CHAPTER 7

General Discussion

7

Overview

Genomic actions of thyroid hormone (TH) are regulated by the binding of TH to thyroid hormone receptors (TRs). Mutations of the genes encoding TRα and TRβ lead to resistance to thyroid hormone (RTH) α and β, respectively. In the first part of this thesis, we describe two novel mutations identified in an RTHα and RTHβ patient. *In silico* and *in vitro* studies confirmed the pathogenic impact of the amino acid substitutions on receptor function. In addition, these studies allowed us to gain more insight into the role of particular amino acid residues in TR functions. In the second part, we focus on the genotype-phenotype correlation in RTHα (1,2). Based on our observations, the severity of the clinical phenotype of these patients is not solely explained by the degree of impairment in T3 binding affinity. Therefore, we conducted studies to evaluate the diverse functional impairment of mutant TRs to correlate the *in vitro* functional impairment to the severity of the phenotype of reported patients. The last part of this thesis focuses on nuclear coregulatory proteins that are involved in TR functions. We asked whether the nuclear coregulatory protein recruitment by TRs have tissue- and isoform-specific patterns. In this chapter, we discuss the relevance of our studies to the current knowledge gaps and further research strategies to confirm our findings and broaden the understanding of the complexity of TR actions.

Role of specific amino acid residue on TR function: lessons from mutated TRs

Pathogenic mutations in TRs that cause RTH are located in the ligand binding domain (LBD) and adjacent hinge region of the receptors and lead to a reduced affinity for TH. The LBD shares a high sequence homology between isoforms and among species (3). However, not all amino acids in the LBD are equally important for T3 binding and T3-induced transcriptional activity. For instance, a study of Hayashi et al. on sixteen TRβ1 mutations located in the LBD (six patient-derived and ten artificial mutations) showed that six artificial mutations did not significantly affect T3 binding affinity (4), suggesting that only certain residues in the LBD play a role in T3 binding and are sensitive to amino acid changes. Subsequent crystal structures of TRα1 and TRβ1 showed that amino acid residues located at the surface of the ligand-binding pocket interact with T3 and mainly determine T3 binding affinity (3,5). This was confirmed by mutations of TRα1 and TRβ1 identified in RTH patients that reduce T3 binding affinity of the receptor (6-8).

In addition to the affinity for T3, studies of mutated TRs also emphasized the role of particular amino acid residues in interaction between TRs and cofactor proteins. For example, Arg243, Arg383, and Pro453 of TRβ1 seem to be important for the interaction with corepressor proteins because mutations at these residues (R243Q and R243W (9), R383H (10), and P453S (11)) impair interaction with the corepressor NCoR1. In addition, Thr277 and

Leu454 of TRβ1 are likely involved in the interaction with coactivator proteins, since mutations of these residues (T277A (12) and L454V (13)) affect interaction with the coactivator SRC1. Therefore, functional studies of the mutant TRs do not only confirm the pathogenicity of a mutation in the diagnosis of RTH in patients, but also allow us to gain more understanding about the role of specific amino acid residues in TR function.

Role of Leu341 in T3 binding affinity of TRβ

In **chapter 2**, we describe the role of Leu341 in TRβ1 function, prompted by the identification of a novel L341V mutation in RTHβ patients. Another mutation at this residue (L341P) has been reported as a cause of RTHβ (14,15). However, the functional importance of this amino acid residue has never been established. Our in-depth inspection of wild-type (WT) TRβ crystal structure showed that Leu341 lines the ligand-binding pocket of TRβ1 and form a direct hydrophobic interaction with the outer ring of the T3 molecule, which is in agreement with previous reports (16-18). This residue also interacts with the surrounded residues such as Phe272 and Leu346 to stabilize the microarchitecture of the ligand-binding pocket. The leucine to valine substitution (L341V) in the *in silico* model showed that the consequential shortening of the aliphatic amino acid side-chain alters the shape of the ligand-binding pocket and abolishes the direct hydrophobic interaction of the side-chain with T3. The functional impairment of TRβ1-L341V predicted by the *in silico* model was confirmed by the *in vitro* studies. The mutant has reduced T3 binding affinity, impaired T3-induced transcriptional activation, and a dominant-negative effect on WT receptor function. The importance of Leu341 was further confirmed by three artificial mutations, L341A, L341I and L341F, which were created based on the *in silico* prediction. The side-chains of these three amino acids (alanine, isoleucine, and phenylalanine) have similar hydrophobic properties but different in size and orientation, resulting in a variable distance to the T3 molecule. We also demonstrated a correlation between the degree of receptor impairment and the side-chain size and orientation of these artificial mutants. Based on this study, we suggested that the direct interaction between Leu341 and T3 and the microarchitecture formed by interactions between Leu341 and its surrounding residues are required for optimal T3 binding affinity and T3-induced transcriptional activation of TRβ1.

