Published in final edited form as:

Mol Cell Endocrinol. 2007 January 29; 264(1-2): 149–156.

Variable RXR requirements for thyroid hormone responsiveness of endogenous genes

Ericka M. Diallo, Kenneth G. Wilhelm Jr., Deborah L. Thompson, and Ronald J. Koenig*

Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor MI 48109-0678, USA

Abstract

Thyroid hormone receptors heterodimerize with retinoid X receptors *in vitro* and it is widely assumed that these heterodimers mediate the T3 induction of target genes. However, the importance of RXR for the T3 induction of endogenous genes has not been assessed. We used cDNA microarrays to identify 54 genes induced by T3 in Neuro2a cells that express thyroid hormone receptor beta. RNA interference-mediated knock down of endogenous RXRs showed that these genes vary from being highly dependent on RXR for T3 induction to being independent of RXR. Thus, the availability of RXR may differentially regulate the T3 induction of subsets of genes within a cell. Furthermore, coregulatory proteins that preferentially interact with TR homodimers or RXR-TR heterodimers may further expand the range of T3 response for genes within the same cell.

Keywords

retinoid X receptor; thyroid hormone receptor; T3; heterodimer; homodimer

1. Introduction

Thyroid hormone receptors (TRs) and retinoid X receptors (RXRs) are members of the nuclear hormone receptor family of ligand activated transcription factors which regulate many important processes in development and physiology. Nuclear hormone receptors mediate gene induction through interactions with specific response elements often located in the promoter sequences of target genes. Nuclear receptors share a canonical structure consisting of an amino terminal domain, a zinc finger DNA binding domain, and a carboxyl terminal ligand binding domain (LBD). The LBD also contains a receptor dimerization interface as well as surfaces that interact with various co-regulatory proteins.

Thyroid hormone response elements (TREs) generally consist of two receptor binding “half sites” related to the sequence AGGTCA arranged as a direct repeat separated by 4 bp, or as an everted repeat or an inverted repeat. By electrophoretic mobility shift assay (EMSA), RXR-TR heterodimers generally are found to bind TREs more strongly than do TR homodimers (Kliwer et al., 1992; Leid et al., 1992; Yu et al., 1991). This difference is especially striking in the presence of thyroid hormone (3,5,3' triiodothyronine, T3), which destabilizes the TR

*Corresponding Author: Ronald J. Koenig, University of Michigan, 5560 MSRB-II, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0678, Telephone: 734-763-3056, Fax: 734-936-6684, Email: rkoenig@umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

homodimer-DNA complex (Yen et al., 1992). Thus, it has generally been assumed that RXR-TR heterodimers mediate the regulation of transcription by T3.

However, the importance of RXR for T3 action can be questioned. Using EMSAs, TREs that contain the optimal TR binding half site TAAGGTCA (Katz and Koenig, 1993) can bind TR homodimers well, and the addition of a coactivator protein such as nuclear receptor coactivator 2 (NCoA2/SRC-2/TIF2) stabilizes this TR homodimer-TRE complex, even in the presence of T3 (Diallo et al., 2005). Yeast expression studies suggest that TREs form a spectrum of RXR independence, roughly correlating with the resemblance of the 5' half site to the optimal TAAGGTCA (Olson and Koenig, 1997). However, it has been difficult to examine the role of RXR in more relevant cellular systems as there are no RXR-null mammalian cells, and it has not been possible to measure TR homodimer versus RXR-TR heterodimer complexes on TREs *in vivo* by chromatin immunoprecipitation.

We therefore employed an RNA interference approach to specifically inhibit RXR activity in a mouse pre-neuronal cell line (Neuro2a) that expresses TR β 1, denoted N2a/TR β (Denver et al., 1999; Lebel et al., 1994). We then measured the T3 induction of endogenous genes in the presence and absence of RXR activity, with the goal of isolating a pool of genes at either end of a spectrum of RXR responsiveness. We show herein that siRNAs against RXRs specifically inhibit RXR activity in this murine cell line, and that there is indeed a hierarchy of RXR requirement among endogenous T3 responsive genes in this system.

2. Materials and methods

2.1 Cell culture

The mouse pre-neuronal cell line N2a/TR β has been described (Denver et al., 1999; Lebel et al., 1994). The cells were grown in F-12 Ham's media supplemented with 10% charcoal stripped fetal bovine serum with penicillin-streptomycin and 0.5 mg/ml hygromycin B (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO₂.

2.2 Transient Transfections

Two annealed double stranded siRNAs directed against mouse RXR α and two directed against mouse RXR β were purchased from Ambion (Austin, TX) as follows: RXR α #1 sense sequence GGAUAUCAAGCCGCCACUAtt, antisense sequence UAGUGGCGGCUUGAUAUCCtc; RXR α #2 sense sequence GGAAUAUGGCCUCCUUCAtt, antisense sequence UGAAGGAGGCCAUUUUCCtg; RXR β #1 sense sequence GGGUUUCUUAAGCGCACctt, antisense sequence GGUGCGCUUGAAGAAACCCtt; RXR β #2 sense sequence GGACCUGACCUACUCGUGUtt, antisense sequence ACACGAGUAGGUCAGGUCCtt). A standard control siRNA which does not recognize any known mammalian gene sequence also was purchased from Ambion (catalog #4611).

