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The *In Vivo* Role of Nuclear Receptor Corepressors in Thyroid Hormone Action

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Abstract

BACKGROUND—The thyroid hormone receptor (TR) isoforms interact with a variety of coregulators depending upon the availability of T₃ to mediate their transcriptional effect. Classically, in the absence of ligand, the TRs recruit the nuclear corepressors, NCoR and SMRT, to mediate transcriptional repression on positively regulated TR target genes. However, new insight into the roles of NCoR and SMRT using *in vivo* models have better defined the role of nuclear corepressors both in the absence and presence of T₃.

SCOPE OF REVIEW—This review will place the variety of *in vivo* nuclear corepressor mouse models developed to date in context of thyroid hormone action. Based on these models, we will also discuss how corepressor availability together with the levels of endogenous nuclear receptor ligands including T₃ controls multiple signaling pathways.

MAJOR CONCLUSIONS—Nuclear corepressors mediate repression of positive TR targets in the absence of T₃ *in vivo*. Even more importantly they attenuate activation of these targets at the normal physiological levels of ligands by TR and other nuclear receptors. While the role of corepressors in the regulation of negative TR targets and HPT axis remains poorly understood, lack of corepressor recruitment to TR in the animals leads to a compensatory change in the set point of HPT axis that allows to balance the increased sensitivity to T₃ action in other tissues.

GENERAL SIGNIFICANCE—Available data indicate that targeting specific interactions between corepressors and TR or other nuclear receptors presents a new therapeutic strategy for endocrine and metabolic disorders.

Introduction

With the cloning of the first thyroid hormone receptors in 1986 it soon became clear that the thyroid hormone receptors (TR) possessed the capability to silence transcription of target genes in the absence of thyroid hormone (TH) [1, 2]. This action of the TRs is consistent with the detrimental clinical effects of hypothyroidism. Work by a number of laboratories determined that this silencing capability was mediated by the C-terminus of the TR via its interactions with unknown cellular protein(s) that were specifically recruited in transfection experiments [3]. The significance of this pathway was validated by the identification of highly homologous high molecular weight proteins that were able to interact with the C-terminus of the unliganded TR (and other nuclear receptors). These two proteins were both

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termed nuclear corepressors (CoRs) and named NCoR (nuclear receptor corepressor, NCoR1) and SMRT (silencing mediator of retinoid and thyroid hormone receptors, NCoR2). Both proteins (Figure 1) share approximately 50% amino acid identity and similar structural domains. The N-terminal portion of both NCoR and SMRT mediates transcriptional repression by recruiting a multiprotein complex through three separate repression domains [4–6]. Included in this complex is histone deacetylase 3 (HDAC3) whose enzymatic activity is activated by binding to CoRs and mediates chromatin remodeling and transcriptional repression [7, 8]. Also, included in the complex is transducin binding protein like 1 (TBL1), its homolog TBLR1 and GPS2. Both TBL1 and TBLR1 bind to histones and appear to be involved in transcriptional activation and the clearance of the CoR complex while the role of GPS2 remains less clear though it is likely structurally required for maintenance of the CoR complex [9, 10].

In contrast to the N-terminus of the NCoR and SMRT, the C-terminus mediates interactions between the CoRs and the TR and other nuclear receptors (NR). Both NCoR and SMRT have exons that encode 3 separate receptor interacting domains (RIDs) that contain a core isoleucine rich motif that is essential for NR-binding [11–13]. However, because of alternative splicing not all RIDs are expressed in all tissues. Interestingly, the most N-terminal of the RIDs (N3 in NCoR and S3 in SMRT) binds the TR with high avidity, while N2 also prefers the TR especially when compared to S2, which prefers the retinoic acid receptor (RAR) [14–17]. Because of the different preferences of the RIDs for the TR it remains possible that NCoR and SMRT have non-redundant roles in the regulation of thyroid hormone action *in vivo*.

While NCoR and SMRT are felt to mediate the repressive functions of the unliganded TR or mutated TRs found in the syndrome of Resistance to Thyroid Hormone (RTH), it is also clear that the unliganded TR activates genes that are negatively regulated by TH. This feature suggests that the corepressors may have paradoxical activating functions on certain target genes. While cell culture studies have attempted to discern the role of both NCoR and SMRT in TH action, they have been complicated by the fact that many cell lines fail to express functional TR isoforms and TR responsive genes. Thus, the real role of NCoR and SMRT in TH-action is best discerned using mouse genetic models and the remainder of this review will focus on the advances made using an *in vivo* approach.

