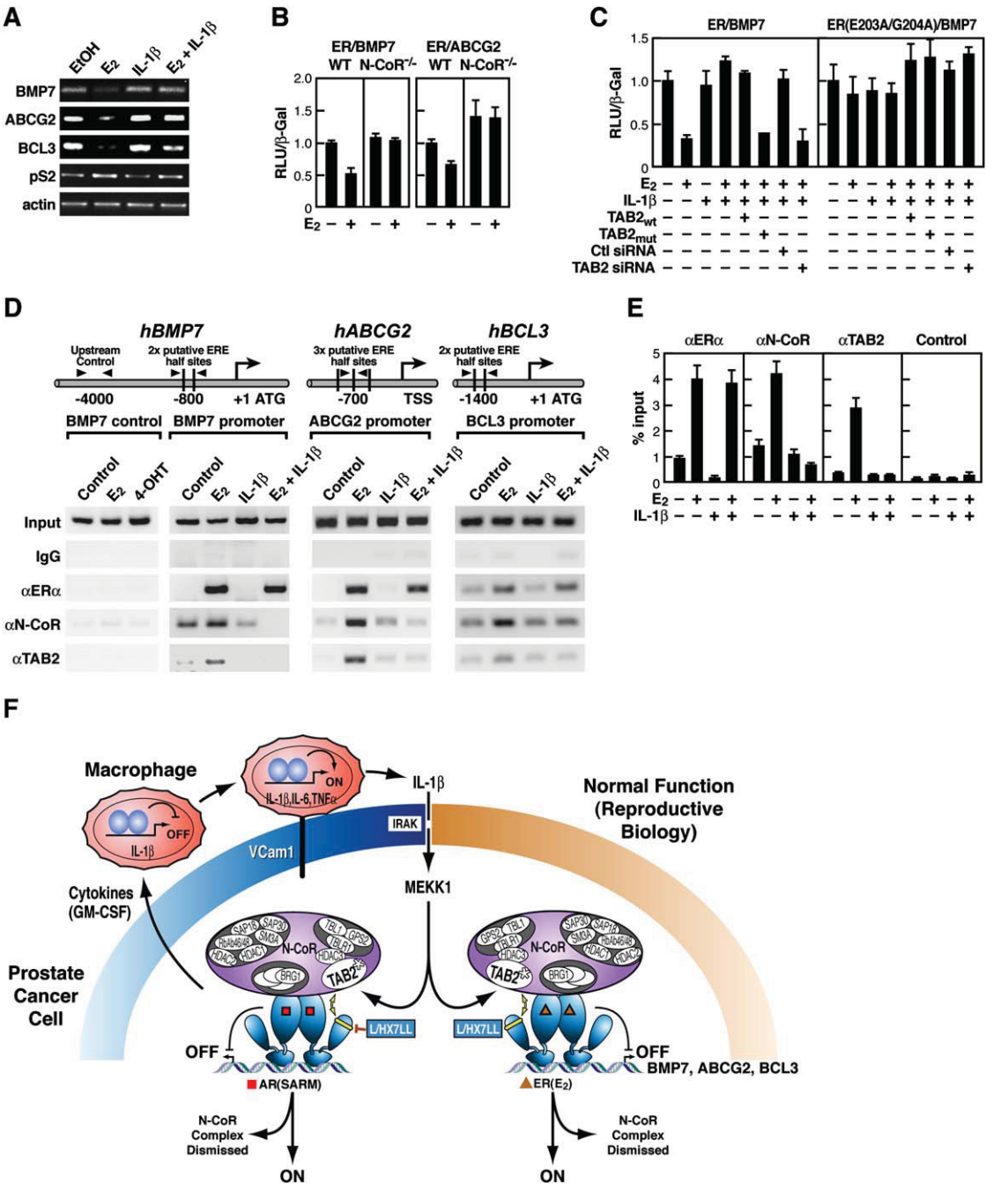

Figure 6. Physiological Roles of Inflammatory Signal-Dependent Derepression in Sex Steroid Receptor Regulation

(A) Semiquantitative RT-PCR analysis of ERα target genes in MCF-7 cells upon different treatments. Actin was used as the loading control.

(B) Reporter assays using luciferase reporters driven by a human *BMP7* or *ABCG2* promoter sequence (*BMP7*: −1091 to −250 and *ABCG2*: −872 to +6; numbers indicate relative positions to ATG and transcription start site, respectively). Reporter activities were tested in wt or *N-CoR*$^{-/-}$ MEFs by transient cotransfection with ERα.

(C) *BMP7* promoter-controlled luciferase reporter assays. Wt ERα and ERα (E203A/G204A) were cotransfected with reporter in 293 cells. Expression levels of wt and mutant ERα were similar, and siRNA knock-down of TAB2 was efficient (>70%) (data not shown).

(D) ChIP analysis of *BMP7*, *ABCG2,* and *BCL3* promoters. The primer pair marked "promoter" flanks putative ERE half sites, and primer pair "control" flanks a region −3 kb upstream of *BMP7* promoter.

(E) Quantitative ChIP analysis of *BMP7* promoter using real-time PCR. Values are relative ratios to corresponding input. Data are represented as mean ± SEM.

(F) Model of nuclear receptor-specific derepression program in response to proinflammatory signals.

adhesion, causes macrophage activation and production of cytokines, including IL-1β, which are sufficient to cause resistance to antagonists (SARMs). These data suggest that the interaction between these two cell types serves, at least, as an important component of the ''resistance'' events in prostate cancer. This is consistent with the idea that, in addition to roles in infection and surveillance, the recruitment and activation of macrophages is probably a key aspect of many diseases. Because we have found that peptides corresponding to the L/HX_7LL motif of AR or ERα can block macrophage-dependent resistance, we suggest that this peptide inhibitor may serve as a prototype for identifying antiagonists that might act to prevent inflammatory cytokine-dependent switch in SARM or SERM function and hence ''block'' resistance. Our data would also suggest that IL-1β-mediated MEKK1 activation is likely to be the major macrophage-induced pathway for SARM resistance.

Molecular Mechanism of Proinflammatory Signal-Induced Derepression of Specific Nuclear Receptors

The macrophage/prostate cancer cell interaction dictates a coordinated program of transcriptional response to an inflammatory cytokine resulting in derepression of a subset of N-CoR/SMRT-repressed genes based on the presence or absence of a specific molecular beacon, TAB2, which acts as a sensor of specific inflammatory signaling pathways. We find that recruitment of an N-CoR ''holocorepressor'' complex, including the TBL1/TBLR1-, mSin3-, and Brg1-containing components that are simultaneously recruited with N-CoR to AR, and multiple, required HDAC enzymatic activities serve to maintain repression on SARM bound AR. However, it is the inclusion of a specific component, TAB2, that underlies macrophage/prostate cancer cell derepression events. This complexity of the holocorepressor machinery may serve primarily to permit an ''integration'' of transcriptional responses to additional regulatory signals, exemplified by the selective derepression of specific cohorts of N-CoR-repressed genes in response to proinflammatory signaling events.

Intriguingly, the molecular basis for recruitment of TAB2 to the N-CoR holocorepressor complex by AR, ER, and PR proves to lie in the specific interaction between TAB2 and an evolutionarily conserved N-terminal ''L/HX_7LL'' sequence that permits recruitment of TAB2 to the N-CoR holocorepressor complex in a receptor/TAB2/N-CoR ternary complex (Figure 5G). This L/HX_7LL TAB2 recognition motif can transfer recruitment of TAB2 and IL-1β-sensitive derepression to RARα that lacks the motif. In the case of p50 gene targets, a similar L/HX_7LL sequence in BCL3 appears to mediate recruitment of TAB2 (our unpublished data). Therefore, the genomic response to IL-1β-dependent actions in derepression appears to be determined by a specific motif that serves to alter the composition of the N-CoR corepressor complex by recruiting the TAB2 component.

While the cytoplasmic function of TAB2 in IL-1β-induced activation of NF-κB p50/p65 heterodimers is unimpaired in TAB2-deficient embryonic fibroblasts, probably due to compensation by the related TAB3 (Sanjo et al., 2003), the nuclear function of TAB2 in permitting IL-1β-dependent corepressor dissociation based on phosphorylation of TAB2 by MEKK1 (Baek et al., 2002) is lost in these cells. The potential conformational change in TAB2 upon phosphorylation simultaneously weakens its association with the AR/ER N terminus while enhancing its association with the N-CoR complex, which probably enables it to mediate dismissal of the entire N-CoR ''holocorepressor'' complex. Thus, DNA binding factor-specific recruitment of certain components of the corepressor holocomplex can serve as the ''molecular beacon'' for integrating nuclear transcriptional responses of different signaling pathways (Figure 6F), accounting for the resistance of other nuclear receptors like RARα to IL-1β signals.