Annealed siRNAs were transfected into N2a/TR β cells in 6-well clusters using siPORT Amine according to the manufacturer's instructions (Ambion). Briefly, siRNAs were diluted into serum free media, then siPORT Amine was added at a ratio of 6 μ l to 1 μ l of each siRNA from a 50 μ M stock. Final siRNA concentrations were 200 nM total for all transfections. N2a/TR β cells were washed and then incubated with the nucleic acid/siPORT Amine mixture for three hours. At the end of the incubation, growth media containing 20% stripped fetal bovine serum and antibiotics were added to the transfection media at a 1:1 ratio to result in standard final concentrations. The cells were harvested and assayed within 72 hours of transfection.

For experiments in which siRNAs were cotransfected with plasmid DNA, Lipofectamine 2000 was utilized according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmids utilized for transfection experiments include a reporter construct consisting a 3x repeat of the

TRE from the rat alpha myosin heavy chain gene (Izumo and Mahdavi, 1988) driving firefly luciferase expression (MHC-Luc), a reporter plasmid consisting of the glucocorticoid responsive MMTV promoter driving firefly luciferase expression (GRE-Luc) (Nordeen, 1988), a transfection control vector expressing *Renilla* luciferase under the control of the CMV promoter (CMV-*Renilla*) (Promega, Madison, WI), an expression vector for murine RXR γ (pCDM-RXR γ), and empty pCDM. Combinations of these plasmids were transfected as noted in the descriptions of individual experiments. siRNAs and plasmids were mixed in serum-free media to which Lipofectamine 2000 was added at a ratio of 2 μ l Lipofectamine 2000 to 200 ng plasmid DNA and 200 nM final concentration siRNAs for each well of a 24 well cluster. N2a/TR β cells were incubated with the nucleic acid/Lipofectamine 2000 mixture for three hours, then cultured in growth media, and harvested after 48 hours for analysis. Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase system (cat. #E1960, Promega). Results are expressed as the ratio of firefly luciferase activity normalized to *Renilla* luciferase activity, and are pooled from at least three independent experiments as described above.

2.3 RNA processing and Northern blotting

RNA was prepared from cell cultures by extraction with Trizol (Invitrogen) followed by passage over an RNeasy column (Qiagen, Valencia, CA) according to the manufacturer's protocols. Northern blotting was performed using standard protocols with probe labeling using a Strip-EZ DNA Kit (Ambion). Detection was with a BioRad phosphorimager.

2.4 Western blot analysis

For western blotting, cells were washed twice in phosphate buffered saline, then trypsinized, centrifuged, and nuclear proteins extracted using NE-PER (Pierce) according to the manufacturer's protocol. The resulting nuclear proteins were separated by SDS-PAGE in 10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and probed using RXR β antibody #06-527 (Upstate Biotechnology, Charlottesville, VA) at 1:2000 followed by horseradish peroxidase conjugated goat anti-rabbit IgG #sc-2301 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100,000. Detection was with Super Signal West Dura enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and a BioRad Fluor-S Max Multiimager.

2.5 Microarray

RNA was processed in the University of Michigan Cancer Center Affymetrix Core Facility (www.michiganmicroarray.com) using Mouse Genome 430 2.0 GeneChips following the manufacturer's standard protocol. Affymetrix GeneChip data were analyzed as follows. Raw probe-level data were converted to expression measures using the Robust Multi-array Average method, which is implemented in the Affymetrix package of Bioconductor. Briefly, the raw perfect match probes were quantile normalized to remove any non biological variability (due to differential binding, chip effects, etc). The normalized probe data were then converted to an expression measure for each gene on each chip using a robust modeling strategy. Differentially expressed genes were detected by fitting a by-gene linear model to the expression values and extracting the contrasts of interest. Standard error estimates were adjusted using an empirical Bayes method to account for small sample size. Contrasts of interest included the comparison of T3 treated and untreated cells with and without siRNA. We also tested for an interaction between these treatments, which tested for genes that reacted differently to the T3 treatment when also treated with siRNA. Genes were selected based on an adjusted *p*-value of 0.005, adjusting for multiplicity using false discovery rate.

2.6 Real time PCR

Total RNA prepared as described above was reverse transcribed using random hexamer primers and SuperScript III (Invitrogen). Real time PCR was performed on an Applied Biosystems 7500 thermal cycler using SYBR Green PCR Master Mix according to the vendor's instructions. Cycling conditions were 95°C for 10 min, then 45 cycles of 94°C for 15 sec, 56°C for 30 sec, and 72°C for 1 min. The primers used for real time PCR are given in Table 1. For each gene on each reaction plate, a standard curve was generated in duplicate using the cDNA from 1, 10 and 100 ng of RNA, and this was used by the instrument's software to convert cycle threshold values to a quantity corresponding to ng equivalents of starting RNA for each sample analyzed (also in duplicate). In addition to running reactions for each of the T3 inducible target genes, three neutral control genes were studied: glyceraldehyde 3-phosphate dehydrogenase, beta actin and selenoprotein M. The expression of these three genes was independent of culture conditions (siRNA or T3). The expression levels of the three were averaged, and this was used to normalize the expression of each T3 target gene under each culture condition. Separately, real time RT/PCR was used to test whether the RXR α and β siRNAs blocked the expression of RXR γ , which was transfected in reporter gene experiments described in section 2.2 to rescue the RXR knock down. The primers used to quantify RXR γ were (top strand) TGCTCTTGGCTCTCCGTATAG and (bottom strand) AGCTGCTGACACTGTTGACC.

