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PRINCIPAL INVESTIGATOR: Bong-Gun Ju, Ph.D.
Doctor Michael G. Rosenfeld

CONTRACTING ORGANIZATION: The University of California, San Diego
San Diego, California 92093-0602

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Bong-Gun Ju, Ph.D.
Doctor Michael G. Rosenfeld

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, San Diego
La Jolla, California 92093-0602

E-Mail: bju@ucsd.edu

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Understanding the regulation of N-CoR and its associated corepressors, in concert with defining the molecular mechanisms that underline 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/HDAC1/2 and also the novel TAB2/HDAC3/N-CoR complex that bind to agonist. The inflammatory signals such as IL-1 causes export of the TAB2/N-CoR/HDAC3 complex from nucleus to cytoplasm by MEKK1 action. Among the N-CoR target genes, c-myc and KAI1 plays important roles in the cell cycle control and metastases during breast cancer. Therefore, 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(s). Furthermore, we demonstrated that specific N-CoR complex containing TBL1/TBLR1 serve as specific adaptors for the recruitment of the ubiquitin conjugating/19S proteasome complex, with TBLR1 selectively serving to mediate a required exchange of the ER corepressors, N-CoR and SMRT, for coactivators upon ligand binding. Therefore, TBL1 and TBLR1 constitute a specific class of coregulators which play important roles in both activation and repression, acting as *n*uclear receptor *co*repressor/coactivator *ex*change factors (N-CoEx), required for modulating regulated gene transcription by ER.

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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 transhydroxytamoxifen (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-N-CoR 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 (API) 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 a thymic 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-actincomplex 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 Dl, 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.

Biochemical purification of the N-CoR and SMRT corepressor complexes showed that they both contain TBL1(Transducin β-like 1) and HDAC3 (Histone Deacetylase 3) (16, 17). It has been subsequently identified GPS2 and TBLR1, which shares very high homology with TBL1, as additional components of the N-CoR/SMRT complex (18). Because TBL1/TBLR1 are putative F box/WD40 repeat proteins, it seemed reasonable to consider that their requirement for nuclear receptor function might reflect a role in the recruitment of the ubiquitin/proteasome machinery. Degradation of nuclear receptors upon ligand binding has been reported for almost all the components of the nuclear receptor superfamily, even though the turnover of the receptor is not unambiguously functionally linked to transcriptional activity (19).

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 and molecular mechanism of N-CoR-ER mediated repression 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 NCoR (-/-)

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 perfonned 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 anti-N-CoR IgG at endogenous levels of both proteins. GST pulldown 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 TNFα (tumor necrosis factor α)/IL-l (interleukin-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-I, 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/TNFα 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/TNFα, 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 HDAC3, but not HDAC1, HDAC2, or mSin3. In parallel, single cell nuclear microinjection assays (11) were performed, initially employing affinity-purified anti-TAB2 IgG (immunoglobulin G), revealing that NCoR 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-l (mitogen-activated protein kinase kinase 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-l/TNFα 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 confonnational change, dependent upon phosphorylation of a specific C-terminal residues serves to initiate a confonnational 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 TAP2 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-1 induced translocation could be assessed in single cell assays. We found that anti-MEKKl IgG blocked IL-1-induced translocation to cytoplasm.

The next issue was to explore the effects of the IL-l/TNFα pathways on actions of specific classes of DNA binding transcription factors. Based on the relationship of the TNFα/IL-1 pathway to activation of NFκB, we will explore NFκB-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 ICAM-1 (intercellular adhesion molecule-1) and IL-6. To address this further, we investigated potential downstream target genes using the chromatin immunoprecipitation (ChIP) assay. The KAIl 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 (20). KAIl 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 KAIl gene is involved in the progression of the cancer, and the KAIl gene appears to be regulated by signaling molecules that activate this NFκB target gene. We found that the p50 component of NFκB 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, TAP2, and N-CoR on the KAIl promoter. In the absence of treatment, p50, but not the p65 NFκB 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 used anti-TAB2 IgG and a KAI-l/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 Tip60. Using MEKK1-/- MEF (mouse embryonic fibroblast) 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 (small interfering RNA) 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 HDACI, 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 histone deacetylases.

Role of the SMRT Corepressor. Because N-CoR and SMRT (silencing mediator for retinoid and thyroid hormone receptors) exhibit complimentary activity in many actions, we have generated SMRT -/- mice, and have investigate the effects on estrogen, as well as androgen receptor actions, eventually using MEFs cells prepared from SMRT-/- and N-CoR-/-/SMRT-/- embryos.

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 genese (c-myc, cyclin D 1, 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 HDAC 1 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 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 N-CoR/SMRT by ubiquitin ligase. N-CoR has been proven to be a component of a large number of distinct complexes, which are probably combinatorially required to mediate repression in a promoter- and cell-specific fashion. TBL1 initially purified as a component of an N-CoR corepressor complex that also contained HDAC3 (16, 17). Analyzing genes regulated by the estrogen receptor, we unexpectedly observed recruitment of TBL1 in response to ligand-dependent gene activation. TBL1 was recruited on the ERE response element of the *pS2* promoter in response to estradiol (E_2) stimulation. Failure of TBL1 recruitment to the pS2 promoter in the ERα null cell line MDA-MB-231 proved that TBL1 recruitment to the ERE response element was dependent on binding of the liganded receptor. Furthermore, endogenous TBL1 could be coimmunoprecipitated with the estrogen receptor in the presence of ligand and could be also coimmunoprecipitated with coactivators such as RIP140 and CBP/p300 both in the presence or absence of ligand, suggesting that receptor-dependent recruitment of TBL1 is probably indirect, based on interactions with other cofactors and, perhaps, with histones (16, 21). We next investigated whether TBL1 was functionally required for estrogen receptor-mediated transcriptional activation. Single cell microinjection of specific purified IgGs against TBL1 blocked the activation of an ERE-dependent reporter upon E_2 treatment, while they did not have any effect with the antagonist 4-hydroxy-tamoxifen (4-OHT). TBL1 was also found to be required for the E_2-dependent activation of the regulatory region of the *pS2* promoter. To confirm these findings by an independent approach, we designed a specific siRNA against human *Tbl1*, effectively reducing the level of *Tbl1* mRNA to undetectable levels, based on RT-PCR analysis of TBL1 expression performed on several hundred microinjected cells. Microinjection of this specific siRNA into MCF7 nuclei abolished activation of the ERE-dependent reporter upon ligand stimulation, while control siRNA did not have any effect, confirming that TBL1 is required for transcriptional activation by the estrogen receptor.

