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Identification of thyroid hormone response elements *in vivo* using mice expressing a tagged thyroid hormone receptor $\alpha 1$

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Synopsis

TR $\alpha 1$ (thyroid hormone receptor $\alpha 1$) is well recognized for its importance in brain development. However, due to the difficulties in predicting TREs (thyroid hormone response elements) *in silico* and the lack of suitable antibodies against TR $\alpha 1$ for ChIP (chromatin immunoprecipitation), only a few direct TR $\alpha 1$ target genes have been identified in the brain. Here we demonstrate that mice expressing a TR $\alpha 1$ -GFP (green fluorescent protein) fusion protein from the endogenous TR α locus provide a valuable animal model to identify TR $\alpha 1$ target genes. To this end, we analysed DNA-TR $\alpha 1$ interactions *in vivo* using ChIP with an anti-GFP antibody. We validated our system using established TREs from neurogranin and hairless, and by verifying additional TREs from known TR $\alpha 1$ target genes in brain and heart. Moreover, our model system enabled the identification of novel TR $\alpha 1$ target genes such as RNF166 (ring finger protein 166). Our results demonstrate that transgenic mice expressing a tagged nuclear receptor constitute a feasible approach to study receptor-DNA interactions *in vivo*, circumventing the need for specific antibodies. Models like the TR $\alpha 1$ -GFP mice may thus pave the way for genome-wide mapping of nuclear receptor-binding sites, and advance the identification of novel target genes *in vivo*.

Key words: brain, hyperthyroidism, hypothyroidism, thyroid hormone receptor, thyroid hormone response element

Cite this article as: Dudazy-Gralla, S., Nordström, K., Hofmann, P.J., Meseh, D.A., Schomburg, L., Vennström, B. and Mittag, J. (2013) Identification of thyroid hormone response elements *in vivo* using mice expressing a tagged thyroid hormone receptor $\alpha 1$. Biosci. Rep. 33(2), art:e00027.doi:10.1042/BSR20120124

INTRODUCTION

The importance of TH (thyroid hormone) for proper brain development and function is widely recognized [1,2]. It becomes most evident in congenital hypothyroidism, which can lead to severe mental retardation if not diagnosed and treated properly [3]. Similarly, low maternal TH levels during pregnancy can interfere with normal brain development in the offspring [4].

Most of the defects arising from the developmental hypothyroidism result from the actions of unliganded (apo-) TR $\alpha 1$ (thyroid hormone receptor $\alpha 1$), the main TR isoform in the brain [5]. To understand the role of apo-TR $\alpha 1$ in brain development better, mice heterozygous for a point mutation in the ligand-binding domain of the receptor (TR $\alpha 1$ + m mice) have been generated [6]. Like in cretinism, these TR $\alpha 1$ + m mice present a

plethora of brain defects, e.g. high anxiety, locomotor dysfunction and defects in the control of autonomic functions [7–9]. At the anatomical level, neuronal defects were observed in the hippocampus, cerebellum and cortex [9,10]. Recently, the first patients carrying a mutation in TR $\alpha 1$ were identified [11,12], who also display central defects, e.g. in memory function or the control of fine-motor movement. Thus, the findings in man and mice collectively underline the importance of TR $\alpha 1$ signalling for proper brain development – unfortunately, the underlying molecular mechanisms have remained largely enigmatic. To date, only a few target genes have been identified in the brain [13], severely hampering the understanding of defects caused by developmental hypothyroidism.

Several specific reasons account for this lack of knowledge in the field of TH action. First, the identification of TH target genes by *in silico* studies is severely complicated by the remarkable

Abbreviations used: 3'-UTR, 3'-untranslated region; ChIP chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GFP green fluorescent protein; HCN2, hyperpolarization-activated cyclic nucleotide-gated ion channel 2; qRT-PCR, quantitative real-time PCR; RNF166, ring finger protein 166; RXR, retinoid X receptor; Sema3a, Semaphorin 3a; TH, thyroid hormone; TRE, thyroid hormone response element; TR $\alpha 1$, thyroid hormone receptor alpha 1; wt, wild-type.

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sequence variety in the structures of the TREs (thyroid hormone response elements) described in previous studies [14,15]. Secondly, the available antibodies for TRs are often unspecific and display strong cross-reactivity with cytosolic proteins. Consequently, the identification of TR α 1 target cells and target genes in the brain is not accessible by techniques relying on antibody quality such as immunohistochemistry or ChIP (chromatin immunoprecipitation).

To overcome this problem, mice expressing a TR α 1–GFP (green fluorescent protein) fusion protein from the endogenous TR α 1 locus were generated recently [16]. The analysis of brains from these animals revealed TR α 1 expression in almost all post-mitotic neurons, with an exclusively nuclear localization [16]. Given the specificity in the detection provided by the tagged TR α 1, we explored if TR α 1–GFP mice could be used for ChIP experiments using GFP antibodies, aiming at the identification of TR α 1 target genes *in vivo*.

Here we show that TR α 1–GFP binds to previously characterized as well as novel TREs of TH target genes in heart and brain, independently of ligand availability. Our candidates were confirmed by electrophoretic mobility shift and reporter gene assays. As a proof of concept, we identify a novel TH target gene in the brain – the RNF166 (ring finger protein 166).

