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Understanding the regulation of N-CoR and its associated corepressors, in concert with defining the molecular mechanisms that underlie regulation by estrogen receptor, offers a reasonable prospect of achieving new approaches to diagnosis and treatment of breast cancer. Here, we demonstrated that estrogen receptor recruits at least two distinct N-CoR-containing complexes as mSin3/HDAC 2/1 and also the novel TAB2/HDAC3/N-CoR complex that bind to antagonist. Also, we identification of a novel N-CoR complex containing a factor TAB2 and HDAC3 as a nuclear repressor complex. The inflammatory signals such as IL-1b causes export of the TAB2/N-CoR/HDAC3 complex from nucleus to cytoplasm by MEKK1 action. Furthermore, our results revealed that a specific complex such as TBL1/TBLR1 is required for activation of ERα, based on its function in antagonizing N-CoR/SMRT by ubiquitin ligase mediated exchange for transcriptional activation.

Among the N-CoR corepressor target genes, c-myc and KA1 plays important roles in the cell cycle control and metastases during breast cancer. In this repor, we suggested that N-CoR is linked to β-catenin signaling pathway and N-CoR/TAB2 might be involved in the regulation of the expression of an important metastasis suppressor gene.

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Table of Contents

INTRODUCTION

Estrogen stimulates ductal morphogenesis and lobuloalveolar proliferation in vivo in the normal breast (reviewed in 1) as well as to stimulate proliferation of estrogen receptor (ER)-positive breast cancer cell lines like MCF-7. While cell lines have provided suitable models for the study of ER mouse genetic models provide a more natural environment in which to investigate the normal physiology of the breast and to identify the molecular alterations that occur during the process of tumorigenesis. The experiments described in my proposal were designed to elucidate the physiological contributions of N-CoR to proliferation, differentiation, and physiology as well as the molecular mechanisms of ER-mediated gene activation and repression.

Classical hormone ablation experiments have demonstrated that the steroid hormone estrogen (E) and progesterone (P) are required for ductal morphogenesis during puberty and proliferation of alveoli during pregnancy (1). Estrogenic compounds can activate either of two receptors; the classical ERα and the more recently discovered ERβ. ERβ shares 95% and 55% homology with DNA-binding and ligand binding domains of ERα, respectively (2). Deletion of ERα, the predominant form of ER expressed in the mammary gland, has shed insight into its role during mammary gland development (3). Deletion of ERα results in a failure of the ductal epithelium to penetrate the stromal fat pad during ductal morphogenesis as a result of defects residing within the stromal component (4). The development of inhibitory ligands for the nuclear receptors yielded important therapeutic treatments, among them the use of the anti-estrogen tamoxifen for endocrine therapy of breast cancer. The tamoxifen-related compounds, including trans-hydroxytamoxifen (TOT) are thought to inhibit estradiol-dependent transactivation by competitive binding to ER (reviewed in 5,6). However, in certain tissues such as uterus and bone, and in patients in long term treatment with breast cancer, tamoxifen exhibits partial agonistic activity, thought to be mediated by the constitutively active activation function 1 (AF1) domain of ER. However, the molecular mechanism by which tamoxifen exerts differential effects in various tissues has remained elusive.

The cloning and biochemical characterization of a protein which functions as a repressor of unliganded thyroid hormone receptor (TR) and retinoic acid receptors (RAR), nuclear receptor corepressor (N-CoR), has led to insights into the molecular mechanisms of nuclear receptor-mediated gene regulation (7,8). Ligand binding causes decreased interaction of N-CoR to TR and RAR on most DNA sites in both biochemical assays (6,7) and intact cells (9). The Rosenfeld laboratory demonstrated that, upon the binding of the anti-estrogen TOT, ER binds to N-CoR effectively. However, in the absence of N-CoR, TOT is converted from an antagonist to an agonist with regards to ER function (10). The ability of N-CoR to function as a repressor of ER-mediated transactivation suggests that N-CoR may play an important role not only during normal mammary gland biology, but also in the process of breast tumorigenesis and the acquired resistance to tamoxifen treatment. To test the hypothesis that N-CoR is a critical component of the repression complex which mediates tamoxifen's ability to act as an inhibitor of ER, I suggested testing these event in a genetic system which could show which ER is activated by agonists in the absence of the N-CoR complexes. Furthermore, it is possible that tamoxifen resistance in breast cancer patients results from either decreased levels of N-CoR expression or through inhibition of N-CoR's interaction with ER so that the co-repressor cannot be effectively recruited.

Using the single cell nuclear microinjection assay with affinity-purified specific anti-NCoR IgG, our experiments have documented that, in the absence of N-CoR, TOT is converted from antagonist to agonist function. This activation was dependent upon the N-terminal (AF1) domain of ER. Additionally, the interaction of N-CoR with ER is impaired in cells in which tamoxifen acts as an activator and in breast tumors derived from MCF-7 cells implanted into athymic nude mice that develop tamoxifen resistance (10). Furthermore, western blot analysis performed on whole cell extracts from these tumors showed that N-CoR levels decrease in tumors that have developed resistance to the antiproliferative effect of TOT. These data suggest that either a decreasing level of N-CoR or the inhibition of the corepressor binding to the receptor, might explain the ability of tamoxifen to induce ER activation in specific cell types, as well as in late stages of breast cancer. Indeed, this hypothesis has been confirmed using cells from an N-CoR gene-deleted mouse model (11). Examining N-CoR levels in normal mouse mammary glands reveals that N-CoR mRNA levels increase during pregnancy, peaking at or around lactation and declining thereafter, indicating a role for N-CoR in normal mammary gland development. The expression of SMRT follows a similar expression profile.

