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Cell, Vol. 116, 511–526, February 20, 2004, Copyright ©2004 by Cell Press

A Corepressor/Coactivator Exchange Complex Required for Transcriptional Activation by Nuclear Receptors and Other Regulated Transcription Factors

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Summary

The mechanisms that control the precisely regulated switch from gene repression to gene activation represent a central question in mammalian development. Here, we report that transcriptional activation mediated by liganded nuclear receptors unexpectedly requires the actions of two highly related F box/WD-40-containing factors, TBL1 and TBLR1, initially identified as components of an N-CoR corepressor complex. 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 nuclear receptor corepressors, N-CoR and SMRT, for coactivators upon ligand binding. *Tbl1* gene deletion in embryonic stem cells severely impairs PPARγ-induced adipogenic differentiation, indicating that TBL1 function is also biologically indispensable for specific nuclear receptor-mediated gene activation events. The role of TBLR1 and TBL1 in cofactor exchange appears to also operate for c-Jun and NFκB and is therefore likely to be prototypic of similar mechanisms for other signal-dependent transcription factors.

Introduction

Nuclear receptors are a class of transcription factors widely used in development and homeostasis to regulate critical processes. Binding of specific ligands regulates many nuclear receptors, and their transcriptional functions are mediated by cofactors that are broadly defined as coactivators and corepressors on the basis of their requirement for gene transcriptional activation or repression (reviewed in Mangelsdorf et al., 1995; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). Ligand-dependent recruitment of coactivators to nuclear receptors is usually mediated by a conserved short LXXLL helical motif present in the majority of the coactivators (Heery et al., 1997; Torchia et al., 1997), while interaction of the corepressor N-CoR/SMRT with the unliganded nuclear receptors is dependent on a conserved motif, referred to as the CoRNR box or as LXXI/HIXXXI/L, that seems to represent a longer helix (Nagy et al., 1999; Perissi et al., 1999; Hu and Lazar, 1999; Xu et al., 2002). The number of cofactor proteins and complexes involved in mediating nuclear receptor transcriptional events is quite large, suggesting that activation and repression require a factor-, promoter-, and cell-specific combination of sequential events mediated by multiple enzymatic activities (reviewed in Glassand Rosenfeld, 2000; Dilworth and Chambon, 2001; McKenna and O'Malley, 2002).

The usage of regulatory corepressors/coactivators is not limited to nuclear receptors, and many other classes of DNA binding transcription factors, such as homeodomain factors (Xu et al., 1998; Asahara et al., 1999; Li et al., 2002) can recruit both coactivators and corepressors, with a correct balance between corepressor and coactivator levels modulating the threshold of transcriptional activation. In the case of nuclear receptors, ligand binding not only induces the dismissal of corepressors and the recruitment of coactivators, but it can also regulate the rapid turnover of receptors and coactivators on DNA (McNally et al., 2000; Shang et al., 2000; Reid et al., 2003).

Furthermore, degradation of nuclear receptors has been linked to transcriptional activity on the basis of observations that inhibition of the proteasome activity abolishes transcriptional activation mediated by estrogen, androgen, and retinoic acid receptor-γ (Lonard et al., 2000; Gianni et al., 2002; Kang et al., 2002; Lin et al., 2002). It has also been reported that the levels of several nuclear receptor coactivators are regulated by ubiquitylation/degradation (Yan et al., 2003).

Interestingly, many transcription factors have their activation domains overlapping with the elements that signal for degradation, suggesting a general correlation between activator potency and instability (Molinari et al., 1999; Salghetti et al., 2000). For example, Myc degradation and transcriptional activity are tightly linked, with the F box protein Skp2 acting as a potent coactivator (Kim et al., 2003; Von der Lehr et al., 2003).

The ubiquitin/proteasome pathway is widely used to control the turnover of many regulatory proteins, and protein polyubiquitylation provides the specific recognition signal that marks the substrate for degradation, but there is increasing evidence that the ubiquitylation of a protein may serve purposes not exclusively linked to its degradation (reviewed in Conaway et al., 2002 and Muratani and Tansey, 2003).

The ubiquitylation process is characterized by an enzymatic cascade with an E1 ubiquitin-activating enzyme, several E2 ubiquitin-conjugating enzymes (Ubc), and multiple E3 ubiquitin ligases (reviewed in Glickman and Ciechanover, 2002). E3 ligases serve as the specific recognition factors, and they can be broadly divided into HECT E3 ligases and RING E3 ligases. RING finger-

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containing E3 ligases can either function as single proteins or in multisubunit complexes (reviewed in Jackson et al., 2000). Specific ring finger (SCF) complexes use F box proteins as variable subunits responsible for specific substrate recognition (reviewed in Patton et al., 1998). The F box domain is a protein-protein interaction motif, usually found in the amino-terminal portion of a protein and often associated with leucine-rich repeats (LRRs) or WD-40 repeats in the carboxy-terminal region (reviewed in Kipreos and Pagano, 2000).

Transducin β-like 1 (TBL1) is an F box/WD-40-containing protein, originally cloned in relationship to an X-linked human disorder called Ocular Albinism with late-onset Sensorineural Deafness (OASD), in which a microdeletion of the C-terminal part of the *Tbl1* gene was suggested to be responsible for the hearing defect (Bassi et al., 1999). In *Drosophila*, the homolog of *TBL1, Ebi,* has been genetically linked to the ubiquitin-dependent degradation of Tramtrack88 by the Phyl/Sina complex in response to EGFR signaling during R7 photoreceptor cell development (Dong et al., 1999; Boulton et al., 2000). Interestingly, biochemical purification of the N-CoR and SMRT corepressor complexes showed that they both contain TBL1 and HDAC3 (Guenther et al., 2000; Li et al. 2000). Zhang and colleagues (2002) subsequently identified GPS2 and TBLR1, which shares very high homology with TBL1, as additional components of the N-CoR/SMRT complex.

In this manuscript, we report that the corepressors-associated F box/WD-40 proteins TBL1 and TBLR1 are unexpectedly required for gene activation by liganded nuclear receptors, as well as by other classes of regulated DNA binding transcription factors, based on their ability to mediate corepressor/coactivator exchange by a ligand-dependent recruitment of the ubiquitin/19S proteasome complex.

Results

TBL1 Is required for Nuclear Receptor-Mediated Activation

N-CoR has been proven to be a component of a large number of distinct complexes (reviewed in Jepsen and Rosenfeld, 2002), which are probably combinatorially required to mediate repression in a promoter- and cell-specific fashion. To further define the role of the F box/WD-40 protein, TBL1, initially purified as a component of an N-CoR corepressor complex that also contained HDAC3 (Guenther et al., 2000; Li et al., 2000), we used the chromatin immunoprecipitation (ChIP) analysis. We found, as expected, that TBL1 was present on the *RARβ* and the *HoxA1* promoters in absence of ligand but was only partially released upon retinoic acid binding, concomitantly with the dismissal of the corepressors N-CoR and HDAC3 and with the recruitment of coactivators such as CBP or SRC-1 and the acetylation of histone H3 (Figure 1A and data not shown).

Analyzing genes regulated by the estrogen receptor (ER) or the androgen receptor (AR), 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 (Figure 1B), together with other expected coactivators, as well as to the androgen-regulated *kallikrein 2* promoter upon 5α-dihydroxytestosterone (DHT) stimulation (Figure 1C). 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 (Figure 1D). 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 (Figure 1E and data not shown), suggesting that receptor-dependent recruitment of TBL1 is probably indirect, based on interactions with other cofactors and, perhaps, with histones (Guenther et al., 2000; Yoon et al., 2003).

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 (Figure 1F), while they did not have any effect with the antagonist 4-hydroxy-tamoxifen (4-OHT) (Figure 1G). TBL1 was also found to be required for the E_2-dependent activation of the regulatory region of the *pS2* promoter (Figure 1H). 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 (Figure 1I), confirming that TBL1 is required for transcriptional activation by the estrogen receptor. Analysis of androgen receptor-mediated activation, using both an artificial ARE-response promoter as well as the regulatory region of the *PSA* promoter, revealed a similar requirement for TBL1 (Figure 1J).

Interestingly, TBL1 proved to be required for activation by thyroid hormone receptor (T_3R) and peroxisome proliferator-activated receptor γ (PPARγ) (Figure 1K), but not required for retinoic acid receptor (RAR)-mediated activation (Figure 1L). In contrast, microinjection of αTBL1 antibody partially released transcriptional repression mediated by the unliganded RAR (Figure 1L), suggesting that, in this case, TBL1 functions only as a corepressor.