Negative Gene Regulation As the Evolutionary Basis of the L/HX_7LL/TAB2 Mechanism

Based on the data presented here, we are tempted to suggest that this regulatory mechanism selective for sex steroid receptors arose in the context of gene inhibition by estrogen, androgen, and/or progesterone agonists. Here, on the promoters of *BMP7*, *ABCG2,* and *BCL3*, E_2 causes recruitment of the ERα/N-CoR/TAB2 complex, and IL-1β reverses this E_2-dependent repression along the N-CoR/TAB2-dependent pathway. In the absence of N-CoR, E_2-dependent repression is abolished, and macrophage/IL-1β-induced derepression is reversed by inhibition of TAB2. This provides at least one physiological explanation for this evolutionarily conserved L/HX_7LL-dependent TAB2 recruitment and suggests that this mechanism, which derepresses a subset of E_2-repressed genes in response to inflammatory signals, may play critical roles in several biological processes, particularly in reproductive biology. Indeed, macrophages are intimately connected with the development of hormone responsive tissues and reproductive organs (Cohen et al., 1999; Gouon-Evans et al., 2000). It is intriguing to note that blastocyst implantation into the uterus constitutes an ''inflammatory event'' with regulated production of cytokines such as IL-1β and the closely related IL-18 (de los Santos et al., 1996) by both maternal cells and the blastocyst itself, which in turn direct remodeling of endometrium at the site of implantation. Strikingly, *BMP7* expression is locally induced in the tissue immediately adjacent to the implanting blastocyst, exactly at the putative site of IL-1 action in the implantation process (Paria et al., 2001). Therefore local induction of estrogen-inhibited *BMP7* levels by inflammatory signals may facilitate the changes in uterine tissue organization necessary for blastocyst invasion and implantation. The induction of parturition has recently been proposed to involve activated, IL-1β-producing macrophages recruited into the uterine wall in response to signals from the maturing fetus (Condon et al., 2004), an event which may also underlie preterm labor invoked by uterine infection; it is tempting to speculate similar TAB2/PR-mediated events. The *ABCG2* gene encodes an ATP binding cassette (ABC) family half-transporter protein that is highly expressed in the placenta, where it has been implicated in protecting the developing fetus from xenobiotics during gestation (Suzuki et al., 2003), consistent

with a role in protecting sensitive tissues from cytotoxic, mutagenic, or hormonal stimuli. BCL3 serves as the transactivating component of transcription factor complexes containing NFκB1/p50 or NFκB2/p52 homodimers, which have been implicated in the pathogenesis of multiple hormone-dependent cancers. Thus, it appears reasonable to suggest that the ability of inflammatory signals to reverse sex hormone-dependent gene repression is an important biological strategy, related to reproduction and probably other critical aspects of mammalian homeostasis.

In conclusion, our findings have provided evidence for a macrophage/prostate cancer regulatory axis that causes derepression of SARM actions by selective inclusion of TAB2 in the recruited N-CoR holocorepressor complex, and they exemplify a powerful sensor-based strategy of integrating genome-wide responses to specific signaling pathways, apparently of particular physiological relevance in reversing negative gene regulation events by the sex steroids. Defining this macrophage/cancer cell functional interaction as a component in resistance may provide additional strategies to modify therapeutic approaches to specific cancers.

EXPERIMENTAL PROCEDURES

Materials and Reagents

The following antibodies were obtained from Santa Cruz Biotechnology: α-AR, ERα, HDAC1, HDAC2, HDAC3, MBD3, MEKK1, MEK1, BRG1, and mSin3A/B. See Supplemental Data for all other antibodies and reagents.

Macrophage Binding Assay

RWPE-1 cells were seeded on glass coverslips at subconfluent density and subsequently treated with cytokines for 6 hr. THP-1 cells were labeled with 3′-O-Acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) (Molecular Probes, Inc) following manufacturer's instructions. The labeled macrophages were resuspended in DMEM and added in a small volume to the RWPE-1 cells and incubated with gentle agitation for 30 min. Coverslips were washed with PBS and either mounted for microscopy or quantitatively analyzed by flourimetry.

Small Interfering RNA

The small interfering RNA (siRNAs) were delivered into cells by single-cell microinjection or transfection using Lipofectamine 2000 (Invitrogen). Information of siRNAs is available in Supplemental Data.

Single-Cell Nuclear Microinjection Assays and Luciferase Reporter Assays

Microinjection assays and Luciferase reporter assays were carried out as previously described (Kamei et al., 1996; Heinzel et al., 1997). Details are available in Supplemental Data.

Chromatin Immunoprecipitation Assays

The ChIP assay was conducted as previously described (Shang et al., 2000; Zhu et al., 2004). Details are described in Supplemental Data.

RNA Profiling

GEArray Q Series Human Androgen Signaling and Prostate Cancer Gene Array (HS-031, Superarray Bioscience, Frederick, MD) was used to perform RNA expression profiling experiments of AR target genes. See Supplemental Data for details.

RT-PCR and Real-time Q-PCR

Semi-quantitative RT-PCR was carried out as described (Zhu et al., 2004). For real-time Q-PCR, standard procedure was followed according to the Mx3000P Real-Time PCR Systems and the Brilliant QPCR reagent kits (Stratagene). Details are available in Supplemental Data.

Tissue Microarray and Immunohistochemistry

Prostate tissue microarrays (Cat# A302) were purchased from ISU Abxis (Seoul, South Korea) and stained as previously described (Zhu et al., 2004).

All other experimental procedures are available in Supplemental Data.

Supplemental Data

Supplemental Data include three figures, one table, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/124/3/615/DC1/.

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Chapter 2:

A TRAF6-Dependent Mechanism For Inflammation-Mediated Reactivation of Nuclear Receptor-Repressed Target Genes

ABSTRACT

Uncovering the linkage between inflammatory signaling pathways and transcriptional regulatory programs is key to understanding the role of inflammation in homeostasis and disease. An intriguing aspect of this connection has emerged with the identification of a cohort of genes that exhibit direct negative regulation by liganded ERα that can be reactivated by specific inflammatory signals. Here, we report that ERα-dependent repression of the *BMP7* gene, which is overexpressed in up to 70% of primary breast tumors and is reactivated by inflammatory signaling, utilizes an LXXLL motif-containing orphan nuclear receptor, SHP, as a corepressor to recruit the NCoR/TAB2 complex to the *BMP7* promoter. In response to inflammatory signals, transient association of TRAF6 and Ubc13 with the TAB2-containing NCoR complex on the *BMP7* promoter catalyzes K63-linked polyubiquitination of TAB2, permitting promoter-specific recruitment of MEKK1 and licensing clearance of the N-CoR complex. We have identified similar inflammation-induced reactivation of additional ERα-repressed genes, including *BAK1*, *NCOA3*, and *ZNF217*, which also exhibit TRAF6-dependent derepression. We suggest that this TAB2/TRAF6/MEKK1 corepressor clearance pathway regulates specific gene expression programs in response to diverse stimuli and in other cell types, including the hematopoietic and nervous systems, identifying a novel pathway for selective gene activation by inflammatory signals.

INTRODUCTION

A key aspect of homeostatic regulation is the activation of repressed gene programs in response to inflammatory signals (Smale, 2010). Precise regulation of gene expression programs is essential for cell differentiation and function and is governed by combinatorial interactions between transcription factors, coactivators and corepressors, epigenetic machinery, and cell signaling pathways. The estrogen receptor, ERα, a member of the steroid hormone receptor family of nuclear transcription factors, is critical in mammary gland formation and is expressed in most primary breast tumors (Sommer and Fuqua 2001). Many studies of ERα have focused on transcriptional activation; however, more than half of genes modulated by estradiol (E_2) are downregulated (Frasor et al., 2003). Some reported mechanisms by which ERα decreases gene expression include squelching (Gilbert et al., 1993), transrepression (Valentine et al., 2000), and recruitment of corepressors including NCoR (Stossi et al., 2006; Zhu et al., 2006), CtBP (Stossi et al., 2009, Perissi et al., 2010), and RIP140/NRIP1 (Carroll et al., 2006). ERα activity is also modulated by cell signaling pathways, in particular the actions of protein kinases including Src, PKA, AKT and MAPKs (Likhite et al., 2006, Perissi et al. 2008), and the availability of cofactors such as the p160 steroid receptor coactivators (Torchia et al., 1997).