The Wnts are a family of secreted proteins whose overexpression results in the accumulation of β-catenin, a protein involved in both cellular adhesion (as a critical component of the E-cadherin-actin complex found at adherens junctions) and oncogenesis including breast cancer (12, 13). Binding of the soluble Wnt protein to the Frizzled receptor results in the inactivation of glycogen synthase kinase-3 β, which normally phosphorylates excess β-catenin and causes its ubiquitination and degradation. Wnt signaling-induced inactivation of glycogen synthase kinase-3β results in the loss of this regulating mechanism, causing an accumulation of β-catenin. This increased pool of β-catenin can translocate to the nucleus, where it functions as a transcriptional coactivator of LEF/TCF transcription factors, resulting in the transcription of the protooncogenes c-myc and cyclin D1, among others (14,15). Interestingly, we found that N-CoR interacts with β-catenin by yeast two hybrid assays. It is very interesting to study the functions of N-CoR and β-catenin signaling with target gene regulation in normal mammary gland and N-CoR(-/-) mammary gland.

BODY:

Specific Aims: To determine the localization of N-CoR protein in relationship to ER-alpha (ERα) during the course of normal development, and to investigate the biological roles of N-CoR in breast development and tumorigenesis. Several areas have been evaluated.

Regulation of N-CoR Function: Understanding the regulation of N-CoR and its associated corepressors, in concert with defining the molecular mechanisms that underlie regulation by estrogen receptor, offers a reasonable prospect of achieving new approaches to diagnosis and treatment of breast cancer. Using the genetic model of an N-CoR (-/-) mouse, we have been able to establish that estrogen receptor antagonists require the actions of N-CoR, in a fashion analogous to the actions of estrogen receptor antagonists, without N-CoR, they can function as full agonists. This surprising result indicates that any mechanism that permits escape from the actions of N-CoR will be effective in generating "resistance" to antagonists. Yeast two hybrid screens were

performed using each of the N-CoR transcription repressor domains which led to several unexpected observations: The first was the identification of a protein that we refer to as N-CoR associated protein 2 (NAP2/TAB2), a 70 kD protein that can be present in both cytoplasm and nucleus. This protein binds strongly to N-CoR, and can be immunoprecipitated with α N-CoR IgG at endogenous levels of both proteins. GST pull-down assay established that the C-terminus of NAP2/TAB2 interact best with Repression Domains 1 and 3 of N-CoR. Cotransfection of N-CoR and NAP2/TAB2 resulted in a striking increase in T3 receptor repressor activity, indicating a corepressor role for NAP2/TAB2. A specific IgG against NAP2/TAB2 marked by recording the expected 70 kDa protein on tissue Western blot analysis, presented us the opportunity to explore the potential regulators of NAP2/TAB2. We find that NAP2/TAB2 is predominantly nuclear in localization in quiescent cultures, but can be regulated to exhibit virtually complete cytoplasmic localization. One of the first pathways we have identified is regulation of TAB2 by TNFa/IL-1. Addition of IL-1 to cell cultures causes a progressive, dramatic relocation of NAP2 from nucleus to cytoplasm which is blocked by leptomycin B, indicating regulation at the level of nuclear export. The C-terminus of TAB2 protein, which itself exhibits similar responses to IL-1, contains a classical nuclear export signal. Mutation of this motif blocks IL-1 dependent relocation from nucleus to cytoplasm. Therefore, given its association with N-CoR, we investigated whether IL-1 also induced nuclear/cytoplasmic relocation of N-CoR. Over a period of ~1 hr, most of the N-CoR was now found to be cytoplasmic. Thus, export commences by several minutes, but translocation of N-CoR continues over 60-90 min, with dramatic redistribution evident by 30-60 min. Because of the presence of a consensus nuclear export signal in TAB2, we mutated four residues, which now eliminated the ability of IL-1/TNFa to cause translocation of TAB2 from nucleus to cytoplasm. Therefore, we tested whether expression of TAB2 containing the mutated nuclear export signal (NES) (TAB2 NES mutant) would inhibit N-CoR translocation in response to IL-1/TNFa, finding that it was itself not translocated and blocked IL-1 dependent translocation of N-CoR. Therefore, TAB2 represents an intriguing candidate for one component of regulation of N-CoR function. We and others have established that the highest affinity N-CoR/SMRT complex contains HDAC3 (16, 17). The TAB2/N-CoR complex appears to contain HDC3, but not HDAC1, HDAC2, or mSin3. In parallel, single cell nuclear microinjection assays (11) were performed, initially employing affinity-purified anti TAB2 IgG, revealing that N-CoR now fails to relocate from nucleus to cytoplasm in response to an IL-1 signal. We conclude that TAB2 is required for IL-1-induced redistribution of N-CoR. Similarly, the loss of specific HDACs could be linked to TAB2, and our initial experiments will emphasize HDAC1, HDAC2, HDAC3, HDAC4, and HDAC5. How does IL-1 signaling regulate TAB2? We hypothesize that NAP2 it is a target of IL-1-induced activation of MEKK-1. While TAB2 contains >18 potential consensus sequences for MEKK1 phosphorylation, the TAB2 C-terminal region that is sufficient to exhibit regulated translocation and interaction with N-CoR contains 8 potential sites flanking the NES. Every potential phosphorylation site (consensus S A) was mutated and assayed for the ability to exhibit IL-1/TNFa induced nuclear-cytoplasmic translocation. Our preliminary data suggest a single site (aa 419-423), N-terminal of the export signal, may exert a specific regulatory role. This would imply that TAB2 is capable of interacting with N-CoR both in the phosphorylated and unphosphorylated state, but that a conformational