Role of TRα1-Met256 and TRβ1-Met310 in T3 versus T4 recognition of TRs

The concept that T4 is a prohormone which has to be converted to the biologically active form T3 to activate TRs in target tissues has been established for several decades based on the seminal studies showing that T3 has a greater biological potency than T4 (19-22). However, molecular and structural mechanisms underlying this concept have never been thoroughly examined. In **chapter 3**, we highlight the role of residues Met256 of TRα1 and Met310 of TRβ1 in determining the differential biological potency of T3 versus T4, by characterizing a novel mutation (TRα1-M256T) identified in an RTHα patient and a mutation at the corresponding position (TRβ1-M310T) identified in RTHβ patients (23-25). The crystal

structure previously showed that the ligand-binding pocket of WT TRβ1 can accommodate both T3 and T4, but that it is more tightly packed in the case of T3 binding than T4 binding (26). Helix 12 of T4-bound TRβ1 is more mobile than that of T3-bound TRβ1. Therefore, T4 is less stably retained in the LBD, resulting in a higher ligand dissociation rate and a lower binding affinity of T4-bound TRβ1. By focusing on Met256 of TRα1, we demonstrated that this residue forms a direct (hydrophobic) interaction with 5' carbon of the outer ring of T3 and interacts with surrounding residues to create a niche that allows the accommodation of T3. The bulky 5' iodine of T4 abolishes these direct interactions between the ligand and Met256 and, consequently, affects the stability of T4 in this niche. Therefore, we predicted based on our *in silico* models that Met256 in TRα1 plays a pivotal role in discriminating between T3 and T4. This prediction was confirmed by the studies on threonine and alanine substitutions. We demonstrated that TRα1-M256T and the artificial mutant TRα1-M256A have a more pronounced distortion of the hydrophobic niche accommodating the outer ring of T3 than that of T4, which suggests a greater impact on the affinity for T3 of these two mutations. The *in vitro* studies confirmed that threonine and alanine substitutions at Met256 selectively reduced the affinity for T3 and had a greater impact on T3- versus T4-dependent transcriptional activation. The naturally occurring mutation at the corresponding residue in the TRβ1 (M310T) also showed the same result. In contrast, amino acid substitutions at other residues of TRα1 (D211G (27), A263S, and R384H (28)) equally affect the transactivation potency of both T3 and T4. These findings confirm that Met256 in TRα1 and Met310 in TRβ1 are important for T3 versus T4 discrimination and shed light on the underlying molecular and structural basis for the role of T4 as a prohormone and T3 as a biologically active hormone in a widely accepted concept of TH physiology.

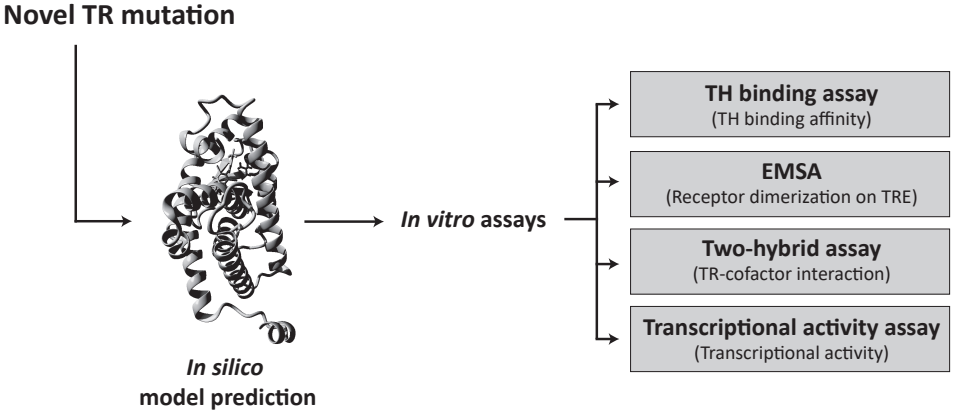

Figure 1. A pipeline of assays for confirming functional impairment of mutant TRs. The assay details are described in chapters 2-4 of this thesis. (EMSA, electrophoretic mobility shift assay; TRE, thyroid hormone response elements)

These studies in **chapters 2 and 3** are based on the identification of novel mutations in RTHα and β patients. Our studies showed that the combination of *in silico* model prediction and the creation of artificial mutations is a highly relevant approach to further explore the role of mutated amino acid residues of TRs in RTH patients and expand the knowledge about underlying structural and molecular basis for interactions of TR with its ligands and protein co-partners. In addition to the *in silico* prediction, we have applied a pipeline of assays, including *in vitro* TH binding assay, electrophoretic mobility shift assay (EMSA), TR-cofactor (two-hybrid) interaction assay, and transcriptional activity assay, in order to rapidly and extensively confirm functional impairment of mutant TRs (Figure 1).

Diverse functional impairment of TRα1 mutants and phenotype variability in RTHα

RTHα caused by mutations of the *THRA* gene was first identified in 2012 (1,2). To date, 25 mutations (in a total of 40 patients) have been reported. These mutations can be categorized into two groups based on the type of mutation. The first group consists of nonsense and frameshift mutations that generate premature stop codons. These mutations lead to truncated receptors that exhibit negligible T3 binding and a lack T3-induced gene expression (1,2,28-32). The second group consists of missense mutations that result in amino acid substitutions (7,27,28,33-39). These missense mutants can bind T3 but with a lower affinity than WT receptors.