In order to independently confirm the unexpected role for TBL1 in gene activation events, we employed a genetic approach deleting the *Tbl1* genomic locus in ES cells to investigate the role of TBL1 in regulated developmental processes. The targeting vector replaced the 5′-terminal part of the gene with EGFP cDNA, introducing a strong splicing acceptor site from the β *-globin* gene in order to avoid any cryptic splicing event over the EGFP coding sequence, and G418-resistant R1-ES cells were screened for homologous recombination events by Southern blot analysis with specific 5′- and 3′- probes; two positive clones were further confirmed by PCR analysis. Because the *Tbl1* gene is located on the X chromosome and R1-ES cells are male, the positive clones identified are *Tbl1* hemizygous clones ($Tbl1^{\Delta}/Y$), and Western blot analysis confirmed that TBL1 protein expression was completely abolished in the recombinant cells. The recombinant $Tbl1^{\Delta}/Y$ ES cells provided a powerful model in which to analyze TBL1 function for the activation of known endogenous nuclear receptor target genes upon ligand stimulation. $Tbl1^{\Delta}/Y$ cells are no longer able to respond to estrogen treatment as shown by the lack of activation of known target genes, such as *pS2*, *ERα*, and *ERβ*. Thus, *Tbl1* gene deletion provides independent confirmation of the observation that TBL1 is required for transcriptional activation by estrogen hormone receptors.

In repeating a purification of N-CoR- and HDAC3-containing complexes, an additional member of the TBL1 family, TBLR1 has been identified, TBLR1 encoded by a distinct

gene located on autosomal murine Chr 3. The TBLR1 protein product is highly homologous to the TBL1 protein encoded by the *Tbl1X* gene. Additionally, the database search showed that in the human genome, *Tbl1X* has a conserved homolog, *Tbl1Y*, on the Y chromosome. However, in the murine genome, the *Tbl1Y* gene does not seem to be conserved as murine *Tbl1X* was mapped to a more proximal chromosomal position, far from its neighboring genes in human Xp22.3 (22) and among genes that do not have conserved homologs on the Y chromosome. Furthermore, our data showing loss of nuclear receptor-mediated transcriptional responses in the murine *Tbl1*$^{\Delta}$*/Y* ES cells also suggest the absence of a TBL1Y protein able to compensate for *Tbl1X* deletion.

Overexpressed *Tbl1*$^{\Delta}$*/Y* ES cells of either Flag-tagged TBL1 or Myc-tagged TBLR1 and Western blots clearly confirmed that our guinea pig α-TBL1 antibody was highly specific for TBL1. An additional rabbit antibody against a peptide specific for TBLR1 was then successfully raised. Chromatin immunoprecipitation analysis revealed that TBL1 is preferentially bound to the unliganded receptor and partially dismissed upon ligand. ERα receptors all required both TBL1 and TBLR1, as removal of either of these factors, by antibody or siRNA microinjection, was sufficient to abrogate transcriptional activation. Thus, TBLR1 function is required for transcriptional activation by ER. Because TBL1/TBLR1 are putative F box/WD40 repeat proteins, it seemed reasonable to consider that their requirement for nuclear receptor function might reflect a role in the recruitment of the ubiquitin/proteasome machinery. Degradation of nuclear receptors upon ligand binding has been reported for almost all the components of the nuclear receptor superfamily, even though the turnover of the receptor is not unambiguously functionally linked to transcriptional activity (19). As expected, microinjection of an antibody against the S1/Rpn2 subunit of the 19S proteasome or pretreatment of the cells with the proteasome inhibitor MG132 blocked ligand-induced transcriptional activation for ER, confirming, in our experimental model, that ubiquitin-dependent proteasomal degradation is required for nuclear receptor-mediated transcription.

To investigate whether the function of TBL1 or TBLR1 in transcriptional activation might be to recruit the ubiquitin-conjugating machinery, we tested whether TBL1 domains important for binding to ubiquitin ligases were also required for transcriptional activation by TBL1-dependent nuclear receptors. Indeed, deletion of the putative F box, ubiquitin ligase-recruiting domain, was enough to abolish the ability of TBL1 or TBLR1 to rescue transcriptional activation by any of the receptors tested when endogenous TBL1 or TBLR1 had been specifically blocked by antibody microinjection. Deletion of the WD-40 C′-terminal region also blocked TBL1 transcriptional function. To confirm the specificity of these results, we also injected specific siRNAs against TBL1 and TBLR1 and the unrelated F box/WD40 proteins *Fbx3* and *Fbx8* and found that only TBL1 and TBLR1 were required for nuclear receptor-mediated transcriptional activation. To confirm that TBL1 is capable of interactions with ubiquitin-conjugating enzymatic activity, we performed immunoprecipitation of endogenous TBL1 from 293 cells followed by an in vitro ubiquitylation assay, assessing ubiquitylation activity by formation of multiubiquitin chains from free ubiquitin. High molecular weight biotin-multiubiquitin chains were detected in presence of recombinant E1 enzyme and TBL1-associated proteins, but not in a control sample for immunoprecipitation of nonspecific complexes. Addition of exogenous recombinant UbcH/E2 enzymes was not necessary, although ubiquitylation activity appeared increased by the specific addition of UbcH5. We further tested whether TBL1 and TBLR1 could interact directly or indirectly with UbcH5, observing coimmunoprecipitation of Myc-tagged TBLR1 with HA-tagged UbcH5 using either the α Myc or the αHA antibody and similar, although weaker, interactions with TBL1.