NCoR and SMRT Knockout Models

The first attempts to elucidate *in vivo* roles of co-repressors revealed the complexity of their physiologic functions beyond NR signaling. In 2000 the first global NCoR knockout mouse was generated [18]. NCoR^{-/-} animals exhibited defects in definitive erythropoiesis and thymocyte and neuronal differentiation by embryonic day (E)13.5. Furthermore the knockout of NCoR led to embryonic lethality by E15.5. However cultured mouse embryonic fibroblasts (MEFs) obtained from NCoR^{-/-} embryos demonstrated the role of NCoR in the ligand-independent repression by RAR and TR, as well as an antagonist-bound estrogen receptor (ER). Interestingly, NCoR^{-/-} embryonic livers showed increased expression of carbonic anhydrase II, a known positive TR target, thus confirming the importance of NCoR for TR-mediated repression in the absence of TH.

Subsequently, a SMRT knock-out was also developed and shown to lead to embryonic lethality [19]. SMRT^{-/-} animals die before E16.5 because of a defect in heart development, namely a hypoplastic ventricular wall and a defect in ventricular septation. This defect is due to a disruption of a specific interaction between SMRT and a forkhead protein, FOXP1 [20]. When the lethality was overcome by myocyte-specific expression of SMRT, the development of SMRT^{-/-} animals also revealed a critical role for SMRT in forebrain

development and maintenance of the neural stem cell state through its interactions with RAR and Notch signaling, but no observations as to the role of SMRT in TR signaling were made.

Another global SMRT-deleted animal has also been shown to be embryonic lethal. However further analysis of haploinsufficient SMRT^{+/-} animals revealed a role for SMRT in adipocyte biology [21]. These mice exhibit significantly increased adiposity when fed normal chow or high fat diet, which is associated with adipocyte hyperplasia but not hypertrophy as the SMRT knock-out adipocytes are smaller than the wild type by 40%. MEFs isolated from SMRT knockout and haploinsufficient mice have enhanced adipogenic potential and increased expression of PPAR γ , C/EBP α and adiponectin. SMRT haploinsufficient mice demonstrate normal T₄, T₃ and TSH levels, normal body temperature but decreased energy expenditure. While on high fat diet they are also protected from hepatic steatosis and insulin resistance, the latter due to improved adipocyte insulin sensitivity. Overall this phenotype suggests a role for SMRT in repression of PPAR γ signaling in adipose tissue as haploinsufficiency of SMRT enhances PPAR γ signaling.

While these studies indicated that both NCoR and SMRT are essential for normal development and postnatal survival, the information on the function of these CoRs in adults, as well as their physiological role in specific NR action, including TH action, remained unknown. Thus, several mutant NCoR and SMRT knock-in and NCoR conditional knock-out models were generated to more precisely evaluate the physiological function of CoR in NR signaling and TH action.

CoRepressor Knock-in Models

Because of the presence of a number of functional domains that recruit either enzymatic activities or nuclear receptors, the CoRs are ideal targets for knock-in mutations that affect only one of many functions, while potentially avoiding the embryonic lethality seen with complete knock-outs. Thus far, investigators have targeted *in vivo* the RIDs and the site of HDAC recruitment. As outlined, NCoR and SMRT recruit NRs through C-terminal RIDs, which are characterized by a presence of an isoleucine-rich motif termed a CoRNR box [11–13]. Both NCoR and SMRT have 3 RIDs that can be expressed alternatively in a tissue specific manner, and the differences in the RID amino acid sequences dictate the preference for specific NRs [14–17]. Furthermore, the CoRs exert their repressive actions by recruiting HDAC3 and a specific N-terminal sequence present in both NCoR and SMRT termed the deacetylase activation domain (DAD) is absolutely required for the recruitment and activation of HDAC3 [8].