The common phenotype of RTHα patients includes growth retardation, delayed bone maturation, macrocephaly, constipation, delayed cognitive and motor development, anemia, and a high (F)T3/(F)T4 ratio (7). An increase in the number of RTHα patients identified and their clinical characterization allows us to investigate the variability in the phenotype of RTHα patients. In general, patients with truncating mutations have more severe phenotype than patients with missense mutations that can still bind T3 (7). However, there are specific differences in the phenotype of patients in each group. For instance, the neurological phenotype of patients with truncating mutations varies from mild cognitive and motor impairment (F397fsx406 (2) and E403X (1)) to severe mental retardation and in some cases inability to walk and no developed speech (C380fsx387 (28), A382fsx388 (33), R384fsx388, and C392X (29)) (Table 1). Likewise, some patients with missense mutations displayed clear motor and cognitive impairment, whereas in a large family with patients carrying an A263S mutation, the affected members only showed mild symptoms and even partially overlapped with unaffected members for some characteristics (Table 2). So far, the underlying molecular mechanism to explain these observations has not yet been clearly established.

In **chapter 4**, we evaluated the differences in the degree of functional impairment of seven TRα1 missense mutants, four of which were derived from RTHα patients (D211G (27),

M256T (37), A263S, and R384H (28)) and the other three were derived from RTHβ patients (P398H, T223A, and L287V). These mutations covered the three CpG-rich regions of the LBD of TRα1 that are homologous to the mutation-prone hotspots of the TRβ1, namely R384H and P398H in cluster 1, M256T, A263S and L287V in cluster 2, and D211G and T223A in cluster 3 (Figure 2). Studies in patient-derived TRβ1 mutants showed that some mutants have severe transcriptional impairment despite only mild T3 binding defect. This finding would be explained by either impaired dimerization (40-42) or defective TR-cofactor interaction (9-13). In addition, a subset of TRβ1 mutants showed a defective transcriptional activation only on specific TRE configurations (12). Since TRα1 and TRβ1 are highly homologous, we asked whether impaired dimerization, defective TR-cofactor interaction, and configuration of TREs (DR4, IR0, and ER6) could contribute to the functional impairment of TRα1 mutants. Our results showed that in these seven TRα1 missense mutants it is predominantly the reduced T3 binding affinity that determines the severity of impaired transcriptional activation and defective interactions with the cofactors (NCoR1 and SRC1). There is no substantial evidence that suggests an additional TRE-specific transcriptional impairment of these mutants.

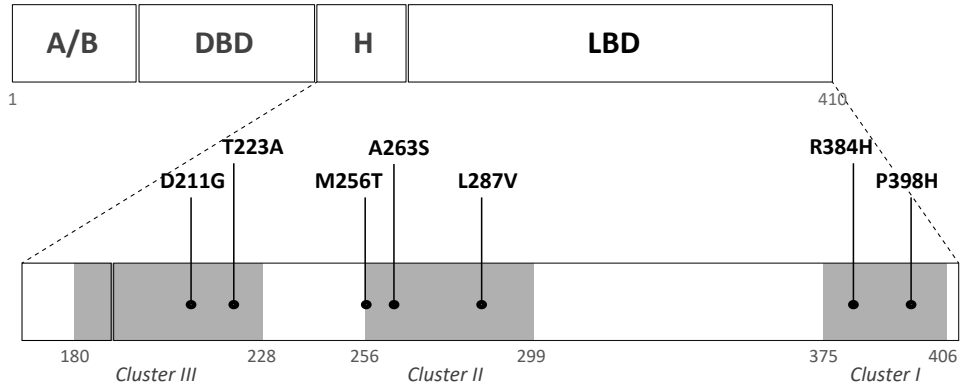

Figure 2. The diagram shows the location of selected mutations in the three CpG-rich regions of the LBD of TRα1 (adapted from Rebai M et al. 2012(43)) [A/B, A/B domain; DBD, DNA-binding domain; H, Hinge region; LBD, ligand binding domain]

The degree of reduced T3 binding affinity and impaired transcriptional activation of the mutants seems to be correlated with the phenotype of the patients. For instance, as previously mentioned, patients carrying D211G and R384H mutations have a more severely impaired motor development than patients carrying the A263S mutation. This is in agreement with the more prominent transcriptional impairment that we showed for the D211G and R384H mutants than for the A263S mutant. This finding is in line with the report of Moran et al. that showed that the degree of transcriptional impairment of two TRα1 missense mutations,

A263V and L274P, correlates with the severity of the clinical phenotype of the patients (36). However, there is an exception. The M256T mutation exhibited the most severe transcriptional impairment, but the available clinical information showed that patient carrying this mutation does not have severe cognitive impairment and delayed motor development (Table 2). There is no clear explanation yet for this paradox. Since our studies did not take the dominant-negative effect of the mutant on WT receptor function into account, it might be that this is one of the factors that further complicates the phenotype of the patients.

