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REGULATION OF GENE TRANSCRIPTION BY THE THYROID HORMONE RECEPTORS

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To my mother

ABSTRACT

Thyroid hormone (TH) has important effects on postnatal development as well as on adult metabolic homeostasis. TH meditates its effects through different isoforms of the thyroid hormone receptors (TRs) encoded by the TRα and TRβ genes, respectively.

TRs belong to the nuclear hormone receptor superfamily. These receptors are ligand-modulated transcription factors that bind to specific DNA elements located in the regulatory regions of target genes. The nature of these elements determines the effect TR will have on transcription. On what is called a positive TH response element (pTRE), the un-liganded receptor binds to DNA and represses transcription. Hormone binding induces a conformational change in the receptor, allowing for activation of transcription. In contrast, on a negative TH response element (nTRE), activation of transcription is mediated by the un-liganded receptor, whereas the liganded receptor represses transcription. The mechanism behind the regulation of a pTRE is quite well understood, while the mechanism regulating nTREs is less elucidated. Interestingly, TH has been found to regulate more genes negatively than positively *in vivo*. In addition, studies of mutant mice that are unable to produce active TH but have intact TR expression, show a lethal phenotype, which contrasts to the viable phenotype of mice lacking all TR isoforms. This further underlines the physiological importance of TRs in the repression of transcription.

The E2F-1 protein is one of the major regulators of cell cycle progression. In Paper I, we identified a putative nTRE in the E2F-1 promoter and showed that TR bound directly to this nTRE. This nTRE was sufficient for TRs to mediate both hormone-independent activation and hormone-dependent repression of transcription. Down-regulation of E2F-1 mRNA expression was observed after TH-treatment, both in P19 embryonic carcinoma cells and in oligodendrocyte precursor cells (OPCs), and correlated with an arrest in cell cycle progression. We further showed that both un-liganded TRα and TRβ activated transcription from the E2F-1 promoter, that DNA binding was required for hormone-independent activation of transcription and that an intact ligand-binding domain was required for hormone-dependent repression of transcription. The results suggest that TH modulates the cell cycle through a direct repression of the E2F-1 gene.

In Paper II, we identified a putative nTRE in the Necdin gene. Necdin is a growth suppressor that facilitates cell cycle exit and neuronal differentiation, and inhibits apoptosis. The Necdin nTRE sequence was similar to the E2F-1 nTRE and identical to the nTRE of the TSH gene, which also is negatively regulated by TH. We showed that TR, in the absence of TH, activated transcription of the Necdin promoter and that TH repressed this activation. We further showed that TR bound as a heterodimer with Retinoic-X-receptor (RXR) to the Necdin nTRE sequence and that RXR and the nuclear receptor corepressor (NCoR) enhanced the TR-mediated transcriptional activation of Necdin in the absence of TH. As Necdin is expressed in postmitotic regions in the mouse brain, and is down-regulated after birth coinciding in time with the crucial postnatal increase of TH, our results suggests that TRs are important to regulation of Necdin expression.

To investigate whether TH mediates post-natal down-regulation of Necdin expression in the mouse brain, *in vivo* studies were performed. In Paper III we showed that TRs regulated expression of Necdin in a cell-type specific manner. *In vivo* gene transfer experiments showed that exogenous TRβ, injected into the hypothalamus, was required for transactivation of the Necdin promoter in the absence of TH. *In situ* hybridization analysis of Necdin expression in the paraventricular nuclei of the hypothalamus in 2-day-old mice showed that TH did not repress Necdin expression. Finally, we showed that the transcription factor NSCL-2 counteracted the regulation of Necdin by TR *in vitro*.

TH down-regulates the production of thyrotropin-releasing hormone (TRH) in a negative feedback mechanism. Both TRβ1 and TRβ2 down-regulate TRH in the presence of TH. However, only TRβ1 participates in activation of TRH transcription in the absence of TH. In Paper IV, we showed that the hsp90-associated co-chaperone protein, XAP2, bound to TRβ1 and was involved in the hormone-independent activation of TRH expression. Furthermore, siRNA-mediated knock-down of XAP2 function *in vivo* abrogated TR-mediated activation of hypothalamic TRH transcription.

In summary, we have identified nTRE sequences in the E2F-1 and Necdin promoters as being TR targets. These nTREs are required for hormone-independent activation of transcription by TR. In addition, factors, such as NCoR, RXR and XAP2, are required for efficient activation of negatively regulated TR target genes in the absence of TH.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Nygård. M., Wahlström. G. M., Gustafsson. M. V., Tokumoto. Y. M. and Bondesson. M. 2003 Hormone-Dependent Repression of the E2F-1 Gene by Thyroid Hormone Receptors. Mol Endo, 17(1):79-92.

II Nygård. M., Becker. N., Demeneix. B., Pettersson. K. and Bondesson. M. 2006. Thyroid Hormone-Mediated Negative Transcriptional Regulation of Necdin Expression. J Mol Endocrinol, 36(3): 517-30.

III Nygård. M., Becker. N., Demeneix. B., Pettersson. K. and Bondesson. M. Regulation of Necdin Expression in the Mouse Brain. Manuscript.

IV Clerget Froidevaux. M. S., Berg. P., Seugnet. I., Becker. N., Nygård. M., Decherf. S., Pongratz. I. and Demeneix. B. The Co-Chaperone XAP2 is Required for Activation of Hypothalamic TRH Transcription *in vivo*. Submitted 2006.

ABBREVIATIONS

AF1, 2	Activation function1, 2
AP-1	Activator protein-1
ApoTR	Unliganded thyroid hormone receptor
AR	Androgen receptor
CBP/p300	CREB-binding protein p300
CNS	Central nervous system
CoA	Coactivator
CoR	Corepressor
D1, D2, D3	Diodothyronine deiodinase type 1, 2, 3
DBD	DNA-binding domain
ER	Estrogen receptor
GR	Glucocorticoid receptor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HoloTR	Liganded thyroid hormone receptor
H-P-T	Hypothalamus-pituitary-thyroid
HRE	Hormone response element
Hsp90	Heat-shock protein 90
LBD	Ligand-binding domain
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
NR	Nuclear receptor
NCoR	Nuclear receptor corepressor
OPC	Oligodendrocyte precursor cell
PKC	Protein kinase C
Pol II	RNA polymerase II
PPAR	Peroxisome proliferator-activated receptor
PVN	Paraventricular nucleus
RAR	Retinoic acid receptor
RID	Receptor interaction domain
RTH	Resistance to thyroid hormone
RXR	Retinoid X Receptor
SMRT	Silencing mediator of retinoic acid and thyroid hormone
SP-1	Specificity protein-1
SRC-1	Steroid receptor coactivator-1
T3	L-3,5,3'-triiodothyronine
T4	L-3,5,3'5'-tetraiodothyronine, thyroxine
TAF	TBP associated factor
TBP	TATA-box binding protein
TF	Transcription factor
TH	Thyroid hormone
TIF-2	Transcriptional intermediary factor-2
TR	Thyroid hormone receptor
TRE	Thyroid response element
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VDR	Vitamin D receptor

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INTRODUCTION

GENE REGULATION

Gene expression is the interpretation of genetic information encoded in the genes to produce a functional protein. A gene consists of a length of deoxyribonucleic acid (DNA), which exerts its influence on the organism's form and function by encoding and directing synthesis of a protein. Each living cell carries a full complement of the genes typical of the species.

A gene in the genome can be silent or expressed; i. e. the genetic information is encoded into mRNA, which is transported out of the nucleus to the cytoplasm, where its information is translated to a protein. All cells contain the same sets of approximately 50,000 – 100,000 genes, but different cell types express only a subset of these genes at a given time-point. Depending on the specific function of the cell and the status of differentiation, the cell integrates different signals into an ordered response, i. e. up- or down-regulation of gene expression. The control of gene expression can be exerted at many different levels from DNA to protein.

General transcription factors

The DNA region involved in initiation of transcription is termed the promoter region. It includes the binding sites for RNA polymerase II (Pol II) and is the start-point of transcription. Pol II transcribes eukaryotic genes together with several other basal transcription factors that recognize the core promoter sequence. The TATA box in the core promoter region is recognized by the basal transcription factor TFIID, which is composed of the TATA box binding protein (TBP) and TBP-associated factors (TAFs). Pol II, along with the TFIID and several other transcription factors, form the pre-initiation complex required for initiation of transcription. Two models have been suggested for assembly of the pre-initiation complex on the promoter: a step-by-step model starting with the recruitment of the TFIID, and a single step model where the pre-initiation complex is pre-formed in the cell before docking at the promoter [1], [2], [3].