TBL1 Gene Deletion in ES Cells and Analysis of Nuclear Receptor-Regulated Genes

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 (Figure 2A), 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 con-

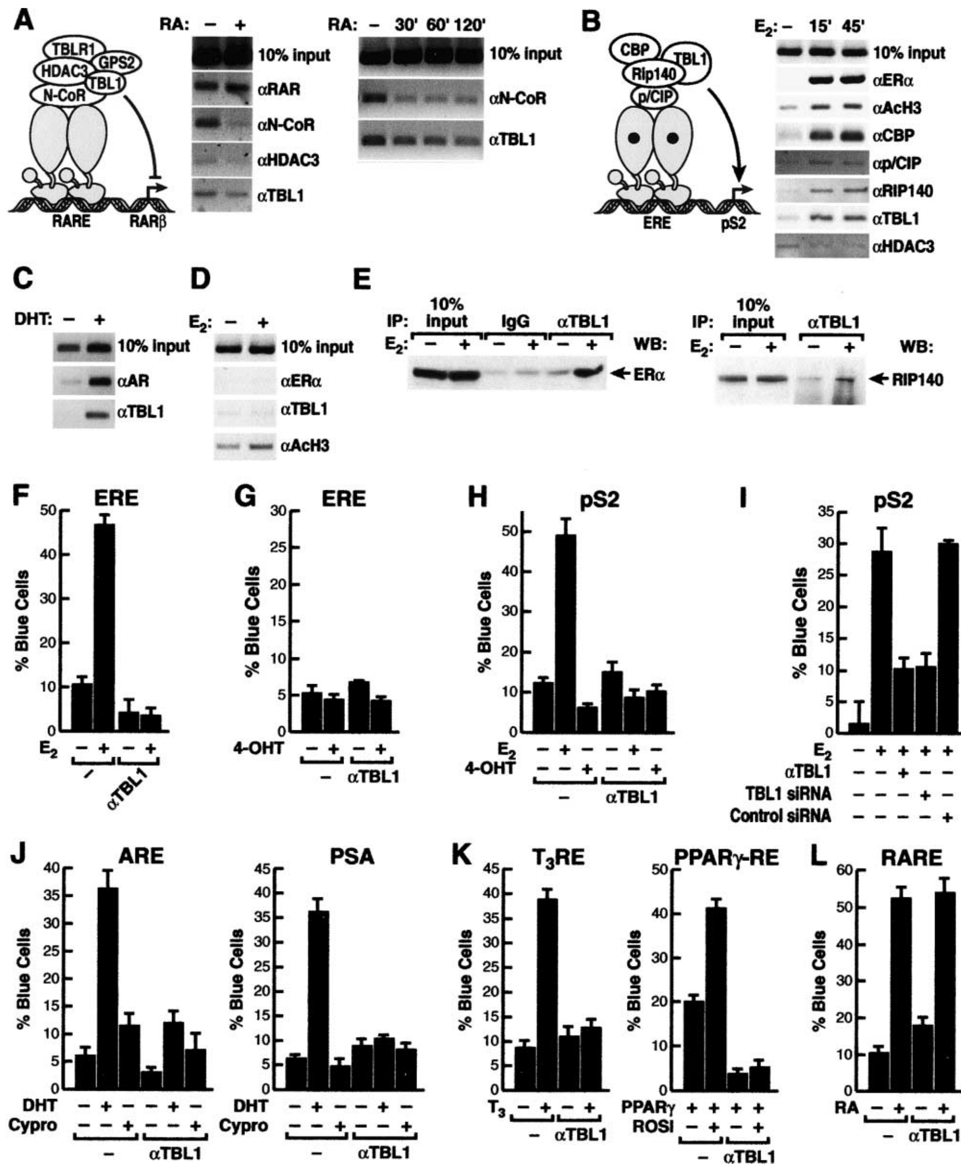


Figure 1. TBL1 Is Required for Transcriptional Activation by Nuclear Receptors

(A) Occupancy of the *RAR*β promoter by RAR, N-CoR, HDAC3, and TBL1 analyzed by chromatin immunoprecipitation (ChIP) in murine P19 cells. Left panel cells were treated for 1 hr with retinoic acid (RA).

(B) ChIP analysis of the occupancy of the *pS2* promoter upon activation by estradiol (E_2). 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 ERα null cell line MDA-MB-231 stimulated by E_2.

(E) Endogenous coimmunoprecipitation of TBL1 with ERα and Rip140 performed in estradiol-stimulated MCF-7 cells.

(F) Single cell nuclear microinjection of purified IgGs against TBL1 inhibited E_2-dependent transcriptional activation in Rat1 cells.

(G) αTBL1 IgGs microinjection did not have any effect of 4-hydroxy-tamoxifen (4-OHT)-induced repression of the ERE/LacZ reporter.

(H) αTBL1 IgGs microinjection blocked activation of a 1.2 kb fragment of the *pS2* promoter upon stimulation with E_2 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 E_2 stimulation. Injection of control siRNA had no effect.

(J) For androgen receptor, TBL1 was required for activation of ARE/LacZ reporter and the *PSA* promoter.

(K) αTBL1 IgGs microinjection also inhibited PPARγ-mediated activation of a PPARγ-RE/LacZ reporter upon Rosiglitazone (ROSI) stimulation and T_3R-mediated activation of the T_3RE/LacZ reporter by T_3.

(L) αTBL1 injection did not have any effect of RAR-mediated activation of the DR5/LacZ reporter in presence of RA.

firmed by PCR analysis (Figures 2B and 2C). 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 (Figure 2D).

The recombinant *Tbl1*$^{\Delta}$/*Y* ES cells provided a powerful model in which to analyze TBL1 function for the activa-