EXPERIMENTAL

Animals and manipulation of TH status

Animal care procedures were conducted according to the guidelines of the EEC (European Community Council Directives 86/609). Required animal permissions were obtained from the local ethical committee. 4–6 month-old mice homozygous for TR α 1–GFP (*gfp/gfp*) (previously described in [16]) and wt (wild-type) mice were housed at 21 °C on a 12 h light, 12 h dark cycle. If required the animals were treated to achieve a hyperthyroid or hypothyroid status, respectively. For the induction of hyperthyroidism 0.5 μ g/ml T3 [3,5,3'-triiodothyronine, in 0.01 % (w/v) BSA] was added to the drinking water for 2 weeks; for hypothyroidism, the mice were treated with the antithyroid drug MMI (methyl-mercapto-imidazol, 0.1 %) and 1 % (w/v) NaClO₄ for 6 weeks in drinking water and food porridge. The efficiency of the treatments and the resulting TH levels have been described previously [17]. The TR α 1–GFP strain has been deposited at the EMMA (European mouse mutant archive) under EM:04369.

TH response elements

Previously characterized TREs for RC3 and hairless were chosen as positive controls in the experiments [18,19]. To identify possible TREs in known T3 target genes, the genomic sequence was obtained from ENSEMBL (www.ensembl.org) and screened using Serial Cloner 1.3 (Serial Basics) by defining TREs as virtual cleavage sites for restriction enzymes. The identified putative TREs, their relative positions to the translation start, and the primers used for their amplification are listed in Table 1.

ChIP

ChIP was based on the manufacturer's instructions (ChIP-IT Express, Active Motif) with some modifications. Briefly, forebrain was prepared by removing olfactory bulb, cerebellum and brain stem; one-half of a brain was used for the preparation of chromatin. The tissue was cut into small pieces (2 mm side length) and moved to ice-cold PBS. Paraformaldehyde was added to a final concentration of 1 % (v/v) and the preparation was rotated at 37 °C for 30 min. After brief centrifugation (8 g at 4 °C for 2 min), the supernatant was removed and the precipitate was washed with 10 ml ice-cold PBS and separated again. Cross-linking was finally stopped by adding glycine to a final concentration of 0.125 M at room temperature (21 °C) for 5 min on rotation. After centrifugation, the tissue was washed twice with 10 ml ice-cold PBS and separated by centrifugation for 10 min at 1250 g. The tissue pellet was homogenized in 5 ml ice-cold PBS. 1 ml of the homogenate was resuspended and incubated in ice-cold lysis buffer, 5 μ l protease inhibitor cocktail and 5 μ l PMSF (all provided by the manufacturer) for 30 min on ice. After centrifugation (10 min at 2100 g at 4 °C) the precipitate was resuspended in shearing buffer (provided by the manufacturer), and the DNA was subsequently sheared by sonication (seven times, interval 0.5, 30 s sonication followed by 30 s break; Bioruptor Sonicator, Diagenode). The shearing efficiency was verified on an agarose gel, showing fragment sizes between 500 and 1000 bp. 10 μ l of the sheared chromatin was used to determine 'input DNA' for normalization. For immunoprecipitation, 25 μ l sheared chromatin (corresponding to \sim 7 μ g DNA) was incubated with the GFP antibody (1:300, rabbit anti-GFP, abcam ab290). The precipitation, washing, reverse cross-linking and proteinase K incubation were conducted according to manufacturer's manual. To recover the precipitated DNA fragments, the solution was incubated at 95 °C for 3 h, and the DNA subsequently extracted twice with phenol–chloroform. The DNA was then precipitated with ethanol, washed and dissolved in 100 μ l buffer containing 10 mM Tris and 1 mM EDTA. For chromatin from heart, the same procedure was applied on whole mouse hearts.

qRT–PCR (quantitative real-time PCR)

To quantify the amount of precipitated DNA, real-time PCR was conducted before (input) and after the ChIP using the primers listed in Table 1. The ratio between precipitated and input DNA was calculated for each TR α 1–GFP animal to correct for differences in input DNA, yielding a percentage pulldown value. The same procedure was performed in wt animals to determine the unspecific pulldown by the GFP antibody (background). The ChIP experiments were independently performed in five pairs consisting of one TR α 1–GFP and one wt animal each.

The results presented show the precipitation in five TR α 1–GFP animals normalized against the corresponding wt brain from the same experiment.

qRT–PCR was performed with the 7300 Real Time PCR System (Applied Biosystems) and the FastStart Universal SYBR Green PCR Master Mix (Roche) with 40 cycles of 95 °C for 15 s and 62 °C for 90 s. Specificity of amplification was verified by melting curve analyses.

Table 1 List of TREs

The different TREs, their location within the respective gene relative to the translation start site (ATG), the primers used for the detection after ChIP and previously published characterizations. n.a., not applicable.