change, dependent upon phosphorylation of a specific C-terminal residues serves to initiate a conformational alteration that exposes the export signal. In this case, overexpression of TAB2 harboring a mutation of this regulatory phosphorylation site should serve as a dominant-negative regulator of IL-1-dependent N-CoR relocalization. Because there appears to be a high affinity interaction between TAB2 and N-CoR, we tested the ability of TAB2 to directly interact with HDAC3 by GST-pull down assay and by immunoprecipitation from LNCaP, 293, Rat-1 cells in the presence and absence of IL-1 or TNFα. Recently, we have found that interaction of the SANT domain in N-CoR with HDAC3 increases its histone deacetylase (17). Thus, the actions of NAP2 on enzymatic function of HDAC3 alone and in concert with N-CoR, will be assessed using coimmunoprecipitation assays, and using biochemical approaches. Using Anti-MEKK1 IgG, the effects on IL-1b induced translocation could be assessed in single cell assays. We found that aMEKK1 IgG blocked IL-1b-induced translocation to cytoplasm.

The next issue was to explore the effects of the IL-1/TNFa pathways on actions of specific classes of DNA binding transcription factors. Based on the relationship of the TNFa/IL-1 pathway to activation of NFkB, we will explore NFkB-regulated genes. In particular, transcription units are described to bind either the p65/p50 heterodimers or p50 homodimers which are suggested to repress gene expression. The search for IL-1 responsive genes has revealed that these include a metastasis suppressor gene, as well as genes such as ICAMI and IL-6. To address this further, we investigated potential downstream target genes using the chromatin immunoprecipitation (ChIP) assay. The KAI1 gene was chosen for study because it has been reported to be a metastasis suppressor gene for prostate cancer, and possibly also for breast and lung cancer (18). KAI1 encodes the membrane tetraspanin that is linked to cell adhesion interactions with transmembrane helix receptors, and growth factor receptors. Metastasis, the leading cause of death for most cancer patients, remains one of the least understood aspects of breast biology. Decreased expression of the human KAI1 gene is involved in the progression of the cancer, and the KAI1 gene appears to be regulated by signaling molecules that activate this NFkB target gene. We found that the p50 component of NFkB did not directly interact with TAB but was found to exhibit N-CoR-dependent binding of TAB2. A chromatin immunoprecipitation assay (ChIP) was used to evaluate the presence of p50, p65, NAP2, and N-CoR on the KAI1 promoter. In the absence of treatment, p50, but not the p65 NFkB subunit, as well N-CoR and TAB2 were present. After IL-1 treatment, the TAB2 and N-CoR were selectively lost, and immunohistochemistry revealed that TAB2 and N-CoR were exported from the nucleus. These data indicate that N-CoR/TAB2 might be involved in the regulation of the expression of an important metastasis suppressor gene. Therefore, a metastasis suppressor gene is a potential target gene repressed by p50 homodimers. To test whether the presence of TAB2 causes active repression, we will use α TAB2 IgG and a KAI-1/LacZ from reporter gene expression, in the single cell nuclear microinjection assay (11). These studies revealed that export from nucleus is blocked by anti-MEKK1 antibody. However, activation required recruitment of specific coactivators including the MYST HAT, Tip60. Using MEKK1-/- MEF cells and anti-MEKK1 antibody, we found that MEKK1 was specifically recruited pS2 promoter in the response IL-1 in the presence of SERM(4-OHT) and converted its actions from antagonist to agonist as siRNA against MEKK1, or use of cells from MEKK1-/- revealed that 4-OHT remained in antagonist after treatment with IL-1. We next explored if more than one

complex is required for repression estrogen receptor activation. We found at least two complexes are simultaneously recruited-the Sin3A and TAB2 complexes. TAB2 complex can also be recruited simultaneously with a third repressor complex. Thus HDAC1, HDAC2, and HDAC3 are simultaneously present on the estrogen receptor in the presence of 4-OHT. To test whether these complexes were required to maintain antagonist function, we used siRNA against each recruited HDACs proving that each siRNA effectively reduced transcripts to undectectable level by 24 hours. In each case 4-OHT now functions as an agonist, revealing that each of the three distinct histonedeacetylases. Now are required for refreshing. The next question I will address is whether each HDAC has a unique, required substrate(s). In this regard I will analyze whether HDAC mutant or only a specific HDAC, if over expressed, can rescue cells treated with siRNA against HDAC1, HDAC2, or HDAC3. Also, using RNA amplification and single cell microinjection technology I will performed RNA profiling experiments to determine whether in the absence of N-CoR, SMRT, N-CoR+SMRT, HDAC1, HDAC2, or HDAC3 whether the pattern of 4-OHT response of knowm estrogen target genes is altered.

Role of the SMRT Corepressor. Because N-CoR and SMRT exhibit complimentary activity in many actions, we have generated SMRT-/- mice, and will investigate the effects on estrogen, as well as androgen receptor actions, eventually using MEFs prepared from SMRT-/- and N-CoR-/-/SMRT-/- embryos. In these experiments, we will utilize single cell nuclear microinjection assays, as previously described (11) with specific reporters, to determine activity of agonist and antagonists. We are analyzing prostate development in SMRT-/- mice, N-CoR+/-, and SMRT+/- are being generated as these mice are viable.