In addition to the variation between different mutations, the severity of clinical phenotype is also diverse between patients who carry the same mutation (28). This phenomenon has also been reported in RTHβ patients who belong to the same family and carry a similar mutation (44-48). The mechanisms underlying this finding is unknown. This may very well be explained by other factors beyond TRs such as genetic variability or epigenetic modification that might modulate the phenotype of RTH patients.

As previously mentioned, the diversity in phenotype severity was also observed in RTHα patients carrying truncating mutations. In **chapter 5**, we analyzed gene transcription regulated by two TRα1 truncating mutations, C380fsx387 and F397fsx406, both of which lack T3-induced gene expression (1,2) but result in a different degree in cognitive impairment of the patient (Table 1). The patient who carries the TRα1-C380fsx387 mutation was severely handicapped and unable to communicate at 12 years of age, suggesting severe cognitive impairment (28). In contrast, the index patient who carries the TRα1-F397fsx406 mutation had borderline cognitive impairment (IQ score 90 at 11 years old) (2). The transcriptome analysis was performed in a human neuronal cell line (SH-SY5Y) overexpressing Flag- and Hemagglutinin double-epitope tagged TRα1 (FHTRα1) to focus on neurological phenotype. The results showed that overexpressing FHTRα1-C380fsx387 and -F397fsx406 mutants altered the transcriptomes when compared to cells overexpressing FHTRα1 WT and abolished T3-induced gene expression. In addition, the transcriptomes of these two mutants were very different from each other, suggesting a differential effect of these two mutations on gene transcription. The genes that were differentially expressed in the two mutant cells were related to nervous system development and neuronal pathfinding. For instance, we identified many genes (*SEMA3A*, *SEMA3C*, *SLIT1*, *EFNB2*, *UNC5A*, and *UNC5D*) that encode proteins that act as extracellular guidance cues for axonal/dendritic growth (49,50). We also identified many genes that are related to cell adhesion molecules (e.g., *NRCAM*, *CNTNs*, *PCDHs*, *ITGs*, and *ASTNs*) and neural growth factor receptors (*NGFR*, *NTRK3*, and *GFRA2*), which are essential for neural growth and migration (51-56). Additionally, the expression of *ASCL1* and *NEUROG2*, which encode two master proteins for neuronal differentiation, Achaete-scute homolog 1 and Neurogenin 2, respectively, was different between the two mutant cells. Evidence in murine models showed that Neurog2 assigns neuron progenitor cells to differentiate into excitatory (glutamatergic) neurons, whereas Ascl1 assigns progenitor cells to differentiate into inhibitory (GABAergic) neurons (57-61). Progenitor cells that highly express Ascl1 also keep proliferating rather than differentiate into a mature neuron. Since the expression of *NEUROG2* and *ASCL1*

was respectively lower and higher in SH-SY5Y/FHTRα1-C380fsx387 cells compared to both SH-SY5Y/FHTRα1-F397fsx406 and SH-SY5Y/FHTRα1 WT cells, this may keep more cells in a proliferating phase or drive them into becoming inhibitory neurons, thereby contribute to the more severe neurological phenotype in the C380fsx387 patient.

The studies in **chapters 4 and 5** showed a relationship between functional impairment of the mutant TRs and the neurological phenotype of the patients. However, the limitation of our studies is that the neurological phenotype of patients was not systematically evaluated, which leads to difficulty in phenotype comparison. For instance, cognitive and motor function was assessed at a different age and by different tests/scores or described only as a qualitative observation in some publications. The test was also performed and interpreted by different investigators without standardization. In addition, the effect of treatments/interventions which had been given to some patients before the RTH diagnosis and might improve the cognitive and motor function were not taken into account. Therefore, our results need to be interpreted cautiously, and further studies with standardized assessment methods are required in order to solve this issue and confirm our findings.

Recruitment of nuclear coregulatory protein by TRs

TRs regulate gene transcription by interacting with nuclear coregulatory proteins that modify local chromatin structure and accessibility of the promotor region of target genes. In the absence of T3, TRs repress gene transcription by binding to the main corepressor proteins, NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors), that form complexes with other nuclear proteins to promote histone deacetylation and nucleosome compaction. Binding of TH to TRs creates a closed-conformation of TR-LBD, leading to the dissociation of the corepressor complex and the association of coactivator proteins. Steroid hormone receptor coactivator 1, 2, and 3 (SRC-1, -2, and -3) directly bind to TRs and recruit other proteins such as histone acetyltransferase (HAT), resulting in histone acetylation, chromatin accessibility, and gene transcription.

Apart from the classical TR cofactors, other nuclear proteins have been identified as corepressors or coactivators. There is evidence indicates that TRs recruit cofactor proteins in a tissue- and isoform-dependent manner, which may further explain the diverse transcriptional regulation of TRs in different tissues (62-65). Understanding the complexity in TR-cofactor interactions will gain more insight into the impact of TR mutations in RTH syndromes in addition to the effect on T3 binding. Therefore, in **chapters 6a and 6b**, we used an unbiased approach to identify TR-interactomes by using a tandem-affinity protein purification method.

