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To my beloved family

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ABSTRACT

The thyroid gland secretes thyroid hormones (TH), which have a crucial role in metabolism, as well as childhood brain growth and development. The thyroid stimulating hormone (TSH) via its G protein-coupled receptor (TSHR) is the main regulator of thyroid function. Overactivation of TSHR leads to hyperthyroidism and thyroid growth, while impaired activity leads to hypothyroidism. These thyroid disorders (TD) are common and affect over 10 % of the population during their lifetime. However, the detailed etiology of these diseases remains largely unknown. In this thesis work, we aimed to identify and study novel genetic mechanisms of congenital thyroid disorders. We first developed a high-throughput sequencing panel to screen and identify genetic etiology of familial and congenital hypothyroidism. Using this screening panel, we found several known mutations in congenital hypothyroidism candidate genes and also several inactivating and activating mutations in *TSHR*, in patients with congenital hypo- and hyperthyroidism. To better understand the molecular mechanisms of TSHR mediated TD, we generated and analyzed the phenotypes of different disease models affecting TSHR signaling. These models included mice with inducible thyroid-specific deletion of $G\alpha_s$ protein, and models carrying gain-or loss-of-function mutations in the *TSHR* gene. In the study, we demonstrate that partial thyroid-specific deletion of $G\alpha_s$ leads to rapid hypothyroidism, as well as hyperplasia and papillary thyroid cancer- like tumors, due to the highly elevated TSH and remaining $G\alpha_s$ in some thyrocytes. Furthermore, we show that congenital hypo- or hyperthyroidism play a role in adrenal development and function. In summary, we have generated new screening tools and identified novel pathogenic mutations in newborns with congenital TD. Furthermore, our novel thyroid disease models have revealed new insights into TSHR signaling in the pathogenesis of thyroid tumors and adrenal function.

Keywords: congenital hypothyroidism, hyperthyroidism, adrenal gland, constitutively activating mutations, papillary thyroid cancer

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Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Kilpirauhasen tuottamat kilpirauhashormonit ovat välttämättömiä kasvun, kehityksen ja aineenvaihdunnan säätelylle. Kilpirauhasen toiminnan keskeinen säätelijä on aivolisäkkeen tuottama tyreotropiini (TSH), jonka vaikutus välittyy TSH reseptorin (TSHR) kautta. Häiriöt kilpirauhashormonien tuotannossa voivat johtaa kilpirauhasen vajaa- tai liikatoimintaan. Nämä kilpirauhassairaudet ovat yleisiä ja koskettavat yli 10% ihmisistä. Kilpirauhassairaudet ovat yleensä autoimmuunisairauksia, mutta mutaatiot *TSHR* ja *GNAS1* geeneissä ovat yleisiä ei-autoimmuuniperäisissä kilpirauhassairauksissa. Kehittyneistä sekvensointitekniikoista huolimatta kilpirauhassairauksien syyt ovat edelleen suurelta osin tuntemattomia. Tässä väitöskirjaprojektissa, tavoitteenamme oli uusien säätelijägeenien etsiminen ja niiden toiminnan tutkiminen. Ensimmäisessä osatyössä kehitimme geenipaneelin tunnettujen geenivariaatioiden löytämiseksi. Geenipaneelin avulla tunnistimme geneettisen syyn suurimmalta osalta familiaalisista tapauksista ja tunnistimme useita *TSHR* mutaatioita. Ymmärtääksemme paremmin TSHR:n toimintaa kehitimme tautimalleja, joissa TSHR:n välittämää signalointireittiä oli geneettisesti muunneltu. Tautimallissa, jossa hiiren kilpirauhassoluista oli poistettu Gs-proteiinin α- alayksikkö ($G\alpha_s$), hiirille kehittyi muutamassa viikossa kilpirauhasen vajaatoiminta. Yllättäen hiirten kilpirauhasiin alkoi muodostua myös kilpirauhasen papillaarisen karsinooman kaltaisia muutoksia. Viimeisessä osatyössä tutkimme TSHR-välitteisiä hypo-ja hypertyreoosiimalleja. Yllättäen hypertyreoosi-mallin naarailla oli voimakkaasti suurentunut lisämunuainen, kun taas vajaatoiminassa lisämunuainen oli vajaakehittynyt. Jatkoanalyyseissä selvisi, että kilpirauhashormonit ovat tärkeitä hiiren ja ihmisen lisämunuaisen kehityksessä. Yhteenvetona väitöskirjatyössä uusina löydöksinä paljastui TSHR:n signaloinnin merkitys kilpirauhasen tuumorigeneesissä ja kilpirauhashormonien rooli lisämunuaisen kehityksessä.