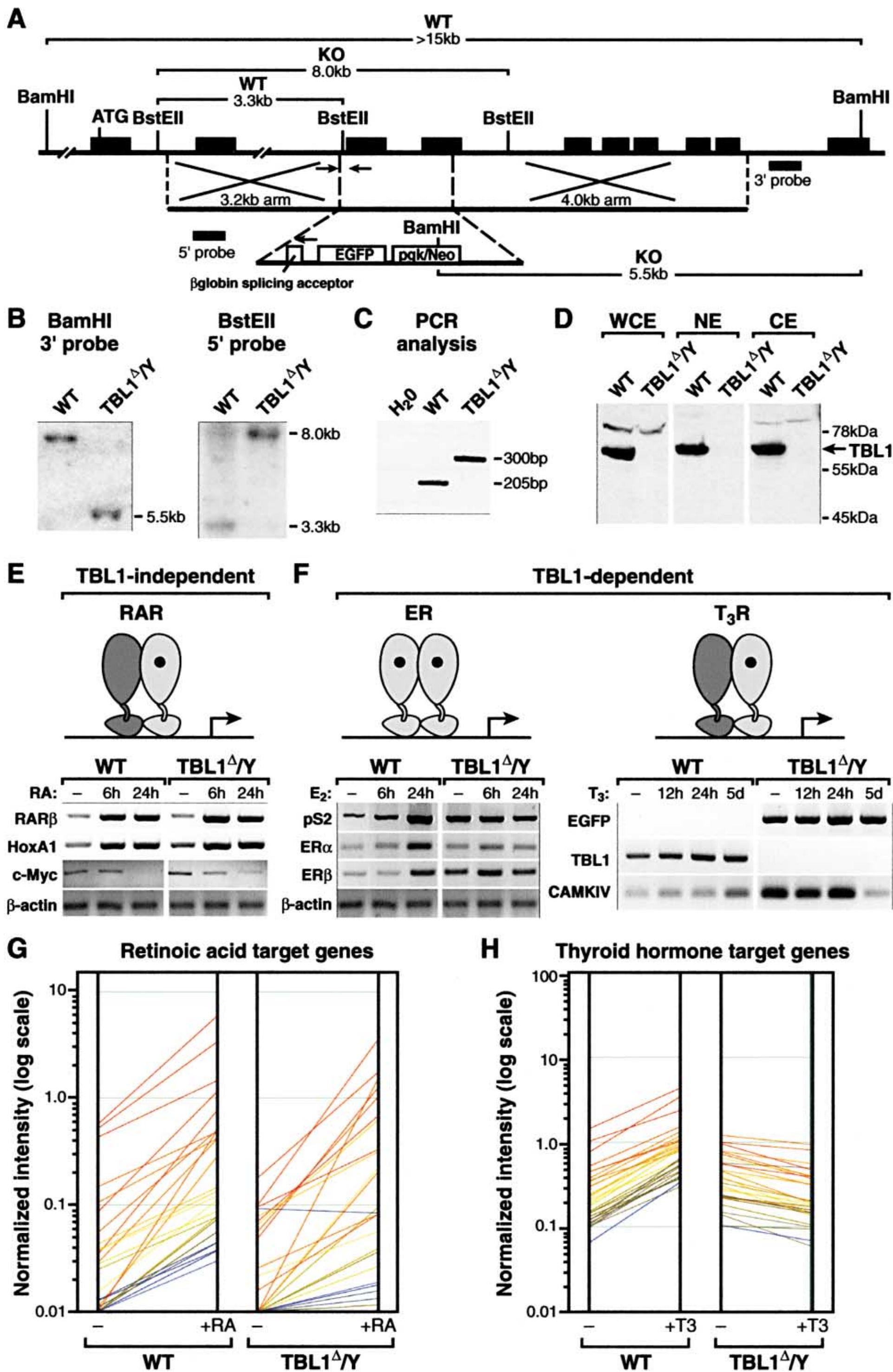


Figure 2. Targeted Disruption of the *Tbl1* Gene in Embryonic Stem Cells

(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 αTBL1 antibody showing that TBL1 protein is absent in the extracts from *Tbli*$^{\Delta}$*/Y* ES cells.

(E) RT-PCR analysis of RAR target genes in ES cells following 6 hr and 24 hr of RA treatment: *RAR*β, *HoxA1*, and *c-Myc* gene expression changes upon ligand stimulation were not affected by *Tbl1* gene deletion.

tion of known endogenous nuclear receptor target genes upon ligand stimulation. Interestingly, retinoic acid (RA)-induced genes, such as *RAR*β and *HoxA1* (de The et al., 1989; Langston et al., 1997), were upregulated equally well in the *Tbl1*$^{\Delta}$*/Y* as in the wild-type ES cells (Figure 2E), confirming the observations that TBL1 is not required for retinoic acid receptor-mediated transcriptional activation. Similarly, a gene downregulated by retinoic acid-induced differentiation, *c-myc* (Dean et al., 1986), showed the same pattern of expression in wild-type and *Tbl1*$^{\Delta}$*/Y* cells, confirming that *Tbl1* hemizygous ES cells are not impaired in their response to retinoic acid. In contrast, *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*, *ER*α, and *ER*β, (Figure 2F) or to T_3 stimulation as exemplified by the *CAMKIV* gene (Figure 2F), which is known to be induced in ES cells by thyroid hormone stimulation (Liu et al., 2002). Thus, *Tbl1* gene deletion provides independent confirmation of the observation that TBL1 is required for transcriptional activation by estrogen and thyroid hormone receptors.

In order to explore whether these results were specific for few targets or reflected a widespread requirement for TBL1, RNA profiling was performed to analyze the ability of the recombinant ES cells to respond to different hormonal stimulations. The results of multiple microarrays performed on the Codelink three-dimensional platform, comparing RNA extracted from wild-type and *Tbl1*$^{\Delta}$*/Y* ES cells treated with retinoic acid and thyroid hormone, are plotted in Figures 2G and 2H. For each treatment, all the genes exhibiting a statistically significant upregulation of more than 3.5-fold (Sasik et al., 2002) in the wild-type were compared to their counterparts in *Tbl1*$^{\Delta}$*/Y* cells. This broader analysis revealed that virtually all the RA-induced genes were activated as well in the *Tbl1*$^{\Delta}$*/Y* ES cells (Figure 2G), including known targets of retinoic-induced neuronal differentiation in embryonic stem cells, such as several *Hox* genes (*HoxA1*, *HoxA2*, *HoxA5*, *Hox-1.4*, *Hox-2.6*) (reviewed in Boncinelli et al., 1991), *Stra6, Stra8, Cyp26*, and *Procollagen type IV*. In contrast, T_3-dependent gene activation was almost completely impaired in *Tbl1*$^{\Delta}$*/Y* cells, and the majority of genes that were found upregulated upon T_3 treatment in wild-type ES cells were not induced in the *Tbl1*$^{\Delta}$*/Y* cells (Figure 2H), including known thyroid hormone targets, such as type I iodothyronine deiodinase, the oncogene JunB, and several Gap junction subunits. Similarly, upregulation of estrogen-dependent target genes was profoundly impaired in *Tbl1*$^{\Delta}$*/Y* cells (data not shown). Thus, TBL1 is broadly required for cellular responses to thyroid hormone and estradiol, while retinoic acid-induced gene activation occurs independently of TBL1.

TBL1 Gene Family: Specificity for Nuclear Receptor Regulation

In repeating a purification of N-CoR- and HDAC3-containing complexes, Zhang and colleagues (2002) have identified an additional member of the TBL1 family, 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 (Figure 3A). 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 (Disteche et al., 1998) 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 shown in Figure 3B 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, permitting us to test whether RAR-mediated activation might require TBLR1 instead of TBL1. Indeed, microinjection of the specific αTBLR1 IgGs blocked retinoic acid-induced activation of a DR5 element-dependent reporter (Figure 3C). Independent confirmation of this result was obtained by microinjection of a specific siRNA for TBLR1, with control siRNA injection having no effect (Figure 3C). Chromatin immunoprecipitation analysis revealed that TBL1 is preferentially bound to the unliganded receptor and partially dismissed upon ligand, while TBLR1 is preferentially recruited to the *RAR*β promoter upon ligand stimulation (Figure 3C). T_3R, ERα and PPARγ 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 (Figure 3D and data not shown). Thus, TBLR1 function is required for transcriptional activation by all the nuclear receptors analyzed; T_3R, ERα, and PPARγ receptors additionally require TBL1, while RAR does not.

In order to establish whether the selective requirement for TBL1 and TBLR1 was specific for nuclear receptors, we also tested several other unrelated transcription factors. Interestingly, NF-κB-mediated activation on canonical sites required both TBL1 and TBLR1, while AP-1-mediated activation depended exclusively on TBLR1 action (Figure 3E). In contrast, CREB-mediated activation did not require either TBL1 or TBLR1 (Figure 3E). Thus, TBL1/TBLR1 usage was not necessarily restricted to nuclear receptors, and a clear specificity between TBL1 and TBLR1 could also be observed in the case of other classes of DNA binding transcription factors.

As an independent approach, we analyzed TBL1 and TBLR1 requirement for NF-κB transcriptional activation by using a pathway-specific gene expression array sys-

(F) RT-PCR analysis of genes activated in response to estradiol and thyroid hormone (5d) stimulation was not observed in the *Tbli*$^{\Delta}$*/Y* ES cells.
(G) RNA profiling of ES cells wild-type and *Tbl1*$^{\Delta}$*/Y* treated with 1 μM retinoic acid for 6 hr, plotting genes upregulated at least 3.5-fold in the wild-type cells.
(H) RNA profiling of ES cells treated for 12 hr with 10 nM thyroid hormone.