2.7 Promoter luciferase plasmids

The polymerase chain reaction and AccuPrime Pfx polymerase (Invitrogen) were used to amplify promoter fragments from 8 genes found to be T3 responsive in the microarray studies. The genes and amplified regions are: *Alas1* -2058 to +17 (+1 is the start of transcription), *Arvcf1* -2425 to +28, *Jup* -2930 to +87, *Tinagl1* (*Lcn7*) -2998 to +74, *Hr* -2213 to +54, *Tgm2* -1634 to +63, *P2ry6* -2278 to +100, and A930021H16Rik (*Mical-L2*) -2756 to +11. The PCR products were ligated into the MluI and XhoI sites of pGL3-basic (Promega, Madison, WI).

3. Results

3.1 siRNAs decrease RXR expression in N2a/TR β cells

By Northern Blot, N2a/TR β cells were found to express substantial levels of RXR β , low levels of RXR α and no RXR γ (data not shown), so RNA interference experiments were directed at silencing the expression of the first two of these three isoforms. As shown in Fig. 1A, RXR β RNA expression was reduced to 36%, 28% and 15% of control levels by RXR β siRNAs 1, 2, and 1 plus 2, respectively. RXR β protein was reduced comparably, to 8, 39 and 7% of control siRNA-treated cells (Fig. 1B). RXR α RNA was expressed at a very low level in the control siRNA-treated cells, and this expression was reduced to 35, 58 and 22% of control levels by RXR α siRNAs 1, 2 and 1 plus 2, respectively (Fig. 1C). RXR α protein expression was below the limit of detection by Western blotting (data not shown), and therefore the effect of the siRNAs could not be measured at the protein level.

3.2 siRNAs directed against RXRs decrease RXR dependent reporter gene activity but do not affect dexamethasone induced reporter gene activity in N2a/TR β cells

To validate the RXR knock down at a functional level, we analyzed the expression of an MHC-luciferase reporter gene. The MHC TRE is thought to be dependent on RXR for T3 induction because, by EMSA, TR binding to this TRE is completely dependent on heterodimerization with RXR (Diallo et al., 2005; Yu et al., 1991), and in yeast, gene expression driven by this TRE requires co-expression of TR and RXR (Olson and Koenig, 1997). As shown in Fig. 2, luciferase was induced 3.7-fold by T3 following cotransfection of N2a/TR β cells with MHC-Luc plus the control siRNA. However, cotransfection with the RXR siRNAs completely

abrogated T3 induction. That this effect is due to loss of RXR is supported by the ability of cotransfected RXR γ to restore T3 induction in the presence of the RXR α and β siRNAs (Fig. 2, right two bars). A separate series of transfections was performed to confirm that the RXR α and β siRNAs did not prevent expression of the RXR γ vector. In fact, as assessed by real time RT/PCR, RXR γ was expressed at a 2.2 ± 0.4 -fold greater level in the presence of the RXR α and β siRNAs than in the presence of the control siRNA ($n=3$).

To further evaluate the specificity of the RXR knock down, we analyzed its effect on glucocorticoid induction of MMTV-Luc. Glucocorticoid receptors activate transcription by similar mechanisms as T3 receptors, except that glucocorticoid receptors bind DNA as homodimers and do not heterodimerize with RXR. Dexamethasone induced luciferase 11.5 ± 1.2 -fold in control siRNA-treated cells, and 13.9 ± 1.0 -fold in RXR siRNA-treated cells ($n=3$). Thus, RXR knock down did not impair dexamethasone induction of MMTV-Luc. Based upon the above data, we conclude that the RXR siRNAs achieve a knock down sufficient to disrupt RXR-dependent gene expression and that they do not impair RXR-independent gene expression. Thus, these RXR siRNAs are appropriate reagents to study the RXR dependence of endogenous T3 responsive genes in N2a/TR β cells.

3.3 Microarray analysis of endogenous T3 responsive genes in N2a/TR β cells treated with and without RXR siRNAs identifies a spectrum of RXR requirements

N2a/TR β cells were transfected with the control or RXR siRNAs. Two days later, T3 was added (or not) for 6 hours, and RNA was harvested for analysis by Affymetrix microarrays. Three independent experiments were performed. Fifty-four genes were induced at least 3-fold by T3 in the control siRNA-treated cells. Table 2 lists these genes sorted according to the ability of the RXR knock down to impair T3 induction. Genes at the top of the list are relatively dependent on RXR for T3 induction. For example, the T3 induction of *Alas1* in the RXR knock down cells is only 44% that in the control cells (4.6 versus 10.3 fold). In contrast, the T3 induction of genes at the bottom of the list is essentially independent of RXR. For example, the T3 induction of *Arvcf* in the RXR knock down cells is at least as large as in the control cells (5.9 versus 5.3 fold). Thus, these studies indicate there is a spectrum of RXR-dependence for T3 induction of endogenous genes in N2a/TR β cells.