Bone Morphogenetic Protein-7 (BMP7) is overexpressed in as many as 70% of primary breast tumors (Alarmo et al., 2006), increases invasion and metastasis in some cell lines (Alarmo et al., 2009), and its expression associates with bone metastasis in breast cancer patients (Alarmo et al., 2008). *BMP7* mRNA is downregulated by E_2

through a non-canonical ER binding site in the promoter region of *BMP7* that binds ERα, TAB2, and the corepressor NCoR in the presence of E_2 (Zhu et al., 2006). TAB2 pre-marks a subset of NCoR-repressed transcription units, including *BMP7*, for reactivation by the pro-inflammatory cytokine IL-1β (Baek et al., 2002, Kim et al., 2005, Zhu et al., 2006). IL-1β-induced phosphorylation of TAB2 by the MAP3K kinase MEKK1 causes dismissal and nuclear export of TAB2 and NCoR, restoring expression of target genes (Baek et al., 2002).

Here we present evidence that repression of *BMP7* by E_2 depends on the orphan nuclear receptor SHP, which is required for ERα-dependent NCoR recruitment to the promoter. We show that inflammatory signals cause TRAF6 to translocate to the nucleus and interact with a conserved motif in NCoR, leading to TAB2 ubiquitination, MEKK1 recruitment, and ultimately NCoR dismissal and gene derepression. Finally we report that a TRAF6-dependent mechanism is required for derepression of other breast cancer-associated genes, exemplified by *BAK1*, *NCOA3*, and *ZNF217*, which are components of the ERα gene repression program. These studies reveal a nuclear role for TRAF6 in gene activation, and uncover a strategy for signal-dependent derepression of specific cohorts of E_2/ERα-repressed genes by inflammatory signals.

RESULTS

SHP Recruits N-CoR to the *BMP7* Promoter

BMP7 mRNA expression is repressed in MCF7 cells treated with E_2, and derepressed upon IL-1β treatment (Figure 2.1A). E_2-dependent recruitment of NCoR to

the *BMP7* promoter has been reported (Zhu et al., 2006), but how agonist-bound ERα adopts a conformation favoring binding of NCoR is unknown. The orphan nuclear receptor SHP, which lacks a functional DNA-binding domain, has been noted to interact with both ERα/β (Johansson et al., 2000) and full-length NCoR (Seol at al. 1997). We have found that E_2 treatment of MCF7 breast cancer cells led to detectable binding of ERα and SHP at the *BMP7* promoter, but not recruitment of RIP140 or CtBP (Figure 2.1B).

Next we evaluated the functional importance of SHP for repression of *BMP7* by E_2, using siRNA to knock down SHP expression. E_2 treatment caused ~60% reduction of *BMP7* mRNA levels in control siRNA-transfected cells, whereas no repression was observed with SHP siRNA (Figure 2.1C). SHP expression is increased approximately 3.5 fold by E_2 (Figure 2.5), suggesting a feed-forward loop in which E_2-induced SHP enforces gene repression by ERα. In order to determine whether SHP plays a more general role in ERα action, qPCR was performed for the classical ERα target genes *GREB1* and *PDZK1*. Induction of both *GREB1* (Figure 2.1D) and *PDZK1* (Figure 2.1E) by E_2 was unaffected by SHP knockdown, suggesting SHP exhibits specific roles in ERα-mediated repression of *BMP7*.

ChIP was performed to determine the kinetics of SHP and NCoR recruitment (Figure 2.1F) to the *BMP7* promoter. After 5 minutes of E_2 treatment, binding of ERα and SHP, but not NCoR, was detected. At 15 minutes, none of these three proteins were present, suggesting a cyclic pattern of cofactor recruitment, as reported for the E_2-

inducible gene *TFF1* (Metivier et al., 2003). At 30 minutes, ERα, SHP, and NCoR were all detected, indicating that recruitment of SHP precedes that of NCoR in the ordered recruitment of factors. We have previously established that ERα is required for NCoR recruitment to the BMP7 gene (Zhu et al., 2006). To determine whether SHP is also required for NCoR recruitment, SHP expression was knocked down by specific siRNA. In control siRNA-transfected cells, ERα, SHP, and NCoR were all detected at the *BMP7* promoter by ChIP at 30 and 60 minutes after E_2 addition (Figure 2.1G). In *SHP* siRNA-transfected cells, ERα binding was slightly delayed/reduced, but NCoR binding was almost completely eliminated, demonstrating that SHP is required for NCoR recruitment to the *BMP7* promoter. NCoR is completely dismissed from the *BMP7* promoter after 30 minutes of IL-1β treatment (Figure 2.1H), while SHP binding is only partially reduced, suggesting that NCoR dismissal occurs independently of SHP.

ER Isotype Determines TAB2 Binding and Response to IL-1β

TAB2 is recruited to the *BMP7* promoter along with NCoR, HDAC2 and ERα in MCF7 cells treated with E_2 (Figure 2.2A). Recruitment of TAB2 to nuclear receptor-repressed transcription units depends on an L/HX_7LL motif present in the N' terminus of the nuclear receptor (Zhu et al., 2006). Two distinct estrogen receptor genes are encoded in the human genome, ERα and ERβ, which play important roles in normal physiology and in hormone-related cancers. A TAB2-binding motif is present in ERα (aa 6-15), but the shorter N' terminal region of ERβ does not contain any similar sequence (Figure 2.6A), suggesting that ERβ would be unable to recruit TAB2 to

chromatin and insensitive to derepression by IL-1β. Consistent with this prediction, the N' terminus of ERα interacts with TAB2 (Figure 2.6B) and recruits TAB2 to the *BMP7* promoter (Figure 2.6C), while the corresponding region of ERβ fails to interact with TAB2 or recruit it to *BMP7* (Figure 2.6B,C). Confirming the functional significance of this difference between the two receptor isotypes, a *BMP7* promoter reporter exhibited derepression by IL-1β in U2OS cells stably expressing ERα, but not in U2OS- cells stably expressing ERβ (Figure 2.6D). MCF7 cells express high levels of ERα and low levels of ERβ (Vladusic et al., 2000), consistent with TAB2 recruitment and derepression of *BMP7* by IL-1β in this cell line.

TRAF6 Translocates to the Nucleus and Interacts with a Conserved Motif in NCoR

Because TRAF6 can rapidly ubiquitinate TAB2 with K63-linked polyubiquitin chains in response to IL-1β (Ishitani et al., 2003), we hypothesized that this modification may occur as a promoter-localized event involved in *BMP7* derepression. Corepressor dismissal was observed as early as 5 minutes after IL-1β treatment and was complete at 30 minutes (Figure 2.2A). We therefore focused on the behavior of TRAF6 during this time period. Nuclear translocation of TRAF6 was detected in MCF7 cells following 5 minutes of treatment with IL-1β (Figure 2.2B), and both TRAF6 and its partner E2 enzyme Ubc13 were detected by ChIP at the *BMP7* promoter at 5 and 15 minutes (Figure 2.2C).

A TRAF6 consensus interaction sequence has been identified in cytokine receptors and signal transduction proteins, consisting of a proline residue followed in

order by any amino acid, a glutamic acid residue, any two amino acids, and an acidic or aromatic residue (PxExxAc/Ar; Ye et al., 2002). Sequence analysis of NCoR revealed a perfect consensus motif (**P**R**E**ER**D**) at aa 690-695 (Figure 2.2D), adjacent to the TAB2-interacting region of NCoR, suggesting that docking of TRAF6 on NCoR could position it to ubiquitinate promoter-bound TAB2. This motif is evolutionarily conserved in species tracing back to Xenopus, and a similar motif is present in zebrafish NCoR. By co-immunoprecipitation (co-IP), we detected an IL-1β-dependent interaction between endogenous TRAF6 and NCoR in MCF7 cells treated for 5 minutes (Figure 2.2E). We observed interaction between TRAF6 and wild type (PxExxD) $NCoR_{516-811}$ by co-IP, which was lost when the three critical residues were mutated to alanine (AxAxxA) (Figure 2.2F).

Promoter-Localized K63-linked Polyubiquitination and MEKK1 Recruitment by TRAF6/Ubc13

Next we wanted to evaluate the putative functional significance of TRAF6/Ubc13 recruitment to the *BMP7* promoter. Knockdown of either TRAF6 or Ubc13 inhibited the ability of IL-1β to reverse E_2 repression of *BMP7* (Figure 2.3A), and expression of a dominant-negative TRAF6 blocked derepression of the *BMP7* promoter reporter (Figure 2.3B). To determine whether ubiquitination of TAB2 plays a role in *BMP7* derepression, we utilized a truncation mutant of TAB2 lacking the last 28 aa, which is defective in polyubiquitination (Figure 2.3C). Expression of the ubiquitination-deficient TAB2 mutant blocked derepression of the *BMP7* promoter

reporter almost completely (Figure 2.3D), suggesting that ubiquitination of TAB2 is required for derepression of *BMP7*.