The association of the general transcription factors to the promoter-region of a certain gene is controlled by gene-specific transcription factors. These factors bind to specific response elements in the promoters and enhancers of the gene. The presence and activity of gene-specific transcription factors, such as nuclear

receptors, in a cell at a certain point in time, determines the subset of genes that will be transcribed.

Chromatin

The DNA in a cell is tightly packed in a condensed form. This is achieved by interactions between the DNA and specific sets of proteins, histones, to form 'chromatin'. However, chromatin does much more than just compact DNA, chromatin has a central role in controlling gene expression. Chromatin represents an additional level of regulation for all DNA metabolic processes (replication, repair and gene expression) [4]. The packing of DNA into a chromatin structure is achieved by the formation of nuleosomes organized by the histone proteins. Nucleosomes consist of 146 base pairs (bp) of DNA wrapped around a protein core of the histones, H2A, H2B, H3 and H4. The N-terminal tails of the histones protrude from the surface of the nucleus.

Modifications of histone tails

Modifications of the histone tails are important in determining the type of chromatin formed (silent or active) and may form the basis of a new level of epigenetic code, i. e. the information contained in chromatin, other than the actual DNA sequence. A variety of post-translational modifications occur on the amino terminal tails of the histones. These modifications include phosphorylation, acetylation, ubiquitination, methylation and SUMOylation [4]. Acetylation of histones occurs at lysin residues by histone acetytransferase (HAT) enzymes. Histone acetylation may facilitate access of transcription factors to promoter elements by disrupting the compact chromatin structure, and is generally associated with gene activation. Histone acetylation is a reversible process. The enzymes that remove acetyl groups from histones, histone deacetylases (HDACs), usually act as repressors of transcription by modifying the chromatin into a compact and transcriptionally silent structure. Lysin and arginin residues on histone tails can also be methylated. In contrast to acetylation, which almost always correlates with transcriptional activation, histone methylation can result in either transcriptional activation or repression. Until recently, the dogma was that methylation is an irreversible process. However, recently the first arginine and lysine demethylases were identified [5]. Phosphorylation is another covalent post-translational modification of histones occurring on serine residues. The lysine residues are also subject to modifications by ubiquitin and ubiquitin-like proteins, such as small ubiquitin-related modifier (SUMO). Histone ubiquitination is generally associated with increased gene transcription, whereas SUMOylation is generally associated with

decreased gene transcription. Both ubiquitination and SUMOylation are reversible histone modifications [4].

THE NUCLEAR RECEPTOR SUPERFAMILY

The nuclear receptor (NR) superfamily in mammals includes 48 structurally and functionally conserved transcription factors. 25 of these NRs regulate transcription in a ligand-dependent manner. 23 NRs are what are called orphan receptors, where no ligand has yet been identified, or for which candidates have only recently been identified. The NRs regulate functions such as reproduction, development, metabolism and homeostasis. Members of the NR superfamily include receptors for steroid hormones, such as the oestrogen receptor (ER), androgen receptor (AR) and glucocorticoid receptor (GR). These receptors are referred to as type I receptors. The receptors for non-steroidal ligands, such as the thyroid hormone receptors (TR), retinoic acid receptors (RAR), peroxisome proliferator activated receptor (PPAR) and liver X receptor (LXR) are referred to as type II receptors (Reviewed in [6]).

Nuclear receptor structure

A typical NR consists of a variable NH2-domain (A/B), a highly conserved DNA-binding domain (DBD), a linker region, a hinge (D), and a conserved E region that contains the ligand-binding domain (LBD). Some receptors also contain a COOH-terminal region (F) of little-known function. (Fig.1)

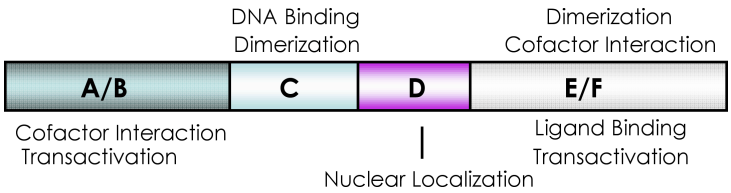

Figure 1. Schematic illustration of the structural and functional organization of nuclear receptors. Two transactivation functions (AFs) have been described in several NRs. AF-1 in the A/B domain is constitutively active and the AF-2 in the E/F domain is ligand-inducible.

N-terminal (A/B) domain

This region is the most variable one, both in size and sequence, but usually contains an activation function-1 (AF-1) domain. Several NRs with multiple isoforms are generated from a single gene by alternative splicing or by the use of alternative promoters, mostly resulting in alternative A/B regions. The A/B domain shows promoter- and cell-specific activity, suggesting that it is likely to contribute to

specificity of action among receptor isoforms and that interaction with cell-type specific factors [6].

DNA-binding domain

The DNA-binding domain (DBD) is located in the central portion of the receptor and has two zinc fingers, each composed of four cysteins coordinated with a zinc ion. Within the first zinc finger, there is a "P-box" important to sequence-specific recognition of HREs by the NRs. The RXR dimerization surfaces are located in the second zinc finger centering on a crucial arginine residue. This is termed the "D-box", a region that has also been shown to be important to a receptor's ability to distinguish spacing between half-sites of hormone response elements [7].

Hinge domain

The hinge region, between the DBD and LBD, is believed to give the receptor flexibility and to enable altered conformation. This region also contains a sequence that is associated with nuclear localization, and interfaces for interaction with cellular chaperone complexes [6].

Ligand-binding domain (E/F)

The ligand-binding domain (LBD), is not only necessary for ligand binding but also plays a critical role for dimerization, transactivation and basal repression by unliganded receptors. The LBD is composed of 12 alpha-helices (H1-H12) that are packed together in a sandwich-like manner. Ligand binding results in a repositioning of the most carboxy-terminal region, H12, which contains the AF-2 motif important to ligand-dependent transactivation [8]. In the unliganded state, helix 12 extends from the ligand-binding domain core, whereas in liganded state, helix 12 folds back towards the ligand binding-domain, realigns with helix 3 and helix 4, and contacts the ligand, thereby closing the ligand binding cavity and generating a surface for coactivator recruitment. The repositioning of helix 12 plays an important role in nuclear receptor function [7].

Hormone Response Elements

NRs regulate transcription by binding to specific DNA sequences in target genes known as hormone response elements, HREs (AGG/TTCA). These elements are located in regulatory sequences usually present in the 5-flanking region of the target gene. The HRE normally consists of a hexameric core recognition motif. Although

some monomeric receptors can bind to a single hexameric motif, most receptors bind as homo- or heterodimes to HREs. HREs are typically composed of two core hexameric motifs that can be configured as palindromes (Pal, ⇒⇐), inverted palindromes (IPs, ⇐⇒) or direct repeats (DRs, ⇒(N)^x⇒). The orientation and the spacing between the hexamers confer the selectivity and specificity of the HRE. Steroid receptors mostly bind as homodimers to the HRE. Two steroid hormone receptor monomers bind cooperatively to their response elements, and dimerization interfaces have been identified both in the DBD and in the LBD. Many non-steroid nuclear receptors bind their HRE as a heterodimeric complex together with the retinoid-X receptor (RXR), such as TR, RAR and vitamin D receptors (VDR) [6].

Nuclear receptors can also modulate gene expression by mechanisms independent of HRE binding. This is accomplished through positive or negative interference with the activity of other transcription factors, a phenomenon known as "cross-talk". For instance, ERs utilize protein-protein interactions to enhance transcription of genes containing AP-1 sites [9]. Other NRs have also been shown to interact with activator protein-1 (AP-1), such as TR, RAR or GR. It has also been suggested that the receptors act as ligand-dependent transrepressors of AP-1 activity. Thus, NRs have the ability to influence expression of genes that do not contain an HRE [10].

Coregulators

NRs perform their many different transcriptional functions through positive and negative regulatory proteins, referred to as coactivators (CoA) and corepressors (CoR). In general, unliganded NRs interact with corepressors to mediate repression of transcription, whereas liganded NRs interact with coactivators to enhance transcriptional activation [11]. Binding of ligand is the crucial molecular event that switches the function of the NR from active repression to activation [12].