Distinct from its ability to activate transcription, T3 represses the transcription of some target genes. The mechanism(s) of transcriptional repression by T3 is not well understood. Therefore, we also examined the effect of RXR knock down on the T3 repression of target genes. Only seven genes were repressed at least 3-fold by T3 (T3 induction ≤ 0.33) in the control siRNA-treated cells, and repression never exceeded 4.5-fold (Table 3). All of these genes showed modestly less T3 repression with RXR knock down, but six of the seven were expressed at very low levels and hence demonstrated substantial inter-experiment variability. Gene repression was not studied further.

3.4 Real time PCR validates the RXR requirements for endogenous T3 inducible genes identified by microarray

We selected 10 genes for further study that spanned the spectrum of RXR dependence for T3 induction and used reverse transcription real time PCR to validate the microarray results. Results from these two methods correlated highly when analyzed either by fold T3 induction (Fig 3A) or level of gene expression in the presence of T3 (Fig. 3B).

3.5 Promoter analysis by transient transfection of genes with T3 inductions that are relatively RXR dependent or independent

Approximately 2 to 3 kb of proximal promoter sequences from 8 of the genes analyzed above were cloned into the promoterless luciferase vector pGL3-basic and were transfected into N2a/

TR β cells. The goal was to characterize the T3 response elements to determine whether features of those elements can explain the variability in RXR dependence. However, although the *Hairless* promoter was induced 4.8 ± 0.2 -fold by T3 (n=3), none of the other luciferase constructs was T3 responsive, and cotransfection of additional TR with or without RXR also failed to induce a T3 response (data not shown). Thus, it was not possible to use this transfection paradigm to study the mechanism of RXR dependence or independence of the endogenous genes.

4. Discussion

RXRs originally were described as nuclear proteins that enhance the binding of TRs to DNA in EMSAs (Murray and Towle, 1989), and were subsequently shown to have similar effects on a broader subset of nuclear receptors, such as retinoic acid and vitamin D receptors (Kliwer et al., 1992; Leid et al., 1992; Yu et al., 1991). However, the precise role of RXRs in T3 action has been difficult to define in part because there are no RXR-null mammalian cells, as noted above. Transfection of mammalian cells with RXR generally only marginally increases T3 induction (Williams et al., 1992), which could suggest that RXR is relatively unimportant or could indicate that it is essential but that endogenous RXR is sufficient.

We approached this question by depleting N2a/TR β cells of endogenous RXRs with RNA interference. We identified 54 genes induced at least 3-fold by T3 in N2a/TR β cells, and showed that they form a spectrum of RXR dependence from genes whose T3 induction was unaffected by RXR knock down to genes whose T3 induction decreased by up to 50–60%. Genes induced less than 3-fold in the control siRNA-treated cells were not studied, since it would be very difficult to quantify a partial loss of T3 induction under those circumstances.

It is perhaps surprising that the T3 induction of endogenous genes was never inhibited by more than 50–60% by RXR knock down, given that the same knock down abolished the T3 induction of transiently transfected MHC-luciferase (the endogenous MHC α gene is not expressed in N2a/TR β cells). This could indicate that no N2a/TR β genes are absolutely RXR dependent for T3 induction. Alternatively, the small level of residual RXR might be sufficient to support a low level of T3 induction by all endogenous N2a/TR β genes, despite it being insufficient for transfected MHC-Luc. In either case, the data clearly indicate that endogenous T3 responsive genes in N2a/TR β cells form a spectrum of RXR dependence, even if the necessity of a minimal level of RXR cannot be assessed with certainty. It is possible that RXR responsiveness would differ for TR α 1, but we do not have data for TR isoforms other than TR β 1.

One potential mechanism to explain the variability of RXR dependence concerns the sequence of the TRE 5' half site, since that is the position potentially bound by RXR (Kurokawa et al., 1993). Based upon TR (Katz and Koenig, 1993) and RXR (Subauste et al., 1994) monomer DNA sequence binding preferences, a 5' half site resembling T(A/G)AGGTCA might facilitate TR homodimerization, whereas a 5' half site resembling GGGTCA might favor RXR-TR heterodimerization. Other mechanisms are possible, as well. RXRs could exert effects independent of TRs, either through interactions with other nuclear receptors or as homodimers. A given promoter might bind additional transcription factors or coregulatory proteins that could preferentially stabilize TR homodimers or RXR-TR heterodimers. In principle, TRs also could regulate target genes without directly binding DNA, thus obviating the need for forming homodimers or RXR-TR heterodimers. For example TRs could be tethered to DNA by other transcription factors, or could function by non-genomic mechanisms. To begin to explore the underlying mechanisms, we created luciferase constructs driven by 2 to 3 kb of the promoters from 8 endogenous genes representing the far ends of the spectrum of RXR dependence or independence. We hoped that these would replicate the RXR dependence of the corresponding endogenous genes, which would allow us to begin to dissect the underlying mechanisms.

Unfortunately, of these 8 constructs, only one (*Hairless*) was T3 responsive when transfected into N2a/TR β cells, and cotransfecting additional TR or RXR did not lead to T3 responses in the others. Thus, it was not possible to use this experimental paradigm to investigate the mechanisms of variable RXR dependence.