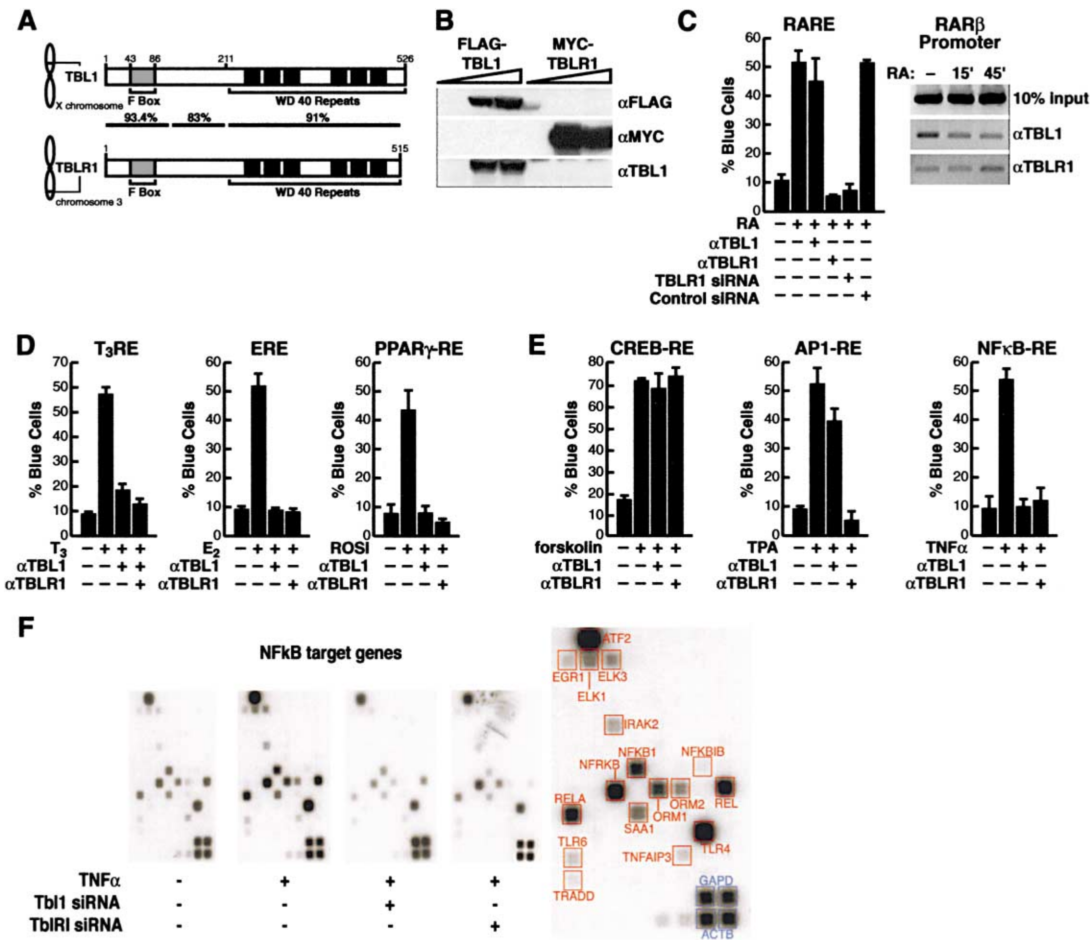


Figure 3. TBL1 Family: TBL1X and TBLR1 Have Specific Non-Overlapping Functions

(A) Schematic representation of murine TBL1 and TBLR1 showing the chromosomal locations of the genes, the position of F box and WD-40 domains, and the homology between TBL1 and TBLR1.

(B) Immunoblot of whole-cell extracts from *Tbl1*$^{\Delta}$*/Y* ES cells transfected with Flag-TBL1 or Myc-TBLR1 showing the specificity of αTBL1 antibody.

(C) Single-cell microinjection of α-TBLR1 antibody specifically blocked activation of the DR5/LacZ reporter upon RA stimulation, while microinjection of αTBL1 antibody did not. ChIP analysis of the occupancy of the RARβ promoter in 293 cells showing TBL1 partial release and TBLR1 recruitment upon RA treatment.

(D) Selective requirement of TBL1 and TBLR1 for transcriptional activation by T_3R, ER, and PPARγ.

(E) Selective requirement of TBL1/TBLR1 by other transcription units in single cell microinjection assay, using a LacZ reporter driven by the NFκB site present in the E-selectin promoter upon TNFα stimulation, an AP1-dependent reporter by TPA and a CREB-dependent reporter by forskolin.

(F) RNA profiling of TNFα-stimulated HUVEC cells either uninjected or injected with specific siRNAs against *Tbl1* and *TblR1* on GE-Arrays.

tem containing 96 gene-specific cDNA fragments, hybridized with RNA extracted from 500 HUVEC endothelial cells, either uninjected or injected with the specific siRNAs for *Tbl1* and *TblR1*. As predicted, the activation of all the NFκB target genes induced in these cells upon TNFα stimulation was lost when either TBL1 or TBLR1 was depleted (Figure 3F).

TBL1 Mediates the Recruitment of the Ubiquitin-Proteasome Complex

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 (reviewed in Dennis et al., 2001).

As expected, microinjection of an antibody against the S1/Rpn2 subunit of the 19S proteasome (Figure 4A) or pretreatment of the cells with the proteasome inhibitor MG132 (data not shown) blocked ligand-induced transcriptional activation for T_3R, ER, PPARγ, and RAR, 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 (Figures 4B and 4C). Deletion of the WD-40 C′-terminal region also blocked TBL1 transcriptional function (Figure 4B). 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 (Figure 4D).

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 (Figure 4E). 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 (Figure 4E). Addition of exogenous recombinant UbcH/E2 enzymes was not necessary, although ubiquitylation activity appeared increased by the specific addition of UbcH5 (Figure 4E). 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 αMyc or the αHA antibody (Figure 4F) and similar, although weaker, interactions with TBL1 (data not shown). 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 (Figure 4F).

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 (Figure 4G). The recruitment of these factors seems to be specific because many ubiquitylation enzymes were not recruited to the same promoter (Figure 4G and data not shown).

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 (Figure 4H), but also appeared to be functionally required for transcriptional activation of an ERE-dependent reporter (Figure 4H). Interestingly, we found that UbcH5 was generally required by all the receptors analyzed (Figure 4H). To use an independent method to confirm this requirement, we designed specific siRNAs that fully abrogated *UbcH5A, B*, and *C* expression and confirmed that UbcH5 is required for transcriptional activation (Figure 4I). Interestingly, we also found receptor-specific recruitment of additional enzymes: the E2 enzyme UbcH7 for ER; the HECT E3 ligases MDM2 and E6AP and UbcH7 in the case of RAR; and UbcH2 and E6AP in the case of thyroid hormone receptor (Figure 4H). Additionally, we showed that transcriptional activation by RAR upon ligand stimulation is dependent on the F box-interacting subunit, SKP1A, of many SCF complexes as specific siRNAs against the two isoforms *SKP1Aa* and *SKP1Ab* fully abrogated SKP1A expression by 72 hr and inhibited RAR-mediated transcriptional activation, while nonspecific siRNAs were without effect (Figure 4I).

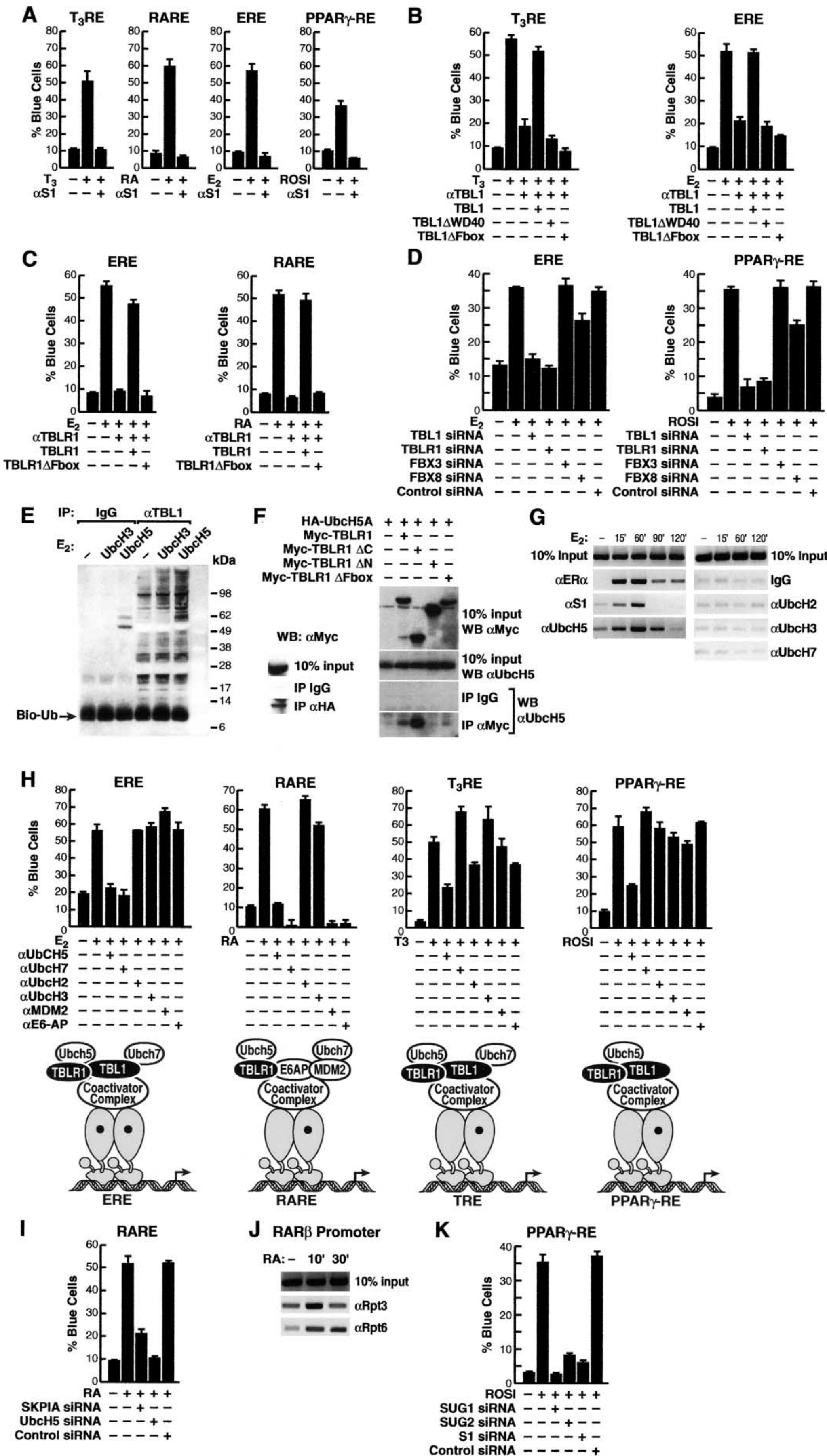
These data are consistent with nuclear receptor activity being modulated by ubiquitylation and suggest that TBL1/TBLR1 are the specific F box/WD-40 adaptor proteins used for the recruitment of the ubiquitin-conjugating enzyme UbcH5 to specific targets. However, a complete and exhaustive description of 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.