Gene	TRE	Location relative to ATG	Primer ChIP	Literature
RC3	GGATTAAGGCGTTCG	- 4192	Fwd_5'-CGAGGAATGGAGATCAGGAG-3' Rev_5'-GCTGGTGGTGGTGGTGTG-3'	Analogous to Arrieta et al. [18]
Hairless TRE1	CCCCCAGGACTCAGGACA GCCCGGGTTCC	- 3523	Fwd_5'-TCCTGAGAGCTCTGGTCTAGC-3' Rev_5'-CCTGACCTCTGGCTCCTG-3'	
Hairless TRE2	AGGGCATCTGAGGACA	- 4220	Fwd_5'-TTCAGCTGTCTGAAGGATGG-3' Rev_5'-GCCAAGCTGCCACTAATCC-3'	Thompson et al. [19]
HCN2	TTGGTTTTCTAAAGGTCA TGATGAGGTTGT	- 2557	Fwd_5'-GCAGGGAAGAGTCTAAAGGAGACCC-3' Rev_5'-GTTTCAACCCAGCCTTTGGGA-3'	
HCN4	GGGAGTCCCCAGGACT	- 508	Fwd_5'-TATGGCAACCCGCGAGCTGC-3' Rev_5'-GGTGTCTGGGGCTGTCAGCG-3'	
Sema3a	AGGACATGACCT	- 204422	Fwd_5'-ACTCTGACCCCTTCTCTTAAGCAGGT-3' Rev_5'-AGTGTCTGGCACCCAGGCT-3'	Perfect inverted repeat far upstream, but the gene also spreads over 200 kb
RNF166	GGGACCACCCGGGTGG	+ 9241	Fwd_5'-GGTGTCTGTGTGGAGAAGG-3' Rev_5'-AATGCTCACGGTGAAGC-3'	
F2T2 TRE	ATTGACCCAGCTGAGG TCAAGTATTGACCCC AGCTGAGGTCAAG	n.a.	n.a.	Wallis et al. [16]
DR4-TRE (synthetic TRE)	TAAGGTCACCTCAGGTCA CTGGATCCTAAGGTCA CTTCAGGTCACT	n.a.	n.a.	Hofmann et al. [20]
Negative control	No TRE	Genomic region on Chr 1	Fwd_5'-GAAGACCTGCTTGTCTTGG-3' Rev_5'-CTCAGCTCTGGCTTCATGC-3'	Between the lysine-specific demethylase 5B and synaptotagmin 2

For gene expression analysis with qRT-PCR, total RNA was isolated from the cortex of juvenile wt mice (treated with T3 or untreated) according to manufacturer's instructions (RNeasy Mini, Qiagen) and cDNA was subsequently synthesized from 4 μ g RNA using oligo dT primers (Transkriptor First Strand cDNA Kit, Roche). The following primers were used to determine RNF 166 expression levels (spanning the intron between exon 5 and 6): fwd 5'-CGGCACAAGTTCTCCTACG-3' and rev 5'-TGCCTCAGTTCTCAGAGAGG-3'. A standard curve was used to correct for PCR efficiency, and the gene expression was normalized using HPRT (hypoxanthine-guanine phosphoribosyltransferase) as housekeeping gene. The specificity of the reaction was confirmed with a melting curve analysis showing a single product. For statistical analysis, a two-tailed Student's *t*-test was performed, and *P*-values <0.05 were considered significant.

Cloning of TREs

Oligonucleotides containing the TREs were designed with KpnI and BglII overhangs for subcloning or EMSAs (electrophoretic mobility shift assays). For cloning, the oligonucleotides were heated to 90°C for 5 min and subsequently cooled for annealing. The TREs were then ligated into the pGI3 promoter luciferase

vector (Promega) between the KpnI and BglII restriction sites using a Quick Ligation kit (New England BioLabs). Positive colonies were identified using restriction analysis and sequencing, and the plasmid DNA for transfection was prepared using the Maxi Prep Kit (Invitrogen).

Luciferase reporter gene assay

Luciferase assays were conducted according to the method of Hofmann et al. [20] with minor modifications. HepG2 cells (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were diluted to a final concentration of 20000 cells per well in DMEM (Dulbecco's modified Eagle's medium)/F12 (Life Technologies) containing 10% (v/v) FBS (fetal bovine serum, Biochrom). Cells were transfected with 70 ng of reporter plasmid pGL3-Promoter (Promega) containing the TRE of interest or empty control, 5 ng pGL4.74 hRLuc (Promega) and 25 ng of pRS-rTR α 1 [21] using FugeneTM HD (Promega) during seeding in white 96-well plates (Nunc). As positive control, p(DR4)2-SV40-luc + [20] and F2T2 [16] were used. After incubation under a 5% (v/v) CO₂ humidified atmosphere at 37°C overnight the solution was replaced by DMEM (Biochrom), and the medium was removed again after 1 h. For the assay 100 nM T3 in DMEM was added to the cells and incubated

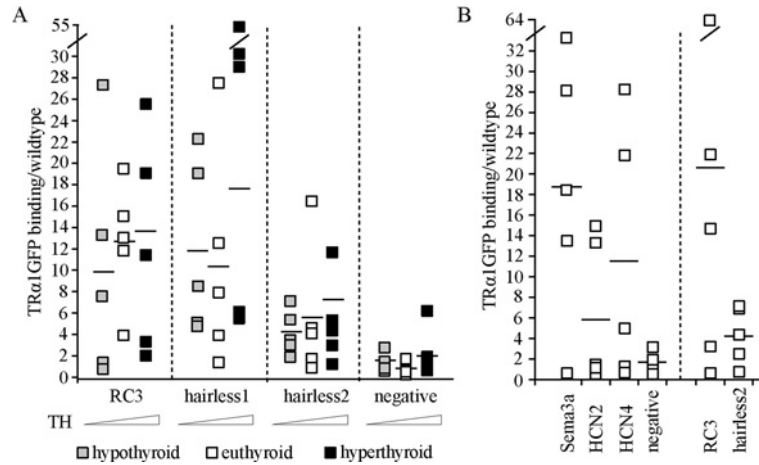


Figure 1 ChIP from brain and heart of TRα1-GFP mice

(A) ChIP from forebrain homogenates of hypo-, eu- or hyperthyroid TRα1-GFP mice with subsequent QPCR detection of the TREs from neurogranin (RC3) and hairless as well as a negative control (squares represent independent experiments, $n = 5$ per group, the mean is indicated by a horizontal line, no significant difference between the groups; $P > 0.05$). (B) ChIP from heart homogenates of euthyroid TRα1-GFP mice with subsequent QPCR detection of TREs from Sema3a, HCN2 and HCN4, including negative control as well as TREs from RC3 and hairless2 (squares represent independent experiments, $n = 5$ per group, the mean is indicated by a horizontal line).

for 24 h. The medium was removed and cells were lysed (passive lysis buffer; Promega) by shaking for 15 min at room temperature (21 °C). Luciferase assays were conducted using the luciferase assay system (Promega) and luminescence was measured with a Berthold Mithras luminometer (Berthold Technology). The results were normalized against the hRluc luminescence.