As opposed to K48-linked polyubiquitination, which triggers proteasomal degradation, K63-linked polyubiquitin chains like those linked to TAB2 can serve as tethers in assembling kinase activation complexes (Chen and Sun 2009). MEKK1, which is required for TAB2-mediated derepression (Baek et al., 2002; Zhu et al., 2006), contains two overlapping ubiquitin-interacting motifs (UIMs) adjacent to its kinase domain. Interestingly the UIM region of MEKK1 (aa 1137-1194) interacts with recombinant K63-linked polyubiquitin chains by GST pulldown, but not with K48-linked chains (Figure 2.3E). IL-1β caused promoter-localized accumulation of K63-linked polyubiquitin and subsequent recruitment of MEKK1, and blocking ubiquitin accumulation by Ubc13 knockdown prevented recruitment of MEKK1 (Figure 2.3F).

Identification of a Broader ERα/TRAF6 Regulatory Program

In order to identify more targets of this ERα repression/TRAF6 derepression program, we correlated data from genome-wide location analyses of ER (Carroll et al., 2006; Kwon et al., 2007; Lin CY et al., 2007) with published gene expression studies (Frasor et al., 2003, Cicatiello et al., 2004, Vendrell et al., 2004) to identify E_2-downregulated genes that harbor or neighbor ER binding sites in the genome. Repression of these genes in MCF7 cells by E_2 was confirmed by realtime RT-PCR (Figure 4A). We found that three of these genes examined were part of the same TRAF6-regulated program as *BMP7*: the Bcl-2 family member *BAK1*, the p160 family coactivator *AIB1/NCOA3/p/CIP* and the anti-apoptotic zinc finger protein *ZNF217*,

which are all associated with growth, survival and drug resistance of breast cancer cells (Zhou et al., 2010, Anzick et al., 1997, Nonet et al., 2001). All three genes were repressed at the mRNA level by E_2 and derepressed by IL-1β in a TRAF6-dependent manner (Figure 4B-D), while SHP was selectively required for repression of *NCOA3* but not the others by E_2 (Figure 2.8).

DISCUSSION

Our findings have uncovered an unrecognized component of negative gene regulation by liganded ERα and describe a novel mechanism for kinase recruitment and corepressor clearance through chromatin-localized K63-linked ubiquitination by Ubc13/TRAF6 (Figure 4E). We have established a novel pathway by which ligand-dependent gene repression, in some cases mediated by the orphan receptor SHP, can be reversed by inflammatory signals through the TAB2 sensor component recruited with the NCoR complex by sex steroid receptors. This strategy proves to involve recruitment and local action of TRAF6 as a K63 ubiquitin ligase at the gene promoter, attracting MEKK1 and licensing the promoter for corepressor clearance. This novel pathway is likely to be exploited in many tissues, probably for diverse classes of DNA-binding transcription factors that recruit TAB2-containing NCoR complexes.

Because tamoxifen-bound ERα and bicalutamide-bound androgen receptors also repress target genes via NCoR and are derepressed by IL-1β via TAB2/MEKK1 (Baek et al., 2002, Zhu et al., 2006), it is not unlikely that a similar TRAF6-dependent mechanism is responsible for MEKK1 recruitment and NCoR dismissal in these

contexts. Our data suggest that the "antagonist-to-agonist switch" should be blocked by inhibitors of TRAF6/Ubc13 activity or expression, such as a chemical inhibitor recently described for Ubc13 (Tsukamoto et al., 2008).

Several other signaling pathways utilize TRAF6, such as Receptor Activator of NF-κB (RANK), the p75 neurotrophin receptor, Toll Like Receptor 4 (TLR4), and the T- and B-cell antigen receptors. TAB2 is highly expressed in both hematopoietic and neural tissues (Orelio and Dzierzak 2007), consistent with the sites of action of these receptor pathways, and a TAB2/NCoR complex containing the presenilin-cleaved intracellular domain of ErbB4 has been linked to repression of astrocytic genes (Sardi et al., 2006). Based on these observations, we speculate that other stimuli such as RANK ligand, nerve growth factor, lipopolysaccharide, and T/B-cell antigens may trigger TAB2-directed derepression of NCoR-repressed gene programs in other cell types.

The gene expression program regulated by ERα/TRAF6 is particularly relevant to breast cancer. *BMP7*, *NCOA3*, and *ZNF217* are overexpressed in many breast cancers (Alarmo et al., 2006, Anzick et al., 1997, Nonet et al., 2001) and regulate cell growth, differentiation, survival and metastasis, while repression of *BAK1* has recently been shown to regulate drug resistance in breast cancer cells (Zhou et al., 2010). Given recent reports of BMP2-mediated microcalcification in animal studies of breast cancer (Liu et al., 2008), it is tempting to speculate that overexpression of BMP7 in primary breast tumors may play a role in the formation of breast tumor microcalcifications. These mineralized lesions, composed of bone-like calcium crystals, are present in pre-cancerous and cancerous breast growths and are a primary mammographic indicator.

Breast cancer microcalcifications have been localized to osteopontin (OPN)-expressing macrophages (Oyama et al., 2002), and BMP7 has been shown to induce OPN expression and calcification in adipose-derived stem cells (Al-Salleeh et al., 2008) and other cell types. We speculate that a macrophage/cancer cell crosstalk may take place in which macrophage-derived IL-1β stimulates breast cancer cells to produce BMP7, which in turn acts on macrophages (or possibly mesenchymal stem cells) to induce osteogenic gene expression and microcalcifications. If this were the case, a blood test for BMP7 might provide an early warning of breast cancer risk before it could be detected by a mammogram.

The identification of nuclear actions of TRAF6 and MEKK1 in licensing inflammatory signal-dependent activation of genes negatively regulated by ERα also suggests potential new therapeutic targets for breast cancer.

MATERIALS AND METHODS

Materials and Reagents

The following antibodies were obtained from Santa Cruz Biotechnology: α-CtBP, ERα, ERβ, GAL4, GST, HDAC2, MEKK1, Myc, RIP140, SHP, TRAF6, and Ubiquitin. See Supplemental Experimental Procedures for all other antibodies and reagents. The following commercially available antibodies were used: anti-TAB2 (Affinity Bio-Reagents), anti-FLAG (Sigma), anti-HA (Covance), anti-Ubc13/Ube2N (Imgenex), anti-K63-linked polyubiquitin (Enzo Life Sciences), anti-Ubiquitin

(Abcam). Anti-NCoR antibody was described previously (Heinzel et al. 1997). 17-β-estradiol (E_2) was purchased from Sigma and used at a final concentration of 10 nM. Recombinant human Interleukin-1β was purchased from Calbiochem and used at a final concentration of 10 ng/mL. Recombinant K48- and K63-linked ubiquitin chains were purchased from Enzo Life Sciences. MCF7 and HEK293T cells were obtained from American Type Culture Collection (ATCC). U2OS-ERα and U2OS-ERβ stable cell lines were a gift from Dr. David Monroe and Dr. Thomas Spelsberg (Mayo Clinic).

RT-PCR and Real-time qPCR

RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was generated using Superscript III RT Kit (Invitrogen). Standard procedure was followed for the MX3000P Real-Time PCR System and the Brilliant QPCR reagent kit (Stratagene). See Supplemental Data for qPCR primer sequences.

Chromatin Immunoprecipitation

Prior to ChIP experiments, cells were cultured for three days in phenol red-free DMEM containing charcoal/dextran stripped serum. ChIP was performed as described previously (Zhu et al., 2006). Briefly, cells were fixed with 1% formaldehyde for 10 minutes, nuclei were pelleted and lysed in SDS buffer, sonicated to an average DNA fragment size of 500 bp, incubated overnight with antibody (~2-4 μg) at 4° C, bound

with Protein A beads, washed, and eluted. ChIP DNA fragments were decrosslinked at 65° C overnight with proteinase K, purified on Qiaquick spin columns (Qiagen) and detected by either qPCR (Stratagene) or PCR followed by gel electrophoresis and ethidium bromide staining.

Small Interfering RNA (siRNA)

The following siRNAs were obtained from Santa Cruz Biotechnology: SHP (sc-44101), TRAF6 (sc-36717), Ubc13 (sc-43551), control (sc-37007). Prior to siRNA transfection, MCF7 cells were trypsinized, mechanically dispersed by repeated pipetting with a P1000, and plated at very low density. The following day, media was replaced with phenol red-free medium with stripped serum, and the cells were transfected with siRNA (10 nM final concentration) using 5 μL (6 well) or 25 μL (10 cm plate) of Lipofectamine 2000 (Invitrogen), and cultured for an additional 48-72 hours to allow for knockdown. Knockdown was confirmed by realtime RT-PCR: for SHP see Figure 2.5; for TRAF6/Ubc13 see Figure 2.7A,B.

Coimmunoprecipitations and GST Pull-Down

Coimmunoprecipitation and GST pull-down assays were performed as described previously (Zhu et al., 2006).