Coactivators

Numerous potential receptor-interacting proteins have been identified and described in the last years. To be classified as a coactivator, the protein must fulfill certain requirements, such as a direct interaction with the activation domain of an NR in an agonist-dependent manner leading to enhancement of the receptor activation function. In addition, a coactivator should interact with components of the basal transcriptional machinery and not enhance the transcriptional activity on its own [12]. Some of the coactivators are specific to one or a few number of

receptors, whereas others act as general NR coactivators. Several coactivators are also common to other signaling pathways, such as CBP (CREB binding protein) and its homologue p300, that are involved in the activation of CREB, AP-1, hematopoetic transcription factor (c-myb) and myogenic determination genes (Myo D) [13]. When a ligand binds to its NR, a conformational change of the LBD occurs, leading to replacement of the corepressor complexes by the coactivator complexes, such as steroid receptor coactivator 1 (SRC-1) and CBP/p300. The coactivator complexes relax the chromatin structure through histone acetylation.

Interaction between coactivators and ligand-bound receptors is generally mediated via conserved NR boxes containing hydrophobic LXXLL motifs (where L corresponds to leucine and X represents any aminoacid residue). NR boxes are present in most coactivators [14], [15], [16]. In general, interactions of coactivators with NRs are both ligand- and AF-2 dependent [17].

In the unliganded state, steroid receptors form a heterocomplex consisting of heat-shock proteins (hsp), immunophlins and p23. Hsp90 interaction is required to maintain GR, mineralcorticoid receptor (MR) and progesterone receptor (PR) in a ligand-binding competent conformation. Unliganded ERα has been shown to associate with the hsp90-complex, but it is not essential for the ligand-binding activity of ERα to be maintained. In addition to maintaining the receptors in a ligand-binding conformation, the hsp90-complex also prevents DNA binding, heterodimerization and nuclear translocation of the receptors. Coactivator binding is required to dissociate the hsp90-complex from the receptor [11], [18].

Corepressors

Unlike coactivators, corepressors interact with the unliganded receptor, resulting in repression of basal transcription. Corepressors also interact with components of the basal transcriptional machinery and possess an autonomous repression domain [12]. Active repression mediated by class II NRs in the absence of hormone has lately attracted a lot of interest. In the absence of ligand, type II receptors actively repress transcription through recruitment of corepressors, such as nuclear receptor corepressor (NCoR) and silencing mediators for retinoid and thyroid receptors (SMRT) that possess histone deacetylase activity and freeze chromatin in an inaccessible conformation, making the DNA inaccessible for transcription factor binding. In the receptor interaction domains (RID) of NCoR and SMRT, there are consensus sequences that resemble LXXLL sequences, enabling coactivators to interact with NRs. In addition, corepressors can form complexes with other repressor proteins, such

as Sin3 and histone deacetylase 1 (HDAC1) and HDAC3, leading to compaction of chromatin and repression of transcription [7].

THE THYROID HORMONE AND ITS RECEPTORS

Thyroid hormone

Thyroid hormones (THs) elicit multiple physiological actions in vertebrates, including metabolic homeostasis, differentiation and development. The two thyroid hormones, L-thyroxine (T4) and 3,3'5-Triiodo-L-Thyronine (T3), are produced in the thyroid gland located in front of the neck, centrally surrounding a major part of the trachea (Fig. 2). T4 is the main product of thyroid secretion, whereas local deiodination in peripheral tissues produce T3, the biologically active thyroid hormone [19].

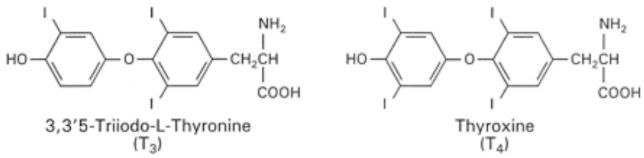

Figure 2. Structure of the thyroid hormones T3 and T4.

The production of T4 and T3 are activated by two other hormones, hypothalamic thyrotropin-releasing hormone (TRH) and pituitary thyroid-stimulating hormone (TSH). In addition, the levels of TH are maintained by a negative feedback loop involving T3 inhibition of TRH and TSK expression (Fig. 3). The concentrations of the hormones are also regulated by tissue-specific expression of three iodothyronine deiondinase enzymes, D1-D3, which activate or metabolize thyroid hormones. In the periphery, type I deiodinase in kidney and liver is responsible for producing most of the circulating T3. In the brain, T3 is produced for local use mainly by the action of deiodinase type II. Interestingly, deiodinases type I and II are differentially regulated in order to protect the brain from both T3 excess and deficiency. In addition, activity of deiodinases themselves is a key step in regulating availability of active T3 [20], [21].

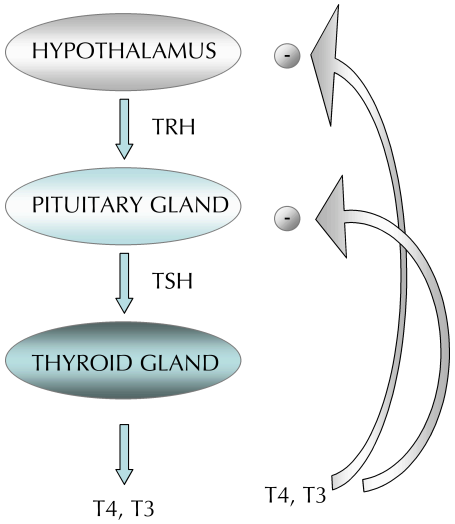

Figure 3. The H-P-T axis
Thyroid hormone negatively regulates its own synthesis and release by feedback inhibition of TRH transcription in the hypothalamus and TSH transcription in the pituitary gland.

The Thyroid Hormone Receptors

TH is circulating in the blood and when it reaches the target cell it proceeds to the nucleus where it binds to TR, which may already be prebound to the target gene. TR was identified in 1986, and belongs to the nuclear receptor superfamily. TRs are expressed in virtually all vertebrate tissues and are involved in regulating diverse physiological processes such as basal metabolic rate, which in turn affects thermogenesis, central nervous system development, and glucose utilization in response to T3 and T4 [7].

TRs are encoded by two separate genes, TRα and TRβ. In humans, they are located on chromosome 17 and 3, respectively (Fig. 4). The TRα gene gives rise to a functional T3 receptor, TRα1 and a splice variant, TRα2, which is unable to bind ligand or transactivate target genes. Recently, Δα1 and Δα2 isoforms have been identified that are transcribed from a novel promoter located in intron 7 [22]. These shorter variants lack the DBD and act as dominant negative regulators [23]. The TRβ locus encodes the proteins TRβ1-3, which are distinguished by variable N-terminals, due to alternative splicing and/or different promoter usage. Recently, internal promoters in the TR genes have been shown to give rise to truncated TR forms, whose functions are largely unknown [22].

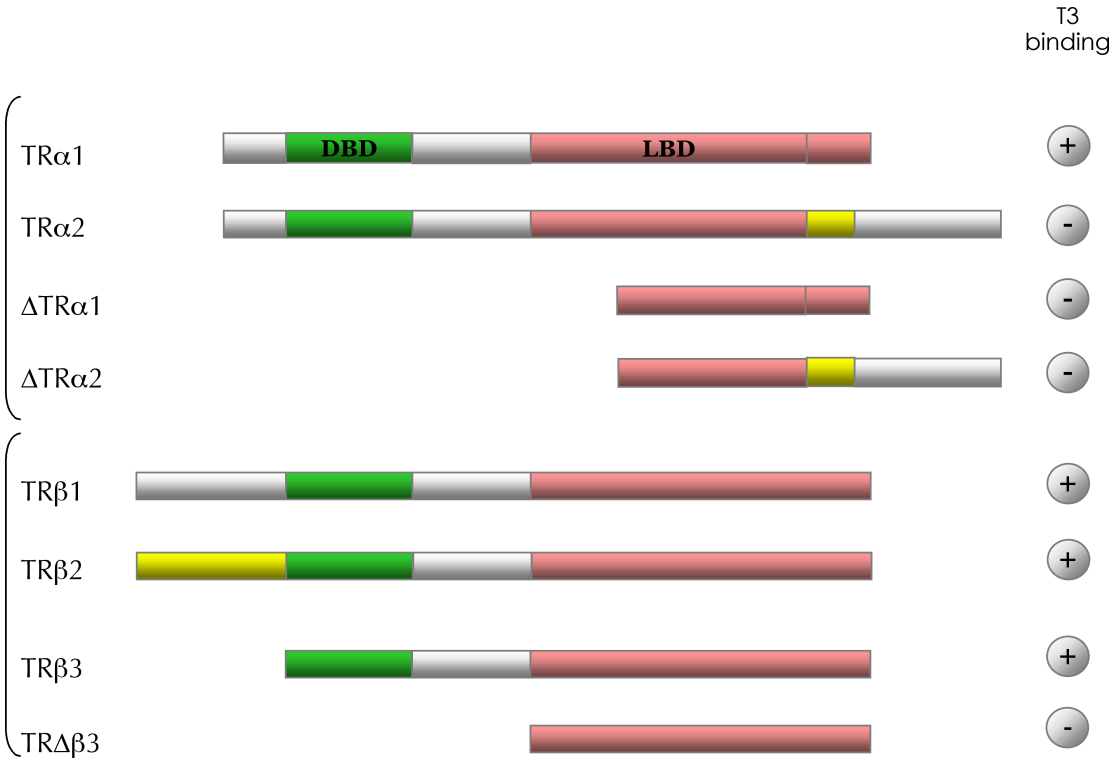

Figure 4. Schematic diagram of the thyroid hormone receptor isoforms.