The lack of T3 induction of the 7 promoter constructs may indicate that the critical *cis* acting elements lie either 5' or 3' to the 2 to 3 kb of promoter sequences cloned into the luciferase vectors. It is extremely difficult to identify TREs by computational methods since there is substantial variability in half site sequence, spacing and orientation. Thus, TREs need to be identified by functional studies. Even though most of the currently identified TREs lie within the proximal 2 to 3 kb promoters, this may reflect a selection bias; i.e., those are the TREs that can be identified easily. It is possible that most T3 responsive genes harbor TREs that lie very distant from the transcription start site. In this regard, recent chromatin immunoprecipitation/whole chromosome microarray studies suggest that the vast majority of endogenous estrogen response elements are found at great distances from basal promoters (Carroll et al., 2005).

There may be important functional consequences of target gene activation by TR homodimers versus RXR-TR heterodimers. If RXR is required, then the magnitude of the T3 response could be regulated by the availability of RXR. Currently it is very difficult to judge the availability of RXR that is free to interact with TR within a cell. RXR can heterodimerize with multiple nuclear receptors and also can form homodimers (Zhang et al., 1992) and homotetramers (Kersten et al., 1995), so just knowing the total amount of RXR does not reveal the amount that is available. Second, it is possible that TR homodimers and RXR-TR heterodimers may prefer to bind different coregulatory proteins, which would provide an additional mechanism to differentially regulate the T3 responsiveness of target genes within the same cell. For example, using an *S. cerevisiae* reconstitution system, steroid receptor coactivator 1 was found to be a much better coactivator for T3 induction from TR homodimers than from RXR-TR heterodimers (Olson and Koenig, 1997). However, the relevance of this yeast model system is unknown. Additional approaches will need to be developed to unravel the mechanisms and potential significance of the variable RXR dependence of T3 responsive genes.

Acknowledgements

This work was supported by NIH grant R01 DK44155 and the Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center, P60 DK20572. The authors thank the University of Michigan Cancer Center Microarray Core for performing the microarray analyses, and Dr. Robert J. Denver for providing the N2a/TR β cells.

References

- Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoutte J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 2005;122:33–43. [PubMed: 16009131]
- Denver RJ, Ouellet L, Furling D, Kobayashi A, Fujii-Kuriyama Y, Puymirat J. Basic transcription element-binding protein (BTEB) is a thyroid hormone-regulated gene in the developing central nervous system - Evidence for a role in neurite outgrowth. *J Biol Chem* 1999;274:23128–23134. [PubMed: 10438482]
- Diallo EM, Thompson DL, Koenig RJ. A method for efficient production of recombinant thyroid hormone receptors reveals that receptor homodimer-DNA binding is enhanced by the coactivator TIF2. *Protein Expr Purif* 2005;40:292–298. [PubMed: 15766871]
- Izumo S, Mahdavi V. Thyroid hormone receptor alpha isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature* 1988;334:539–542. [PubMed: 2841611]
- Katz RW, Koenig RJ. Non-biased identification of DNA sequences which bind thyroid hormone receptor α 1 with high affinity. *J Biol Chem* 1993;268:19392–19397. [PubMed: 8366086]

- Kersten S, Kelleher D, Chambon P, Gronemeyer H, Noy N. Retinoid X receptor alpha forms tetramers in solution. *Proc Natl Acad Sci U S A* 1995;92:8645–8649. [PubMed: 7567990]
- Kliwer SA, Umesono K, Mangelsdorf DJ, Evans RM. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* 1992;355:446–449. [PubMed: 1310351]
- Kurokawa R, Yu VC, Naar A, Kyakumoto S, Han Z, Silverman S, Rosenfeld MG, Glass CK. Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. *Genes Dev* 1993;7:1423–1435. [PubMed: 8392479]
- Lebel JM, Dussault JH, Puymirat J. Overexpression of the beta 1 thyroid receptor induces differentiation in neuro-2a cells. *Proc Natl Acad Sci U S A* 1994;91:2644–2648. [PubMed: 8146169]
- Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen JY, Staub A, Garnier JM, Mader S, Chambon P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 1992;68:377–395. [PubMed: 1310259]
- Murray MB, Towle HC. Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. *Mol Endocrinol* 1989;3:1434–1442. [PubMed: 2608066]
- Nordeen SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* 1988;6:454–458. [PubMed: 2908509]
- Olson DP, Koenig RJ. 5' Flanking sequences in thyroid hormone response element half sites determine the requirement of RXR for receptor mediated gene expression. *J Biol Chem* 1997;272:9907–9914. [PubMed: 9092529]
- Subauste JS, Katz RW, Koenig RJ. DNA binding specificity and function of retinoid X receptor α . *J Biol Chem* 1994;269:30232–30237. [PubMed: 7982932]
- Williams GR, Harney JW, Moore DD, Larsen PR, Brent GA. Differential capacity of wild type promoter elements for binding and *trans*-activation by retinoic acid and thyroid hormone receptors. *Mol Endocrinol* 1992;6:1527–1537. [PubMed: 1333048]
- Yen PM, Darling DS, Carter RL, Forgione M, Umeda PK, Chin WW. Triiodothyronine (T3) decreases binding to DNA by T3-receptor homodimers but not receptor-auxiliary protein heterodimers. *J Biol Chem* 1992;267:3565–3568. [PubMed: 1740410]
- Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Naar AM, Kim SY, Boutin JM, Glass CK, Rosenfeld MG. RXR β - A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 1991;67:1251–1266. [PubMed: 1662118]
- Zhang XK, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner G, Hermann T, Tran P, Pfahl M. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* 1992;358:587–591. [PubMed: 1323763]

Fig. 1.

siRNA-mediated knock down of RXR in N2a/TR β cells. The cells were transfected with siRNAs as described in Methods. The cells in (A) and (B) received siRNAs directed against RXR β (or control siRNA), and the cells in (C) received siRNAs directed against RXR α (or control siRNA). RNA expression was measured by Northern blot and protein expression by Western blot.