β-catenin, N-CoR, cell cycle gene regulation. From yeast two hybrid screens, repressor domain III of N-CoR as bait yielded multiple, independent isolated encompassing the armadillo repeat region of β-catenin. It is very interesting that the cytoplasmic and nuclear redistribution of β-catenin and downstream target genes(c-myc, cyclin D1, etc.,) are frequently involved in the epithelial-to-mesenchymal transion associated with increased invasive/migratory properties during breast cancer development. Coimmunoprecipitation assays performed by anti-N-CoR or anti- β-catenin antibodies revealed a strong interaction between N-CoR and β-catenin. Also, we evaluated the potential effects of β-catenin on the HDAC enzymatic activity of N-CoR or HDAC complexes by coimmunoprecipitation assay by expressing Flag-tagged HDACs in the presence or absence of constitutively active β-catenin (β-catenin$_c$). The HDAC activity associated with immunoprecipitation of Flag-tagged N-CoR was inhibited 3- to -4 fold by expression of β-catenin$_c$. The activity of HDAC1 complexes also was inhibited 4- to -5 fold by β-catenin$_c$, but the activities of HDAC2,3,4, and 5 were not affected. Among the target genes of β-catenin, c-myc plays important role in the cell cycle control of the mammary gland and clinical studies of breast cancer confirm their importance. Base on the complex regulation between E2F factor changes and the known association of HDAC1 with p130, we evaluated the potential association of E2Fs with β-catenin. Interestingly, coimunoprecipitation assays revealed a selective interaction between E2F4 and β-catenin. Furthermore the presence of β-catenin virtually eliminated the deacetylase activity normally associated with the immunoprecipitated E2F4/p130 complex, because

coimunoprecipitation of E2F4, p107, or p130 in the presence of β-catenin$_c$ caused a striking inhibition of associated enzyme activity. If HDAC1 enzymatic activity is a critical component of E2F4-dependent repression, then anti-HDAC1 antibody might be expected to increase expression of c-myc promoter-driven reporter. Indeed, we found that nuclear microinjection of anti-HDAC1 antibody caused a stimulation of the c-myc promoter-driven reporter.

To determine whether β-catenin caused a dismissal of HDAC1 or p130 from c-myc promoter in response to the activation of the Wnt/ β-catenin pathway, we performed the ChIP assay. Without LiCl treatment, no β-catenin was detected initially on the c-myc promoter, consistent with its down regulation. However, E2F4, p130, and HDAC1 all were present on the c-myc promoter. With LiCl treatment, β-catenin now was present on the c-myc promoter, but both p130 and E2F4 remained bound. However, HDAC1 was almost completely dismissed. These data suggest that β-catenin does not interfere with binding of E2F4 and does not displace the pocket proteins from E2F4, but recruitment if β-catenin does result in dismissal of HDAC1 from E2F4 on the c-myc promoter.

TBL1/TBLR1's function in antagonizing NCo-R/SMRT by ubiquitin ligase. In repeating a purification of N-CoR and HDAC3 containing complexs, TBL1/ TBLR1 are identified as components of N-CoR complex (19). Because TBL1/TBLR1 is a F-box/WD-repeat protein, it seemed reasonable to consider that their requirement for nuclear receptor function might reflect a role in the requirement of the ubiquitin/proteosome machinery (20). To confirm that TBL1 is capable of interactions with ubiquitin-conjugating enzymatic activity, we performed immunoprecipitation of endogeneous TBL1 followed by an in vitro ubiquitylation assay. High molecular weight biotin-multiubiquitin chains were detected in the presence of recombinant E1 enzyme and TBL1 associated proteins, but not in a control sample. We tested the hypothesis that TBL1/TBLR1 mediated recruitment of the ubiquitin/19S proteosome would be required for N-CoR ubiquitylation, dismissal and degradation. The level of the N-CoR and HDAC3 were downregulated by the TBL1/Sigh-mediated degradation mechanism upon UV stimulation. Therefore, it reveals that TBL1/TBLR1 complex is required for activation of ERα, based on its function in antagonizing N-CoR/SMRT by ubiquitin ligase mediated exhange for transcriptional activation.

Summary of last year tasks. Task1. We successfully developed anti-N-CoR and SMRT antibodies. In according to preliminary data, N-CoR and SMRT expression are upregulated during late pregnancy when proliferation of mammary epithelial cells (MEC) slows and the alveoli begin to functionally differentiate by producing milk protein. This expression profile is consistent with N-CoR and SMRT's functions as repressors of ER-mediated transcription since estrogen is required for proliferation of MEC, the burst of which occurs between 2-6 days of pregnancy. Also, we have established protocol for immunohistochemistry and in situ hybridization using anti-N-CoR/SMRT antibodies and RNA probes, respectively.

Task2a-2e; We have generated three knout independent lines exhibited germ-line transmission, and the resulting N-CoR (+/-) mice exhibited no gross deformities. Heterozygous mice are capable of breeding and nursing their young, but when heterozygous mice were bred together, no N-CoR (-/-) pups are born. Majority of N-CoR

(-/-) embryos dying around E13.5. We have not been successfully rescue embryonic mammary bud according to transplantation protocol. Now, we are generating conditional N-CoR and SMRT knout mice to permit this experiment.