Thyroid hormone action

TRs regulate transcription by binding to regulatory elements in the promoter regions of target gene, i. e. TR response elements (TRE). TRs can bind to TREs arranged as palindromes (Pal), direct repeats (DR) and inverted palindromes (IP) (Fig. 5) [7]. TRs typically bind to direct repeats spaced by four nucleotides (DR4) as a heterodimer with RXR both in the presence and absence of ligand. In the absence of TH, this binding actively represses basal target gene transcription.

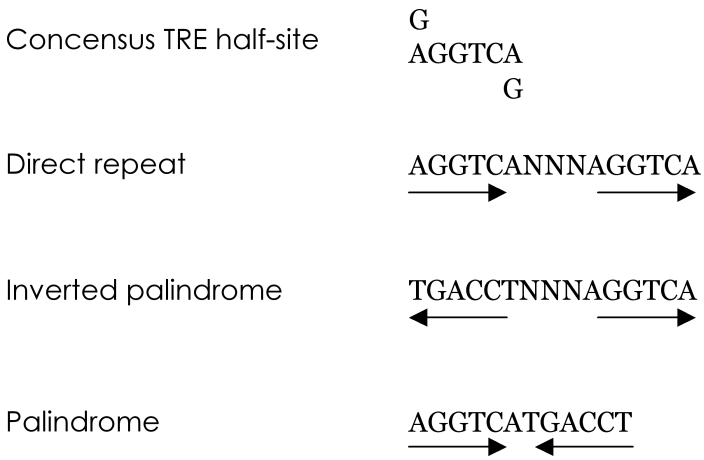

Figure 5. Half-site orientation and optimal spacing between half-sites. N refers to nucleotides and the arrows show the direction of half-sites on the sense strand.

Positive TREs

Promoter regions of genes that are positively regulated by TRs, i. e. activated by liganded TRs, contain a positive TRE (pTRE). In the absence of ligand, TR is bound to pTRE in concert with CoRs, such as SMRT and NCoR and gene transcription is silent. These CoRs in turn form complexes with the binding protein Sin3 and histone deacetylases (HDACs), which remodel chromatin into a closed, transcriptionally inactive conformation. In addition, TRs communicate directly with transcription factor (TF) TFIIB and SMRT interacts with TFIIB, TAFII32, and TAFII70 [24]. Upon hormone binding, the conformation of the TR-complex is altered and the CoR-complex is replaced by a CoA-complex. The SRC or p160 family of coactivators (SRC1, TIF1/GRIP1/SRC2 and RAC3/ACTR/pCIP/AIB-1/SRC3 proteins) possess or recruit histone acetyltransferases (HATs) that modulate chromatin to make it more accessible for binding of transcription factors, thus allowing increased transcriptional activity [25] (Fig. 6).

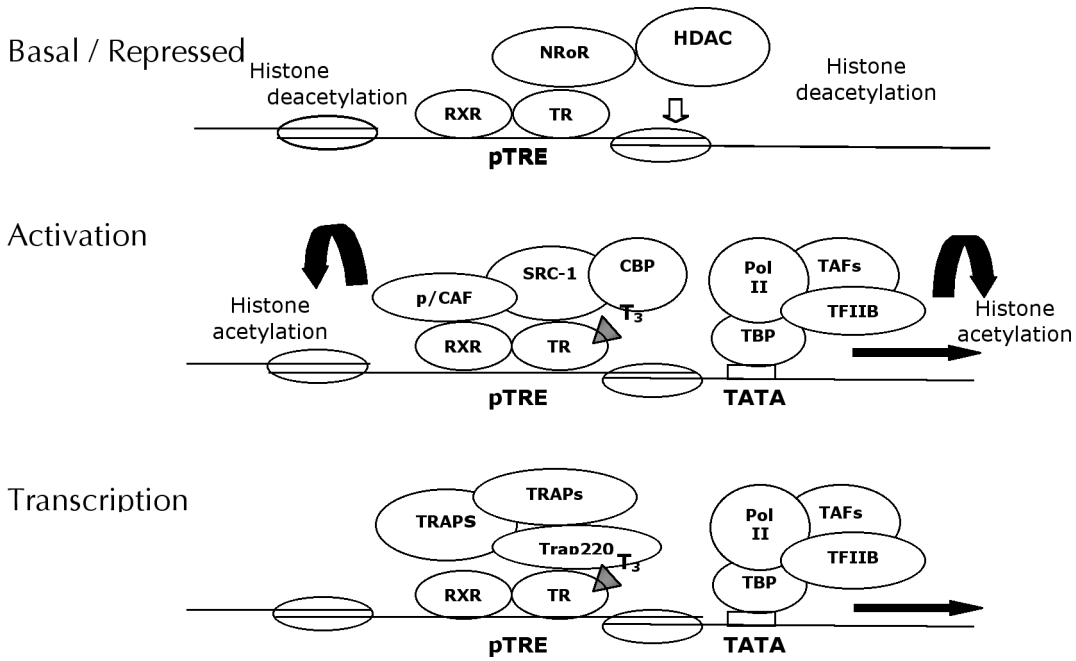

Figure 6. Model of TR-mediated transcriptional activation and repression on pTRE.

Negative TREs

Several genes have been identified that are hormone-independently activated by TRs and repressed by TH. The promoter region of those genes contains a negative TRE (nTRE). The mechanism of transcriptional regulation of genes containing an nTRE is still not completely understood. Conflicting results have been published on the requirement of DNA binding of TRs to nTREs and the roles played by different cofactors for regulation of genes driven by nTREs. In summary, these reports suggest that several mechanisms may exist that operate differentially on genes negatively

regulated by TRs (summarized in table 1). One mechanism for how T3 represses transcription is that TRs exert inhibitory effects on other transcription factors at certain promoters. Furthermore, it has been suggested that ligand-dependent recruitment of HDAC2 contributes to negative regulation of the TSHβ-promoter [26]. A third mechanism involves an overlap of the nTRE with SP-1 sites, in which liganded TR binds to DNA thereby precluding binding by SP-1 [27]. By a similar mechanism, a number of promoters have been reported to contain composite sites for TR and CTCF-binding factor, in which mutations in the CTCF-binding factor response element (CRE) abolish the negative regulation by TR [28]. Whether NCoR and SMRT are involved in the regulation of transcription from genes driven by nTREs or not has been discussed in numerous reports [29], [30], [31], [32], [33] and [34].

Table 1. Summary of nTRE described and the suggested mechanism of action.