Luciferase Assays

Luciferase reporter assays were carried out as described previously (Zhu et al., 2006). Briefly, U2OS stable cell lines expressing ERα or ERβ, or HEK293T cells transfected with an ERα plasmid, were cotransfected with a luciferase reporter driven by the human *BMP7* proximal promoter and a β-Gal expression plasmid as an internal control. Cells were then cultured in dextran/charcoal-stripped medium for 36 hrs and treated with ligand/cytokine for 36 hrs. Luciferase activities were normalized to the corresponding β-galactosidase activities.

DNA Plasmids

Myc-TRAF6 and Myc-DN-TRAF6 plasmids were obtained from Dr. Kristin Johnson (UCSD). Wild type TAB2 plasmid and 3xHA-ERα-N' plasmid were described previously (Zhu et al. 2006). 3xHA-ERβ-N' plasmid was generated by PCR cloning of the region encoding the first 138 aa of ERβ from pCXN2-ERβ (Dr. Sumito Ogawa, UCSD). TAB2 mutant construct was generated by PCR cloning of the region encoding aa 1-665 from the wild type TAB2 plasmid above. GST-MEKK1-UIM was generated by PCR cloning of the region encoding aa 1137-1194 of MEKK1 from MCF7 cDNA into the pGEX vector. GAL4-$NCoR_{516-811}$ plasmid was described previously (Heinzel et al. 1997). GAL4-$NCoR_{516-811}$ mutant (AXAXXA) plasmid was generated by site-directed mutagenesis of the GAL4-$NCoR_{516-811}$ plasmid using QuickChange Mutagenesis Kit (Stratagene).

QPCR Primers

The following primer pairs were used: *ABCG2,* 5’-AGCAGTGTTTCAGCCGTGGAA-3’ and 5’- AGGCATCTGCCTTTGGCTTCA-3’; *β-Actin*, 5’-GGCACCCAGCACAATGAAGATCAA-3’ and 5’-ACTCGTCATACTCCTGCTTGCTGA-3’; *BAK1,* 5’-ATCAGCAGGAACAGGAGGCTGAA-3’ and 5’-AAACAGGCTGGTGGCAATCTTGG-3’*;* *BCL3,* 5’-ACCGAGTGCCAAGAAACCGT-3’ and 5’-ACATTTGCGCGTTCACGTTGG-3’*;* *BHLHB2,* 5’-TACAAATTGCCGCACCGGCT-3’ and 5’-AACCACTCTGCAGGGCAATGA-3’*;* *BMP7,* 5’-TTGACATCACAGCCACCAGCA-3’ and 5’-TGGCCTTGAAGAAAGCCACCA-3’*;* *CGI-85,* 5’-TCCTTCGAGGAGCTCAAGGCAT-3’ and 5’-TGCCCATTCGCCTGAAGTCAA-3’*;* *ERBB3,* 5’-AAAGTGCTTGGCTCGGGTGT-3’ and 5’-AGCAGCCTTACAATGTGGGCA-3’*;* *FOXA1,* 5’-GGCATGAAACCAGCGACTGGAA-3’ and 5’-ATGTTGCCGCTCGTAGTCATGG-3’*;* *GREB1,* 5’-GGCAGGACCAGCTTCTGA-3’ and 5’-CTGTTCCCACCACCTTGG-3’*;* *HBP1,* 5’-AGAGTGAACCAGCCTTCCCTCA-3’ and 5’-TTGAAGGCCAGGAATTGCACCA-3’*;* *INHBB,* 5’-AGGAGCGCGTTTCCGAAATCAT-3’ and 5’-ACGTAGGGCAGGAGTTTCAGGT-3’*;* *PDZK1,* 5’-GAGCTTTTGGTTTGCTGAGG-3’ and 5’-TAGGCTTGCAACTCACTGGA-3’*;* *NCOA3,* 5’-AATCCCTATGGCCAAGCAGCA-3’ and 5’-TTGGAAGGTGGCCCAACAAGA-3’*;* *ZNF217,* 5’-

AGGTGGTTCTGAAGACGGATCTGA-3’ and 5’-CTTCTGGGCTGCAGCATATTCACA-3’.

Cell Fractionation

For separation of nuclear and cytoplasmic fractions, cells were washed with PBS, collected, and pelleted; hypotonic lysis buffer was added (50mM Tris-HCl pH 7.6, 20mM HEPES pH 7.6, 10mM KCl, 1mM $MgCl_2$, 0.3% NP-40 substitute, 10% glycerol, protease inhibitor cocktail (Roche) on ice for 5 minutes, and centrifuged at 1000 x g for 5 minutes at 4° C. The supernatant was harvested as cytoplasmic extract. The remaining pellet was washed once with hypotonic lysis buffer, pelleted again, and nuclear extract buffer was added (hypotonic lysis buffer + 420mM NaCl) for 30 minutes at 4° C with rotation. Samples were centrifuged at full speed for 10 minutes at 4° C, and the supernatant was harvested as nuclear extract. Cytoplasmic extracts and nuclear extracts were mixed with an appropriate volume of 4xSDS gel loading buffer and 2-mercaptoethanol. HDAC2 was used as a nuclear marker, and β-tubulin as a cytoplasmic marker, to assess fractionation.

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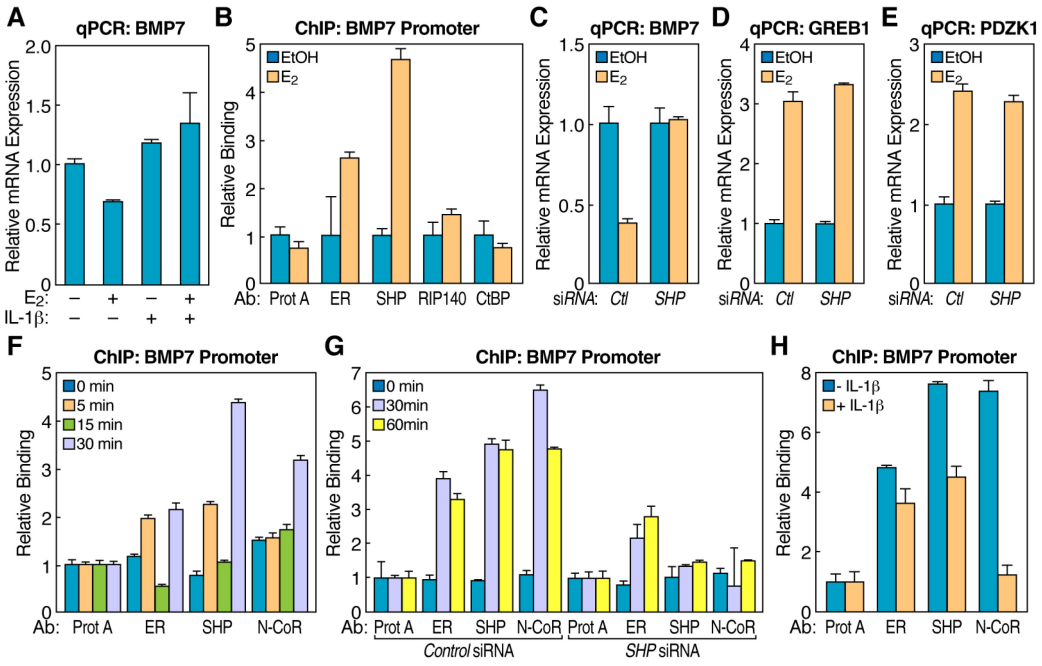

Figure 2.1. E_2-dependent repression of *BMP7* by SHP. (A) *BMP7* mRNA expression is repressed by E_2 and derepressed by IL-1β. MCF7 cells were treated with E_2 and/or IL-1β for 6 hrs. Realtime RT-PCR was performed for *BMP7* and normalized to *β-actin*. (B) Ligand-dependent recruitment of ER and SHP, but not RIP140 or CtBP, to the *BMP7* promoter. MCF7 cells were treated with vehicle (EtOH) or E_2 for 45 min. ChIP was performed with αER, αSHP, αRIP140, or αCtBP Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (C) SHP knockdown abrogates repression of *BMP7* by E_2. MCF7 cells transfected with control (ctl) or SHP siRNA were treated with E_2 for 6 hours. Realtime RT-PCR was performed for *BMP7* and normalized to *β-actin*. (D),(E) SHP knockdown does not affect induction of *GREB1* or *PDZK1* by E_2. MCF7 cells transfected with control (ctl) or SHP siRNA were treated with E_2 for 6 hrs. Realtime RT-PCR was performed for *GREB1* or *PDZK1* and normalized to β-actin. (F) SHP is recruited to the *BMP7* promoter prior to NCoR. MCF7 cells were treated with E_2 for 0, 5, 15 or 30 min. ChIP was performed with αER, αSHP, or αNCoR Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (G) SHP knockdown prevents NCoR recruitment to the *BMP7* promoter. MCF7 cells transfected with control or SHP siRNA were treated with E_2 for 0, 30 or 60 min. ChIP was performed with αER, αSHP, or αNCoR Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (H) IL-1β causes full dismissal of NCoR but not SHP from the *BMP7* promoter. MCF7 cells were pretreated with E_2 for 1 hr. followed by 30 min. with vehicle (PBS) or IL-1β. ChIP was performed with αER, αSHP, or αNCoR Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. Data are represented as mean ± SEM.