Furthermore, deletion of the F box impairs TBLR1 ability to interact with UbcH5 and deletion of the entire N terminus completely abrogates it, while deletion of the C terminus results in increased binding. Chromatin immunoprecipitation analysis performed on the *pS2* promoter after estrogen stimulation revealed that specific components such as the UbcH5 enzyme and the S1 subunit of the 19S proteasome were recruited to the promoter in a ligand-dependent fashion, with kinetics of recruitment very similar to those observed for the receptor itself. The recruitment of these factors seems to be specific because many ubiquitylation enzymes were not recruited to the same promoter. Microinjection of specific antibodies against the different UbcH enzymes revealed that, in the case of estrogen receptor, UbcH5 was not only physically recruited on the promoter, as shown on the *pS2* promoter, but also appeared to be functionally required for transcriptional activation of an ERE-dependent reporter. These data are consistent with nuclear receptor activity being modulated by ubiquitylation and suggest that TBL1/TBLR1 are the specific F box/WD-40 adaptor proteins used for the recruitment of the ubiquitin-conjugating enzyme UbcH5 to specific targets. However, a complete and exhaustive description of the network of enzymes used by each receptor will clearly require further analysis in the future because specific interactions among E3 ligases and specific ubiquitin-conjugating E2 enzymes have yet to be fully defined.

Next, we wished to determine the mechanisms by which TBL1-mediated recruitment of the ubiquitin-machinery regulates gene activation events. In the case of estrogen, it has been reported that binding of the receptor to the promoter is not stable but undergoes a local turnover (23, 24, 25), and it is well established that nuclear receptors are targeted to protein degradation upon ligand binding, as several reports have described a global downregulation of the receptor protein level after ligand stimulation. Thus, a possible explanation for TBL1 function in gene activation could be in mediating the degradation of the receptor, which has been suggested to be the reason for which the proteasome is required for receptor-mediated transcriptional activation. We tested this hypothesis by analyzing whether the global downregulation of the estrogen receptor protein level upon ligand treatment would be affected by the deletion of the *Tbl1* gene. First, we confirmed that ER protein could be downregulated in a proteasome-dependent fashion, following estrogen stimulation, in both MCF7 and in NIH-3T3 cells; however, in the pituitary cell line αT3, only limited degradation occurs and only at much later times after ligand stimulation. Surprisingly, we did not observe any downregulation of ERα in ES cells, even though in these cells TBL1 is fully required for E_2-dependent gene activation; indeed we found that ER protein level was actually upregulated, while it remained essentially unchanged in the $Tbl1^{\Delta}/Y$ cells. Because we observed impairment of the ES cells to respond to estrogen stimulation in absence of TBL1, these data reveal that the requirement for TBL1 for ER-mediated transcriptional activation cannot be explained as a consequence of TBL1 being responsible for estrogen receptor degradation. These data raised the intriguing possibility that TBL1 is responsible for ubiquitylation/degradation of other cofactors and/or components of the transcriptional machinery required for nuclear receptor functions.

Because TBL1/TBLR1 are components of an N-CoR corepressor complex, we tested the hypothesis that TBL1/TBLR1-mediated recruitment of the ubiquitin/19S proteasome would be required for N-CoR ubiquitylation, dismissal, and degradation. Interestingly, it has been reported proteasomal degradation of N-CoR, based on its interaction with the E3 ligase Siah2 (26). In the case of β-catenin and Tramtrack88, both mammalian Siah and its *Drosophila* homolog, Sina, have been described to use TBL1 as their adaptor subunit responsible for specific substrate recognition (27, 28, 29); thus, we investigated whether

N-CoR degradation could also be TBL1 dependent. First, we analyzed the levels of the corepressors N-CoR and HDAC3 upon UV stimulation in the same biological system in which TBL1/Siah-mediated degradation of β-catenin, via p53 activation, was reported (29). Interestingly, in NIH-3T3 cells, the levels of both proteins were downregulated upon UV treatment. To test whether this downregulation was TBL1 dependent, we induced N-CoR degradation by overexpression of Siah1 or Siah2, p53-inducible genes that are the rate-limiting factors of the SCF^{Tbl1} complex (29, 30). As predicted, the N′-terminal region of TBL1 behaves as a dominant-negative and its overexpression is sufficient to abrogate N-CoR degradation, suggesting that N-CoR proteasomal degradation is TBL1 dependent. Similarly, HDAC3 protein level is highly decreased upon Siah2 overexpression, and this effect is partially abrogated by overexpression of TBL1 ΔN, suggesting that HDAC3 may also be a target of TBL1-induced degradation.

Summary of 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 still trying to generate 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 1/2 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-1causes export of the TAB2/N-CoR/HDAC3 complex from nucleus to cytoplasm.
· Evidence that IL-1 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-1 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 function.
- Initial demonstration that tetraspanins, such as KAI1, can exert in vivo anti-metasteses function.
- Lingkage of β-catenin to a repressor function favoring metastatic disease.
- Generation of TBL1-/- embryonic stem cells
- Generation of TBL1 and TBLR1-specific antibodies
- Evidence that TBL1 is required for ER-mediated activation
- Linkage of TBL1 to the recruitment of the ubiquitin-proteasome complex
- Evidence that a specific complex is required for activation of ER, based on its function in antagonizing N-CoR/SMRT by ubiquitin ligase-mediated corepressor/coactivator exchange

Reproducible Outcomes:
Abstract: Nuclear receptor corepressor regulation of estrogen receptor antagonist function. September 25-28: Era of Hope Meeting.

The Regulation of Corepressor/Coactivator Exchange in Estrogen Receptor-mediated Transcription, In preparartion.

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. Furthermore, TBL1/TBLR1 serve as specific adaptors for the recruitment of the ubiquitin conjugating/19S proteasome complex, with TBLR1 selectively serving to mediate a required exchange of the ER corepressors, N-CoR and SMRT, for coactivators upon ligand binding. Therefore, TBL1 and TBLR1 constitute a specific class of coregulators, distinct from other classes of coactivators and corepressors, which play important roles in both activation and repression, acting as *n*uclear receptor *co*repressor/coactivator *ex*change factors (N-CoEx), required for modulating regulated gene transcription by ER.