Gene/ Promoter	nTRE sequence	Mechanism of action	DNA binding	Ref.
TSHβ rTSHβ	CAAAGTAA	T3 dependent recruitment of HDAC2	+ Homodimer	[26] [35]
Necdin	CAAAGTAA	NCoR enhances activation	+ Heterodimer with RXR	[36]
E2F-1	CAAAGTAA	CoR involved in activation	+	[37]
TSHα	CRE	NCoR and SMRT involved in activation, activation caused by apoTR recruitment of HDACs from basal promoter, CREB-activation dependent	- Indirect DNA-binding via CREB	[31]
GHF-1/ Pit-1	CREs adjacent to the TATA-box	HATs req. for activation Transcriptional interference with cAMP response elements, T3 binding causes release of TR from the promoter	+	[38] [39]
Kerain	Keratin RE (KRE)	CoR and HDACs involved in activation, CoA, CBP and HATs involved in repression	+ Homodimers	[40]
Cyclin D1	CRE	Transcriptional interference	- Indirect DNA-binding via CREB	[41]
α-fetoprotein (AFP)	DR4-like	Corepressor/HDACs requried for activation/prevent coactivator p/CAF binding to HNF1	-	[42] [43]

Rat prohormone convertace 1 PC1	TRE like seq. positioned adjacent to the TATA-box	TR together with cis-acting elements recruit transcription cofactors	-/+ Both direct and indirect binding as monomer or as heterodimer with RXR	[44] [45]
Rat PC2	TRE like seq. positioned adjacent to the TATA-box (half-site hexamer)	Inhibition of SP-1 stimulated transcrtption	+ Monomer/ homodimer/ heterodimer with RXR	[46]
TRE genomic element 144	TRE in combination with CTCT	Cross-talk with CoR CTCF	+	[28]
ppTRH	TGACCT	NCoR involved in activation	+	[34]
β-Amyloid Percursor Protein (β-APP)	CGGGCAGAGCAAGGACG (Present in the first exon)	Intact AF-2 required for repression but not for activation (CoA involved in the repression?)	+ Heterodimer with RXR	[47]
c-myc	IP with 3 nucleotide gap (IP3) located in the first exon	TR binds and prevents polII from completing transcritpion-> permature termination, CoR CTCF involved	+ Heterodimer with RXR	[48]
rat Na-K ATPase α	Adjacent to the TATA-box	Interference with TATA binding proteins, cell-density dependent	+ Both as monomer and as heterodimer with RXR	[49]
Glycoprotein hormone α	-	Protein-protein interaction with promoter spec. TFs	-	[50]
Rat CD44	TAGGTCAC	NCoR and GAF requried for T3-independent activation	Weak DNA binding	[32]
Epidermal growth factor receptor (EGFR)	GGGACTC Overlap with SP-1 sites	Inhibition of SP-1 stimulated transcription competition between TR/RXR and SP-1	+	[51] [52]

Does each TR isoform have a unique function?

TRs are expressed in a tissue-dependent and developmentally regulated manner [53]. Although TRα and TRβ genes are differentially expressed, at least one receptor seems to be expressed at any given time in virtually all cells [54]. However, the relative abundance of any TR isoform varies and this may represent a possible mechanism underlying tissue-specific responses to T3. For example, TRα1 is the major isoform expressed in heart, whereas TRβ1 predominates in liver. In the brain, TRα1 is the major foetal isoform, but after birth there is a marked increase in TRβ1 expression, which is maintained throughout adult life. TRβ2 expression is restricted to anterior pituitary, hypothalamic TRH neurons, developing inner ear and retina. TRα2 is the most widely expressed mRNA, although the significance of this abundance in mRNA is unknown. TRβ3 shows widespread expression, with the highest levels found in liver, kidney and lung. The TRΔβ3 is highly expressed in skeletal muscle, heart, spleen and

lung. The TRΔα1 and TRΔα2 are found mainly in the small intestine epithelium, lung and during early development [54].

Different knock-out and knock-in mutant model systems in mice demonstrate a significant redundancy between receptor isoforms (summarized in table 2). However, specific functions for TRα isoforms during postnatal development are involved in maintaining normal cardiac function and adaptation of body temperature to external environment. A major function for TRβ is to regulate the hormone levels (H-P-T-axis). Moreover, TRβ is required for normal development of retina and ear.

Interestingly, mice devoid of all TR receptors are viable, in contrast to *Pax8*$^{-/-}$ knock-out mice, lacking T3 due to the absence of thyroid follicular cells, which die postnatally, due to congenital hypothyroidism. However, the Pax8 mice can be rescued when TRα1 in combination with Pax8 are knocked out. This indicates that lethality associated with Pax8 mutation results from adverse effect of unliganded TRα1 [55]. Also it indicates that the effects of unliganded receptors, for instance due to hypothyroidism or RTH, are important regulatory mechanisms in the body and hence an understanding of the mechanism of TR regulation on nTRE is important.
To study whether TR isoforms mediate specific function *in vivo*, a dominant negative mutation (PV), identified from a patient with RTH, was targeted to TRβ and TRα gene [56]. PV has a frame-shift mutation resulting in the loss of T3 binding and transcriptional activities. TRβPV mice reproduce human RTH syndrome with dysfunction of the pituitary-thyroid axis, impairment in weight gain, accelerated bone development, hearing defects, abnormal regulation of serum cholesterol and increased physical activity. In contrast, TRαPV mice show no abnormalities in the pituitary-thyroid axis or other RTH phenotypes. TRαPV had high mortality, dwarf-like stature, reduced fertility and survival, reduced glucose utilization in the brain and marked delay in bone development [56]. Introducing a point mutation, originally described in TRβ (R438C) of a RTH patient family, into the TRα gene locus, led to reduced T3-binding affinity and serious retardation in postnatal development where observed. Those deficiencies were largely absent in adult mice, suggesting other mechanisms acting to overcome the impediment caused by mutant receptor [57].

Table 2. Genotypes and major phenotypes of TR null and related knock-outs (Adapted from [58] and [59]).

Knockout	Expressed TR mRNA	Deleted TR mRNA	Major phenotypes	Ref.
TR$\alpha1^{-/-}$	$\alpha2$, $\Delta\alpha2$ All β isoforms	$\alpha1$, $\Delta\alpha1$	Mildly hypothyroid Low heart rate Low body temperature	[60] [61]
TR$\alpha2^{-/-}$	$\alpha1$ (overexpressed) $\Delta\alpha1$ All β isoforms	$\alpha2$, $\Delta\alpha2$	Mixed hyper/hypothyroid	[62]
TR$\alpha^{0/0}$	All β isoforms	all α isoforms	Euthyroid	[63]
TR$\beta^{-/-}$	All α isoforms	all β isoforms	Goiter RTH Excess T4, T3 and TSH Deafness	[64] [65]
TR$\beta2^{-/-}$	$\beta1$, $\beta3$, $\Delta\beta3$, All α isoforms	$\beta2$	RTH Excess T4, T3 and TSH	[66] [67]
TR$\alpha^{0/0}\beta^{-/-}$	None	all α isoforms all β isoforms	Profound RTH Large goiter Excess T4, T3, and TSH Growth retardation Retarded bone development Intestine malformation	[68]
TR$\alpha^{-/-}\beta^{-/-}$	$\Delta\alpha1$ and $\Delta\alpha2$	all α isoforms all β isoforms	Large goiter Excess T4, T3, and TSH Growth retardation Retarded brain development Female infertility	[69]
TR$\alpha^{-/-}$	$\Delta\alpha1$ and $\Delta\alpha2$ All β isoforms	$\alpha1$, $\alpha2$	Progressive to severely hypothyroid, lethal Small thyroid gland Growth retardation Retarded bone development Intestine malformations	[70] [22]
Pax$8^{-/-}$	Deficiency of thyroid follicular cells following thyroid gland agenesis		Congenital hypothyroidism, lethal	[71]

The role of thyroid hormone in the brain

TH has long been known to be important to neurodevelopment. TH does not influence major early developmental processes such as neuronal induction, neurulation, establishment of polarity and segmentation. TH is instead involved in regulation of later events of brain development, such as neural cell migration, differentiation and cell signaling [72], [73]. Hypothyroidism during embryonic development causes the cretinism, a syndrome where the brain in particular is severely affected. Functional symptoms of cretinism include mental retardation, ataxia, spasticity and deafness [74]. In mammals, the post-natal period is a critical window of time for central nervous system (CNS) development. During this period, there is a major increase in TH concentration in the brain. It is well known that TH must act within a critical developmental window, beyond which hormone replacement cannot recover normal functions. In adults, thyroid hormone deficiency or excess is associated with psychiatric manifestations. TH is transferred to the brain through the blood-brain barrier, by a poorly defined mechanism. The concentration of T3 in the brain is also regulated by activities of diodinases D1 and D2 [72, 73, 75]. The presence of TH in different locations of the brain is heterozygous during development. Different functions for T3 in the brain have been suggested. T3 operates not only as a trophic factor, it may also constitute an informative signal able to control cell fate during brain development [76].