Avainsanat: Kilpirauhashormoni, kilpirauhassairaus, tyreotropiini reseptori, lisämunuainen, aktivoiva mutaatio, hypotyreoosi, hypertyreoosi

Table of Contents

Abbreviations

TH	Thyroid hormones
T_4	Thyroxine
T_3	3,3',5-triiodo-L-thyronine
TSH	Thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
CH	Congenital hypothyroidism
IP3	Inositol 1,4,5-triphosphate
DAG	diacylglycerol
TD	Thyroid disorders
NKX2.1	NK2 Homeobox 1
PAX8	Paired box 8
FOXE1	Forkhead Box E1
TG	Thyroglobulin
TPO	Thyroid peroxidase
SLC26A7	Solute Carrier Family 26 Member 7
SLC5A5	Solute Carrier Family 5 member 5,
NIS	Sodium-iodide symporter
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
HPT	Hypothalamic-pituitary-thyroid
TRH	Thyrotropin-releasing hormone
PVN	Paraventricular nuclei
TSHβ	Thyroid-stimulating hormone subunit beta
rT_3	Reverse T_3
Dio (;-1;-2;-3)	Deiodinase; -1; -2; -3
TRα; - β	Thyroid hormone receptor alpha ; - beta
GPCRs	G protein coupled receptors
GRKs	G protein-coupled kinases
FSHR	Follicle-stimulating hormone receptor
GNAS	Guanine nucleotide binding protein, alpha subunit
XLαs	$G\alpha_s$, extra-large
NESP55	Neuroendocrine secretory protein 55

GNAS-AS1	GNAS-antisense RNA 1
AHO	Albright Hereditary Osteodystrophy
MAS	McCune-Albright
PHP	pseudohypoparathyroidism
GH	Growing hormone
FD	Fibrous dysplasia
p44/42 MAPK	p44/42 mitogen-activated protein kinase
c-Fos	Fos Proto-oncogene
I^-	iodide
SLC26A4	Solute Carrier Family 26 Member 4 (pendrin)
ANO1	Anoctamin-1
DUOX	Dual oxidase
ThOX2	Thyroid oxidase
MIT	monoiodotyrosine
DIT	diiodotyrosine
MCT8; -10	Monocarboxylase transporter 8; -10
DEHAL1	Iodotyrosine dehalogenase 1
IYD	Iodotyrosine deiodinase
T1	monoiodothyronine, I-Tyr
T2	diiodothyronine, I_2-Tyr
GPx3	Glutathione peroxidase 3
TREs	Thyroid response elements
RXR	Retinoid X receptor
MAPK1; -2	Mitogen-activated protein kinase 1; -2
PI3K	Phosphatidylinositol 3-kinase
OATP1C1	Organic anion-transporting polypeptide 1C1
SLC17A4	Solute carrier family 17 member 4
LAT	Large neutral amino acid transporter
SLC10A1/NTCP	Solute carrier family 10 member 1 (NTCP)
PTC	Papillary thyroid cancer
FTC	Follicular thyroid cancer
ATC	Anaplastic thyroid cancer
MTC	Medullary thyroid cancer
BRAF	B-Raf proto-oncogene
RET	RET gene rearrangements
PPARγ	Peroxisome proliferator-activated receptor γ
NTRK	Neurotrophic tyrosine receptor kinase
ERT2	Estrogen receptor triple mutant 2
CRISPR	Clustered regularly interspaced short palindromic repeats
PTU	propylthiouracil
bTSH	Bovine TSH
HOM	Homozygous
ZG	Zona glomerulosa
ZF	Zona fasciculata