Increasing evidence has suggested a direct recruitment of the 19S proteasome to specific promoters (reviewed in Lipford and Deshaies, 2003). Thus, having observed requirement of the S1/Rpn2 subunit for nuclear receptor-mediated transcriptional activation (Figure 4A), we further tested other subunits. Chromatin immunoprecipitation analysis in retinoic acid-induced 293 cells confirmed that two other AAA-ATPase subunits, SUG1/Rpt6 and Tbp7/Rpt3, were locally recruited to the RARβ promoter upon ligand stimulation (Figure 4J). Also, specific siRNAs against the S1/Rpn2 or against the AAA-ATPases subunits SUG1/Rpt6 and SUG2/Rpt4 were designed and tested in microinjection analysis, in which all three subunits proved to be required for PPARγ-mediated transcriptional activation (Figure 4K).

TBL1/TBLR1 Function in Transcriptional Activation

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 and androgen receptors, it has been reported that binding of the receptor to the promoter is not stable but undergoes a local turnover (Figure 4D and Shang et al., 2000, 2002; Kang et al., 2002), 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 ERα 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 (Figure 5A and data not shown). 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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6
Bio-Ub
F
HA-UbcH5A
Myc-TBLR1
Myc-TBLR1 ΔC
Myc-TBLR1 ΔN
Myc-TBLR1 ΔFbox
WB: αMyc
10% input
IP IgG
IP αHA
10% input WB αMyc
10% input WB αUbcH5
IP IgG
IP αMyc
WB αUbcH5
G
E2:
15'
60'
90'
120'
10% Input
αERα
αS1
αUbcH5
IgG
αUbcH2
αUbcH3
αUbcH7
H
ERE
RARE
T3RE
PPARγ-RE
αUbCH5
αUbcH7
αUbcH2
αUbcH3
αMDM2
αE6-AP
T3
Ubch5
TBL1
Ubch7
TBLR1
Coactivator Complex
E6AP
MDM2
ERE
RARE
TRE
PPARγ-RE
I
RARE
SKPIA siRNA
UbcH5 siRNA
Control siRNA
J
RARβ Promoter
RA:
10'
30'
10% input
αRpt3
αRpt6
K
PPARγ-RE
SUG1 siRNA
SUG2 siRNA
S1 siRNA
Control siRNA

activation; indeed we found that ERα protein level was actually upregulated, while it remained essentially unchanged in the *Tbl1*$^{\Delta}$*/Y* cells (Figure 5B). Because we observed impairment of the ES cells to respond to estrogen stimulation in absence of TBL1 (Figure 2F and data not shown), 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, Zhang et al. (1998) have reported proteasomal degradation of N-CoR, based on its interaction with the E3 ligase Siah2. 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 (Dong et al., 1999; Boulton et al., 2000; Matsuzawa and Reed, 2001); 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 (Matsuzawa and Reed, 2001). Interestingly, in NIH-3T3 cells, the levels of both proteins were downregulated upon UV treatment (Figure 5C). 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 (Amson et al., 1996; Matsuzawa and Reed, 2001). As predicted, the N′-terminal region of TBL1 behaves as a dominant-negative and its overexpression is sufficient to abrogate N-CoR degradation (Figure 5D), 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 (Figure 5D).

If the dismissal or degradation of the N-CoR corepressor complex was the required step mediated by TBL1 and/or TBLR1, then TBL1 and TBLR1 would no longer be required for activation in absence of N-CoR. Thus, using siRNAs against N-CoR and SMRT, which are effective in bringing them to undetectable levels by 24 hr after siRNA nuclear microinjection (data not shown), we tested whether either TBL1 or TBLR1 were required for transcriptional activation in the absence of these two corepressors. We found that activation of T_3R in response to ligand was still completely dependent on TBL1 independent of the presence of N-CoR/SMRT (Figure 5E). However, transcriptional activation upon thyroid hormone treatment in the absence of both N-CoR and SMRT now became independent of TBLR1, even though removal of either N-CoR or SMRT alone was not sufficient to cause ligand-independent activation in absence of TBLR1 (Figure 5E). Similar analysis of PPARγ-mediated transcriptional activation showed that TBLR1 requirement could be overcome by eliminating both N-CoR and SMRT (Figure 5E). To explore this further, we analyzed the two transcription units that are exclusively dependent on TBLR1, but not on TBL1, for transcriptional activation. In the case of both RAR- and AP1-mediated transcriptional activation, removal of N-CoR and SMRT by siRNAs injection was also sufficient to make transcriptional activation independent from TBLR1 (Figure 5F). Thus, loss of the corepressors N-CoR and SMRT serves in all the cases we have analyzed as a "reverting mutation," restoring transcriptional activation in response to ligand in the absence of TBLR1, but not in the absence of TBL1.

Together, these data suggest that the concomitant requirement for both TBL1 and TBLR1 by many nuclear receptors reflects a complex series of steps, with TBLR1 action being required by all the nuclear receptors analyzed because of its ability to mediate the dismissal of specific corepressors and their subsequent degradation. The requirement for TBL1 in transcriptional activation, on the other hand, does not exclusively reflect

Figure 4. 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 nuclear receptor-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 T_3R, ER, or PPARγ when this was blocked by α-TBL1 IgGs microinjection.

(C) In a similar assay, the F box of TBLR1 is required for ER- and RAR-mediated activation.

(D) Microinjection of specific siRNAs against *Tbl1* and *TblR1* abrogated ER- and PPARγ-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 α-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 nuclear receptor specificity. The schematic indicates factors required for transcriptional activation by each nuclear receptor in quiescent Rat1 cells.

(I) Depletion of UbcH5 and SKP1A by specific siRNAs microinjection abrogated RAR-mediated transcriptional activation.

(J) Recruitment of the AAA-ATPase subunits of the 19S proteasome SUG1/Rpt6 and Tbp7/Rpt3 to the RARβ promoter upon ligand stimulation.

(K) Microinjection of specific siRNAs against the 19S proteasome subunits SUG1/Rpt6, SUG2/Rpt4, and S1/Rpn2 blocks activation of a PPARγ-dependent reporter upon ligand stimulation.