In **chapter 6a**, we compared the unliganded and liganded TRα1-interactomes in a human liver cell model (HepG2) and a human neuronal cell model (SH-SY5Y). The main objective was to evaluate the tissue-dependency of TR-cofactor interactions. FHTRα1 WT

was stably expressed in these two cell types by lentiviral transduction. To identify nuclear proteins that interact with TRα1 in the absence and presence of T3, we purified FHTRα1 and its associated proteins from the nuclear extracts of HepG2 and SH-SY5Y (after stimulating with 0 or 100 nM T3) using a tandem-affinity purification. The TRα1-interactomes were identified by LC-MS/MS and confirmed by co-immunoprecipitation. By this approach, we were able to identify several proteins and protein complexes that are associated with TRα1. The number of hits is larger than in previous TR-interactome studies (63,65). The composition of TRα1-interactomes is strongly dependent on ligand-binding state and largely overlapped between HepG2 and SH-SY5Y cells, suggesting that TRα1 uses common nuclear coregulatory proteins to regulate gene transcription in these two cell types. However, some proteins are likely to interact with TRα1 in a cell-type specific manner, including nuclear receptor interacting protein 1 (NRIP), as known as RIP140, in HepG2 cells and transcription factor 4 (TCF4) in SH-SY5Y cells. Given that NRIP and TCF4 are exclusively expressed in HepG2 and SH-SY5Y cells, respectively, the cell-type specific TR-cofactor recruitment found in our study may be mainly explained by the differential availability of these particular cofactors and may not necessarily prove a tissue-specific interaction.

In addition to the cell-type specificity, we also identified a novel putative binding partner, transcription factor Prospero homeobox 1 (Prox1), in both HepG2 and SH-SY5Y cells. The interaction of this transcription factor with TR was increased in the presence of T3. Prox1 is known to be involved in cell fate specification and metabolism. Prox1 can interact with many nuclear receptors, including chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) in lymphatic endothelial cells (66), and hepatic nuclear factor 4 alpha (HNF4α), liver receptor homologue-1 (LRH-1), and Retinoid Orphan Receptor (ROR) α and γ in the liver (67-69). The interaction between TR and Prox1 had not previously been described, although Broekema et al. recently showed the interaction between TRs and a binding motif of Prox1 by using the Microarray Assay for Realtime Coregulator-Nuclear Receptor Interaction (MARCoNI) technology (70). Our finding further confirms that Prox1 may work as TR coregulatory protein to regulate gene transcription. In addition, we identified several proteins belonging to multisubunit chromatin remodelling complexes that help to rearrange local chromatin architecture and regulate gene transcription. The most notable one was the nuclear remodelling and deacetylase (NuRD) complex that was identified in both HepG2 and SH-SY5Y cells. The NuRD complex was initially identified as a transcriptional repression complex (71-73). However, it was later shown that this complex can also be involved in transcriptional gene activation (74), which is in line with our finding that components of the NuRD complex identified in our study were slightly enriched in the presence of T3.

In **chapter 6b**, we studied the isoform-specific recruitment of coregulatory proteins for TRs in SH-SY5Y cells in order to understand the complexity of TR actions in the brain. Since previous studies in other cell models showed that TRs recruit a subset of coregulatory proteins in an isoform-specific manner (63,65), we asked whether the isoform-specific coregulatory protein recruitment exists in SH-SY5Y cells as well. Therefore, we compared

the interactomes for the two major TR isoforms (TRα1 and TRβ1) using the same tandem-affinity purification method and LC-MS/MS analysis as in chapter 6a. The result showed that the majority of identified proteins were associated with both TRα1 and TRβ1, suggesting that both TR isoforms associate with common nuclear coregulatory proteins to regulate gene transcription. However, a subset of nuclear proteins interacted with TRs in an isoform-specific manner. The interesting hits are lysine-specific demethylase 6A (KDM6A, as known as UTX), set1/Ash2 histone methyltransferase complex subunit ASH2 (ASH2L), and retinoblastoma-binding protein 5 (RBBP5), all of which were identified exclusively with TRα1 in the presence of T3. These proteins are likely to work together to establish transcriptional permissive chromatin for many key developmental genes such as HOX genes (75-78).

In agreement with the study in chapter 6a, we identified many components of multisubunit chromatin remodelling complexes that regulate gene transcription together with both TR isoforms in SH-SY5Y cells, such as the NuRD complex and the Spt-Ada-Gcn5-Acetyltransferase (SAGA) complex, both of which seems to interact with TR more prominently in the presence of T3. Identifying the NuRD complex again in this study confirms that this protein complex is a common co-partner that plays a crucial role in TR actions, regardless of TR isoform and tissue context. The proteins in the SAGA complex were also co-purified with TRs exclusively in the presence of T3, suggesting the role of this complex in transcriptional activation. To our knowledge, this is the first study suggesting a relationship between SAGA complex and TRs. Other novel binding co-partners of TRs, Foxhead box transcription factor K1 and K2 (FOXK1 and FOXK2), were identified in the absence of T3. The FOXK1 and FOXK2 are the proteins that positively regulate the Wnt/β-catenin signaling pathway (79). These studies indicate crosstalk between TH-TR and the Wnt/β-catenin pathway at multiple levels, such as an effect of TH on the Wnt and β-catenin protein expression, a physical interaction between TRs and β-catenin in specific tissues, and synergistic effect of TH-TRs and Wnt/β-catenin signaling on cell proliferation and differentiation (80). However, the role of FOXK1 and FOXK2 in this crosstalk has not yet been established. Our finding suggests that these proteins may also take part in the interaction between TRs and Wnt/β-catenin signaling.