NCoRAID Mice

To determine the role of NCoR in TH-action *in vivo* our group utilized a Cre-Lox system to generate an allele that upon Cre-mediated recombination will encode for a mutant NCoR missing its two most N-terminal RID domains termed RID3 and RID2 (Figure 1) [22]. This mutant NCoR (NCoRAID) is unable to interact with certain NRs, and in particular, TR isoforms, as RID3 is absolutely required for NCoR-TR interactions. Microarray analysis of hepatic gene expression in mice that express NCoRAID exclusively in the liver (L-NCoRAID) confirmed for the first time the important role of NCoR in TR-mediated repression in hypothyroid state *in vivo*. Indeed, out of 173 genes that were repressed (positive TH targets) during hypothyroidism in wild type (WT) mice, 16% were significantly de-repressed in L-NCoRAID animals. At the same time, out of 326 transcripts that were significantly upregulated in hypothyroid WT animals (representing negative TH targets) only 3 were significantly downregulated in hypothyroid L-NCoRAID compared to WT. The study also found significant up-regulation of a number of positive TH targets in L-NCoRAID livers compared to controls in the euthyroid state. Therefore, this model showed

that NCoR plays a key role in repression of positive TH targets by TR in the hypothyroid state, and also determines the sensitivity and responsiveness of gene expression to TH in euthyroid livers. At the same time, the role of NCoR in negative regulation by TH in the liver appears limited.

To further delineate the role of NCoR globally in TH-signaling we next were able to express NCoRAID globally in lieu of wild-type NCoR [23]. This model allowed us to gain further insight into the role of this CoR in TR signaling, in particular, in the regulation of hypothalamic-pituitary-thyroid (HPT) axis. Surprisingly, NCoRAID animals are born at expected Mendelian ratios and appear phenotypically normal demonstrating that interactions between NCoR and NRs mediated by RID3 and RID2 are not required for normal development and survival. NCoRAID mice have low circulating T₄ and T₃ levels accompanied by normal serum TSH and normal expression of TSH subunit mRNA in the pituitary. Such a constellation of thyroid function tests would be consistent with central hypothyroidism in humans. However NCoRAID mice do not appear hypothyroid, as they grow normally, have normal body temperature and increased energy expenditure (EE). Furthermore in the setting of low circulating TH levels they have either normal or elevated expression of positive TR target genes in peripheral tissues and the pituitary. These findings again confirm the increased peripheral sensitivity to TH, previously found in the liver of L-NCoRAID mice. However, the sensitivity of HPT axis is not altered in NCoRAID animals as it responds normally to manipulations of TH levels. Interestingly, the thyroid gland shows decreased responsiveness to TSH stimulation and reduced levels of stored free T₄ and T₃. Therefore, in the absence of NCoR-TR interactions the HPT axis appears to be reset to recognized lower TH levels as normal and adjust TH production accordingly in order to balance the increased sensitivity in peripheral tissues.

Further understanding of the role of NCoR in TH action can be gathered from studying RTH. This syndrome is caused by mutations in the ligand-binding domain of the TR β isoform and characterized by different degrees of resistance to TH in peripheral tissues and the HPT axis, such that high levels of TH are accompanied by inappropriately elevated or normal levels of TSH. Inability of the mutant TR to release CoRs in the presence of ligand has been postulated to be the molecular mechanism underlying RTH. Thus, the availability of NCoRAID mice provided an excellent tool to test this hypothesis. When crossed with TR β PV mutant mice, a mouse model of RTH that introduces a human mutation into the mouse TR β locus, the disruption of TR-NCoR interactions in NCoRAID mice led to a modest but significant relief of RTH symptoms [24]. The NCoRAID-TR β PV mice had decreased serum T₄ and T₃ levels as well as lower circulating TSH and TSH β subunit expression, especially in the presence of only one mutant PV allele (akin to human RTH). Furthermore, weight loss, severe thyroid hyperplasia and repression of certain TH target genes in the liver were also reversed. Therefore, recruitment of NCoR by mutant TR β is, at least in part, responsible for the clinical manifestations of RTH.