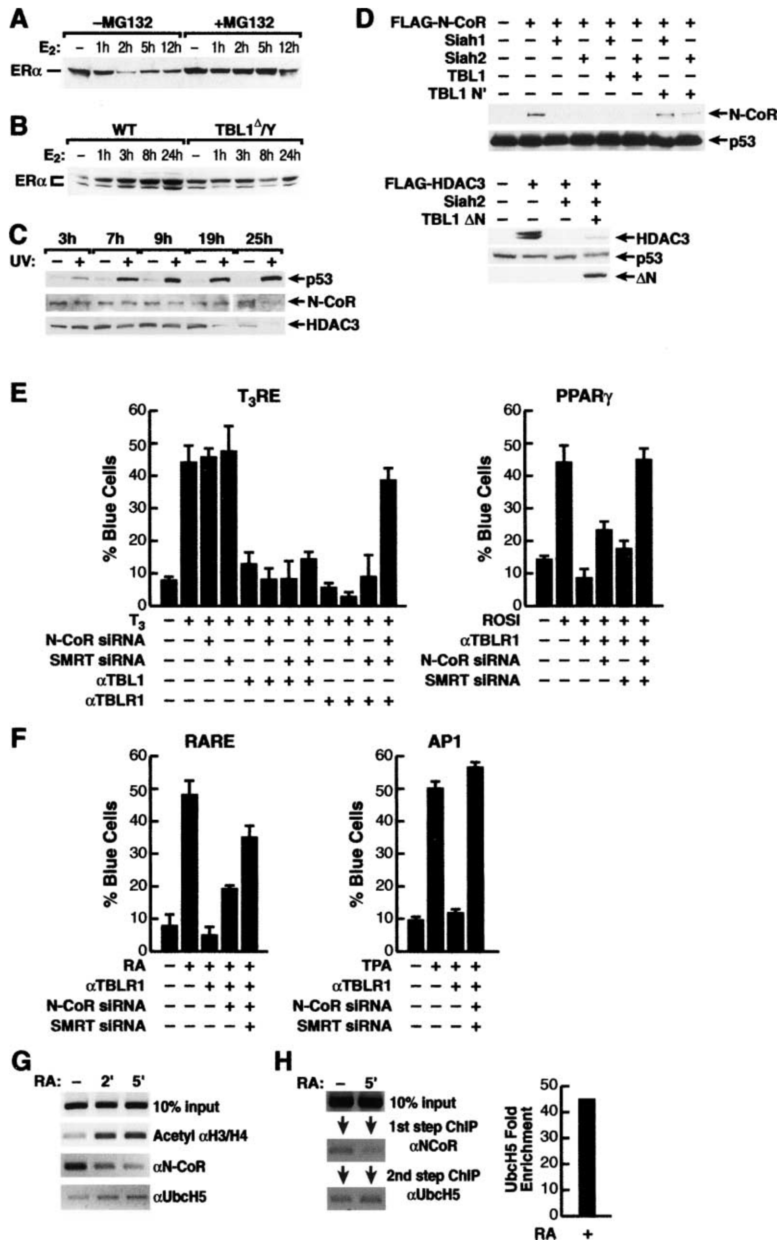

Figure 5. TBL1/TBLR1 Function in Mediating N-CoR Degradation

(A) Inhibition of the proteasome activity by MG132 treatment blocked ERα protein degradation upon estrogen stimulation.

(B) Immunoblot analysis of extracts from E_2-treated embryonic stem cells. The global level of ERα protein in wild-type ES cells increased upon estrogen treatment and remained unchanged in *Tbl1*$^{\Delta}$/*Y* ES cells.

(C) Immunoblot with αp53, αN-CoR, and αHDAC3 of NIH-3T3 cell extracts lysed at different times after irradiation with 50 J/cm^2 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 αFlag 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.

(E) Single cell nuclear microinjection of specific siRNAs against N-CoR and SMRT abolishes the requirement for TBLR1 for T_3R- and PPARγ-mediated transcriptional activation. TBL1 is required for transcriptional activation even in absence of N-CoR/SMRT.

(F) N-CoR/SMRT depletion by siRNAs microinjection abolishes the need for TBLR1 in AP1- and RAR-mediated transcriptional activation events.

(G) Analysis of the occupancy of RARβ promoter showed that N-CoR dismissal and UbcH5 occur by 2 to 5 min after ligand stimulation.

(H) Two-step chromatin immunoprecipitation was performed showing that UbcH5 is recruited, upon ligand binding to N-CoR bound RARβ promoters with a 45-fold enrichment of UbcH5 bound chromatin pulled down in the second step, compared with non-N-CoR bound promoters.

either the dismissal/degradation of corepressors or the turnover of the receptor, suggesting that TBL1 plays additional roles, possibly in the exchange of other coregulators, either corepressors and/or coactivators.

The idea that TBLR1 is required for activation because of the necessity of mediating ligand-dependent dismissal/degradation of the corepressors raises the interesting question of whether ligand causes the recruitment of the UbcH5-containing ubiquitination complex to the N-CoR/SMRT bound receptor. First, to confirm the recruitment of UbcH5-containing ubiquitin machinery upon ligand stimulation, we examined the early kinetics following RA stimulation, finding that UbcH5 is recruited to the RARβ promoter upon ligand stimulation and that most N-CoR dismissal occurs by 2–5 min (Figure 5G). Interestingly, by performing a sequential, two-step chromatin immunoprecipitation experiment in 293 cells, first using α-N-CoR IgG and subsequently α-UbcH5, we were able to observe UbcH5 recruitment to N-CoR-occupied RARβ promoter following 5 min of retinoic acid stimulation (Figure 5H). Because the N-CoR/SMRT corepressor complex had been almost completely dismissed (Figures 5H and 5G), the material pulled down by ChIP during the second step with α-UbcH5 IgG represented a highly enriched population of UbcH5/N-CoR-containing promoters (∼45-fold) (Figure 5H). These results suggest that the UbcH5-containing ubiquitin machinery is recruited by TBLR1 to the N-CoR bound receptor upon ligand stimulation, permitting corepressor dismissal and cofactor exchange.

Adipogenic Differentiation Is Inhibited in TBL1$^{\Delta}$/Y Embryonic Stem Cells

Thus, as TBL1 is required for transcriptional activation by many nuclear receptors, independently of the role of TBLR1 in N-CoR/SMRT exchange, we investigated whether the deletion of *Tbl1* would be biologically relevant for regulated developmental processes. As embryonic stem cells can be induced to differentiate in vitro to form a variety of cell types, essentially recapitulating different stages of murine embryogenesis (reviewed in Weiss and Orkin, 1996), we took advantage of the ES cell model system to investigate the role of TBL1 in physiological processes and we tested whether *Tbl1* gene deletion would render ES cells unable to undergo specific differentiation pathways known to be regulated by nuclear receptors.

First, we analyzed the role of TBL1 for developmental events mediated by the retinoic acid receptor. Retinoic acid is required for normal development of the mammalian brain and promotes neuronal differentiation of murine ES cells (Bain et al., 1996; Fraichard et al., 1995; Strubing et al., 1995). Consistent with the data showing that retinoic acid receptor activation is not dependent on TBL1, we found that embryo bodies derived from wild-type or *Tbl1$^{\Delta}$/Y* ES cells were equally able to differentiate in response to retinoic acid stimulation. Cells with neuronal morphology and expression of specific neuronal markers could be identified in the outgrowths of both wild-type and *Tbl1$^{\Delta}$/Y* embryo bodies, as shown by immunostaining of 7-day-old embryo bodies with antibodies against β-tubulin and TuJ1 (Figure 6A).

Because estrogen and thyroid hormone receptors are not clearly and exclusively associated with specific differentiation pathways, we focused on a PPARγ-regulated differentiation process to test whether differentiation pathways regulated by nuclear receptors that are TBL1 dependent, such as ER, T_3R, or PPARγ, were affected by *Tbl1* gene deletion. Adipogenesis is probably one of the most well described in vitro developmental processes, during which a well-defined sequence of events accounts for the differentiation into mature adipocyte (reviewed in Ntambi and Young-Cheul, 2000; Rosen et al., 2000). In order for ES cells to be able to commit to the adipogenic lineage, embryo bodies require a short exposure to retinoic acid, after which terminal differentiation is induced by stimulation of PPARγ. Retinoic acid treatment followed by stimulation with adipogenic factors leads to differentiation of 60%–80% of the embryo body outgrowth into fat cells, compared to a 2%–5% of differentiated cells that are obtained by spontaneous differentiation in the absence of any stimulus (reviewed in Rosen et al., 2000; Grimaldi, 2001).

Interestingly, Oil Red O staining of the fat droplets showed a marked decrease (to less than 5%–10%) in the number of mature, fully differentiated adipocytes in the outgrowth of *Tbl1$^{\Delta}$/Y* EBs compared to what was observed in their wild-type counterpart (Figure 6B). Furthermore, the expression of a downstream PPARγ target gene, such as the fat cell marker *adipsin*, was clearly reduced in the absence of TBL1 (Figure 6C). Together, these data suggest that TBL1 is required for effective commitment of ES cells to the adipogenic fate, probably because of its requirement for effective PPARγ-induced gene activation.