EMSA

Single-stranded oligonucleotide pairs (2.5 μg) were denatured at 95 °C for 5 min and annealed by slow cooling. Annealed oligonucleotides (10 pmol) were labelled with ³²P-dCTP (NEN 3000Ci/mmol; PerkinElmer) using Klenow DNA polymerase (Fermentas) to fill in the overhanging ends [22]. EMSA was conducted according to Wahlström et al. [23] with minor alterations. Receptor/DNA complex formation was done in an incubation buffer consisting of 4% Ficoll, 60 nM KCl, 1 mM EDTA, 10 mM HEPES pH 7.9 and a final concentration of 0.1 μg/μl of carrier poly dIdC DNA with 1–3 μg receptor-nuclear extracts (obtained from overexpressing of receptors in vaccinia virus, [23]). T3 was added to a final concentration of 250 nM to saturate the TRs, and incubated for 1 min on ice. The mixture was complemented with 1500–10000 cpm of labelled oligonucleotides and incubated for 15 min on ice. A 6% PAGE was pre-run for 60 min at 4 °C (250 V). The buffer was changed, 10 μl of receptor/DNA complexes were loaded on to the gel and the gel was run for an additional 60–90 min. To visualize the band shift, the gel was fixed in 20% (v/v) methanol and 10% (v/v) acetic acid and dried at 80 °C for 30 min. Radiolabelled bands were visualized with a phosphor imager.

RESULTS

To identify native TR-binding sites, we prepared chromatin from the TRα1 expressing tissues brain and heart of TRα1-GFP mice, and performed ChIP with an anti-GFP antibody. As controls we used chromatin from wt mice with the anti-GFP antibody – a procedure that is superior to other methods, which omit the primary antibody or use an unspecific primary IgG for control, as our approach generates the same background in sample and control. We initially tested the ChIP on previously characterized TREs from the known TH target genes neurogranin (RC3) and hairless [18] in brains of hypo-, eu- and hyper-thyroid adult mice (Figure 1A, Table 1). We detected TRα1-GFP binding on all three TREs; however, there was no significant difference when comparing the three TH levels ($P > 0.05$ for all genes). Low binding was detected with a fragment of genomic DNA that contained no TRE-like elements and served as a negative control (Table 1). We then analysed chromatin from heart as a second tissue expressing predominantly TRα1 [24]. Our results identified TRα1 binding to novel TREs in the promoter region of three known cardiac TH target genes (Figure 1B), namely Sema3a (Semaphorin 3a), and potassium/sodium HCN2 and HCN4 (hyperpolarization-activated cyclic nucleotide-gated ion channels 2 and 4). Interestingly, although the chromatin was prepared from heart, TRα1 binding also occurred on the TREs of RC3 and hairless – genes that have not been reported to be expressed in this tissue.

To confirm the results obtained by the ChIP, we used EMSA as an independent method for detecting TRα1 binding to TREs (Figure 2). We radioactively labelled the TREs from F2T2 (positive control, see [16]), hairless, Sema3a, HCN2 and HCN4, and

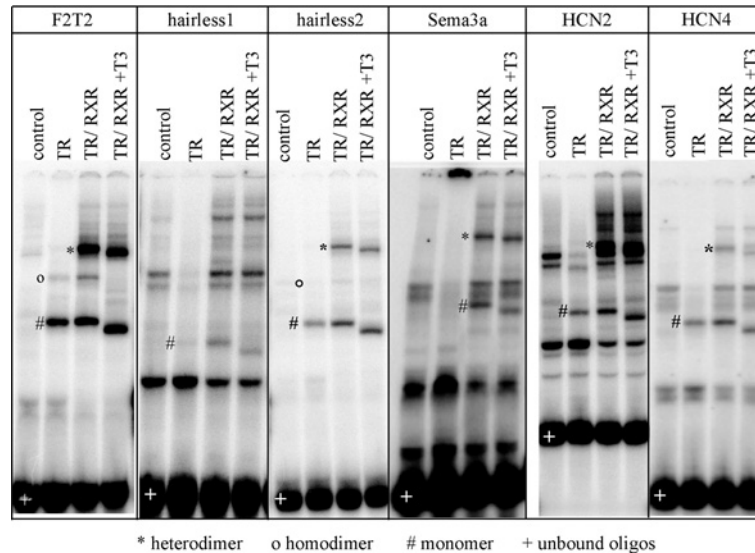


Figure 2 EMSA confirming TR α 1 binding to TREs

An EMSA with radiolabelled oligonucleotides was used to test for TR α 1 binding to TREs from F2T2 (positive control), hairless, Sema3a, HCN2 and HCN4. Every sample was run without TRs (lane 1), with TR α 1 (lane 2), with TR α 1 and RXR (lane 3) or with TR α 1, RXR and T3 (lane 4). *: band shift resulting from TRE binding to heterodimer TR/RXR; o: TRE binding to TR/TR; #: TRE binding to TR monomer; +: unbound labelled TRE.

analysed their interaction with TR, RXR (retinoid X receptor) or T3 by gel electrophoresis. Monomeric TR binding (indicated with #) was observed for all TREs when compared with controls, and all TREs except for hairless1 also displayed a shift caused by the TR/RXR heterodimer (indicated with *). The addition of T3 caused an additional minor shift of the TRE bound to TR or TR/RXR, due to a conformational change of the complex as described previously [22,25]. Collectively, the results of the EMSA confirm the TR α 1 binding to the TREs identified in the ChIP, and underline that the TR α 1-GFP-binding properties to TREs are comparable with those of wt TR α 1.