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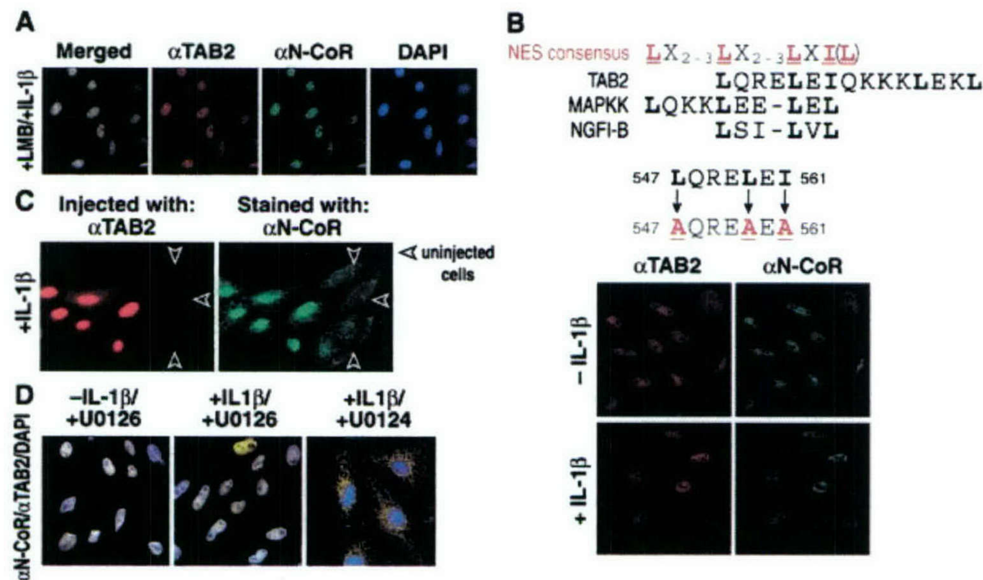
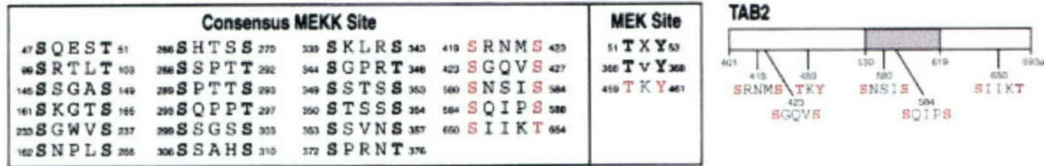


Figure 1. Identification of the Nuclear Export Signal (NES) and MEKK1 Target Sites on TAB2(A) CV-1 cells pretreated with leptomycin B (10 ng/ml) for 30 min were examined for effects of IL-1 for 2 hr on localization of N-CoR and TAB2.(B) Alignment of NES and block of the nuclear export by mutation of 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 TAB2 IgG, using TRITC-conjugated dextran to mark injected cells prior to IL-1 treatment 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.

A



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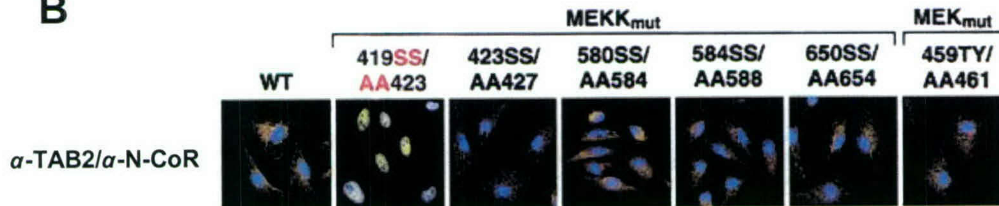


Figure2. Effects of mutagenesis of MEKK and MEK target sites (A) Schematic representation of MEKK and MEK target sites on TAB2. Site-directed mutagenesis was performed to replace serine residues by alanine residues.(B) TAB2 holoprotein harboring the indicated MEKK1 target site disruption mutants of TAB2 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.