Discussion

Regulated association of DNA binding transcription factors with a series of coactivators and corepressors dictates the precisely regulated levels of target gene expression (reviewed in Grandori et al., 2000; Glass and Rosenfeld, 2000; McKinsey et al., 2001; Levine and Tjian, 2003). In the case of nuclear receptors, the use of a common binding pocket by many coactivators and corepressors (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999; Xu et al., 2002) itself implies a critical regulation of this corepressor/coactivator exchange. Here, we show that while the F box/WD-40 domain proteins TBL1 and TBLR1 as intrinsic components of an N-CoR complex can modulate some basal repression events, ligand-regulated nuclear receptors unexpectedly require TBLR1, and often TBL1, for effective gene activation. The recruitment of TBL1/TBLR1 to both unliganded and liganded nuclear receptors suggests that TBL1/TBLR1 may be recruited as an integral component of complexes playing opposite roles in transcriptional regulation. The stability of TBL1/TBLR1 recruitment to liganded receptors may also reflect the ability of TBL1/TBLR1 to interact with histones, possibly depending on specific covalent modifications, in a fashion analogous to the epigenetic code used in the ordered recruitment of components of the HO activation machinery (Cosma et al., 1999).

TBL1 and TBLR1 appear to exert their functions in transcriptional activation, based on their ability to recruit the ubiquitin/19S proteasome complex to nuclear receptor-regulated transcription units upon ligand binding, in concert with the mounting evidence of the important role of ubiquitylation/proteasomal degradation events in gene expression (reviewed in Conaway et al., 2002; Muratani and Tansey, 2003).

An intriguing aspect of the role of TBLR1 is that all nuclear receptors analyzed, as well as AP1 and NFκB, require TBLR1 for transcriptional activation, but that many of these factors, including PPARγ, estrogen receptor, thyroid hormone receptor, and NFκB, also require TBL1. We propose that the most critical, specific role of TBLR1 is to mediate the exchange of N-CoR/SMRT corepressor for coactivator complexes, an exchange required for transcriptional activation to occur, based on the fully "revertant" phenotype observed when N-CoR and SMRT expression is abolished. Thus, in the absence of N-CoR and SMRT, TBLR1 is no longer required for gene activation by thyroid hormone and retinoic acid receptors. However, TBL1 is required for transcriptional activation independent of the presence of N-CoR and SMRT, suggesting that TBL1 plays a role in the exchange of other corepressors and/or in subsequent steps in transcriptional activation.

Furthermore, the requirement for TBLR1 in activation of AP1-dependent targets, and of both TBLR1 and TBL1 for activation of NFκB targets, indicates that, as in the case of nuclear receptors, the TBLR1-mediated exchange of corepressors is also a central aspect in growth and inflammatory signaling events.

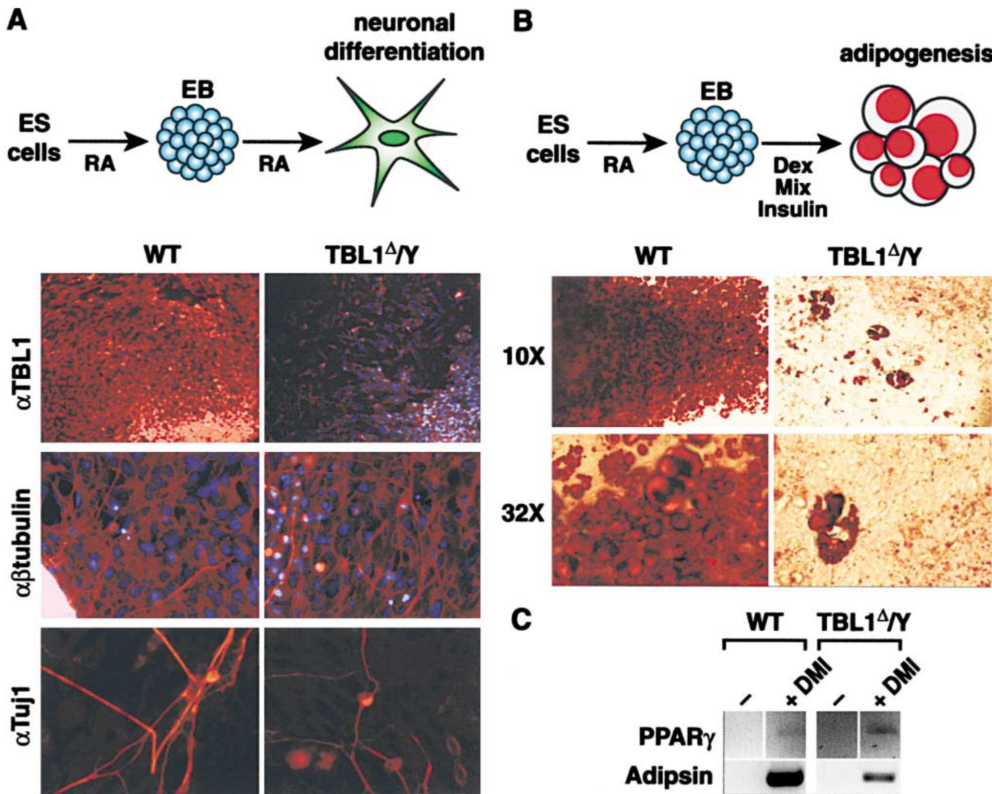

Figure 6. TBL1 Is Required for ES Cells Differentiation into the Adipogenic Lineage and Not for Differentiation into the Neuronal Lineage

(A) Immunofluorescence staining of 7-day-old embryo bodies (EBs) treated with RA to induce neuronal differentiation, as shown in the schematic. β-TBL1 immunostaining in the top panel confirms that TBL1 expression is absent in *Tbli*$^{\Delta}$*/Y* ES cells. Immununofluorescence with αβ-tubulin and αTuj1 in the lower panels shows the presence of neuronal-like cells in the outgrowths of differentiated wild-type and *Tbli*$^{\Delta}$*/Y* EBs. In the upper four panels, nuclear 4,6-diamidino-2-phenylindole (DAPI) staining is indicated in blue.

(B) Red Oil O staining of EBs 5 days after the adipogenic differentiation is induced with Dex/MIX/insulin as indicated in the schematic (10× or 32× magnification).

(C) RT-PCR analysis of adipogenic markers *PPAR*γ and *adipsin* in wild-type, and *Tbli*$^{\Delta}$*/Y* EBs were examined in embryo bodies induced to differentiate with Insulin/Dex/MIX or left untreated.

Zhang and colleagues (1998) have reported that N-CoR degradation by the 26S proteasome is based on the recruitment of the E3 ubiquitin ligase Siah1/2. We have shown here that TBL1/TBLR1 mediates N-CoR proteasomal degradation in vitro, and thus suspect that in vivo Siah1/2 may require TBLR1 for specific recognition of N-CoR as a substrate, analogous to the case of β-catenin and Tramtrack88 (Dong et al., 1999; Matsuzawa and Reed, 2001). This is consistent with the genetic data presented by Tsuda et al. (2002), which suggested that in *Drosophila* eye development, Ebi/TBL1 is required for EGFR-induced expression of the Notch ligand, Delta, because of the necessity to antagonize the repressor function of Suppressor of Hairless [Su(H)] by nuclear export and perhaps proteasomal degradation of the *Drosophila* N-CoR homolog, SMRTER. Thus, the presence of TBL1/TBLR1/Ebi in repressor complexes, such as the Sno/Ebi/SMRTER/Su(H) (Tsuda et al., 2002) or the TBL1/N-CoR/HDAC3/Six6 that we have previously described in the case of Six6-mediated repression of the p27^{Kip1} gene (Li et al., 2002), may reflect the fact that TBL1/TBLR1 play a role for the effective dismissal of the corepressors for these DNA binding transcription factors.

Interestingly, modulation of degradation events is often achieved by phosphorylation of the substrate. For example, the F box/WD-40 protein β-TrCP recognizes β-catenin phosphorylated by GSK3 (at Ser32 and Ser36), targeting it for ubiquitylation and destruction (Wu et al., 2003). Interestingly, during UV stress, the binding of β-catenin to Ebi, the *Drosophila* homolog of TBL1/TBLR1, is independent of the phosphorylation sites recognized by β-TrCP. However, the TBLR1 putative target, N-CoR, has multiple regulatory phosphorylation sites, some of which have been linked to nuclear-cytoplasmic translocation and possibly to degradation (Hong and Privalsky, 2000; Hermanson et al., 2002), suggesting that N-CoR phosphorylation may represent a mark for protein ubiquitylation and degradation.