In summary, our results in **chapters 6a and 6b** showed that TRs interact with not only classic nuclear coregulatory proteins, such as NCoR/SMRT and SRC complexes, but also several potential novel binding partners. TRs also interact with a number of transcription factors and chromatin remodeling complexes, which highlights the role of TRs in local chromatin accessibility. Although the majority of identified proteins are able to interact with both TR isoforms and regardless of cellular context, we also found that a small subset of nuclear proteins seems to interact with TRs in tissue- and isoform-specific manner. These findings expand the knowledge about the interaction between TRs and cofactor proteins in transcriptional gene regulation and may lead to a more understanding of the impact of TR mutations in RTH syndrome.

Concluding remarks and future perspective

TRs are key controllers for transcriptional regulation (genomic actions) by TH. Research on molecular functions of TRs provides more understanding about the physiology of TR actions which are more complicated than merely binding of TH to TRs.

The knowledge of TR actions has been broadened by the identification of mutations in RTH patients. All identified mutants help to confirm a crucial role of that particular residue/domain in TR functions, which includes interactions with TH in the ligand-binding pocket and interactions with regulatory proteins, such as RXR, corepressors, and coactivators. In this thesis, we provide more information about the importance of particular amino acid residues in TR functions prompted by identifying two RTH patients who carry novel mutations. The combination of *in silico* model prediction and *in vitro* studies of mutated TRs allows us to understand not only the effect of the mutation but also the role of the residue of interest in TR function. We, therefore, suggest using this approach to further explore the role of mutated amino acid residues of TRs in RTH patients. According to this, we have applied a pipeline of assays for a novel TR mutation by combining all of the techniques we have used, including *in silico* protein modeling and several *in vitro* functional assays. Since we are now capable of performing TR transcriptome and interactome analyses, we could also incorporate these techniques in order to explore all aspects of transcriptional regulation by TRs and effect of TR mutations in RTH syndromes.

One of the interesting issues is the factor that determine the functional impairment of the mutated TRs and the severity of phenotype of RTHα patients. Our study with the TRα missense mutants showed that the reduced affinity for T3 is the main factor that determines the severity of impaired transcriptional activity of mutants and seems to define patients' phenotypes. However, the transcriptome analysis of two TRα1 truncating mutations (C380fsx387 and F397fsx406), both of which exhibit negligible T3 binding, showed that there is a difference in baseline RNA expression between mutants. This finding suggests that additional factors, apart from the affinity for T3, could also influence the severity of patients' phenotype in RTH, which is confirmed by the diversity of the cognitive phenotype in these patients. We are aware that our studies have some limitations. For instance, the *in vitro* overexpression system we used may not perfectly represent the *in vivo* situation. The system also not allow us to study the effect of the mutant on WT receptor function (i.e., dominant-negative effect) since it was difficult to mimic heterozygosity. For this, other cell models, such as patient-derived primary cells or CRISPR-Cas9 genome editing, need to be developed. There is also a difficulty in phenotype comparison of RTHα patients since the phenotype description is often incomplete, and clinical assessment methods sometimes vary among publications. This may affect the reliability of the relationship between genotype and phenotype we reported. A patient registry for RTH syndrome, which provides a guideline and standardized methods for clinical assessment should overcome this limitation and will lead to a more comprehensive understanding of the genotype-phenotype correlation in RTH syndromes.

Interaction between TR and cofactors is important for transcriptional gene regulation by TH. Our studies showed that TRs interact with not only classical nuclear coregulatory proteins but also several potential novel binding partners to regulate gene transcriptions. A small subset of proteins interacts with TRs in tissue- and isoform-specific manner. These findings provide more insight into the complexity of TR actions and may expand the understanding about the impact of mutated TRs on phenotype of RTH patients, especially in patients who carry mutated TRs that have a greater impaired transcriptional activation than the degree of T3 binding defect. However, further studies are still needed to confirm the interaction between WT TRs and novel binding protein copartners and the functional importance of these interactions in both the physiological actions of TRs and RTH syndromes.

Table 1. Clinical characteristics and thyroid function tests (TFTs) of RTHα patients (index cases) carrying **truncating mutation** in the *THRA* gene.