Key Research Accomplishments:

- Generation of N-CoR-specific antibodies and probes.
- Temporal/Spatial pattern of N-CoR expression defined.
- Generation of mice deleted for the related gene SMRT.
- Analysis of SMRT expression during development.
- Generation of MEF lines from wild type and N-CoR-/- mutant mice.
- Generation of MEF lines from SMRT-/- mutant mice.
- Demonstration that estrogen receptor recruits at least two distinct N-CoR-containing complexes as mSin/HDAC 2/1 and also the novel TAB2/HDAC3/N-CoR complex that bind to antagonist.
- Identification of a novel N-CoR complex containing a factor TAB2 and HDAC3 as a nuclear repressor complex.
- Evidence that inflammatory signals such as IL-1b causes export of the TAB2/N-CoR/HDAC3 complex from nucleus to cytoplasm.
- Evidence that IL-1 b activates MEKK1, which phosphorylates TAB2, causing exposure of nuclear export signals and permitting the N-CoR/TAB2/HDAC complex to be exported.
- Evidence that IL-1b with export of the TAB2/N-CoR/HDAC complex causes estrogen antagonists to function as full agonists.
- Proof that the histone deacetylase activity of HDAC1, HDAC2, and HDAC3 are all required for antagonist fuction.
- Evidence that a specific complex is required for activation of ER α, based on its function in antagonizing N-CoR/SMRT by ubiquitin ligase mediated exchange.
- Initial demonstration that tetraspanins, such as KA1, can exert in vivo anti-metasteses function.
- Lingkage of β-catenin to a repressor function favoring metastatic disease.

Conclusions: We can conclude that clinically appropriate response to estrogen antagonists can be linked to activation on binding of an N-CoR/HDAC2/TAB2 complex, required to prevent antagonist as agonists. The definition of this pathway could be extended in the coming year and uses to provide export of the complex will be explored. Finally, in these studies, the role of the TSE has been uncovered and I will explore this over the coming year.

Reproducible Outcomes: The corepressor functions on the KA1 regulation. In preparation.

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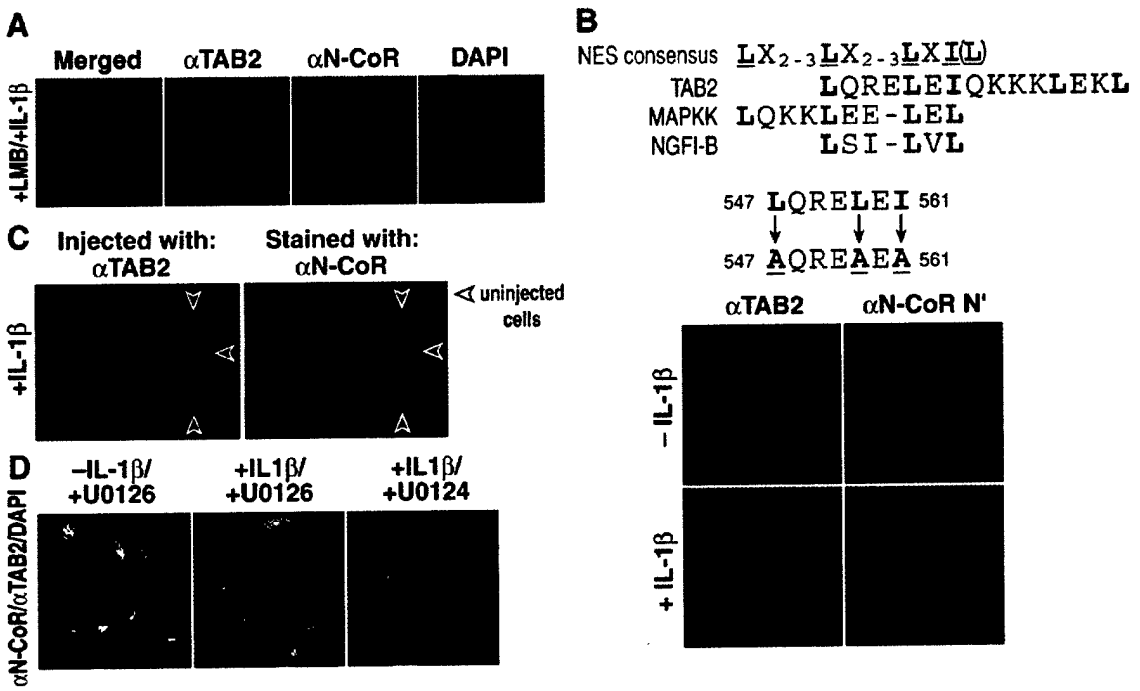

Figure 1. Identification of the Nuclear Export Signal (NES) and MEKK1 Target Sites on TAB2. (A) CV-1 cells pretreated with leptomycin B (10 ng/ml) for 30 min were examined for effects of IL-1or 2 hr on localization of N-CoR and TAB2. (B) Alignment of NES and block of the nuclear export by mutation of the NES of TAB2. NES sequences are compared with MAPKK and NGFI-B. Consensus residues are underlined and indicated in "red." Transiently transfected cells with NES disruption mutant were treated with IL-1 for 6 hr, and localization of N-CoR- and TAB2-containing NES disruption mutants was examined. (C) Microinjection of anti-TAB2 IgG, using TRITC-conjugated dextran to mark injected cells prior to IL-1treatment for 2 hr, largely blocks most nuclear N-CoR export. Uninjected cells (arrows) show N-CoR export to cytoplasm. (D) CV-1 cells were pretreated with U0124 (negative control) or U0126 (MEK/MEKK inhibitor) for 30 min, followed by IL-1 treatment for 2 hr. Localization of N-CoR and TAB2 were examined.