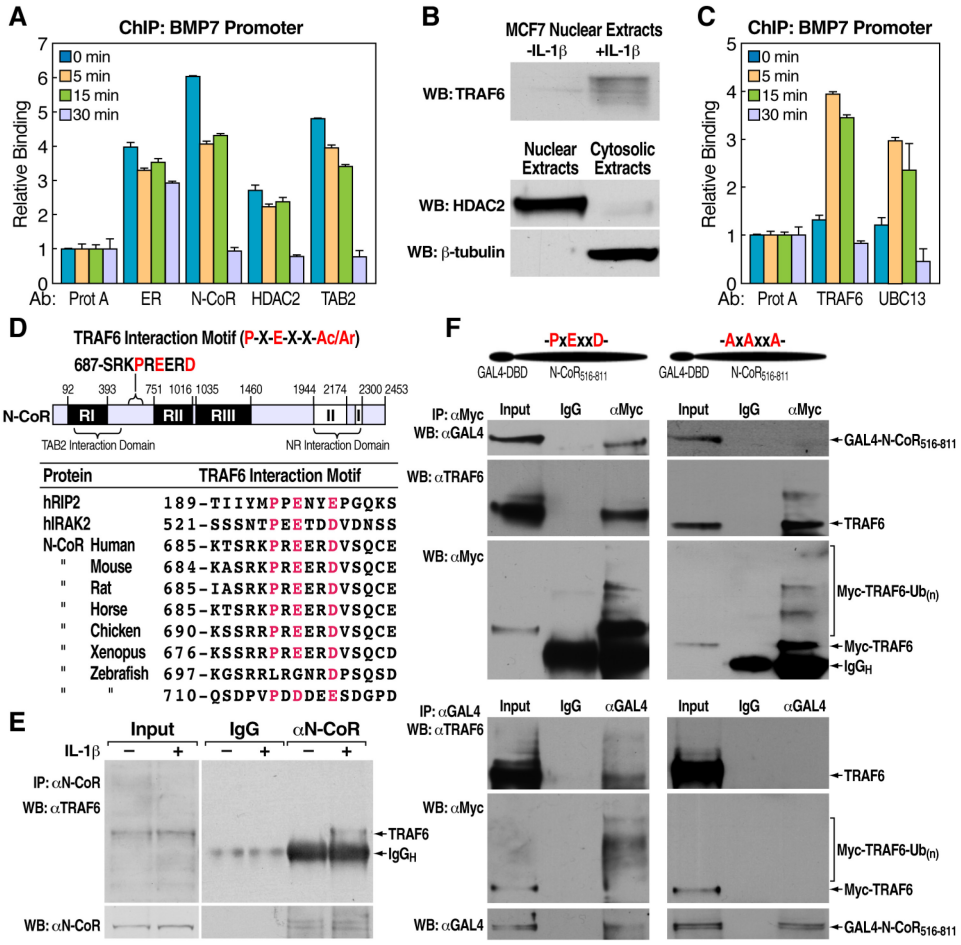

Figure 2.2. TRAF6 is recruited to the *BMP7* promoter, interacts with a conserved motif in N-CoR, and is required for *BMP7* derepression. (A) Kinetics of IL-1β-mediated dismissal of NCoR, HDAC2 and TAB2 from the *BMP7* promoter. MCF7 cells were pretreated with E_2 for 1 hr. followed by 0, 5, 15, or 30 min. with IL-1β. ChIP was performed with αER, αNCoR, αHDAC2, or αTAB2 Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (B) IL-1β causes nuclear translocation of TRAF6. MCF7 cells were treated with IL-1β for 5 min., fractionated into cytoplasmic and nuclear extracts, and subjected to western blotting with αTRAF6 Ab. HDAC2 and β-tubulin were used as fractionation controls. (C) TRAF6 and Ubc13 are rapidly and transiently recruited to the *BMP7* promoter in response to IL-1β. MCF7 cells were pretreated with E_2 for 1 hr. followed by 0, 5, 15, or 30 min. treatment with IL-1β. ChIP was performed with αER, αTRAF6, or αUbc13 Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (D) Map of NCoR showing putative TRAF6 interaction motif, and sequence alignment of similar motifs in human RIP2 and IRAK2 with NCoR from several species. (E) TRAF6 interacts with NCoR in response to IL-1β. MCF7 cells were treated with IL-1β for 5 min., immunoprecipitated with αNCoR Ab, and subjected to western blotting with αTRAF6 or αNCoR Ab. (F) TRAF6 interacts with a conserved motif in NCoR. HEK293T cells were transfected with Myc-TRAF6 and wild type (left) or PxExxD→AxAxxA mutant (right) GAL4-$NCoR_{516-811}$ plasmids, immunoprecipitated with αMyc, αGAL4, or control IgG, and subjected to western blotting with αGAL4, αTRAF6, or αMyc Ab. Data are represented as mean ± SEM.