Fig. 2.

Functional validation of RXR knock down. N2a/TR β cells were transfected with siRNAs directed against RXRs α and β (or control siRNA), as well as the T3-responsive reporter plasmid MHC-Luc and a control *Renilla* luciferase plasmid. For the right two bars, the cells also received an RXR γ expression plasmid. The cells were cultured $\pm$ T3. Fold T3 induction is firefly luciferase/*Renilla* luciferase for cells treated +T3, divided by firefly luciferase/*Renilla* luciferase for cells treated -T3.

Fig. 3. Correlation of gene expression measured by microarray and real time reverse transcription PCR. Ten genes were selected from the microarray data based upon their T3 inductions spanning the range of RXR dependence. The expression levels of these genes were then measured by real time RTPCR. (A) The data represent the fold T3 inductions in cells treated with RXR siRNAs divided by the fold T3 inductions in cells treated with control siRNA. The *x*-axis plots these ratios measured by real time RTPCR (qRTPCR), and the *y*-axis plots these ratios measured by microarray. (B) The data represent the expression levels +T3 in cells treated with RXR siRNAs divided by those in cells treated with control siRNA. The *x*-axis plots these ratios measured by real time RTPCR, and the *y*-axis plots these ratios measured by microarray.

Table 1

Real time PCR primers

Gene Name	Gene symbol	5' primer	3' primer
<i>Aminolevulinate, delta-, synthase 1</i>	<i>Alas1</i>	GGCTTTCCTGTTAT CCCCTGTC	CATAGATGCTGTGC TTGGAGAGC
<i>Armadillo repeat gene deletes in velocardiofacial syndrome</i>	<i>Arvcf1</i>	CCAGATGGTTACGC CACAGTTG	TGCTCGGACTCTTC ACTCTCAATAG
<i>Hairless</i>	<i>Hr</i>	AGTTTGACATTCGG GGGCAC	TGGGGGAGTTTCT CTGTTGG
<i>Junction plakoglobin</i>	<i>Jup</i>	TGTGCCCTCTGTAA GCAGCAAG	CCGTTGTGGACATC TGGTATTC
<i>LIM and cysteine-rich domains 1</i>	<i>Lmcd1</i>	ACTGCCCCAACCAC CAATG	GTATCCAGCCCTGT CAGCGTAG
<i>Pyrimidinergic receptor P2Y, G-protein coupled, 6</i>	<i>P2ry6</i>	CACCAAATCTGGCA CTCCTCC	CCATTGTCCTGCTC CATAACTGC
<i>Transglutaminase 2</i>	<i>Tgm2</i>	TTACGCAACAGGG CTCATCTAC	CAACAGCATCAGG CAGGTATCC
<i>Tubulointerstitial nephritis antigen-like 1</i>	<i>Tinagl1 (Lcn7)</i>	CCCCACTCCTCGTT GTATGATG	GACCTGGTAGATGT CGTTGGAATC
<i>Zinc finger protein 467</i>	<i>Zfp467</i>	GAACTCCTTGGGAT TCTCAGTGG	AGTGTGCTCTCTCC TCTGGATGAC
<i>Mical-like 2</i>	A930021H16 <i>Rik (Mical-L2)</i>	TGCTTCAGGTGTAA ACAGTGCTCC	TGATGACTTGGGGC TCACAGAG
<i>Glyceraldehyde 3-phosphate dehydrogenase</i>	<i>Gapdh</i>	TGACATCAAGAAG GTGGTGAAGC	CCCTGTTGCTGTAG CCGTATTC
<i>Actin, beta</i>	<i>Actb</i>	TATTGGCAACGAGC GGTTC	GGCATAGAGGTCTT TACGGATGTC
<i>Selenoprotein M</i>	<i>Selm</i>	TGACAGTTGAATCG CCTAAAGGAG	AACAGCACGAGTT CGGGGTC