To test whether the TREs are capable of functionally regulating gene expression, we used a luciferase reporter assay as described in detail previously [20]. All TREs including the novel ones (Sema3a, HCN2, HCN4 and hairless1) were capable of inducing luciferase expression upon T3 stimulation (Figure 3), with the strongest induction observed for the F2T2 control and the hairless TREs.

Since the *in vitro* studies substantiated that all TREs identified by ChIP assay were functional, we tested if this approach could also identify novel TH responsive genes. Therefore we conducted ChIP assays on chromatin from brains of TR α 1-GFP mice, reversed the cross-linking and incubated the precipitated shared DNA fragments with DNA polymerase to fill in possible gaps and add an additional 5'-adenosine. After cloning the fragments into pGEM T-easy vector, we tested clones for positive inserts using restriction analysis and sequenced the fragments in random clones. The most frequent clone contained a region adjacent to the *RNF166* gene, which was subsequently screened for putative TREs by *in silico* analysis. Surprisingly, we identified a possible TRE in the *RNF166* genomic sequence coding for the 3'-UTR

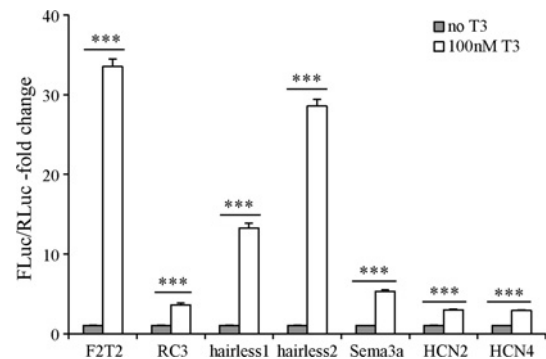


Figure 3 Luciferase assay demonstrating T3 regulation *in vitro*

TREs from F2T2 (positive control), RC3, hairless, Sema3a, HCN2 and HCN4 were tested for regulation of luciferase expression upon T3 treatment ($n=8$, means \pm S.E.M., *** $P < 0.001$, unpaired two-tailed Student's *t*-test).

(3'-untranslated region) (Table 1). Subsequent ChIP analysis indeed confirmed TR α 1 binding to this TRE in brain chromatin of hypo-, eu- and hyperthyroid mice (Figure 4A), with no significant binding differences between the three conditions. This result was confirmed by binding of the putative TRE to TR (#) and TR/RXR (*) in the EMSA (Figure 4B). The TRE was also found to be functional in regulating luciferase expression *in vitro* (Figure 4C). Consequently, we tested whether *RNF166* would be regulated *in vivo* as well, and found a significant approx. 50% reduction of *RNF166* mRNA levels in the cortex of T3-treated wt mice compared with untreated controls (Figure 4D), identifying *RNF166* as a novel TH target gene.

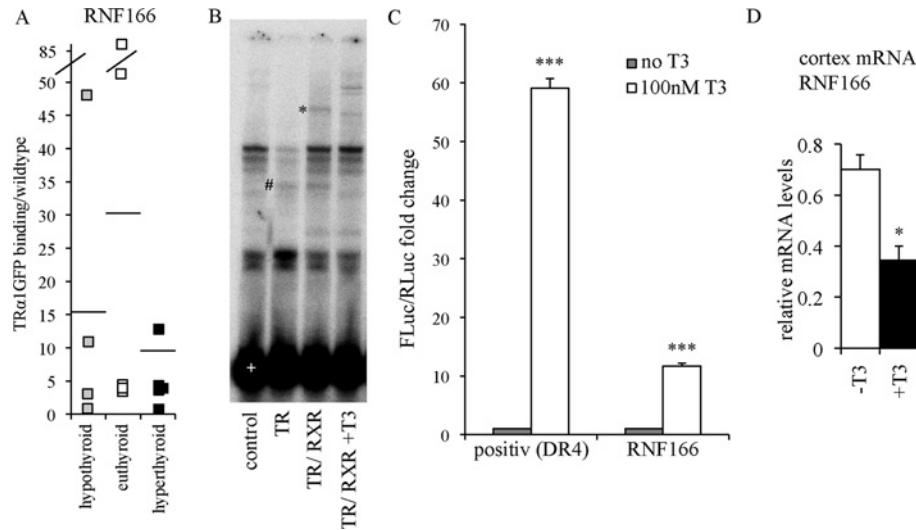


Figure 4 Identification of RNF166 as novel *TRα1* target gene

(A) ChIP from forebrain homogenates of hypo-, eu- and hyper-thyroid *TRα1*-GFP mice (squares represent independent experiments, $n=5$ per group, the mean is indicated by a horizontal line, no significant difference between the groups; $P > 0.05$). (B) EMSA to detect *TRα1* binding to the RNF166 TRE run without TRs (lane 1), with *TRα1* (lane 2), with *TRα1* and RXR (lane 3) or with *TRα1*, RXR and T3 (lane 4). *: band shift resulting from TRE binding to heterodimer TR/RXR; #: TRE binding to TR monomer; +: unbound labelled TRE. (C) Luciferase assay to confirm the functionality of the RNF166 TRE *in vitro* ($n=8$, means \pm S.E.M., *** $P < 0.001$, unpaired two-tailed Student's *t*-test). (D) Relative gene expression of RNF166 mRNA in wt mice with and without T3 treatment ($n=3-5$ per group, means \pm S.E.M., * $P < 0.05$, unpaired two-tailed Student's *t*-test).