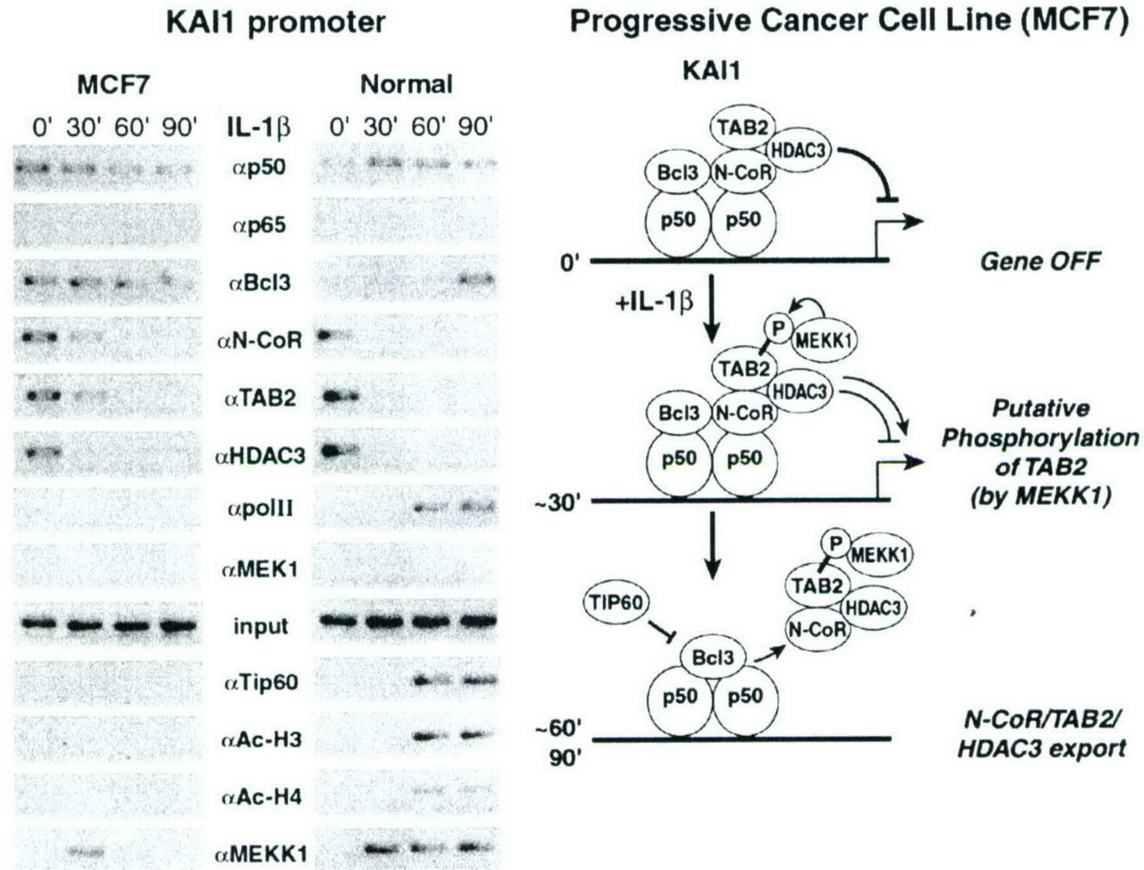


Figure 4. ChIP Analysis of IL-1-Induced Cofactor Association with the KA1 Promoter (A) Recruitment patterns of p50, p65, and different coactivators on KA1 promoter in MCF7 cells treated with IL-1. (B). Schematic representation showing KA1 promoter occupancy by various coactivators induced by IL-1 in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

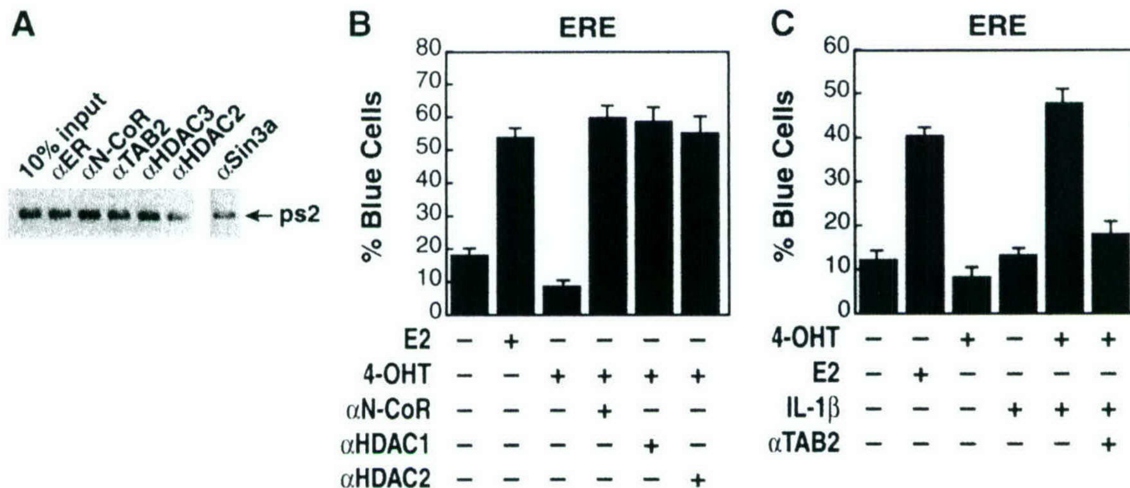


Figure 5. Functions of IL-1 Signaling to Relieve N-CoR-Mediated Repression. (A)ChIP assay on the *pS2* promoter shows promoter occupancy by N-CoR/HDAC2/mSin3 complex, as well as N-CoR/TAB2/HDAC3 complexes in 4-OH-tamoxifen (4-OHT)-treated cells. (B) Microinjection of IgG against HDAC1, HDAC2, or N-CoR 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.

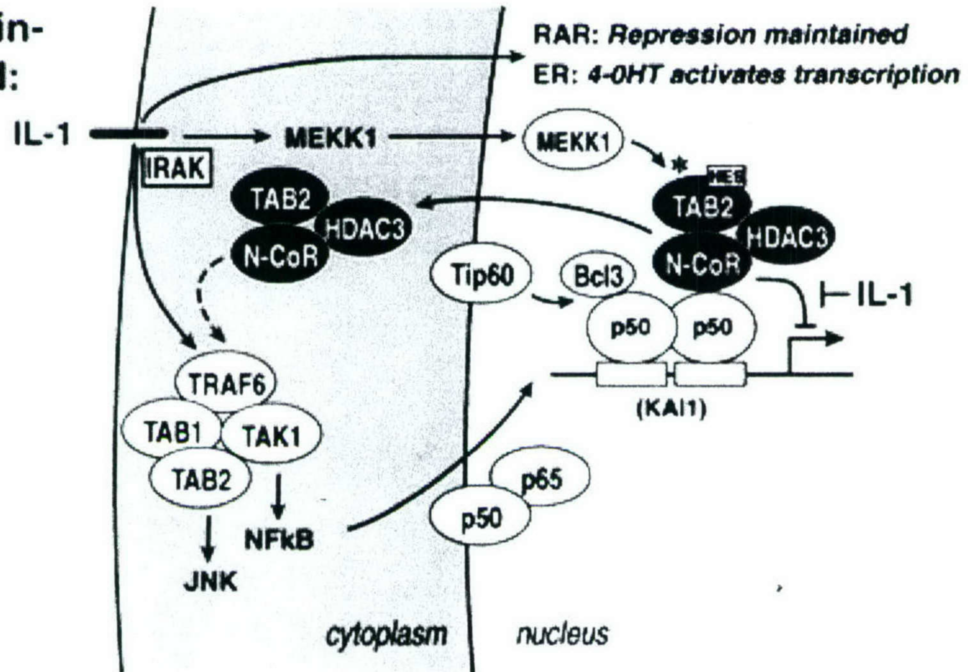


Figure 6. Integration of Signaling Responses by IL-1-Induced Derepression and by a Physiological/Pathological Mechanism of Derepression/Activation. IL-1 signaling activates MEKK1-dependent translocation of an N-CoR/TAB2/HDAC3 complex based on phosphorylation of a specific residue on TAB2, causing derepression 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.