Table 2

Effect of RXR knock down on the T3 inducibility of genes

Probe	Symbol	Expression -T3 siRNA	Expression +T3 siRNA	Expression -T3 siRNA	Expression +T3 siRNA	Expression -T3 siRNA	Expression +T3 siRNA	Fold T3 Induction control siRNA	Fold T3 Induction RXR siRNA	Fold T3 Induction RXR/control siRNA
1424126 at	Alas1	2.03±15	2078±127	235±7	1070±91	10.3±0.9	4.6±0.5	10.3±0.9	4.6±0.5	0.44±0.04
1424596 s at	Lmed1	21±4	710±61	22±3	364±57	36.1±8.9	17.3±4.4	36.1±8.9	17.3±4.4	0.48±0.12
1434322 at	A930021H16Rik (MICAL- L2)	31±5	107±21	23±2	42±3	3.7±1.0	1.8±0.1	3.7±1.0	1.8±0.1	0.49±0.15
1427351 s at	Igh-4	25±1	204±30	22±1	93±23	8.2±1.2	4.2±0.8	8.2±1.2	4.2±0.8	0.52±0.02
1460582 x at	AA517566	32±4	128±41	37±2	86±18	4.4±1.9	2.3±0.4	4.4±1.9	2.3±0.4	0.53±0.18
1424378 at	AA691260	108±7	344±31	137±14	243±18	243±0.2	1.8±0.2	243±0.2	1.8±0.2	0.57±0.08
1436932 at	A1561912	22±2	108±10	26±2	74±13	4.9±0.1	2.8±0.3	4.9±0.1	2.8±0.3	0.58±0.06
1427329 a at	Igh-6	16±1	55±10	15±0	30±7	3.6±0.7	2.1±0.5	3.6±0.7	2.1±0.5	0.58±0.04
1417109 at	Len7 (Tina11)	43±5	476±60	35±3	226±10	11.3±1.2	6.5±0.3	11.3±1.2	6.5±0.3	0.58±0.04
1435950 at	Hr	34±5	377±43	36±4	257±9	11.9±2.9	7.4±0.8	11.9±2.9	7.4±0.8	0.63±0.16
1418090 at	Plvap	108±15	415±32	105±3	268±43	4.0±0.6	2.5±0.3	4.0±0.6	2.5±0.3	0.64±0.19
1421469 a at	Stat5a	93±5	341±16	103±10	257±14	3.7±0.4	2.6±0.4	3.7±0.4	2.6±0.4	0.70±0.07
1460634 at	Ralgds	134±2	457±19	161±9	380±19	3.4±0.2	2.4±0.1	3.4±0.2	2.4±0.1	0.70±0.06
1415936 at	Bcat3	95±3	285±22	95±5	200±9	3.0±0.3	2.1±0.2	3.0±0.3	2.1±0.2	0.70±0.13
1455447 at	D430019H16Rik	22±3	84±8	29±1	79±12	3.9±0.2	2.8±0.4	3.9±0.2	2.8±0.4	0.70±0.14
1455452 x at	A1449310	16±2	48±5	19±1	39±7	3.0±0.2	2.1±0.5	3.0±0.2	2.1±0.5	0.70±0.13
1428996 at	4833426J09Rik	47±3	270±8	64±6	251±18	5.7±0.3	4.1±0.7	5.7±0.3	4.1±0.7	0.71±0.09
1434957 at	Cdon	262±18	1222±46	331±14	1101±55	4.7±0.2	3.3±0.2	4.7±0.2	3.3±0.2	0.71±0.04
1417801 a at	Pfifbp2	178±13	766±30	195±10	602±22	4.3±0.2	3.1±0.2	4.3±0.2	3.1±0.2	0.71±0.05
1424067 at	Icam1	45±5	193±6	45±2	146±7	4.4±0.7	3.2±0.0	4.4±0.7	3.2±0.0	0.72±0.11
1429144 at	D12Mgi15	65±10	292±1	68±5	235±39	4.7±0.6	3.4±0.4	4.7±0.6	3.4±0.4	0.73±0.11
1416273 at	Tnfai2	61±8	533±28	48±2	317±39	9.1±1.5	6.6±0.6	9.1±1.5	6.6±0.6	0.73±0.11
1420826 at	AA522022	121±10	434±9	144±9	384±17	3.7±0.4	2.7±0.3	3.7±0.4	2.7±0.3	0.74±0.02
1417343 at	Fxyd6	12±1	41±4	10±1	26±1	3.4±0.2	2.6±0.1	3.4±0.2	2.6±0.1	0.75±0.06
1428623 at	Pikna1	251±20	757±62	266±27	602±39	3.0±0.1	2.3±0.2	3.0±0.1	2.3±0.2	0.75±0.09
1418687 at	Arc	74±5	335±26	75±10	246±18	4.5±0.0	3.4±0.6	4.5±0.0	3.4±0.6	0.76±0.12
1422698 s at	AU018574	224±22	838±80	221±20	628±48	3.7±0.2	2.9±0.0	3.7±0.2	2.9±0.0	0.76±0.05
1424737 at	Thrsp	12±1	62±11	12±1	49±13	5.3±0.4	4.1±1.0	5.3±0.4	4.1±1.0	0.79±0.13
1450710 at	Jarid2	62±8	253±45	59±10	182±3	4.0±0.2	3.3±0.7	4.0±0.2	3.3±0.7	0.82±0.22
1451932 a at	Tsrc1	39±1	290±46	42±4	261±25	7.6±1.5	6.2±0.4	7.6±1.5	6.2±0.4	0.82±0.16
1451674 at	Slc12a5	41±2	135±2	40±6	108±14	3.3±0.1	2.7±0.1	3.3±0.1	2.7±0.1	0.83±0.02
1419185 a at	Whscr14	36±2	123±15	35±4	100±14	3.4±0.3	2.9±0.2	3.4±0.3	2.9±0.2	0.85±0.05
1428250 at	Gpr30	65±10	318±24	108±5	464±60	5.1±0.7	4.3±0.6	5.1±0.7	4.3±0.6	0.85±0.12
1433968 a at	Egfl5	30±5	96±22	36±4	105±11	3.6±1.3	3.0±0.7	3.6±1.3	3.0±0.7	0.85±0.78
1418219 at	Il15	58±4	279±18	60±6	241±19	4.9±0.5	4.2±0.8	4.9±0.5	4.2±0.8	0.85±0.23
1418025 at	Bhlhb2	218±5	1347±40	221±11	1162±84	6.2±0.1	5.3±0.5	6.2±0.1	5.3±0.5	0.85±0.07
1423603 at	Zfpml	58±5	187±14	46±2	126±3	3.2±0.2	2.8±0.1	3.2±0.2	2.8±0.1	0.85±0.08
1427293 a at	Auts2	43±5	128±8	49±5	133±7	3.1±0.5	2.8±0.4	3.1±0.5	2.8±0.4	0.89±0.29
1417288 at	Plekha2	400±15	1333±17	361±35	1069±85	3.3±0.1	3.0±0.3	3.3±0.1	3.0±0.3	0.90±0.10
1418620 at	Phox2a	745±35	2231±82	818±22	2211±23	3.0±0.1	2.7±0.1	3.0±0.1	2.7±0.1	0.90±0.06
1433428 x at	Tgm2	97±7	1082±35	90±3	906±42	11.2±0.5	10.1±0.4	11.2±0.5	10.1±0.4	0.90±0.08
1435971 at	Rims3	111±5	418±28	103±2	348±31	3.8±0.3	3.4±0.4	3.8±0.3	3.4±0.4	0.90±0.16
1456341 a at	Btbl	36±1	115±17	39±2	113±8	3.2±0.6	2.9±0.1	3.2±0.6	2.9±0.1	0.91±0.15
1452646 at	Trp53inp2	132±10	717±33	160±7	807±15	5.5±0.6	5.1±0.2	5.5±0.6	5.1±0.2	0.92±0.11
1455854 a at	Ssh1	226±12	757±89	223±21	678±56	3.3±0.3	3.1±0.3	3.3±0.3	3.1±0.3	0.92±0.14
1427844 a at	Cebpb	203±38	621±104	168±21	493±50	3.1±0.4	3.0±0.1	3.1±0.4	3.0±0.1	0.95±0.09