DISCUSSION

The results presented in this study demonstrate that a transgenic mouse strain expressing a TR-GFP fusion protein represents a valuable tool to investigate previously described and novel TREs in chromatin, taking advantage of the high quality of GFP antibodies in the ChIP assays. Our approach was carefully validated using previously characterized TREs and complemented by studies employing EMSA or reporter gene assays.

Advantages and limitations of the *TRα1*-GFP ChIP assay

Compared with the frequently used *in vitro* systems, the *TRα1*-GFP animal model has several distinct advantages. First, it represents the exact *in vivo* situation of an intact animal and normal tissue with regard to the molecular repertoire of the cells. This is in stark contrast to the several cell line-based approaches used for the analysis of TH responsiveness, which often do not express TRs or required cofactors, or are poorly characterized with regard to their regulatory machinery. Consequently, they often have to be transfected to express the molecular components needed to obtain a detectable response to TH. This strategy usually results in supraphysiological expression levels leading to false positive results from TRs binding to DNA sites, which they might not necessarily occupy *in vivo*. Furthermore, transfected and proliferating cells often contain poorly chromatinized DNA, which could alter the accessibility of TREs on the DNA [26,27]. These

epigenetic differences between tissue samples and cell culture-based studies constitute another cause for potentially discrepant findings as recently shown for the T3-effects on the amyloid precursor protein [28].

It needs to be acknowledged, however, that the levels of the fusion protein are also slightly elevated in *TRα1*-GFP mice [16]. This is an inevitable consequence of targeting the *TRα1* locus, as it eliminates the alternative splicing variant *TRα2* and therefore results in a mildly increased production of the *TRα1*-GFP, about 2.5-fold [16]. However, *TRα1*-GFP mice do not display obvious phenotypical defects resulting from impaired *TRα1* signalling [16], suggesting that this mild elevation seems to be of negligible biological relevance. Moreover, these findings imply that the chimaeric *TRα1*-GFP receptor is functionally equivalent to the wt *TRα1*, which concurs well with the similar transactivation properties of both constructs *in vitro* [16]. In fact, previous *in vitro* studies convincingly demonstrated that the GFP tag does not interfere with T3 or TRE binding, transcriptional activation or subcellular localization of TH receptors [29,30].

Secondly, a distinct advantage of the *TRα1*-GFP mouse strain is the possibility to use high-quality GFP antibodies to precipitate the fusion protein. This option not only circumvents the problem of the well-known lack of specificity of commercially available TR antibodies, but it also allows the use of the same primary antibody in wt animals as ideal controls. In contrast to other methods that often omit the primary antibody or use a different IgG as control, this approach enables the detection and quantitative correction of the endogenous background effect of the primary antibody. Moreover, as the GFP tag is outside the

functional domains of the TR, it is not affected by any structural changes that occur upon ligand or cofactor binding, and the affinity of the antibody for the apo- or the holo-TR is probably identical. This is an absolute prerequisite for the analysis of TR–DNA interactions in dependence of the hormone availability [27]: if the affinity differed, false occupancy rates of the TREs would likely be obtained under hypo- or hyper-thyroid conditions.

Gene regulation by TH receptors

Our findings that TREs seem to be similarly occupied by the TR α 1–GFP in the hypo-, hyper-, or eu-thyroid state is not surprising, as it concurs with the current model of TH gene regulation in the literature [14,31]. It is assumed that the availability of the hormone constitutes the major variable controlling the expression rate of target genes. For positively regulated genes, the model predicts that in hypothyroid conditions the apo-TR is bound to the TRE and associates with a co-repressor complex thereby suppressing target gene transcription. If TH becomes available and binds to the receptor, a conformational change is induced, the co-repressors are released and co-activators are recruited, thereby stimulating gene expression [14,31]. This model is supported by our data, showing a similar degree of TR binding in hypo-, eu- and hyper-thyroid states. However, as the ChIP procedure requires a strong fixation, which permanently crosslinks DNA and TR, it can only provide a snapshot of the highly dynamic events taking place *in vivo*.

Identification of novel TH target genes

As proof of principle, we demonstrate here that the TR α 1–GFP mice are valuable for the identification of novel genes regulated by TH. We detected a TR α 1-binding site in the *RNF166* gene, which was biologically active as a functional TRE in a reporter gene assay. Although the genomic location coding for the 3'-UTR of *RNF166* seems to represent an unusual binding site for a transcription factor, such a location for a regulatory site has been described previously in TH-regulated genes [32]. In fact, it is not uncommon that a genomic 3'-UTR region contains transcriptional regulatory elements, which can loop back to the promoter to regulate gene expression [33,34]. Interestingly, the location of the TRE also seems crucial for its regulatory effects: *in vivo*, we observe suppression of cortical *RNF166* mRNA levels by T3, whereas *in vitro* when the TRE is located in the promoter region, a T3-dependent induction of luciferase expression is observed. Similar effects have been reported previously for a different TRE in the 3'-UTR [32], underlining that the relative location plays a decisive role for the type of regulation. However, the precise mechanisms underlying this positional effect remain yet to be elucidated. Also, as the function of *RNF166* *in vivo* is poorly understood, we can only speculate that the protein could constitute a link between TH and the regulation of ubiquitin ligation [35]. Nevertheless, the example of *RNF166* demonstrates that mice expressing a tagged nuclear receptor isoform can be used for identifying novel DNA-binding sites *in vivo*, while circumventing the need for specific antibodies or artificial expression

systems. Moreover, with regard to TH action, our study opens the road for genome-wide analyses of TR-binding sites, e.g. by using ChIP-sequencing.