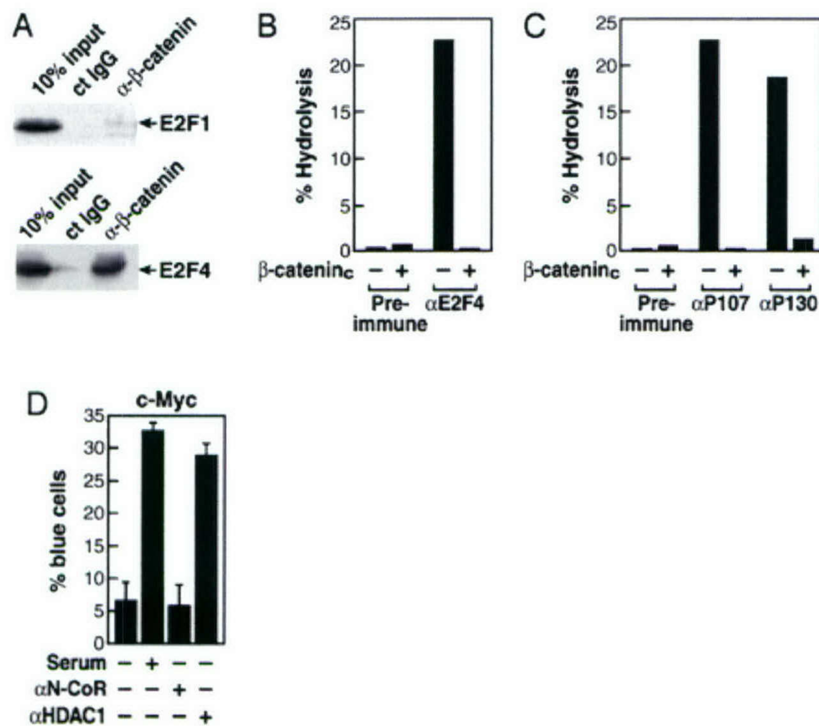


Figure7. Role of β-catenin in derepression. (*A*) β-Catenin interacts with E2F4. HA-E2F1 and HA-E2F4 expression vectors were cotransfected with β-cateninc-expressing vectors, and immunoprecipitation was performed with control IgG of β-catenin IgG. β-Catenin was interacting strongly with E2F4. (*B*)β -Catenin dismisses HDAC activity associated with E2F4. 293 cells were transfected with β-cateninc expression vectors and immunoprecipitated with preimmune IgG or E2F4 IgG, and HDAC activity was measured. (*C*) β-Cateninc also inhibits HDAC activity on p107- and p130-immunoprecipitated material from 293 cells. (*D*) Effects of HDAC1 and N-CoR IgGs on expression of the c-*Myc* promoter in Rat1 cells. After 48 h under serum-free conditions, cells were microinjected with either N-CoR or HDAC1 IgGs, and serum was added 8 h before assay. Each point is mean ± SEM of >300 microinjected cells; similar results were obtained in two additional experiments of similar design by using C2C12 or Rat1 cells.