Phenotype	C380fs387X	A382fs388X	R384fs388X	C392X	E395X	E403X	F397fs406X
Sex	F	F	F	M	M	F	F
Age (year)	1.3	45	19	18	2	6	6
GA (week)	Term	N/A	Term	41	Term	N/A	N/A
Birth weight (gm)	3200	N/A	N/A	**4200**	**4000**	N/A	N/A
Skeletal							
-Ht SDS or percentile (P)	**-2.46**	**-2.34**	**<P3**	**-6**	**<P3**	**Ht deficit**	**Ht deficit**
-Sitting/ total Ht SDS	N/A	+0.29	N/A	N/A	N/A	N/A	N/A
-Subischial length SDS	N/A	**-3.87**	N/A	N/A	N/A	**<P3**	N/A
-HC SDS or percentile (P)	**+2.29**	**+9.0**	**Macrocephaly**	**Macrocephaly**	No	N/A	**+1.65**
-Delayed bone maturation	N/A	N/A	N/A	N/A	**Yes**	**Yes**	**Yes**
-Delayed dentition	**Yes**	N/A	N/A	N/A	N/A	**Yes**	**Yes**
Appearance							
-Coarse facies	**Yes**	**Yes**	**Yes**	**Yes**	N/A	N/A	N/A
-Skin tags	N/A	**Yes**	N/A	N/A	No	N/A	N/A
-Macroglossia	**Yes**	**Yes**	**Yes**	N/A	No	N/A	**Yes**
-Umbilical hernia	**Yes**	**Yes**	**Yes**	N/A	N/A	N/A	N/A
Gastrointestinal							
-Constipation	**Yes**	**Yes**	N/A	**Yes**	**Yes**	**Yes**	**Yes**
Neurocognitive							
-Delayed milestone	**Yes**	**Yes**	**Yes**	N/A	**Yes**	**Yes**	**Yes**
-Cognitive impairment	**Severe***	**Yes** (IQ 52)	**Severe**	**Severe** (IQ 22)	N/A	**Yes**	**Mild** (IQ 90)
-Delayed motor development	**Severe***	**Yes**	**Yes**	N/A	**Yes**	**Yes**	**Yes**

Phenotype	C380fs387X	A382fs388X	R384fs388X	C392X	E395X	E403X	F397fs406X
Hematological							
-Anemia	**Yes**	No	N/A	**Yes**	**Yes**	N/A	**Yes**
Additional phenotype	Hoarse-sounding cry, Hypertrophic obstructive cardiomyopathy, pericardial effusion, nephrolithiasis	Epilepsy	Atonic seizure, myelination disorder, dysgerminoma, hypertelorism, smooth philtrum, thin upper lip, wide nasal base/ ridge, downslant palpebral fissure	Hypertelorism, palpebral ptosis, flat nasal bridge, elongated thorax, club foot, tortusity of arteries, puffy hands, rough skin texture	Disproportionate short stature, short arm span, broad face and nasal bridge	Mild hypermobility, ligamentous laxity, wormian bones, femoral epiphyseal dysgenesis, restrict adaptive behavior	Congenital hip dislocation
TFTs [NR]							
-TSH (mU/L)	1.4 [0.4-4.3]	**5.8** [0.35-5.5]	N/A	2.76	1.37 [0.38-7.31]	1.04 [0.8-6.2]	Normal
-FT4 (pmol/L)	**5.1** [11-25]	10.0 [10.0-19.8]	N/A	10.0 [9.0-11.6]	**11.2** [15.4-22.3]	**6.5** [10.3-21.9]	**Low**
-TT4 (nmol/L)	**53** [58-128]	85 [69-141]	N/A	N/A	101.8 [66.7-157.7]	**42.5** [95.3-155.8]	Low normal
-FT3 (pmol/L)	**12.4** [3.8-7.6]	4.9 [3.5-6.5]	N/A	**7.96** [2.23-5.38]	**8.06** [4.22-7.18]	6.1 [4.6-7.7]	**High**
-TT3 (nmol/L)	**2.76** [1.4-2.5]	1.7 [0.9-2.8]	N/A	N/A	3.34 [1.52-3.49]	2.4 [2.0-3.4]	N/A
-rT3 (nmol/L)	N/A	**0.15** [0.17-0.49]	N/A	N/A	N/A	**0.11** [0.32-0.57]	**Low**
-T3/T4 ratio	N/A	N/A	N/A	N/A	N/A	N/A	**High**
Reference	*(28)*	*(30)*	*(31)*	*(29)*	*(32)*	*(1)*	*(2)*

Abnormal values are indicated in bold. [GA, gestational age; Ht, height; HC, head circumference; SDS, standard deviation score; M, male; F, female; NR, normal range; N/A, data not available]

*Severely handicapped (unable to walk and communicate) at the age of 12.7 years

Table 2. Clinical characteristics and thyroid function tests (TFTs) of RTHα patients (index cases) carrying **missense mutation** in the *THRA* gene.