AUTHOR CONTRIBUTION

Susi Dudazy-Gralla, Kristina Nordström, Peter Josef Hofmann and Dina Abdul Meseh performed the experiments. Susi Dudazy-Gralla, Peter Josef Hofmann, Lutz Schomburg, Björn Vennström and Jens Mittag designed the experiments. Susi Dudazy-Gralla, Kristina Nordström, Peter Josef Hofmann, Lutz Schomburg, Björn Vennström and Jens Mittag analysed the data. Susi Dudazy-Gralla, Peter Josef Hofmann, Björn Vennström and Jens Mittag drafted the paper; all authors discussed and corrected the paper.

ACKNOWLEDGEMENTS

We thank the people working in our animal house for excellent animal caretaking, and Dr Amy Warner for language editing. No competing financial interests exist.

FUNDING

This work was supported by the Vetenskapsrådet (to J.M. and B.V.); the Karolinska Institutet Foundation (to J.M. and B.V.) and the Deutsche Forschungsgemeinschaft DFG [grant numbers SCH0849/4-1 and GraKo 1208 (to P.J.H. and L.S.)].

REFERENCES

- Bernal, J. (2007) Thyroid hormone receptors in brain development and function. *Nat. Clin. Pract.* **3**, 249–259
- Koibuchi, N. and Chin, W. W. (2000) Thyroid hormone action and brain development. *Trends Endocrinol. Metab.* **11**, 123–128
- Porterfield, S. P. and Hendrich, C. E. (1993) The role of thyroid hormones in prenatal and neonatal neurological development – current perspectives. *Endocr. Rev.* **14**, 94–106
- de Escobar, G. M., Obregon, M. J. and del Rey, F. E. (2007) Iodine deficiency and brain development in the first half of pregnancy. *Public Health Nutr.* **10**, 1554–1570
- Schwartz, H. L., Strait, K. A., Ling, N. C. and Oppenheimer, J. H. (1992) Quantitation of rat tissue thyroid hormone binding receptor isoforms by immunoprecipitation of nuclear triiodothyronine binding capacity. *J. Biol. Chem.* **267**, 11794–11799
- Tinnikov, A., Nordstrom, K., Thoren, P., Kindblom, J. M., Malin, S., Rozell, B., Adams, M., Rajanayagam, O., Pettersson, S., Ohlsson, C. et al. (2002) Retardation of post-natal development caused by a negatively acting thyroid hormone receptor alpha1. *EMBO J.* **21**, 5079–5087
- Mittag, J., Davis, B., Vujovic, M., Arner, A. and Vennstrom, B. (2010) Adaptations of the autonomous nervous system controlling heart rate are impaired by a mutant thyroid hormone receptor-alpha1. *Endocrinology* **151**, 2388–2395
- Sjogren, M., Alkemade, A., Mittag, J., Nordstrom, K., Katz, A., Rozell, B., Westerblad, H., Arner, A. and Vennstrom, B. (2007) Hypermetabolism in mice caused by the central action of an unliganded thyroid hormone receptor alpha1. *EMBO J.* **26**, 4535–4545