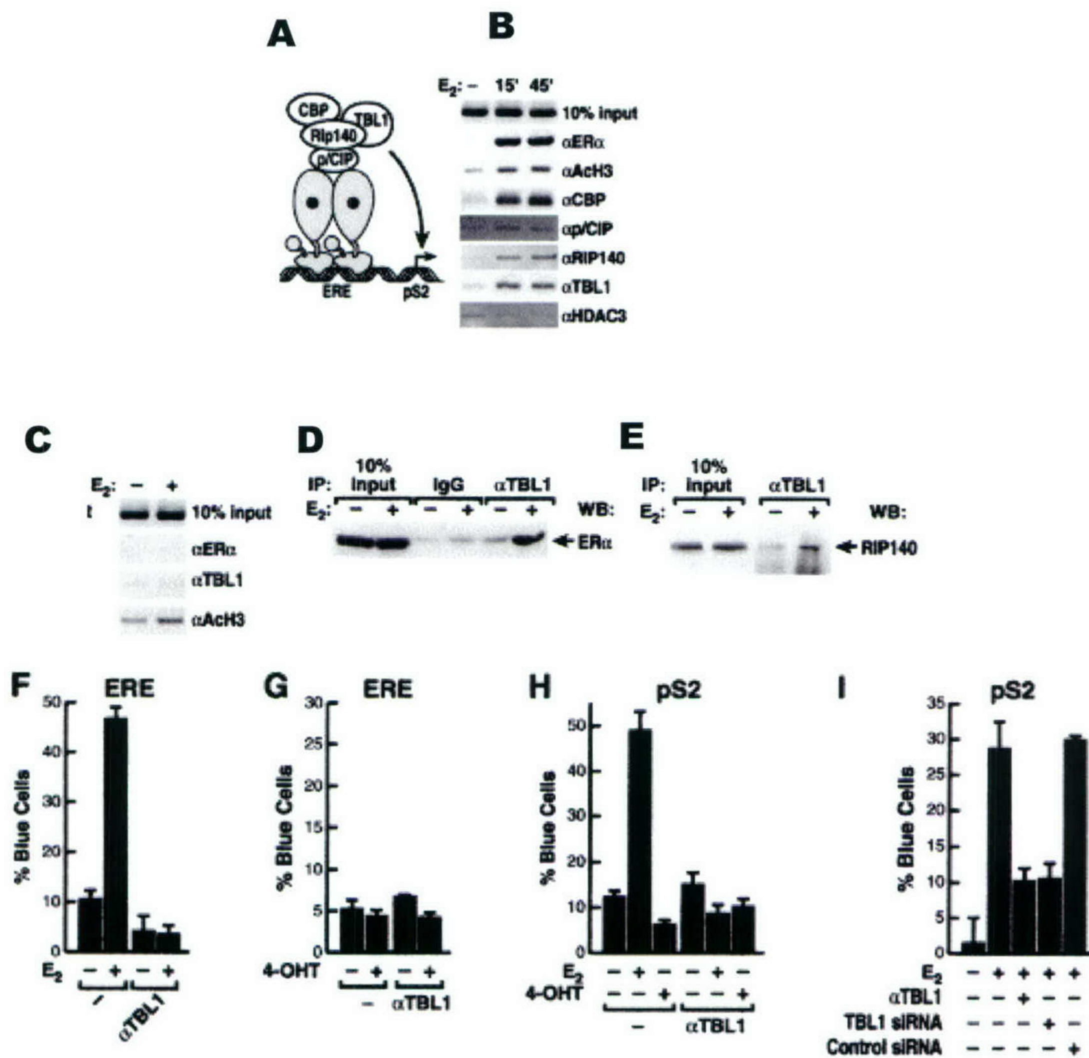


Figure 8. TBL1 Is Required for Transcriptional Activation by Nuclear Receptors. (A,B) ChIP analysis of the occupancy of the *pS2* promoter upon activation by estradiol (E2). TBL1 was recruited to the *pS2* promoter within 15′ after ligand stimulation.(C) TBL1 recruitment to the androgen-regulated *kallikrein 2* promoter upon dihydroxytestosterone (DHT) binding.(D) TBL1 was not found by ChIP on the *pS2* promoter in the ER null cell line MDA-MB-231 stimulated by E2.(E) Endogenous coimmunoprecipitation of TBL1 with ER and Rip140 performed in estradiol-stimulated MCF-7 cells.(F) Single cell nuclear microinjection of purified IgGs against TBL1 inhibited E2-dependent transcriptional activation in Rat1 cells.(G) TBL1 IgGs microinjection did not have any effect of 4-hydroxy-tamoxifen (4-OHT)-induced repression of the ERE/LacZ reporter.(H) TBL1 IgGs microinjection blocked activation of a 1.2 kb fragment of the *pS2* promoter upon stimulation with E2 in MCF7 cells. (I) Single cell microinjection of a specific siRNA in MCF7 nuclei induced selective downregulation of TBL1 RNA, as measured by RT-PCR analysis in 100 injected cells, and blocked activation of the *pS2* promoter upon E2 stimulation. Injection of control siRNA had no effect.

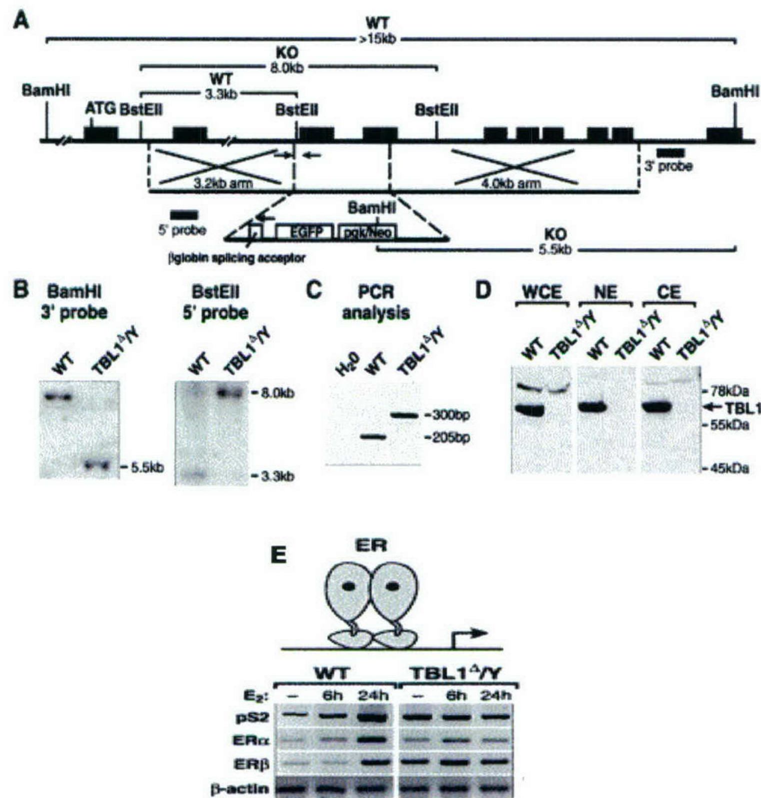
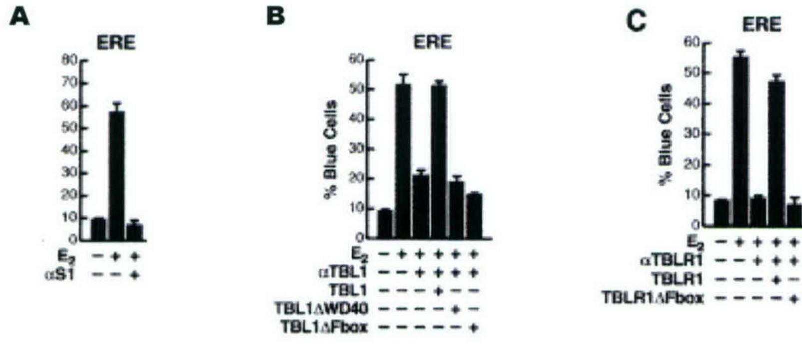