Probe	Symbol	Expression -T3 control siRNA	Expression +T3 control siRNA	Expression -T3 RXR siRNA	Expression +T3 RXR siRNA	Fold T3 Induction control siRNA	Fold T3 Induction RXR siRNA	Fold T3 Induction RXR/control siRNA
1453851 a at	Gadd45g	107±22	435±56	90±4	368±29	4.2±0.6	4.1±0.3	0.96±0.11
1427356 at	2310031A18Rik	129±9	430±20	143±17	483±61	3.3±0.2	3.4±0.2	1.01±0.11
1425214 at	P2ty6	70±10	215±26	74±4	232±34	3.1±0.1	3.1±0.3	1.02±0.11
1419564 at	Zfp467	76±17	270±11	78±4	324±15	3.9±0.9	4.2±0.1	1.05±0.25
1454880 s at	Bmf	77±15	252±32	98±16	352±59	3.4±0.6	3.6±0.1	1.05±0.21
1426873 s at	Jup	92±6	354±16	94±6	389±12	3.9±0.3	4.2±0.2	1.08±0.12
1450855 at	Arvcf	142±12	742±19	123±11	713±46	5.3±0.3	5.9±0.8	1.12±0.13
1422573 at	Ampd3	15±1	122±9	16±1	179±25	8.1±0.3	11.2±1.2	1.38±0.11

N2a/TRβ cells were transfected with control or RXR siRNAs, and then were treated ±T3 for 6 hours. Gene expression was analyzed by microarray in 3 independent experiments. Genes induced 3-fold or more by T3 in the control siRNA-treated cells are shown. The list is sorted from those genes most dependent on RXR for T3 induction (far right column).

Table 3

Effect of RXR knock down on the T3 repression of genes

		Expression -T3 control siRNA	Expression +T3 control siRNA	Expression -T3 RXR siRNA	Expression +T3 RXR siRNA	Fold T3 Repression control siRNA	Fold T3 Repression RXR siRNA	Fold T3 Repression RXR/ control siRNA
Probe	Symbol	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
1450297 at	Il6	92±20	20±3	43±7	21±1	4.46±.34	2.06±.32	0.46±.07
1455436 at	Diras2	175±31	44±3	183±29	77±9	3.93±.44	2.35±.19	0.60±.02
1434572 at	Hdac9	48±3	13±1	52±3	20±3	3.85±.20	2.73±.39	0.70±.06
1440973 at	C230001G10Rik	156±38	45±8	146±33	57±8	3.36±.23	2.51±.23	0.74±.02
1425483 at	Tox	88±12	25±2	108±12	46±5	3.63±.72	2.46±.44	0.75±.21
1438796 at	Nr4a3	762±29	234±18	712±33	287±22	3.31±.36	2.52±.26	0.77±.06
1419420 at	St6galnac5	199±68	43±25	185±38	48±11	4.46±.80	3.98±.26	0.94±.14

N2a/TRβ cells were transfected with control or RXR siRNAs, and then were treated ±T3 for 6 hours. Gene expression was analyzed by microarray in 3 independent experiments. Genes repressed 3-fold or more by T3 (fold induction ≤0.33) in the control siRNA-treated cells are shown. The list is sorted from those genes most dependent to those least dependent on RXR for T3 repression (far right column).