ZR	Zona reticularis
DHEA;-S	dehydroepiandrosterone; - sulfate
CYPs	Cytochrome P450
Cyp51	Lanosterol 14α-demethylase
StAR	Steroid acute regulatory protein
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP21A2	Cytochrome P450 family 21 Subfamily A Member 2
11-DOC	11-deoxycorticosterone
HSD3B	3β-hydroxysteroid dehydrogenase
CYP17A1	Cytochrome P450 family 17 subfamily A member 1
CYP11B2;-B1	Cytochrome P450 family 11 subfamily B member 2
HET	Heterozygous
fT_4	Free T_4
20αHSD	20-α-hydroxysteroid dehydrogenase
PA	Premature adrenarche
uS-TSH	Umbilical serum - TSH
FCS	Fetal calf serum
iBMX	3-isobutyl-1-methylxanthine

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Löf C., Patyra K., Kuulasmaa T., Vangipurapu J., Undeutsch H., Jaeschke H., Pajunen T., Kero A., Krude H., Biebermann H., Kleinau G., Kuhnen P., Rantakari K., Miettinen P., Kirjavainen T., Pursiheimo J.P., Mustila T., Jääskeläinen J., Ojaniemi M., Toppari J., Ignatius J., Laakso M., Kero J. 2016. Detection of Novel Gene Variants Associated with Congenital Hypothyroidism in a Finnish Patient Cohort. Thyroid. 2016. 26(9): 1215–24.

II Patyra K., Jaeschke H., Löf C., Jännäri M., Ruohonen S.T., Undeutsch H., Khalil M., Kero A., Poutanen M., Toppari J., Chen M., Weinstein L.S., Paschke R., Kero J. Partial thyrocyte-specific Gs deficiency leads to rapid-onset hypothyroidism, hyperplasia, and papillary thyroid carcinoma-like lesions in mice. FASEB J. 2018. 32(11): 5761-6354.

III Patyra K., Löf C., Jaeschke H., Undeutsch H., Zheng H., Tyystjärvi S., Pulawska K., Doroszko M., Chrusciel M., Loo B.M., Kurkijärvi R., Zhang FP., Huang C.J., Ohlsson C., Kero A., Poutanen M., Toppari J., Paschke R., Rahman N., Huhtaniemi I., Jääskeläinen J., Kero J. Congenital hypothyroidism and hyperthyroidism alters adrenal gene-expression, development, and function. Thyroid. 2022. 32(4): 459-471.

1 Introduction

The thyroid gland produces the thyroid hormones (TH) thyroxine (T_4) and 3,3',5-triiodo-L-thyronine (T_3), which play an important role in the development and physiology of many organs. The main regulator of thyroid growth and function is the thyroid-stimulating hormone (TSH) or thyrotropin, which acts via its G-protein-coupled receptor (TSHR) on the basolateral membrane of the thyrocyte. By binding to its receptor, TSH activates an adenylyl cyclase-cAMP system mediated by the $G\alpha_s$ protein. Moreover, at high concentrations TSH can also trigger signaling via inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) as a result of $G\alpha_q$ coupling to the TSHR (Szkudlinski et al., 2002).