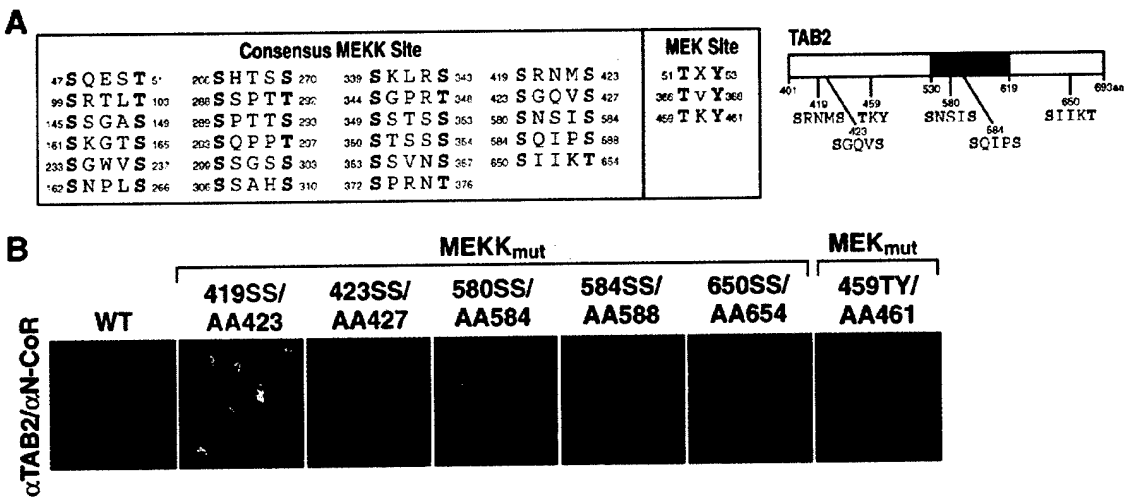

Figure 2. Effects of mutagenesis of MEKK and MEK target sites. (A) Schematic representation of MEKK and MEK target sites on TAB2. Site-directed mutagenesis was performed to replace serine residues by alanine residues.(B) TAB2 holoprotein harboring the indicated MEKK1 target site disruption mutants of TAB2 were transiently transfected, the cells were treated with IL-1_for 6 hr, and cellular distribution of N-CoR and TAB2 was analyzed by staining with anti-N-CoR IgG and anti-TAB2 IgGs.