Phenotype	D211G	M256T	A263S	A263V	L274P	G291S	N359Y	R384H	P398R	E403K
Sex	F	M	M	M	M	M	F	M	F	F
Age (year)	1.4	19	2.6	17	15	4	27	0.9	8	12
GA (week)	Term	**43**	Term	39	41	38	37	N/A	42	42
Birth weight (gm)	**4000**	**5000**	3000	**4580**	3260	2900	N/A	3400	3450	3650
Skeletal										
-Ht SDS or percentile (P)	**-2.77**	-1	-1.18	P50	**P0.4**	**-2.47**	**-6**	-0.63	-0.5	**-3**
-Sitting/ total Ht SDS	N/A	**+2.5**	**+2.71**	N/A	N/A	N/A	**Short limbs**	-1.00	**Short limbs**	**Short limbs**
-Subischial length SDS	N/A	-1	N/A	+1	**-2**	N/A	N/A	N/A	N/A	N/A
-HC SDS or percentile (P)	0	**+2.5**	+1.14	**P99**	**P99**	**+2.08**	**Macrocephaly**	**+2.02**	N/A	**Macrocephaly**
-Delayed bone maturation	N/A	N/A	**Yes**	**Yes**	**Yes**	**Yes**	N/A	**Yes**	N/A	**Yes**
-Delayed dentition	N/A	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	No	No	N/A	N/A
Appearance										
-Coarse facies	**Yes**	**Yes**	N/A	**Yes**	**Yes**	**Yes**	No	**Yes**	**Yes**	**Yes**
-Skin tags	No	**Yes**	No	**Yes**	**Yes**	N/A	N/A	No	N/A	N/A
-Macroglossia	No	N/A	No	**Yes**	**Yes**	N/A	N/A	**Yes**	No	No
-Umbilical hernia	N/A	**Yes**	No	**Yes**	**Yes**	**Yes**	N/A	No	N/A	N/A
Gastrointestinal										
-Constipation	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	N/A	No	**Yes**	**Yes**

Phenotype	D211G	M256T	A263S	A263V	L274P	G291S	N359Y	R384H	P398R	E403K
Neurocognitive										
-Delayed milestone	**Yes**	N/A	No	**Mild**	**Severe**	**Yes**	N/A	**Yes**	**Yes**	**Yes**
-Cognitive impairment	No	**Mild (Special school)**	No	Regular school	**Special needs school**	**Yes**	No (obtained an advanced academic degree)	**Moderate**	Normal (IQ 95)	**Mild** (IQ 80)
-Delayed motor development	**Severe**	**Yes**	No	**Mild**	**Moderate**	**Yes**	N/A	**Severe**	**Yes**	**Yes**
Hematological										
-Anemia	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Additional phenotype	-	Mild hypertelo-rism, striking blue eyes, mild learning problems, mild autistic spectrum disorder	-	Bilateral inguinal hernia	Genu valgum, coxa valga, femoral epiphyseal dysgenesis, mesomelic short limbs	-	Hypertelorism, clavicular & 12th ribs agenesis, humero-radial synostosis, syndactyly, scoliosis, hip dislocation, hypercalcemia	-	Hypertelo-rism, wide valgus foot with sandal gap, puffy hands	Hypertelorism, micrognathia, short neck, wide valgus foot with sandal gap, puffy hands
TFTs [NR]										
-TSH (mU/L)	4.40 [0.5-5.0]	1.83 [0.4-4.3]	2.10 [0.4-4.3]	3.6 [0.35-5.50]	2.07 [0.35-5.50]	3.89 [0.35-5.5]	**0.34** [0.4-3.6]	1.89 [0.4-4.3]	0.45 [0.4-0.6]	1.89 [0.4-0.6]
-FT4 (pmol/L)	**9.0** [10-23]	**10.6** [11-25]	16.4 [11-25]	10 [10-19.8]	**8.4** [10-19.8]	12.0 [11.4-22.7]	10.3 [9.0-15.4]	13.9 [11-25]	9.05 [12.5-21.5]	13.35 [12.6-21.5]
-TT4 (nmol/L)	110 [70-150]	67 [58-128]	85 [58-128]	N/A	N/A	N/A	N/A	107 [58-128]	N/A	N/A

Phenotype	D211G	M256T	A263S	A263V	L274P	G291S	N359Y	R384H	P398R	E403K
-FT3 (pmol/L)	N/A	N/A	7.28 [3.8-7.6]	**7.6** [3.5-6.5]	**9.1** [3.5-6.5]	**7.7** [3.5-6.5]	6.14 [3.07-6.14]	**8.08** [3.8-7.6]	5.62 [3.88-8.02]	6.94 [3.93-7.70]
-TT3 (nmol/L)	**3.6** [1.3-2.7]	**2.9** [1.4-2.5]	**3.65** [1.4-2.5]	N/A	N/A	N/A	N/A	**5.20** [1.4-2.5]	N/A	N/A
-rT3 (nmol/L)	**0.09** [0.11-0.44]	**0.18** [0.22-0.54]	0.31 [0.22-0.52]	**<0.07** [0.12-0.36]	**<0.07** [0.12-0.36]	N/A	0.26 [0.22-0.83]	0.31 [0.22-0.52]	N/A	N/A
-T3/T4 ratio	**0.033** [0.01-0.04]	**0.043** [0.01-0.03]	**0.043** [0.01-0.03]	N/A	N/A	N/A	N/A	**0.049** [0.01-0.03]	N/A	N/A
Reference	*(27)*	*(37)*	*(28)*	*(36)*	*(36)*	*(39)*	*(34)*	*(28)*	*(29)*	*(29)*

Abnormal values are indicated in bold. [GA, gestational age; Ht, height; HC, head circumference; SDS, standard deviation score; M, male; F, female; NR, normal range; N/A, data not available]

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