NCoR Deacetylase Activation Domain Mutant (DADm) Mice

Another NCoR knock-in model (NCoR-DADm mice) utilized a strategy where the interaction between NCoR and HDAC3 was disrupted by mutation of the NCoR deacetylase activation domain (DAD, see Figure 1) [25]. These mice, similar to NCoRAID animals, are viable and display no gross abnormalities. However, disruption of NCoR-HDAC3 interactions results in derepression of the clock gene *Bmal1* by *Rev-erba* and abnormal circadian behavior. The NCoR-DADm mice also demonstrated a favorable metabolic phenotype characterized by reduced fat mass, increased insulin sensitivity, increased EE and protection from diet-induced obesity and insulin resistance. The observed metabolic changes were not due to a sustained increase or decrease in the expression of certain genes but resulted from the alterations in the oscillatory expression patterns of several metabolic genes

implicated in lipid metabolism. Overall these findings demonstrate that recruitment and activation of HDAC3 by NCoR plays an important role in the regulation of circadian and metabolic physiology.

More detailed studies of NCoR-DADm mice also revealed alterations in TH signaling [26]. Similarly to NCoRΔID animals, NCoR-DADm mice showed derepression of several TH-activated genes in the liver both in euthyroid and hypothyroid states. Interestingly, while these mice also demonstrated dysregulation of HPT axis, it was different from that observed in NCoRΔID animals. NCoR-DADm mice presented with normal T₄ and T₃ levels but significantly elevated serum TSH and TSHα subunit expression. TSH levels responded normally to manipulations with circulating TH levels, but expression of TSHα as well as pituitary Dio2 remained modestly but significantly elevated in all conditions, suggesting that HPT axis in this case is reset in such a way that it recognizes normal TH levels as low – ie. in the opposite direction than in NCoRΔID mice. To understand the discrepancy in the phenotype related to HPT axis between NCoRΔID and NCoR-DADm models further insight into molecular mechanisms of negative regulation by TRs and the precise role of histone acetylation in this process will be required.

SMRT Receptor Interacting Domain Mutant Mice (SMRT^{mRID})

The SMRT^{mRID} knock-in model is similar to NCoRΔID as the mutations were introduced into two RIDs (RID1 and RID2) that are present in the most commonly expressed SMRT isoform (Figure 1) [27]. The mutant SMRT^{mRID} loses its ability to interact with RAR, TR and PPAR isoforms. SMRT^{mRID} mice are born at expected Mendelian ratios, and on a mixed C57BL/6-Sv129 background have slightly reduced viability, as only 14% of total number of pups (instead of 25% that were born) survived weaning. Surviving animals did not exhibit any gross phenotypical abnormalities. SMRT^{mRID} animals present with normal T₄, T₃ and TSH levels, but when made hypothyroid show significant derepression of some TH target genes and reduction of hypothyroidism-induced hypercholesterolemia, indicating that while SMRT plays a role in repression of positive TR targets during hypothyroidism, its recruitment to TR is not required for the regulation of negative targets such as TSH subunit genes.

When fed normal chow, these animals have reduced body weight, decreased EE and RER. They also demonstrate dysregulation of glucose metabolism characterized by elevated fasting glucose and glucose intolerance due to increased basal hepatic glucose production and a decrease in insulin-stimulated glucose disposal rate. Despite decreased body weight, when on normal chow, SMRT^{mRID} animals fed both chow and high fat diet have dramatically increased adiposity (by 70% compared to WT), which can be at least partly explained by greatly increased adipogenic potential. MEFs isolated from these animals, similar to SMRT KO MEFs show increased PPARγ expression during adipogenic differentiation, elevated PPARγ target gene expression, and increased basal and rosiglitazone-induced PPARγ activity in reporter assays. This indicates that SMRT-mediated repression by NRs, and particularly PPARγ, determines the adipogenic set point, and also serves as an integrator of glucose metabolism and metabolic homeostasis.

Interestingly, when SMRT^{mRID} mice were backcrossed to a pure C57BL/6 background, the survival of SMRT^{mRID} pups dramatically decreased to less than 1% [28]. These pups die soon after birth due to a previously unidentified respiratory distress syndrome (RDS) characterized by a defect in the development of type 1 pneumocytes. If SMRT-dependent repression of one or more NRs was required for establishment of type I pneumocyte lineage, inhibition of those NRs would rescue these animals. Antagonists of RARα, ER, GR and PR administered to pregnant dams did not improve the survival of the pups. However when treated with the antithyroid drugs PTU or MMI in utero SMRT^{mRID} mice are born alive,