TRs are distributed widely throughout the brain, in neurons and glial cells [58], [77]. It has been suggested that the profile of TR distribution in different brain regions, implicates specific functions for different isoforms in neurodevelopment [78], [79]. However, knock-out data suggest that TRα and TRβ partly mediate redundant actions in the central nervous system (CNS) [58].

Non-genomic action of thyroid hormone

Nongenomic or extranuclear effects of THs do not appear to require nuclear receptor interaction, but are probably mediated by specific membrane receptors. The non-genomic action shows a time-course of seconds to minutes compared to the genomic action, which requires a longer period of time for protein synthesis and biological response to manifest itself. A cell surface receptor for TH has recently been identified. This receptor is connected via one or more signal transduction pathways both to nuclear events and to rapid local changes of cell membrane function [80]. The non-genomic signal transduction mechanisms involve modulations in the

phosphatidylinositol, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) pathways and the alterations in solute transport (Ca^{2+}, Na^{+}, H^{+}, glucose) [81]. TH non-genomically affects the activity of plasma membrane ion channels and ion pumps. However, the mechanisms are not well understood [82], [83], [84].

Diseases associated with thyroid hormone

Hypothyroidism

If TH amounts are insufficient to support normal body functions, the condition is called hypothyroidism. The production of TH in the thyroid gland is dependent on dietary iodine intake. In many countries, iodine is therefore added to various dietary products, for example table salt. Worldwide, the most common cause of thyroid disorder is iodine deficiency. Inadequate intake of iodine causes enlargement of the thyroid gland (goiter) due to increased TSH secretion. The syndrome cretinism is associated with endemic goiter and severe iodine deficiency. Cretenism in infants results in irreversible brain defects and marked growth retardation. In developing countries, congenital hypothyroidism is usually detected by perinatal screening and is curable by treatment with T4 during early infancy.

Hypothyroidism sometimes occurs as a consequence of autoimmune disease where endogenous antibodies block TH synthesis and/or growth of the thyroid gland, i. e. Hashimoto's disease. In addition, cancer in the thyroid gland is often associated with low production of TH. The clinical symptoms of hypothyroidism are fatigue, decreased body temperature, weight gain, and reduced heart rate (bradycardia) [85].

Hyperthyroidism

The term hyperthyroidism is used to describe an abnormal increase in TH synthesis and secretion. Thyrotoxicosis, on the other hand, refers to the clinical manifestations of increased serum TH levels, regardless of whether TH is endogenously produced or exogenously distributed. The most common cause of hyperthyroidism is Graves' autoimmune disease, where a certain type of antibody occupies the TSH binding site on the TSH receptor, thereby causing over-stimulation of TSH receptor and concomitant increased serum levels of TH [86]. Clinical symptoms associated with hyperthyroidism are increased metabolic rate, hyperactivity, weight loss, increased food intake and heat production (thermogenesis), as well as increased heart rate (tachycardia) [87].

Resistance to thyroid hormone

Resistance to thyroid hormone (RTH) is a syndrome characterized by reduction in sensitivity to THs. RTH is mostly caused by mutations in the LBD of the TRβ gene, leading to reduced or absent T3 binding and the predicted reduction in transcriptional capacity. The hallmark of RTH is an elevated level of circulating TH associated with nonsuppressible serum TSH. Other clinical features include short stature, weight loss, tachycardia, hearing loss, frequent ear infections, attention deficit hyperactivity disorder (ADHD), decreased IQ, and dyslexia. [56], [88].

AIMS OF THE THESIS

Many genes are activated by unliganded TRs (apoTRs) and repressed by liganded TRs (holoTRs). Considering the fact that TR knock-out animals are surprisingly normal compared to the severe phenotypes of the congenital hypothyroid-like mouse model, the distinctions between T3 deficiency and receptor deficiency suggest a significant function *in vivo* for T3-independent actions by TRs. In addition, the mechanism of TR-mediated gene regulation on nTREs is not completely understood.

The aims of the thesis have been:

- To find novel genes that are activated T3-independently by TR.
- To functionally characterize the nTRE present in the identified genes that was T3 independently activated by TR, with respect to cofactors involved.
- To investigate T3-independent/-dependent actions of TR on nTRE *in vivo*.
- To identify isoform-specific cofactors involved in TRH regulation.

RESULTS AND DISCUSSION

Hormone-Dependent Repression of the E2F-1 Gene by Thyroid Hormone Receptors (Paper I)

Mouse embryonic carcinoma P19 cells have the ability to differentiate into different cell-types depending on external stimuli. Retinoic acid (RA) induces differentiation of P19 cells into a neuron-like phenotype [89]. Treatment with low concentrations of T3 induces differentiation of P19 cells into beating cardiac muscle cells [90]. We have seen that T3 treatment induces the differentiation of P19 cells into neurons (Fig. 7) (Nygård *et al*. unpublished data).

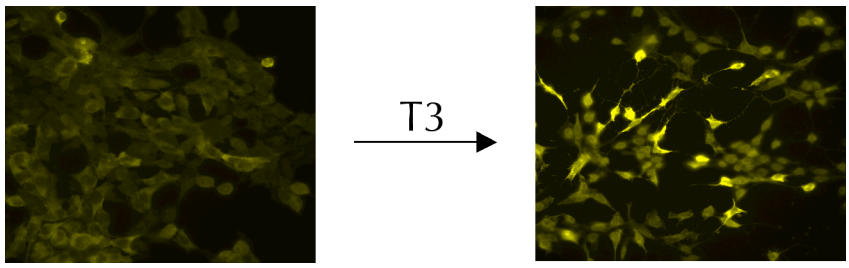

Fig. 7. P19 cells differentiate into neurons upon treatment with T3. Cells are stained with a neuron-specific antibody directed against βIII-tubulin.

Neuronal differentiation is accompanied or preceded by decreased cell proliferation. In this paper, we investigate whether TR affects cell proliferation by regulating factors involved in cell cycle progression. One of the most important transcription factor families involved in cell cycle regulation is the E2F family of transcription factors that drives transcription of S-phase specific genes. The E2F family consists of six different E2F factors, five of which (E2F-1 to –5) activate transcription, while E2F-6 represses transcription [91], [92]. E2F DNA-binding sites are found in the promoters of genes involved in DNA synthesis, such as DNA polymerase α, thymidine kinase (TK) and dihydropholate reductase (DHFR), as well as in genes regulating the cell cycle, such as the cyclin A, cyclin E and cdc2 genes [93].

We show in the paper that TR regulates transcription from the E2F-1 promoter. T3 treatment leads to an increasing number of cells in G1 cell cycle phase, indicating a cell cycle arrest P19 cells. Also, mRNA levels of E2F-1 decreased after T3 treatment, in both P19 cells and oligodendrocyte precursor cells (OPCs). Furthermore, protein levels of E2F-1 decreased after T3 treatment. In addition, we identified an nTRE (Z-element) in the E2F-1 promoter, which resembles the nTREs found in the TSH-promoter. We showed that TR binds directly to this Z-element in the E2F-1 promoter, and that the Z-element is sufficient for apoTR-mediated activation and for holoTR-mediated

repression of E2F-1 transcription. In transient transfection studies of different TR mutant constructs, we were able to show that DNA binding of TR to the E2F-1 promoter is required for T3-independent activation of E2F-1. Also, ligand binding of T3 to TR is required for T3-dependent repression of the E2F-1 promoter. A mutant, deficient in corepressor binding, showed a decreased ability to activate hormone-independent transcription, whereas a mutant unable to bind coactivators was still able to activate transcription from the E2F-1 promoter. This suggests that corepressors, rather than coactivators, are involved in the T3-independent transcriptional activation of the E2F-1 promoter. We also show that TR, in the presence of T3, represses expression of DNA polymerase α, TK and DHFR, which are transcriptionally regulated by E2F-1 during the S-phase.

T3 induces differentiation in many cell types, for instance erythrocytes [94], myocytes [90], B-lymphocytes [95] and nerve cells, such as OPCs and neural cells [96], [97]. Differentiation is linked to proliferation arrest and exit of the cell cycle. Furthermore, TH regulates oligodendrocyte cell proliferation and survival. Hypothyroidism has been shown to reduce the number of oligodendrocytes in the brain [98]. T3 also plays an integral role in Sertoli cell development. Cell cycle progression is regulated by cyclins and cyclin-dependent kinases, which in turn, are regulated by cyclin-dependent kinase inhibitors (CDKIs), such as $p27^{Kip1}$. T3 has been shown to affect $p27^{Kip1}$ and this may be a critical mechanism by which T3 induces cessation of Sertoli cell proliferation. Neonatal hypothyroidism in rats results in increased Sertoli cell number [99]. TH has important functions in controlling cell cycle progression in many different cell types during development. Consequently, impaired regulation of T3-responsive genes, such as E2F-1, due to TH deficiency, may lead to developmental defects as seen in cretinism.