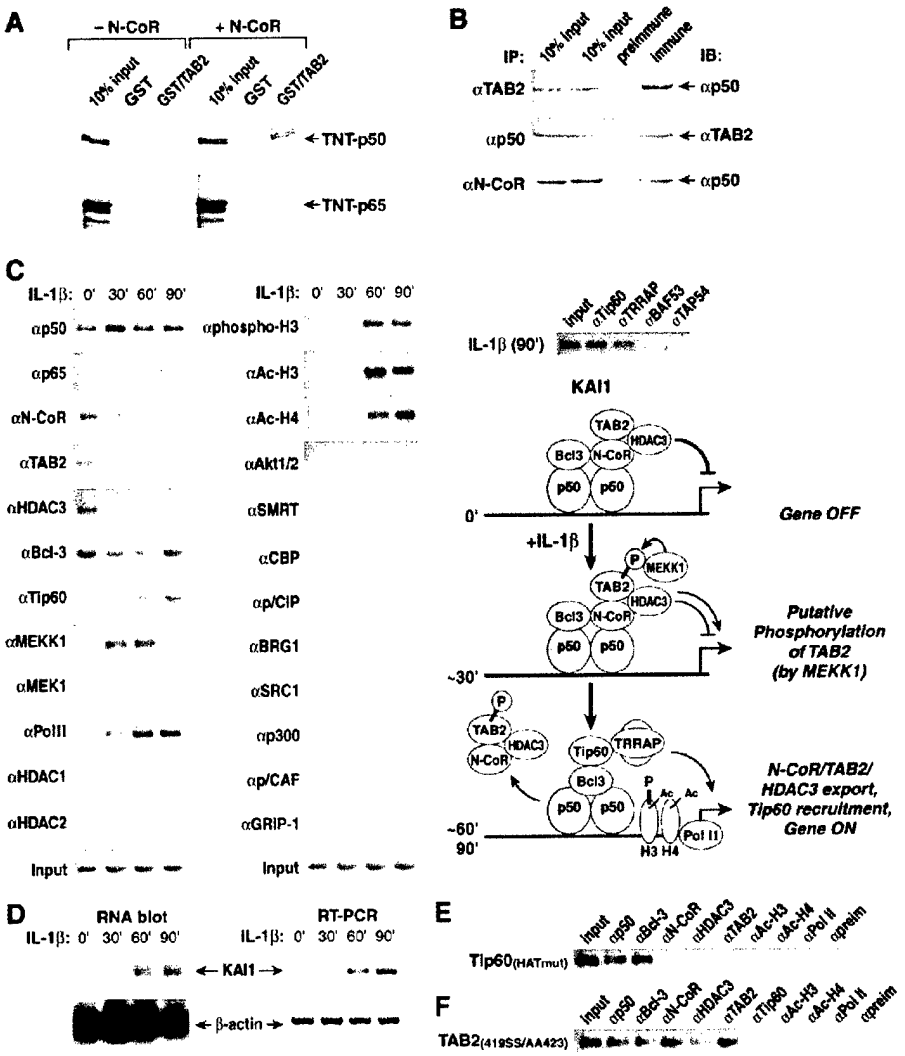

Figure 3. Identification of a p50-Dependent Target Gene, *KAI1*, Recruiting N-CoR/TAB2/HDAC3 Complex. (A) TAB2 interacts with p50 but not p65 in the presence of N-CoR. A GST/TAB2 fusion protein was incubated with 35S-labeled p50 or p65 from rabbit reticulocyte lysate alone or with unlabeled in vitro translated N-CoR. (B) Intracellular interactions between p50 and N-CoR or TAB2 shown in 293 cell extracts immunoprecipitated with either anti-TAB2 or anti-N-CoR IgG and coprecipitated were detected with anti-p50 IgG. (C) Chromatin immunoprecipitation (ChIP) assay on *KAI1* promoter. Occupancy of *KAI1* promoter by p50, N-CoR/TAB2/HDAC3, Bcl3, Tip60, acetylated histones H3 and 4, phosphorylated histone H3, RNA polymerase (Pol II), MEKK1, TRRAP, and other factors at the indicated times after IL-1 treatment in 293 cells. Schematic representation shows the dynamics of cofactor occupancy on the *KAI1* promoter. (D) RNA blot and RT-PCR of KAI1 and beta-actin transcripts following IL-1 stimulation. (E) Overexpression of Tip60 HATmut causes the failure of Pol II binding and acetylation of histones H3 and H4 after IL-1 treatment for 1 hr. (F) Overexpression of TAB2 S419/423A blocks dismissal of the N-CoR complex and gene activation events in response to IL-1 for 1 hr.

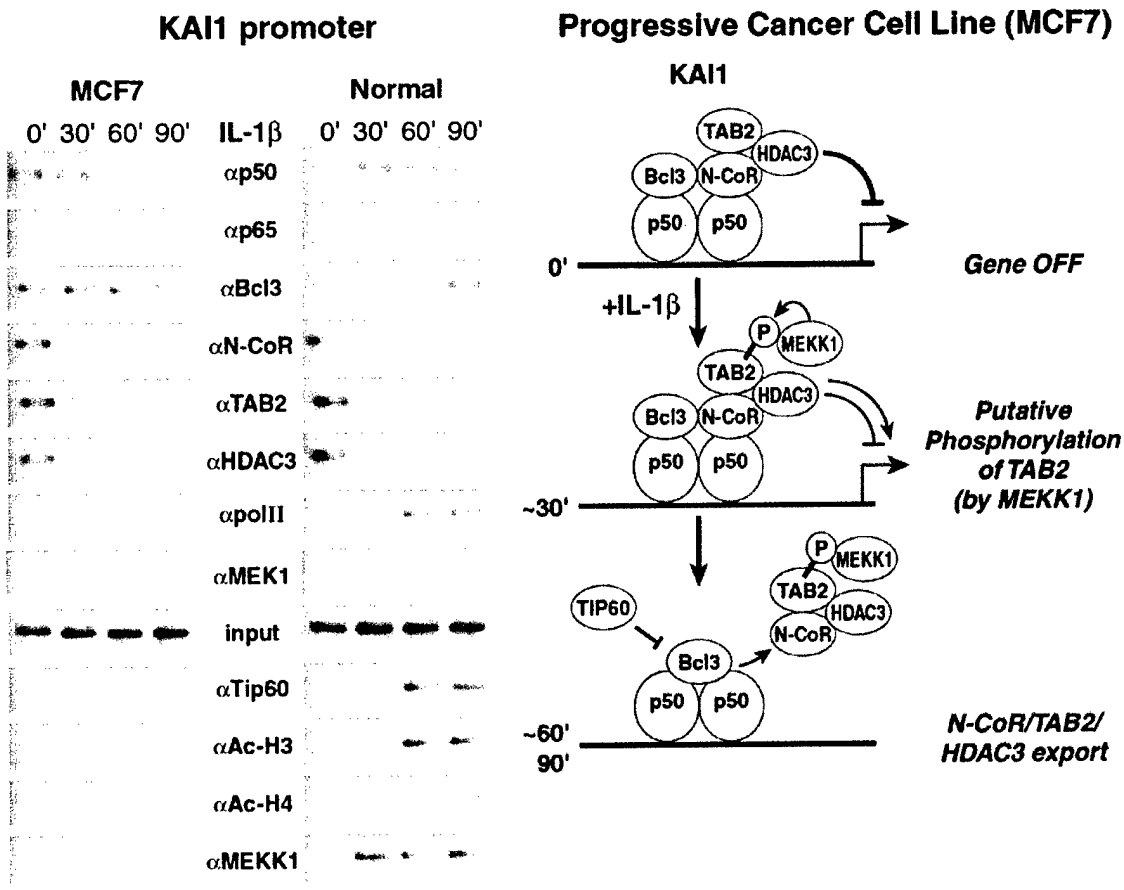

Figure 4. ChIP Analysis of IL-1-Induced Cofactor Association with the KA1 Promoter (A) Recruitment patterns of p50, p65, and different coactivators on KA1 promoter in MCF7 cells treated with IL-1. (B). Schematic representation showing KA1 promoter occupancy by various coactivators induced by IL-1 in MCF7 cells.