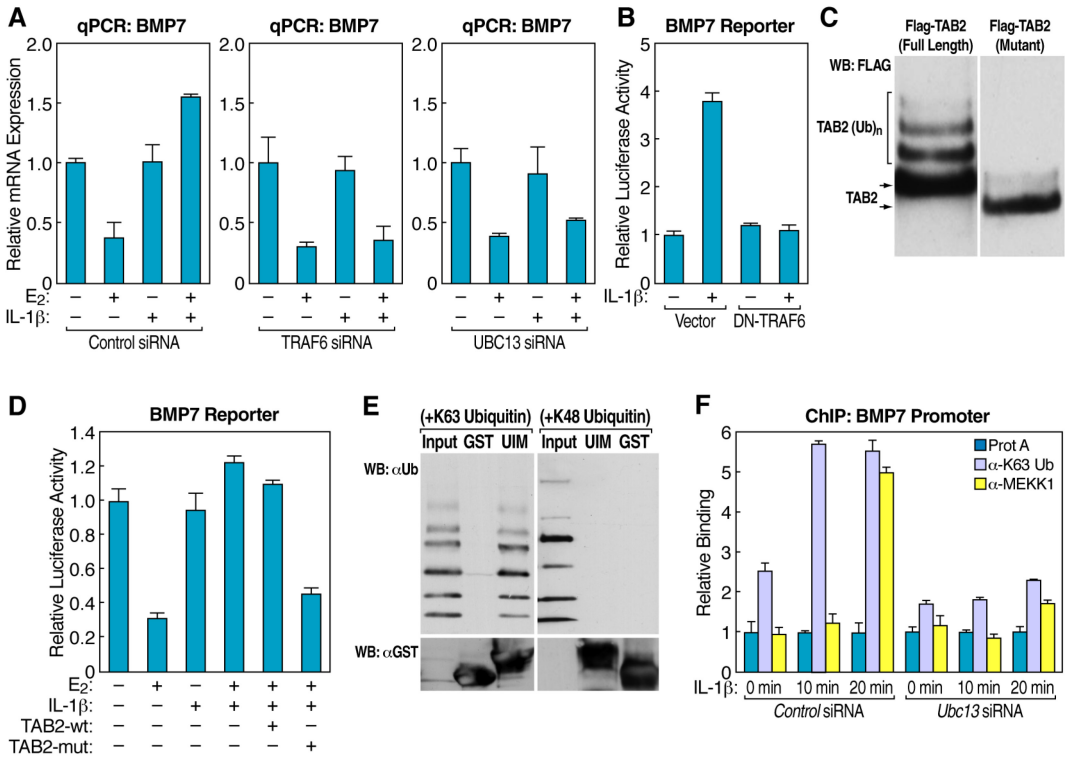

Figure 2.3. Ubiquitin-dependent recruitment of MEKK1 is required for *BMP7* derepression by IL-1β. (A) *BMP7* derepression by IL-1β requires TRAF6 and Ubc13. MCF7 cells were transfected with control, TRAF6, or Ubc13 siRNA, and treated with E_2 and/or IL-1β for 6 hrs. Realtime RT-PCR was performed for *BMP7* and normalized to *β-actin*. (B) Dominant-negative TRAF6 abrogates *BMP7* promoter reporter derepression by IL-1β. U2OS-ERα cells were transfected with *BMP7* reporter, β-gal expression plasmid, and either DN-TRAF6 or vector control, and treated with E_2 and vehicle (PBS) or IL-1β for 36 hrs. Luciferase activity was assayed and normalized to β-gal activity. (C) TAB2 truncation mutant (TAB2-mut) is deficient in polyubiquitination. HEK293T cells transfected with FLAG-tagged TAB2-wt or TAB2-mut plasmid were treated with IL-1β for 5 min., immunoprecipitated with αFLAG Ab, and subjected to western blotting with αFLAG Ab. (D) Ubiquitination-deficient TAB2 mutant attenuates derepression of *BMP7* reporter by IL-1β. HEK293T cells were transfected with ER, *BMP7* reporter, and β-gal expression plasmids, and either TAB2-wt, TAB2-mut, or vector control, and treated with E_2 and/or IL-1β for 36 hrs. Luciferase activity was assayed and normalized to β-gal activity. (E) MEKK1's ubiquitin interacting motif (UIM) interacts with K63-linked, but not K48-linked, polyubiquitin. GST and GST-MEKK1-UIM proteins were expressed in bacteria, purified, and used for GST pulldown with recombinant K63- (left) or K48-linked (right) polyubiquitin chains, followed by western blotting with αUbiquitin or αGST Ab. (F) Ubc13 is required for accumulation of K63-linked polyubiquitin and subsequent MEKK1 recruitment to the *BMP7* promoter. MCF7 cells were transfected with control or Ubc13 siRNA and pretreated with E_2 for 1 hr. followed by 0, 10, or 20 min. treatment with IL-1β. ChIP was performed with αK63-ubiquitin or αMEKK1 Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. Data are represented as mean ± SEM.

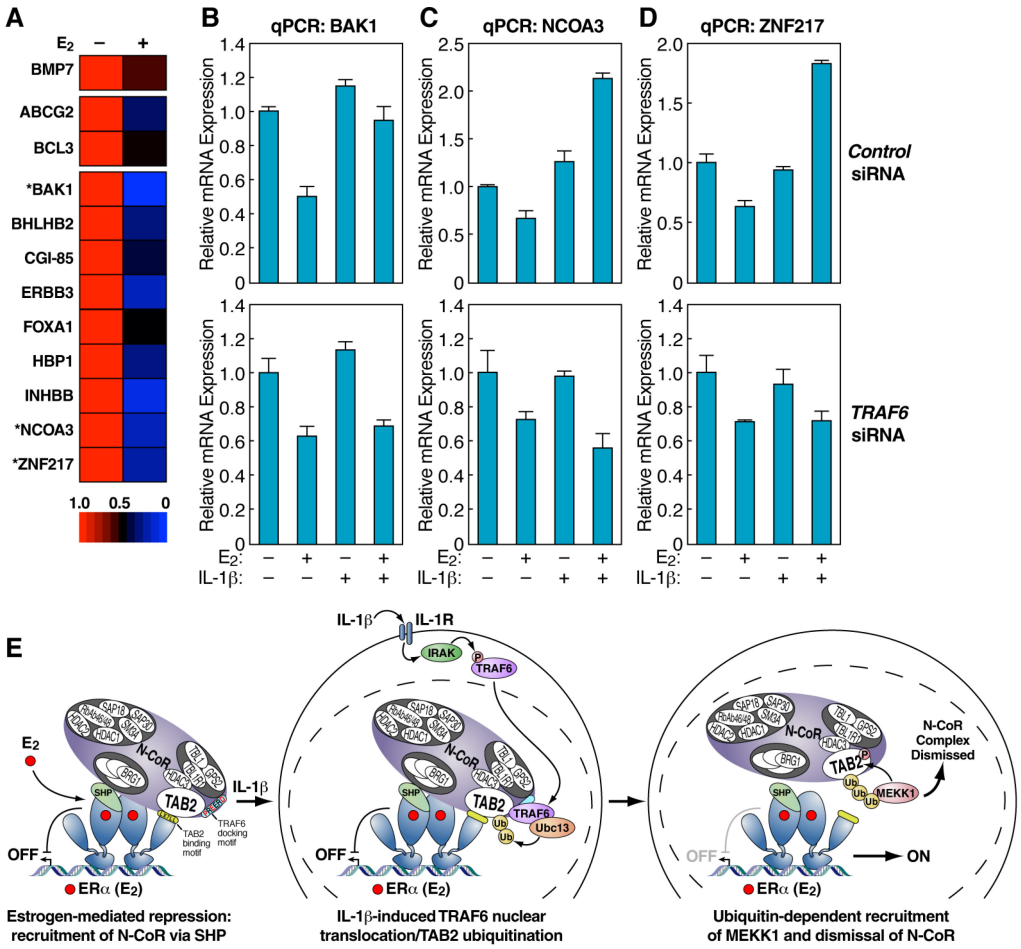

Figure 2.4. TRAF6 mediates derepression of a subprogram of ER-repressed genes. (A) Realtime RT-PCR analysis of negative ER target genes identified by correlation of location/expression data confirms their repression in MCF7 cells. MCF7 cells were treated with E_2 for 12 hrs. Realtime RT-PCR was performed with gene-specific primers and normalized to β-actin. (B)-(D) *BAK1*, *NCOA3* and *ZNF217* are repressed by E_2 and derepressed by IL-1β through TRAF6. MCF7 cells transfected with control or TRAF6 siRNA were treated with E_2 and/or IL-1β for 6 hours. Realtime RT-PCR was performed for *BMP7* and values were normalized to β-actin. (E) Model depicting repression by ER/SHP, and the TAB2/TRAF6/MEKK1 derepression pathway. Data are represented as mean ± SEM.

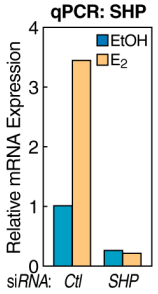

Figure 2.5. SHP induction by E_2 and knockdown by siRNA. MCF7 cells transfected with either control (Ctl) or SHP siRNA were treated with E_2 for 6 hours. Realtime RT-PCR was performed for *SHP* and normalized to β-actin.

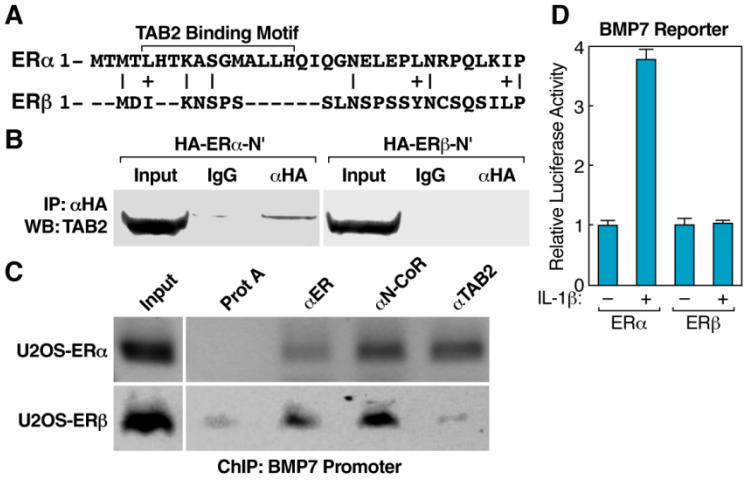

Figure 2.6. ER isotype determines TAB2 binding and derepression by IL-1β. (A) Alignment of ERα N'-terminal TAB2-binding motif with ERβ N' terminus. (B) HEK293T cells transfected with 3xHA-ERa-N' or -ERβ-N' were immunoprecipitated with αHA Ab or control IgG, and subjected to western blotting with αTAB2 Ab. (C) ERβ recruits NCoR but not TAB2 to the *BMP7* promoter. U2OS-ERα or U2OS-ERβ stable cell lines were treated with E_2 for 45 minutes. ChIP was performed with αER, αNCoR, or αTAB2 Ab or protein A beads alone as control, and realtime PCR was performed with primers specific for the *BMP7* promoter. (D) Derepression of *BMP7* promoter reporter by IL-1β is specific for ERα and not ERβ. U2OS-ERα or U2OS-ERβ stable cell lines transfected with *BMP7* promoter-driven luciferase reporter plasmid and β-gal expression plasmid were treated with E_2 and vehicle (PBS) or IL-1β for 36 hours. Luciferase activity was assayed and normalized to β-gal activity. Data are represented as mean ± SEM.