show no evidence of respiratory distress and survive into adulthood. Furthermore, their lungs appear normal and expression of type 1 pneumocyte markers is restored. On this background serum T₄ concentrations in SMRT^{mRID} mice were reported to be lower than in WT animals. As both TR isoforms are expressed in late embryogenesis and in P0 type 1 pneumocytes, with TR β being more abundant, genome-wide gene expression analysis by microarray was performed at E18.5 with or without PTU treatment. After PCR verification the authors identified one gene, Klf2 (Kruppel-like factor 2), that was significantly downregulated in SMRT^{mRID} lungs compared to WT mice and remained unaffected by PTU. Both SMRT and TR were found to bind an enhancer region downstream of Klf2 locus, which is repressed by T₃, identifying Klf2 as a negative TR target. Klf2 is expressed predominantly in lung and its overexpression in mouse lung carcinoma cell line induces expression of type I pneumocyte markers. Moreover, mice with a high percentage of chimerism for Klf2^{-/-} in the lungs die soon after birth from RDS resembling the SMRT^{mRID} pups. These observations identify a key role of TR in the development of neonatal lung through regulation of transcription factor Klf2, and demonstrate a role of SMRT in the ligand-independent activation of Klf2.

SMRT^{mRID1} Mice

In SMRT^{mRID1} mice the mutation that disrupts SMRT-NR interaction was only introduced into one of the two RIDs – termed RID1 by the authors but shown as RID 2 in Figure 1. This leads to a shift in the interaction patterns, increasing the repression by NRs that preferably utilize the most C-terminal RID (RID1 in Figure 1), particularly PPARs [29, 30]. This leads to premature aging, decreased lifespan and related metabolic diseases, accompanied by reduced mitochondrial function. SMRT^{mRID1} demonstrate certain similarities to SMRT^{mRID} mice, such as increased susceptibility to diet-induced obesity, insulin resistance and decreased RER. However, they also display marked hepatic steatosis, and are refractory to glucose lowering effects of TZDs and AICAR in contrast with SMRT^{mRID} mice which demonstrated enhanced response to PPAR γ stimulation. The similarities in the phenotypes of these two SMRT mutants must be due to the relief of repression through NRs interacting with the similarly mutated RID, but the differences are the result of increased repression through PPAR isoforms in SMRT^{mRID1} animals, while in SMRT^{mRID} mice this repression is also relieved.

Tissue-specific NCoR knock-out models

Very recently two models of tissue-specific NCoR knock-out have been published with both groups employing the same conditionally targeted NCoR allele to delete NCoR completely in either skeletal muscle or adipose tissue. In both cases the presence of Cre recombinase completely abrogated expression of NCoR in the tissue of interest. The skeletal muscle-specific NCoR knock-out identified NCoR as a negative regulator of fatty acid oxidation in the muscle [31]. These mice demonstrate enhanced exercise endurance due to an increase in overall muscle mass and the number of oxidative fibers. NCoR-deleted muscle has higher mitochondrial number and activity and overall increased oxidative capacity. These changes result from the increased activity of transcription factors that play a key role in the muscle function, such as MEF2, PPAR δ and ERR isoforms. This study also showed that NCoR expression is regulated by physiological conditions that favor fatty acid oxidation, so that the cellular NCoR levels are diminished by low glucose, low insulin and high fatty acid concentrations. In the muscle NCoR mRNA levels are decreased during aging, as a result of fasting, high-fat meal or exercise.

Adipocyte-specific deletion of NCoR demonstrated that this CoR plays a role in the repression of PPAR γ signaling in adipocytes [32], as has been shown previously for SMRT in SMRT^{+/-} mice. When fed high fat diet, adipocyte-specific NCoR knock-out mice develop

more pronounced obesity accompanied by increased food intake compared to the wt animals. However, they demonstrate an improved metabolic phenotype characterized by decreased insulin levels, improved insulin sensitivity and glucose tolerance. Their adipose tissue consists of a greater number of smaller adipocytes that show increased glucose uptake and decreased lipolysis, as well as decreased macrophage infiltration and inflammation. This phenotype is similar to the phenotype of animals treated with PPAR γ agonists. Indeed, PPAR γ response genes were activated in NCoR knockout adipose tissue and CDK5-mediated phosphorylation of PPAR γ was diminished, thus increasing the levels of transcriptionally active PPAR γ [33]. Moreover, adipocyte-specific NCoR knock-out mice were refractory to further PPAR γ stimulation with rosiglitazone, confirming that NCoR normally represses PPAR γ function in adipocytes, so that deletion of NCoR increases its sensitivity to endogenous ligands.