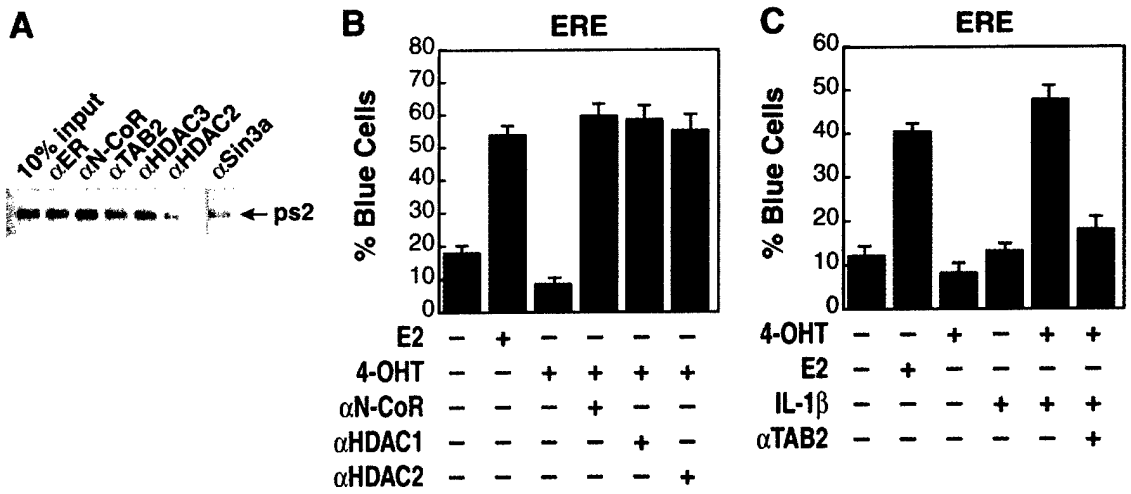

Figure 5. Functions of IL-1 Signaling to Relieve N-CoR-Mediated Repression. (A)ChIP assay on the *pS2* promoter shows promoter occupancy by N-CoR/HDAC2/mSin3 complex, as well as N-CoR/TAB2/HDAC3 complexes in 4-OH-tamoxifen (4-OHT)-treated cells. (B) Microinjection of IgG against HDAC1, HDAC2, or N-CoR fully relieved the repression by 4-OHT bound estrogen receptor a. (C) Pretreatment with IL-1 abolished 4-OHT-mediated repression of a reporter containing ERE, and nuclear microinjection of anti-TAB2 IgG blocked IL-1-dependent derepression. Results are mean ± SEM >200 cells injected for each data point.

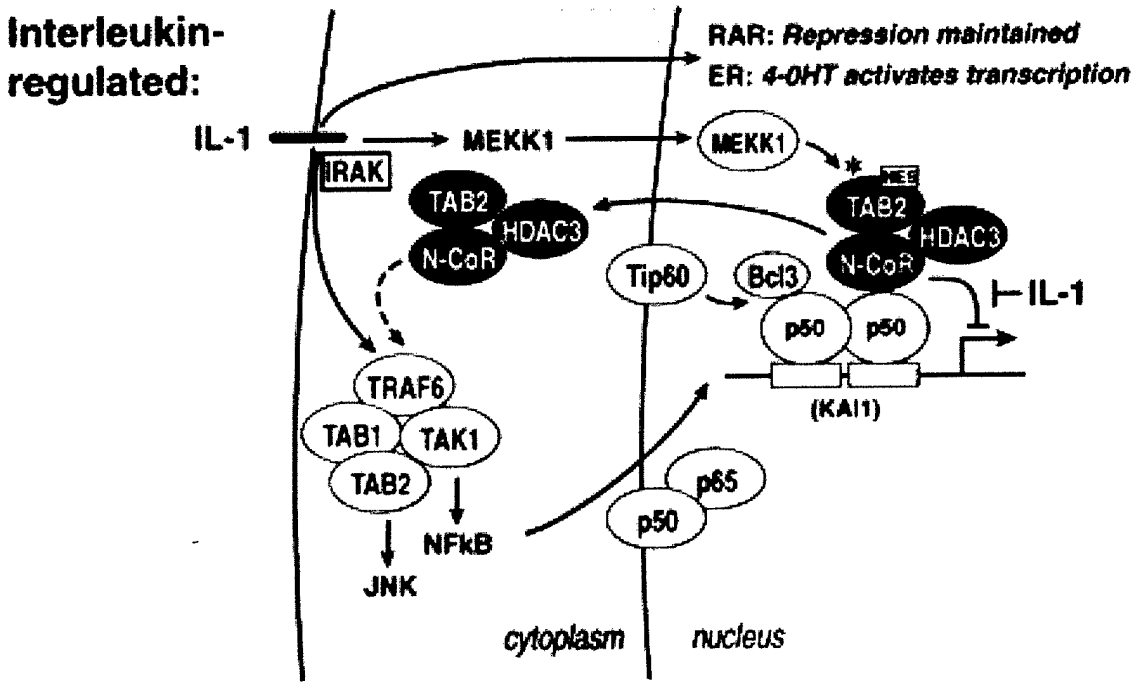

Figure 6. Integration of Signaling Responses by IL-1-Induced Derepression and by a Physiological/Pathological Mechanism of Derepression/Activation. IL-1 signaling activates MEKK1-dependent translocation of an N-CoR/TAB2/HDAC3 complex based on phosphorylation of a specific residue on TAB2, causing derepression of p50-dependent transcription units, exemplified by *KAI1*. However, antagonist actions of tamoxifen are lost upon IL-1 stimulation, indicating a specific program of derepression integrated by this signaling pathway. This complex, formation of which requires Tip60 HAT function, in transfected cells displaces N-CoR/TAB2/HDAC3, activating the *KAI1* promoter and providing a potential mechanism for pathophysiological regulation of gene expression in the breast cancer.