We are tempted to speculate that TBL1-selective requirement by some of the DNA binding transcription factors examined may reflect the exchange of corepressors other than N-CoR/SMRT, or perhaps one or more of the coactivators, or ligand-dependent corepressors such as L-CoR or REA (Fernandes et al., 2003; Delage-Mourroux et al., 2000). The TBLR1- and TBL1-recruited ubiquitin/19S proteasome complexes may also have nonproteolytic functions, including possibly the modula-

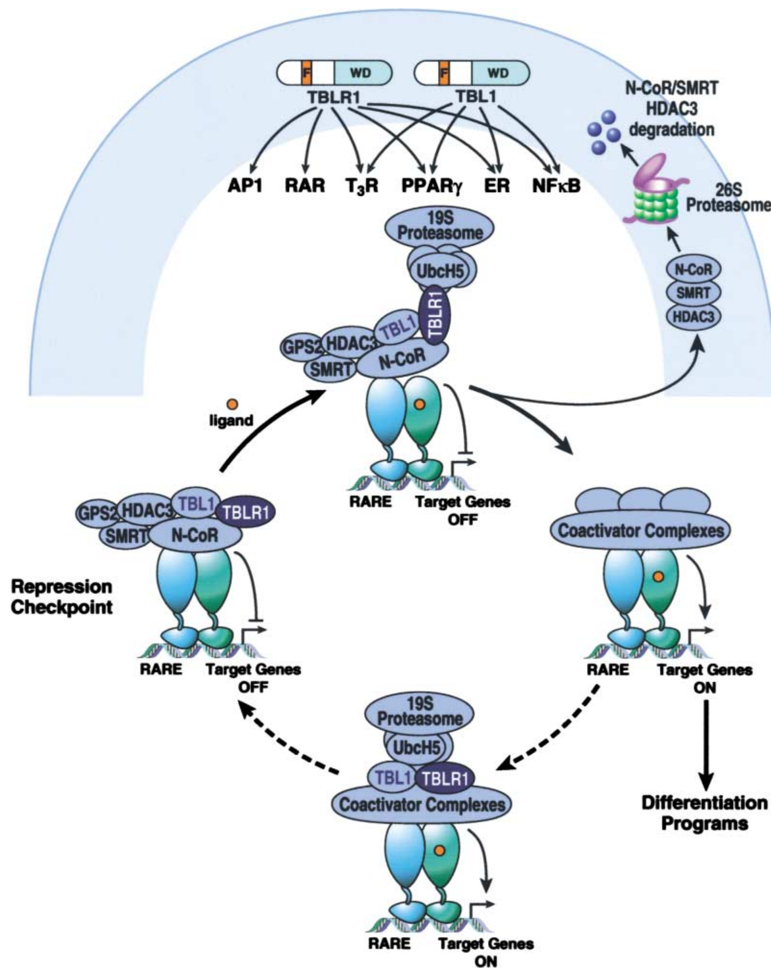

Figure 7. Model of TBL1/TBLR1 Functions in Transcriptional Regulation

TBLR1 is required for transcriptional activation mediated by all nuclear receptors examined, as well as in regulation of AP1 and NFκB, while TBL1 is required by a subset of nuclear receptors. Regulation of transcriptional activation by retinoic acid receptor upon ligand binding, where TBLR1 specifically serves as a *n*uclear receptor *co*repressor/coactivator *ex*change factor (N-CoEx), required for the dismissal and subsequent degradation of the corepressors N-CoR/SMRT and for the subsequent recruitment of the coactivator complexes, which will lead to target genes activation and differentiation programs. Analogous exchange functions are suggested for TBL1.

tion and recruitment of other enzymatic activities, as well as the reconfiguration of chromatin. Indeed, it is becoming increasingly clear that multiple coactivator and corepressor complexes are required for transcriptional events because of the necessity for multiple, distinct catalytic activities. Correspondingly, actions of distinct ubiquitin/proteasome complexes are also likely to be central to different points of control of transcriptional activation, suggesting that recruitment of the 19S proteasome is needed for multiple functions and probably achieved through more than a single specific mechanism and leaving open the possibility that the exchange and dismissal of N-CoR/SMRT is not inevitably linked to their degradation. Analysis of the requirement for different ubiquitin-conjugating enzymes and ubiquitin ligases showed that, while UbcH5, as well as TBL1/TBLR1, was required for transcriptional activation by all the nuclear receptors examined, additional components of the ubiquitin/19S proteasome machinery could be required by specific receptors. This is consistent with the idea that receptors, coactivators, and other components of the transcriptional machinery are undergoing continuous turnover.

As an independent confirmation of this overlapping, but distinct specificity between TBL1 and TBLR1, we have shown a TBL1-selective requirement for effective gene activation by specific nuclear receptors during important developmental programs. The biological consequences of the loss of *Tbl1* include a 90%–95% loss in adipocyte differentiation triggered by PPARγ induction in *Tbl1*$^{\Delta}$*/Y* ES cells and a significant loss in thyroid hormone receptor-dependent activation of target genes, as assessed by RNA profiling. In contrast, retinoic acid receptor, which is TBL1 independent, was able to activate a normal program of neuronal differentiation in *Tbl1*$^{\Delta}$*/Y* ES cells, including a correct regulation of virtually all retinoic acid-dependent gene targets, as revealed by RNA profiling. Depletion of either TBL1 or TBLR1 by specific siRNAs in TNFα-stimulated endothelial cells appeared to result in significant inhibition of the NFκB-dependent gene activation program.

In conclusion, we suggest that 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 nuclear receptors and other classes of DNA binding transcription factors (Figure 7).

Experimental Procedures

Reagents and Antibodies

Anti-TBL1 antibody was generated in guinea pigs against bacterially expressed N′-terminal region of murine TBL1. Anti-

TBLR1 was generated in rabbit against the specific peptide 5′-TIANNHTDMMEVDGDVEIPSN-3′. Other antibodies are listed in the Supplemental Data at http://www.cell.com/cgi/content/full/116/4/511/DC1.

Cell Culture, Cell Staining

For ES cell transfection, the TransIT-LT1 reagent was used following the manufacturer's protocol (Mirus). Induction of adipogenesis was performed as described by Rosen et al. (2000) (see Supplemental Data on *Cell* website). Immunostaining was performed following standard protocols on cells fixed in 4% paraformaldehyde/PBS, using Alexa Fluor-conjugated secondary antibodies (Molecular Probes).

Immunoprecipitation and Chromatin Immunoprecipitation

Immunoprecipitation was performed by standard methods (see Supplemental Data online). Chromatin immunoprecipitation was performed as described in Shang et al., 2000; Baek et al., 2002 (see *Cell* website for Supplemental Data). The primers used to amplify the pS2 and the RARβ promoters were previously described (Shang et al., 2000; Perissi et al., 1999).

Single Cell Nuclear Microinjection Assay

The single cell nuclear microinjection assays were performed as described (McInerney et al., 1998). Each experiment was performed on three independent coverslips with >300 injected cells per point, and rhodamine-conjugated dextran was used as a negative control in each experiment. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24–36 hr. See the *Cell* website for Supplemental Data on plasmids and SiRNAs design.

RNA Isolation, RT-PCR Analysis, and RNA Profiling

RT-PCR products were prepared as described in the Supplemental Data and were analyzed by agarose gel electrophoresis. RNA profiling for NFκB-specific target genes was performed on GEArray gene expression arrays following the manufacturer's instructions (SuperArray Bioscience). Genome-wide RNA profiling of embryonic stem cells was performed on Codelink three-dimensional platforms (BioGem core, UCSD).

Genetic Manipulation

The targeting vector was designed to replace the 5′-terminal part of the *Tbl1* gene with EGFP cDNA; a strong splicing acceptor site from the β-*globin* gene was inserted to avoid cryptic splicing and the *Neo* resistance gene was placed under control of the PGK promoter. Homologous recombination was screened by Southern blot analysis.

Acknowledgments

We are very grateful to K. A. Ohgi, F. Liu and C. Nelson for excellent technical assistance; J. Lozach for microarray analysis; E. Miller for antibody production in guinea pigs; R. Dickins for providing Siah1 and Siah2 expression vectors; C. Mak for siRNAs designing and C. Cheng for technical help; L. Erkman for critical reading of the manuscript; X. Li, O. Hermanson and the other members of the Rosenfeld lab for discussions and valuable advice during the course of this study; J. Hightower and P. Myer for figure preparation; M. Gonzalez (Santa Cruz) for advice on reagents. M.G.R. is an investigator with the Howard Hughes Medical Institute and V.P. is supported by Telethon-Italy (grant number 484/b). These studies were supported by grants from NIH to M.G.R., C.K.G. and D.W.R., by the DIRK award, by the Prostate Cancer Foundation and by the Sandler Foundation.

Received: July 25, 2003
Revised: January 5, 2004
Accepted: January 6, 2004
Published: February 19, 2004

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