Summary and Future Perspectives

Both nuclear corepressors, NCoR and SMRT, are absolutely required for normal development and postnatal survival in mammals. However, the recruitment of corepressors or other components of the repression complex, such as HDAC3, to NRs, does not appear to be critical for these processes. The only exception is SMRT^{mRID} knock-in mice on a pure C57BL/6 background, where the absence of SMRT recruitment to the TR leads to RDS and lethality in newborn pups. The reason for such drastic differences in phenotype depending on the genetic background remains unknown, while one can speculate that it might be due to the strain-specific differences in the expression levels of the second CoR, NCoR, in the lung.

While mouse models with targeted disruption of CoR-NR or CoR-HDAC3 interactions or tissue specific knock-outs display different phenotypes, they point to a similar role of CoRs in thyroid hormone action. As was previously postulated, CoRs mediate repression of positive targets by the TR. However according to accumulated data, this takes place not only in situations when the ligand is unavailable, but also in normal physiologic conditions and concentrations of ligand. This applies not only to the TR and RAR, but also to “metabolic” NRs, such as PPAR isoforms and others. Removal of CoRs or their ability to recruit other members of the repression complex leads to a shift in the dynamic activation/repression balance that underlies transcriptional actions of NRs, and leads to augmented transcriptional activation by NRs.

In case of TR, the absence of CoR recruitment increases availability of the receptors to the present hormone, thus enhancing their sensitivity and transcriptional activation in response to the same hormone concentrations. While this is clearly the case for the positive TR targets, the role of CoRs in the regulation of negative targets is still poorly understood. It has been proposed that CoRs are paradoxically required for the activation of negative targets by TRs in the absence of ligand. The only definitive *in vivo* proof of the role of CoRs in such activation is regulation of Klf2 expression in the neonatal lung [28]. While all models with disrupted CoR/HDAC recruitment to the TR demonstrate dysregulation of the HPT axis, none of them show impaired dynamic regulation of TSH subunits by TH. Rather, these models are characterized by a change in the set point of HPT which, in some cases adjusts the circulating TH levels to accommodate increased peripheral sensitivity to TH signaling [23, 28]. New models are needed to provide further mechanistic insight into the role of CoRs in negative regulation by NRs.

Overall the possibility to manipulate NR signaling by changing the cellular availability of CoRs or by selectively disrupting/enhancing interactions between NRs and specific RIDs using novel ligands presents an attractive therapeutic opportunity for treatment of various endocrine and metabolic disorders.

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Highlights

- Available mouse models allow understanding of specific function of CoRs in NR signaling.
- CoRs mediate repression of positive targets by TR in the absence of T_3 .
- CoRs play a role in establishing a set point of HPT axis.
- CoRs attenuate transcriptional activity of NRs in the presence of ligands.
- CoR might be new drug targets for endocrine and metabolic disorders.

Figure 1.

Schematic representation of the structure of wild type NCoR and SMRT as well as various mutants discussed in this review. Repression domains (RD), deacetylase activation domain (DAD) and receptor-interacting domains (RID) are indicated. RID3 of SMRT is shown in lighter color to reflect the fact that the predominantly expressed SMRT isoform contains only RID2 and RID1.

Figure 2.

A model of TR-mediated regulation of transcription. On a positively regulated target gene, in the absence of thyroid hormone (upper left) TR-RXR heterodimer recruits the corepressor complex to repress transcription. In the presence of high concentrations of TH (upper right) most of the receptors bind TH which induces the conformational change that leads to release of corepressors and recruitment of coactivators to maximally activate transcription. At intermediate concentrations of TH corresponding to a normal euthyroid state (upper middle) only some of the receptors bind the ligand. The complex between TR, hormone, coactivators and corepressors is in a dynamic balance that achieves moderate activation of transcription. Removal of corepressors at this state results to the increased availability of TR for the ligand and coactivators, leading to the increased sensitivity to the hormone. Paradoxically, on negatively regulated targets, corepressor complexes recruited in the absence of hormone may activate transcription, while coactivators that are recruited when TR binds TH, mediate transcriptional repression.