- 9 Venero, C., Guadano-Ferraz, A., Herrero, A. I., Nordstrom, K., Manzano, J., de Escobar, G. M., Bernal, J. and Vennstrom, B. (2005) Anxiety, memory impairment, and locomotor dysfunction caused by a mutant thyroid hormone receptor alpha1 can be ameliorated by T3 treatment. *Genes Dev.* **19**, 2152–2163
- 10 Wallis, K., Sjogren, M., van Hogerlinden, M., Silberberg, G., Fisahn, A., Nordstrom, K., Larsson, L., Westerblad, H., Morreale de Escobar, G., Shupliakov, O. and Vennstrom, B. (2008) Locomotor deficiencies and aberrant development of subtype-specific GABAergic interneurons caused by an unliganded thyroid hormone receptor alpha1. *J. Neurosci.* **28**, 1904–1915
- 11 Bochukova, E., Schoenmakers, N., Agostini, M., Schoenmakers, E., Rajanayagam, O., Keogh, J. M., Henning, E., Reinemund, J., Gevers, E., Sarri, M. et al. (2012) A mutation in the thyroid hormone receptor alpha gene. *N. Engl. J. Med.* **366**, 243–249
- 12 van Mullem, A., van Heerebeek, R., Chrysis, D., Visser, E., Medici, M., Andrikoula, M., Tsatsoulis, A., Peeters, R. and Visser, T. J. (2012) Clinical phenotype and mutant TRalpha1. *N. Engl. J. Med.* **366**, 1451–1453
- 13 Thompson, C. C. and Potter, G. B. (2000) Thyroid hormone action in neural development. *Cereb. Cortex* **10**, 939–945
- 14 Cheng, S. Y., Leonard, J. L. and Davis, P. J. (2010) Molecular aspects of thyroid hormone actions. *Endocr. Rev.* **31**, 139–170
- 15 Santos, G. M., Fairall, L. and Schwabe, J. W. (2011) Negative regulation by nuclear receptors: a plethora of mechanisms. *Trends Endocrinol. Metab.* **22**, 87–93
- 16 Wallis, K., Dudazy, S., van Hogerlinden, M., Nordstrom, K., Mittag, J. and Vennstrom, B. (2010) The thyroid hormone receptor alpha1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Mol. Endocrinol.* **24**, 1904–1916
- 17 Gullberg, H., Rudling, M., Forrest, D., Angelin, B. and Vennstrom, B. (2000) Thyroid hormone receptor beta-deficient mice show complete loss of the normal cholesterol 7alpha-hydroxylase (CYP7A) response to thyroid hormone but display enhanced resistance to dietary cholesterol. *Mol. Endocrinol.* **14**, 1739–1749
- 18 Martinez de Arrieta, C., Morte, B., Coloma, A. and Bernal, J. (1999) The human RC3 gene homolog, NRG1 contains a thyroid hormone-responsive element located in the first intron. *Endocrinology* **140**, 335–343
- 19 Thompson, C. C. and Bottcher, M. C. (1997) The product of a thyroid hormone-responsive gene interacts with thyroid hormone receptors. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8527–8532
- 20 Hofmann, P. J., Schomburg, L. and Kohrle, J. (2009) Interference of endocrine disrupters with thyroid hormone receptor-dependent transactivation. *Toxicol. Sci.* **110**, 125–137
- 21 Thompson, C. C. and Evans, R. M. (1989) Trans-activation by thyroid hormone receptors: functional parallels with steroid hormone receptors. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3494–3498
- 22 Andersson, M. L., Nordstrom, K., Demczuk, S., Harbers, M. and Vennstrom, B. (1992) Thyroid hormone alters the DNA binding properties of chicken thyroid hormone receptors alpha and beta. *Nucleic Acids Res.* **20**, 4803–4810
- 23 Wahlstrom, G. M., Sjoberg, M., Andersson, M., Nordstrom, K. and Vennstrom, B. (1992) Binding characteristics of the thyroid hormone receptor homo- and heterodimers to consensus AGGTCA repeat motifs. *Mol. Endocrinol.* **6**, 1013–1022
- 24 Dillmann, W. (2010) Cardiac hypertrophy and thyroid hormone signaling. *Heart Fail. Rev.* **15**, 125–132
- 25 Takeshita, A., Yen, P. M., Ikeda, M., Cardona, G. R., Liu, Y., Koibuchi, N., Norwitz, E. R. and Chin, W. W. (1998) Thyroid hormone response elements differentially modulate the interactions of thyroid hormone receptors with two receptor binding domains in the steroid receptor coactivator-1. *J. Biol. Chem.* **273**, 21554–21562
- 26 Collingwood, T. N., Rajanayagam, O., Adams, M., Wagner, R., Cavailles, V., Kalkhoven, E., Matthews, C., Nystrom, E., Stenlof, K., Lindstedt, G. et al. (1997) A natural transactivation mutation in the thyroid hormone beta receptor: impaired interaction with putative transcriptional mediators. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 248–253
- 27 Flamant, F. and Gauthier, K. (2012) Thyroid hormone receptors: the challenge of elucidating isotype-specific functions and cell-specific response. *Biochim. Biophys. Acta*, in the press
- 28 Belakavadi, M., Dell, J., Grover, G. J. and Fondell, J. D. (2011) Thyroid hormone suppression of beta-amyloid precursor protein gene expression in the brain involves multiple epigenetic regulatory events. *Mol. Cell. Endocrinol.* **339**, 72–80
- 29 Zhu, X. G., Hanover, J. A., Hager, G. L. and Cheng, S. Y. (1998) Hormone-induced translocation of thyroid hormone receptors in living cells visualized using a receptor green fluorescent protein chimera. *J. Biol. Chem.* **273**, 27058–27063
- 30 Mengeling, B. J., Pan, F. and Privalsky, M. L. (2005) Novel mode of deoxyribonucleic acid recognition by thyroid hormone receptors: thyroid hormone receptor beta-isoforms can bind as trimers to natural response elements comprised of reiterated half-sites. *Mol. Endocrinol.* **19**, 35–51
- 31 Yen, P. M., Ando, S., Feng, X., Liu, Y., Maruvada, P. and Xia, X. (2006) Thyroid hormone action at the cellular, genomic and target gene levels. *Mol. Cell. Endocrinol.* **246**, 121–127
- 32 Bigler, J. and Eisenman, R. N. (1995) Novel location and function of a thyroid hormone response element. *EMBO J.* **14**, 5710–5723
- 33 Jash, A., Yun, K., Sahoo, A., So, J. S. and Im, S. H. (2012) Looping mediated interaction between the promoter and 3' UTR regulates type II collagen expression in chondrocytes. *PLoS ONE* **7**, e40828
- 34 Zhang, W., Brooks, R. L., Silversides, D. W., West, B. L., Leidig, F., Baxter, J. D. and Eberhardt, N. L. (1992) Negative thyroid hormone control of human growth hormone gene expression is mediated by 3'-untranslated/3'-flanking DNA. *J. Biol. Chem.* **267**, 15056–15063
- 35 Giannini, A. L., Gao, Y. and Bijlmakers, M. J. (2008) T-cell regulator RNF125/TRAC-1 belongs to a novel family of ubiquitin ligases with zinc fingers and a ubiquitin-binding domain. *Biochem. J.* **410**, 101–111

Received 4 December 2012/29 January 2013; accepted 12 February 2013

Published as Immediate Publication 12 February 2013, doi 10.1042/BSR20120124