In addition, impaired regulation of E2F-1 and other S-phase specific genes due to mutations in TR might be involved in cancer development and TR may function as a tumour-suppressor gene. Interestingly, high frequencies of mutant TRs were identified in human hepatocellular carcinoma [81]. Moreover, in human renal clear cell carcinoma, multiple mutations in both TRα1 and TRβ1 were identified [100]. Recently, high frequencies of mutations in both TRα1 and TRβ1 were identified in human papillary thyroid carcinomas. The majority of mutations found in TRs from different cancers exhibit strong dominant negative activity. In most cases, the binding of T3 to TR is impaired and in some instances the mutations cause impaired DNA binding to the TRE [100]. Recently, Porlan *et al* showed that TRβ1 is involved in the molecular mechanisms that control cell proliferation. In contrast to our paper, they reported

that unliganded TRβ1 induced growth arrest by decreasing the cyclin D and E levels, and Cdk2 activity, preventing phosphorylation of pRB and leading to decreased E2F-1 transcriptional activity [101]. However, both Porlan's paper and ours show that TR and T3 regulate both expression and activity of E2F-1. Interestingly, it has also been shown that TRβ1 physically and functionally interacts with the tumor suppressor p53 gene and this interaction is mediated by the DBD of TR and several domains of p53 [102], [103]. Accordingly, mutations in either TR or p53 might cause dysregulation of target genes.

Thyroid Hormone-Mediated Negative Transcriptional Regulation of Necdin Expression (Paper II)

In this paper, we investigate whether other genes contain the same nTRE sequence as those present in the E2F-1 and TSH genes, and if so, whether TR was able to regulate those genes. We performed database homology searches for potential nTRE. One interesting gene containing an identical nTRE sequence is the Necdin gene (Fig. 8).

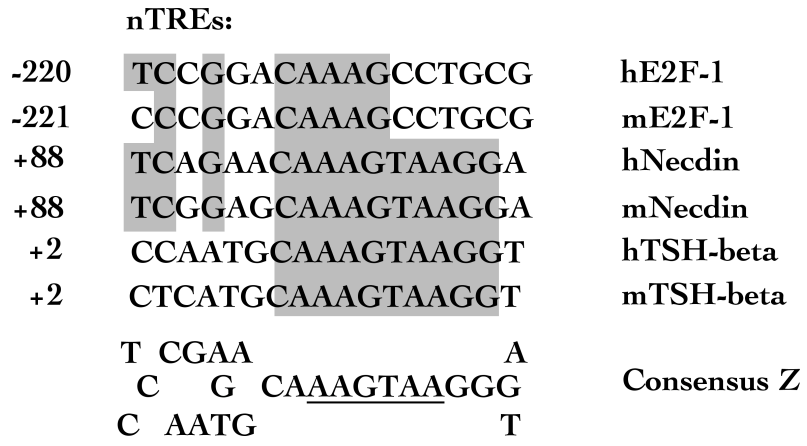

Figure 8. Sequence comparison of the negative thyroid hormone response elements, nTRE, from human and mouse E2F-1, Necdin and TSH promoters.

Necdin (Neurally differentiated embryonal carcinoma-derived protein) is a nuclear 325-amino acid protein expressed predominantly in post-mitotic cells, such as neurons and skeletal muscle cells. Studies *in vitro* have suggested that Necdin may be a neuron-specific growth suppressor that facilitates cell cycle exit and neuronal differentiation, and inhibits apoptosis [104]. Ectopic expression of Necdin has been shown to induce growth arrest [105]. Necdin is maternally imprinted and expressed only from the paternal allele, and has been shown to be involved in the ethiology of the neurobehavioral disorder Prader-Willi syndrome (PWS). The Necdin gene is not expressed in individuals with PWS [106].

In this study, we showed that unliganded TR activated transcription from the Necdin promoter. In the presence of T3, Necdin expression was suppressed. In addition we showed that the Z-element present in the Necdin promoter is sufficient for TR to mediate regulation of Necdin expression and also that Necdin mRNA expression is suppressed by T3 in P19 cells. Furthermore, TR was found to bind as a heterodimer with RXR to the nTRE. In addition, RXR was also required for transcription from the Necdin promoter to occur in the absence of T3. A heterodimerization-deficient TR mutant was unable to activate transcription from the Necdin promoter, supporting a role for RXR in T3-independent activation of Necdin. Furthermore, the corepressor NCoR enhanced the T3-independent transcriptional activation of Necdin expression. T3-independent activation of Necdin expression was blocked using an HDAC inhibitor, trichostatin A (TSA). This result indicates that the corepressor NCoR functions as a coactivator for TR, on the nTRE present in the Necdin promoter. We also showed, using chromatin immunoprecipitation (ChIP) assays, that TR, RXR and NCoR bind to the Necdin promoter in the absence of T3. In the presence of T3, less TR and RXR were associated with the promoter, whereas NCoR was found still to bind to the promoter. We also showed that both acetylated histone H3 and H4 were bound to the promoter, whether T3 was absent or present.

Others have described similar phenomena, where corepressors can function as coactivators. For example, Jho *et al*. showed that in the context of keratin gene expression, corepressors (NCoR and SMRT) function as coactivators for unliganded-TR. In addition, the HDACs were shown to participate in activation of keratin gene expression [40]. Also, HDACs have been shown to play a crucial role in the activation of interferon-stimulated transcriptional activation and a subset of CREB target genes [107], [108], [109], [110]. Jho *et al.* suggest that the interaction between coregulators and the receptor (liganded or unliganded) is constant and that the transcriptional output depends on the sequence of the response element, which in turn, determines receptor configurations. As a result, this plays a crucial role in determining signal specificity and transcriptional output [40].

Necdin is a growth suppressor that promotes neuronal differentiation and inhibits apoptosis by facilitating cell cycle exit. Down-regulation of Necdin is required for normal development [104]. Hence, Necdin is a potential mediator of the adverse effects of TH deficiency.

Regulation of Necdin Expression *in vivo* (Paper III)

Thyroid hormones have many important functions in the brain. As a consequence, T3 deficiency during foetal development causes mental retardation and reduced growth. The levels of T3 and T4 in the brain are relatively low at birth, but substantially increase at the end of first postnatal week. The levels of both hormones peak at day 15 postnatally [111]. In this study, we wanted to investigate whether TR could regulate Necdin expression *in vivo*. To this end, we analyzed the expression of Necdin in the hypothalamus during the postnatal period when the levels of T3 increase in the brain. *In situ* hybridization experiments of hypothalamus-sections with a probe against Necdin revealed decreased Necdin expression between postnatal day 2 and 15. To further investigate whether this down-regulation of Necdin expression is mediated by T3, *in vivo* gene transfer assay was performed. A Necdin reporter construct was injected into the hypothalamus of 2-day-old hypothyroid mice treated with or without T3. The Necdin reporter construct was not activated in hypothyroid mice, nor repressed in the T3-treated mice. To further examine whether endogenous levels of TR are sufficient to mediate regulation of Necdin, *in vivo* gene transfer of exogenous TR and the Necdin reporter construct was performed. Exogenous expression of TR mediated T3-dependent repression of Necdin expression in the hypothalamus of 2-day-old mice. Furthermore, we studied mRNA levels of Necdin in hypothyroid mice treated with or without T3. We did not detect any decrease in mRNA levels of Necdin in T3-treated mice. This could be due to factors counteracting the effect of endogenous TR on Necdin in the hypothalamus of 2-day-old mice.