Figure 9. Targeted Disruption of the *Tbl1* Gene in Embryonic Stem Cells(A) Schematic representation of the *Tbl1* genomic locus and of the targeting vector showing the restriction sites relevant for the use of specific 5′ and 3′ probes in Southern blot analysis and the primers (arrows) used for PCR analysis.(B) Southern blot analysis of ES cell clones digested with BamHI and hybridized with the 3′-external probe or digested with BstEII and hybridized with a 5′-specific internal probe.(C) PCR analysis of genomic DNA extracted from ES cells using the primers indicated in (A), designed to amplify fragments of different sizes in the wild-type or the recombinant clones.(D) Immunoblot of whole-cell extracts (WCE), nuclear extracts (NE), and cytoplasmic extracts (CE) with TBL1 antibody showing that TBL1 protein is absent in the extracts from *TbliΔ/Y* ES cells.(E) RT-PCR analysis of genes activated in response to estradiol stimulation was not observed in the *TbliΔ/Y* ES cells.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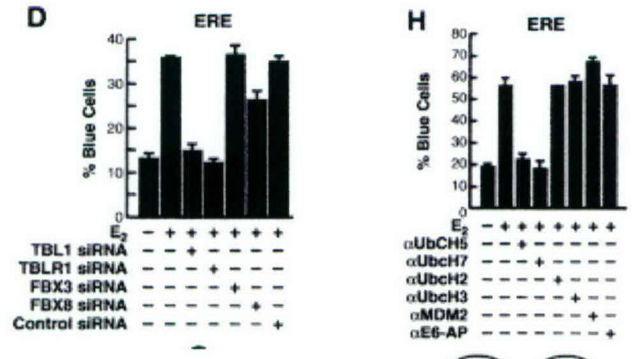
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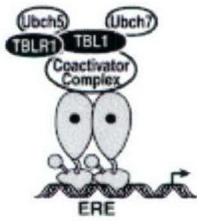
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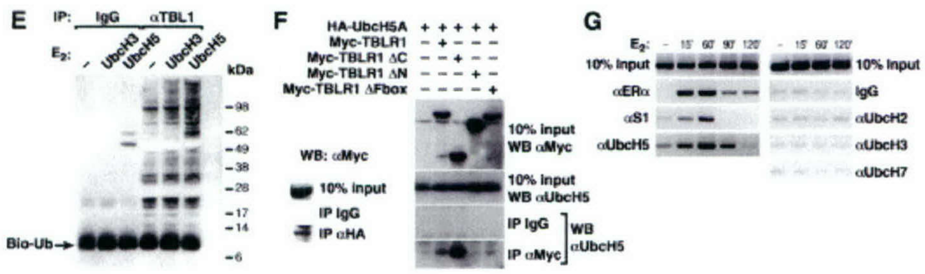
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αUbcH3
αUbcH7

Figure 10. TBL1 Recruits the Ubiquitin/Proteasome Complex, Required for Nuclear Receptors Transcriptional Activity(A) In Rat1 cells, nuclear microinjection of IgGs against the S1 subunit of the 19S proteasome blocked ER -mediated transcriptional activation. (B) F box and WD-40 domains of TBL1 are needed for mediating transcriptional activation, as expression vectors in which those domains have been deleted could not rescue transcriptional activation by ER when this was blocked by -TBL1 IgGs microinjection.(C) In a similar assay, the F box of TBLR1 is required for ER-mediated activation. (D) Microinjection of specific siRNAs against *Tbl1* and *TblR1* abrogated ER-mediated activation, while depletion of the unrelated F box/WD40 factors FBX3 and FBX8 did not have any effect.(E) In vitro ubiquitin-conjugation assay performed on MCF7 whole-cell extracts immunoprecipitated with -TBL1 antibody. The protein complex associated with TBL1 had intrinsic ubiquitylation enzymatic activity without the need of adding recombinant E2 enzymes.(F) Coimmunoprecipitation in either direction of HA-UbcH5 with Myc-TBLR1 full-length or N terminus, but not with TBLR1 mutants in which either the C terminus or the F box have been deleted.(G) ChIP analysis of the kinetics of occupancy of the *pS2* promoter by ER, the proteasome subunit S1, and various E2/UbcH enzymes following estradiol stimulation.(H) Nuclear microinjection of IgG specific for different E2 ubiquitin-conjugating enzymes and E3 ligases revealed ER specificity. The schematic indicates factors required for transcriptional activation by ER in quiescent Rat1 cells.

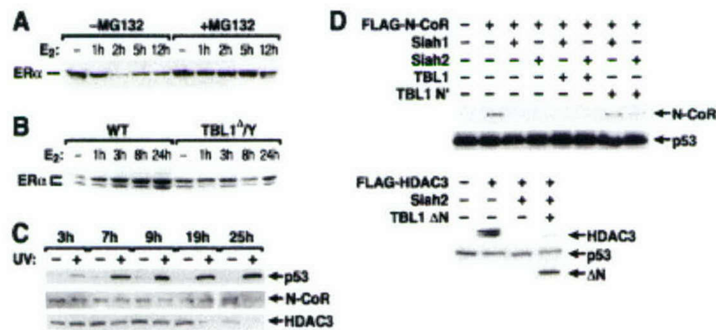

Figure 11. TBL1/TBLR1 Function in Mediating N-CoR Degradation (A) Inhibition of the proteasome activity by MG132 treatment blocked ER protein degradation upon estrogen stimulation.(B) Immunoblot analysis of extracts from E2-treated embryonic stem cells. The global level of ER protein in wild-type ES cells increased upon estrogen treatment and remained unchanged in *TbliΔ/Y* ES cells.(C) Immunoblot with p53, N-CoR, and HDAC3 of NIH-3T3 cell extracts lysed at different times after irradiation with 50 J/cm2 UV. p53 induction can be first observed after 3 hr, while N-CoR and HDAC3 downregulation occur at 19 and 24 hr, respectively.(D) Immunoblot with Flag antibody of 293 cell extracts upon transient transfection with Flag-N-CoR/HA-Siah1/HA-Siah2/Flag-TBL1 and Flag-TBL1ΔWD40. The N′ terminus of TBL1, acting as a dominant-negative, restores N-CoR protein level downregulated by Siah1/2 overexpression. Similar analysis was performed for Flag-HDAC3 degradation.