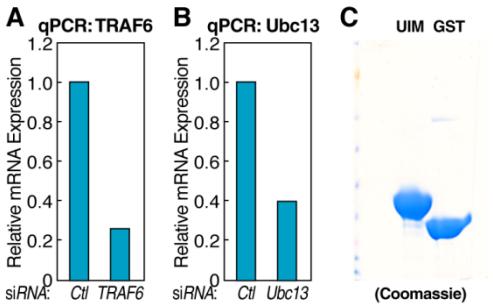

Figure 2.7. TRAF6/Ubc13 siRNA knockdown and GST/UIM protein expression. (A),(B) MCF7 cells were transfected with TRAF6 or Ubc13 siRNA. Realtime RT-PCR was performed for *TRAF6* or *Ubc13* and normalized to *β-actin*. (C) Coomassie-stained SDS-PAGE gel loaded with purified GST-MEKK1-UIM protein ("UIM") or GST alone.

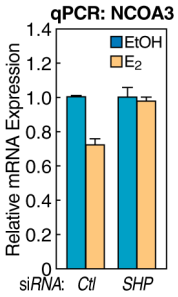

Figure 2.8. SHP knockdown prevents repression of *NCOA3* by E_2. MCF7 cells transfected with either control (Ctl) or SHP siRNA were treated with E_2 for 6 hours. Realtime RT-PCR was performed for *NCOA3* and normalized to *β-actin*. Data are represented as mean ± SEM.

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Chapter 2, in full, has been submitted for publication of the material as it may appear in Molecular Cell, 2010, Bourk, Eliot M.; Zhu, Ping; Hutt, Kasey R.; Garcia-Bassets, I.; Glass, Christopher K.; Rosenfeld, Michael G., Cell Press, 2010. The dissertation author was the primary investigator and author of this material.

CONCLUSION

Dysregulation of gene expression is a common feature of many diseases. In cancer, the overexpression or underexpression of certain genes can make tumor cells grow more aggressively, resist chemotherapeutic drugs and the immune system, or invade the circulatory system and metastasize. Determining the molecular mechanisms by which gene expression patterns are altered in cancerous cells is critical to understanding tumor development and progression, and will hopefully lead to development of targeted cancer therapeutics with enhanced specificity and efficacy.

In Chapter 1, my colleagues and I identified an evolutionarily-conserved nuclear receptor derepression pathway through which macrophage/cancer cell interactions induce resistance to hormone therapy in breast and prostate cancer cells. Transcriptional repression through the androgen and estrogen receptors, induced by selective androgen/estrogen receptor modulators (SARMs/SERMs) such as bicalutamide and tamoxifen, was mediated by the corepressor N-CoR and reversed by the pro-inflammatory cytokine IL-1β. Furthermore, we showed that this TAB2/MEKK1-dependent pathway reactivates the estrogen-repressed ER target genes *BMP7*, *ABCG2*, and *BCL3*, which play roles in reproduction and hormone-dependent cancers.

In Chapter 2, I expanded on our understanding of ligand-dependent repression by ER, highlighting the role of the orphan nuclear receptor SHP in repression of *BMP7* and *NCOA3* by estrogen and demonstrating that derepression of *BMP7* by inflammatory signaling is ER isotype-specific, based on the inability of ERβ to interact with TAB2. I identified a mechanism by which TRAF6 nuclear translocation and promoter

recruitment leads to chromatin-localized, Ubc13-mediated, K63-linked polyubiquitination, which is required for promoter recruitment of the kinase MEKK1, triggering corepressor clearance and transcriptional reactivation. Finally I uncovered additional genes in the ER-repressed/TRAF6-derepressed program that include the breast cancer-related genes *NCOA3*, *ZNF217*, and *BAK1*.

Several possible therapeutic strategies targeting this pathway could be envisioned, including: inhibition of pro-inflammatory cytokine production by anti-inflammatory therapies; antibodies or fusion proteins against IL-1 or IL-1R; IRAK or MEKK1 kinase inhibitors; small molecule Ubc13 inhibitors; cell-permeable decoy peptides for TRAF6 (PxExxD) or TAB2 (LX_7LL); and/or RNAi-mediated downregulation of any of the pathway components, including IL-1, IL-1R, MyD88, IRAKs, TRAF6, Ubc13, TAB2, or MEKK1. I speculate that combinations of one or more of these approaches may yield synergistic results, and that the compounds in the combination could potentially then be given at lower doses to reduce side effects in peripheral tissues.

Another possible trigger for this pathway in breast cancer could be the Receptor Activator of NF-κB (RANK) and its ligand, RANKL. RANKL is an osteoclast differentiation factor and enhances T-cell growth and dendritic cell function (Yasuda et al., 1998), but also acts on breast cancer cells (Thomas et al., 1999). RANK utilizes a similar signal transduction pathway to the IL-1 receptor, involving K63-linked ubiquitination by TRAF6 and Ubc13 (Lamothe et al., 2007) and nuclear translocation of TRAF6 (Bai et al., 2008), raising the possibility that RANKL could reactivate the subset of NCoR-repressed genes that is pre-marked by TAB2. RANKL is upregulated

in stromal cells by breast cancer (Mancino et al., 2001) and could supplant or augment the actions of IL-1β in the primary tumor or in other RANKL-expressing microenvironments such as osteolytic lesions in breast cancer bone metastases.

Three of the genes in the ER/TRAF6-regulated program – *BMP7*, *NCOA3*, and *ZNF217* – are commonly amplified in breast cancer (Alarmo et al., 2006, Anzick et al., 1997, Collins et al., 1998). Given recent studies on the occurrence of DNA damage and chromosomal translocations at nuclear receptor binding sites in the genome (Lin et al., 2009), the production of free radicals by inflammatory cells, and the involvement of Ubc13 and K63-linked ubiquitin in the DNA damage response, it is tempting to speculate that persistent inflammation-mediated nuclear receptor activation could play a role in promoting amplification of these genes in breast cancer.

Hormone therapy for ER-positive breast cancer consists of two main options: selective estrogen receptor modulators/antagonists (SERMs), which compete with estrogen for binding to ER and promote interaction with corepressors such as N-CoR, leading to repression of ER target genes; and estrogen ablation therapy using aromatase inhibitors, which inhibit the natural production of estradiol in the body. In the experiments detailed in Chapter 1 we observed an antagonist-to-agonist switch in SERM activity in response to inflammatory signaling, reversing the ability of SERMs to downregulate ER target genes, however no effect was seen on cells merely cultured in estrogen-depleted media. Therefore, I speculate that inflammation may have a selective effect on ER-regulated gene expression patterns in patients undergoing SERM therapy. In the presence of SERMs, ER-positive breast cancer cells may thrive in microenvironments rich in pro-inflammatory cytokines, such as lymph nodes. While

inflammation may influence tumor behavior through other mechanisms in patients treated with aromatase inhibitors, it likely does not affect the ER-regulated gene program. However, the absence of estrogen in these patients will reverse ER-mediated repression of the entire estrogen-repression program, including BMP7, ABCG2, BCL3, BAK1, NCOA3, ZNF217, and several others such as ESR1, FOXA1, CORO2A, BHLHB2, ERBB3, HBP1, INHBB, PDGFB, and SUV420H1.

A great deal of attention in the field has been paid to mechanisms of gene activation by the estrogen receptor, but relatively little is known about ligand-dependent repression by ER. While I have shown in the preceding chapters that estrogen causes SHP-dependent recruitment of NCoR to the *BMP7* promoter, it is still unclear why this site preferentially recruits these corepressors rather than the coactivators typically recruited to ER-induced genes like *TFF1*, *GREB1*, and *PDZK1*. The ER-binding regions of the *BMP7* promoter and other negatively regulated genes do not contain canonical, palindromic ER binding sites, but rather ER half-sites (GGTCA) with divergent flanking sequences. We speculate that binding to the imperfect ERE sequences may cause allosteric changes in receptor conformation that prefer binding to corepressors like SHP rather than coactivators, and this would be an area of great interest for future study.

SIRT1 has recently been implicated in SHP-dependent repression in other systems (Chanda et al., 2010), and also interacts with NCoR (Hisahara et al., 2008), raising the possibility that NCoR and SIRT1 may function together in the repression of ER downregulated genes as has been shown for antagonist-induced repression of AR-responsive genes (Dai et al., 2006).

The findings presented in the preceding chapters emphasize the ability of the tumor microenvironment to modify critical gene expression patterns in tumor cells, and may help explain the role of inflammation in cancer development and progression.

Future studies of this pathway should include genome-wide location analysis for several of the components, including SHP and NCoR in ligand-dependent repression, TAB2 in pre-marking transcription units for derepression, and TRAF6 and MEKK1 in derepression. This approach would likely uncover additional transcription factors and gene programs that are regulated by TAB2/TRAF6/MEKK1-mediated corepressor clearance and derepression in response to inflammatory signals. These studies could be carried out in macrophages treated with LPS, neural cells treated with NGF, osteoclasts treated with RANKL, or T or B cells activated through antigen receptor stimulation. Further study will increase our understanding of transcriptional regulation by hormone receptors and signal transduction pathways, and may yield additional molecular targets for therapeutic intervention in breast cancer and other diseases.

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