The neuronal stem cell leukemia (NSCL) basic helix-loop-helix factors are neural cell-specific transcription factors [112]. Recently, it was shown that in discrete regions of the hypothalamus, Necdin expression is directly controlled by NSCLs together with other Lim-domain-only (LMO) cofactors [113]. NSCL-1 and NSCL-2 factors are expressed in CNS and PNS during embryonic and perinatal stages [114]. The phenotype of NSCL-2 knock-out animals resembles the major symptoms of PWS, such as obesity and hypogonadism, supporting the notion that Necdin is regulated by NSCL-2. Most of the Necdin-deficient mice die of apparent respiratory insufficiency caused by abnormal neuronal activity within the putative respiratory centre [115]. These findings suggest that Necdin plays roles in differentiation and development of subsets of neurons in the brain. We wanted to test whether NSCL-2 could interfere with TR-mediated regulation of Necdin expression in cells. Interestingly, transient transfection experiments showed that T3-independent activation of Necdin by TR was abolished in JEG-3 cells by increasing concentrations of NSCL-2. Hence, the

effect of NSCL-2 might counteract the effect of TR on Necdin expression in the hypothalamus. Competition for common transcriptional mediators might explain the antagonism seen between NSCL-2 and TR.

Collectively, these data indicate that the down-regulation of Necdin expression seen in mouse brain between day 2 and day 15 postnatally is not mediated by T3. Furthermore, in the hypothalamus TR and NSCL-2 may compete in the regulation of Necdin expression. Recently, it was shown that Necdin is also expressed in non-neural cells, such as skeletal myocytes, chondrocytes, adipocytes and skin fibroblasts [116], [117], [118]. Hence, TR might regulate Necdin in those tissues.

The co-chaperone XAP2 is required for activation of hypothalamic TRH transcription *in vivo* (paper IV)

The TH homeostasis is of vital importance during embryonoic development and maturity and is maintained by negative feedback exerted by T3 on TRH and TSH production. TRH is expressed in the paraventricular nuclei (PVN) in the hypothalamus. Both TRβ1 and TRβ2 are involved in the T3-dependent repression of TRH, whereas only TRβ1 is involved in both repression and activation [119], [120], [121]. Since TRH is activated by TRβ1, but not TRβ2, we wanted to find TRβ1-specific binding partners that might be involved in the T3-independent activation of TRH (paper IV). To this end, we used a yeast two-hybrid assay based on cDNA library from the PVN with TRβ1 as bait. Interestingly, we identified Hepatitis Virus B X-associated Protein 2 (XAP2, also known as AIP or ARA 9) as a TRβ1 partner protein. XAP2 is an ubiquitously expressed immunophilin-like protein that shares considerable sequence homology with classical immunophilins like FKBP51 and FKBP52 [122].

Numerous studies have shown that immunophilins interact with the mature hsp90 chaperone complex. Hsp90 is an evolutionary conserved protein abundantly expressed in all cells. Human cells express several members of the hsp90 family; two soluble hsp90 isoforms, hsp90-α and β, expressed in the cytosol, the related Grp94, located in the endoplasmatic reticulum, and a fourth member TRAP1, located in the mitochondria [123]. Heat shock proteins have been shown to interact with and regulate the functional activity of numerous cellular proteins involved in critical signaling cascades, including protein kinases, phosphatases, and transcription factors, such as certain members of the NR superfamily and of the bHLH-PAS family. The interaction between hsp90 and these factors has been shown to be critical for proper transcriptional regulation.

One example of a transcription factor, regulated by the hsp90 complex, is the Aryl hydrocarbon Receptor (AhR). The AhR is a ligand-dependent transcription factor, which in the absence of ligand, is found in the cytoplasmic compartment of the cell or, depending on cell type, evenly distributed between cytoplasm and nucleus [124], [125]. The non-activated form of the receptor interacts with the hsp90-dependent molecular chaperone complex and its associated proteins, such as the hsp90-associated factor p23, and XAP2 [126]. In the presence of ligand, the receptor accumulates in the nucleus where it interacts with the general dimerization partner factor, aryl hydrocarbon nuclear translocator (ARNT). This event induces a release of the hsp90-complex [125], [127], [128]. Interestingly, the ligand binding activity of the AhR is dependent on the presence of the hsp90 complex, which in addition regulates the intracellular localization of the AhR. The ligand-activated AhR mediates induction of several genes encoding drug metabolizing enzymes [129]. XAP2 has been shown to associate with hsp90 to regulate intracellular localization of AhR by anchoring the non-activated form of AhR in the cytoplasm by locking the receptor complex to actin filaments [125], [130]. In addition, XAP2 has been shown to stabilize AhR by protecting the receptor from ubiquitination, clearly showing that XAP2 is a critical regulator of AhR mediated transcriptional regulation.

Intriguingly, in yeast two-hybrid experiments, we identified XAP2 as a TRβ1 interacting partner. Therefore we decided to study the functional impact of the interaction between XAP2 and TRβ1. First, we investigated if TRβ1 interacts with XAP2 in mammalian cells *in vivo*. Using co-immunoprecipitation experiments, we observe an efficient interaction between XAP2 and TRβ1, but not between TRα and XAP2 demonstrating a high degree of specificity. In addition, *in situ* hybridization assay revealed that XAP2 and TRβ1 are expressed in the same neurons. In addition, our experiments revealed that not only XAP2 but also hsp90 interacts with TRβ1, thus adding TRβ1 as an hsp90 client protein. To further study if XAP2 is involved in the TR mediated regulation of TRH, *in vivo* siRNA-knockdown of XAP2 was performed. Knockdown of XAP2 abolished T3-independent activation of TRH expression. However, the knockdown of XAP2 did not interfere with the T3-dependent repression of TRH expression. Thus, this Indicates that XAP2 is necessary for activation but not repression of TRH transcription.

The XAP2 related immmunophilin FKPB52 has been shown to interact with the steroid hormone receptor GR. FKBP52 selectively potentiates hormone-dependent reporter gene activation, and this potentiation is readily blocked by co-expression of the closely related FKBP51 [131]. However, no previous study has shown a physiological

role for the immunophilin-like protein XAP2. Thus, this study provides the first *in vivo* demonstration of a regulatory, physiological role for a co-chaperone protein.

CONCLUDING REMARKS

The aims of this thesis have been to find novel genes that are T3-independently activated by TR and to further characterize the mechanism by which TR negatively regulates genes. We have shown *in vitro* that both E2F-1 and Necdin promoters are activated by unliganded TR and repressed by liganded TR. Furthermore, we have identified a potential nTRE, Z-element, present in both promoters to which TR directly binds. We then investigated the role of different cofactors in the regulation of the E2F-1 and Necdin genes as well as the TRH gene. We found that both RXR and the corepressor NCoR are important to hormone-independent activation of negatively regulated TH-target genes.

Our finding that TH represses expression of E2F-1 describes one mechanism by which TH represses proliferation of cells. This implicates functions for TR both in differentiation of for example OPCs and other stem cells, as well as for development of tumors. The regulation of Necdin expression by TR might also reflect a mechanism for TR in differentiation of cells, since Necdin expression is regulated during differentiation.

To further investigate T3-independent activation of gene transcription, we used the *in vivo* mouse model system. Since Necdin is expressed predominantly in post-mitotic neurons in the brain, we investigated the effect of T3 on Necdin expression in mouse brain. Un-liganded endogenous TR did not activate Necdin expression. However, coexpression of the NSCL-2 transcription factor abolished the TR-mediated activation of Necdin in transient transfection experiments, indicating that competition may exist between TR and NSCL-2 in the regulation of Necdin expression.

Both TRβ1 and TRβ2 are involved in T3-dependent repression of TRH expression, whereas only TRβ1 is involved in T3-independent activation of TSH expression. To establish whether isoform-specific cofactors involved TRβ1-mediated regulation of TRH expression, two-hybrid assays were performed. We showed that immunophilin-like XAP-2 specifically bound the TRβ1 isoform. Furthermore, XAP-2 has an important function in the hormone-independent transcriptional activation of the TRH gene. However, the molecular mechanism for this remains to be investigated

The mechanism behind T3-independent activation and T3-dependent repression of gene transcription is still not completely understood. It is clear that corepressors

function as coactivators on nTRE containing genes, but the exact mechanism remains to be elucidated. It would be very interesting to further investigate the effect of un-liganded TR on the expression of nTRE containing genes, *in vivo*. Conditional Pax-8-/- mice would be useful in studying the *in vivo* regulation of putative negatively regulated TH-target genes at different developmental stages. To identify windows in time during development for regulation of genes by unliganded TRs, as well as to identify which cells this regulation takes place, it would be helpful to construct transgenic mice containing a reporter gene driven by an nTRE. Finally, the siRNA technique combined with *in vivo* gene transfer could be used to elucidate the specific roles of different cofactors in regulation of nTREs.

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