Disturbances in thyroid function can lead to thyroid disorders (TD), which are one of the most common endocrine diseases. TH deficiency (hypothyroidism) affects up to 10% of the general population (Chaker et al., 2017; Chiovato et al., 2019; Lebastchi & Callender, 2014; Leo et al., 2016). It decreases the metabolism rate and associates with significant excess of comorbidity and mortality (Chaker et al., 2017; Taylor et al., 2018). Furthermore, lack of TH at birth leads to congenital hypothyroidism (CH), which can impair normal growth and brain development. The etiology of hypothyroidism can be linked to a lack of iodine, genetic mutations, or damage to the thyroid gland. The excess of TH leads to hyperthyroidism which affects up to 3% of individuals in the general population and it is 5-10 times more common in women than men. Hyperthyroidism is typically characterized by tachycardia, nervousness, an increased metabolic rate and weight loss (Devereaux & Tewelde, 2014; Guerri et al., 2019; Leo et al., 2016). An overactive thyroid function may affect virtually any organ, and lead to excess comorbidity and mortality. It is potentially lethal due to cardiac complications, if not treated appropriately (Brandt et al., 2011). Hypo- and hyperthyroidism are mainly related to autoimmune diseases and less commonly, to genetic disorders (Chaker et al., 2017; Leo et al., 2016), but the cause for these diseases remains largely unknown. In addition to hypo - and hyperthyroidism, thyroid goiter, nodules, and cancer are common manifestations of TD. Overall, thyroid cancer represents 95% of all diagnosed endocrine tumors, with an increasing incidence number every year. In 2021, 52,890 cases were diagnosed in

the United States and 4.1% died from the disease (J. Hu et al., 2021). In general, the diagnosis of TD is crucial especially in newborns as e.g., it can prevent the destructive consequences of TH deficiency leading to irreversible impairment of brain function or growth (Kurian & Jungbluth, 2014). Understanding the genetic etiology or the pathophysiology of TD in more detail, would help to develop novel treatment and prevention options for TD.

In this PhD thesis, we aimed to identify and study novel genetic mechanisms of TD. We first developed a high-throughput sequencing panel to screen genetic variations in patients with syndromic, familial, or congenital hypo- and hyperthyroidism. In addition, to better understand the molecular mechanisms of TSHR mediated TD, we generated and analyzed the phenotypes of different disease models affecting TSHR signaling. These models included mice with inducible thyroid-specific deletion of $G\alpha_s$ protein, and models carrying a gain-or loss-of-function mutations in the TSHR gene. Using these models, we describe the impact of thyroid-specific deletion of $G\alpha_s$ protein on thyroid function and growth in more detail. In addition, we characterized the unexpected finding of enlarged adrenal glands in hyperthyroid mice carrying an activating mutation in the TSHR and evaluate the impact of congenital hypo- or hyperthyroidism in adrenal development and function. Finally, to understand the possible role of TH in human adrenals, the TSH, TH and adrenal function was evaluated in patients with CH and premature adrenarche (PA).

To conclude, this thesis presents the new tools we generated to screen the genetic etiology of congenital TD and the novel disease models developed to reveal more insights into TSHR signaling.

2 Review of the Literature

2.1 Development of the thyroid gland

An overview of the thyroid gland development is illustrated in Figure 1. Its development starts from foregut endoderm around post-fertilization day 20 and embryonic day 8.5 in humans and mice, respectively. The cells located on the floor of the primitive pharynx, start to express thyroid -specific transcription factors including mainly Nkx2.1, Pax8 and Foxe1, then form thyroid anlage which subsequently emerges as a bud. This thyroid primordium, then detaches and migrates to the pharyngeal endoderm located in the trachea. During a lobulation process, the thyroid cells proliferate and expand laterally to the moment when the thyroid reaches the correct position. In the last step of the thyroid development termed folliculogenesis, the thyroid follicles are formed and followed by the expression of thyroid-specific genes like *TG*, *TPO*, *TSHR*, *SLC26A7* and *SLC5A5* and the initiation of TH synthesis. A mature thyroid gland consists of functional units, colloid filled thyroid follicles surrounded by thyroid follicular cells, thyrocytes. Thyroid follicles are organized in humans into two lobes connected by an isthmus. In addition to the thyroid follicles and stromal cells, the thyroid gland consists of parafollicular cells (C-cells), which produce calcitonin (Fernández et al., 2014; M. Nilsson & Fagman, 2017; M. Nilsson & Williams, 2016). Any failure in the thyroid development process can appear as thyroid dysgenesis, which can be present as thyroid agenesis, hypoplasia or hemithyroid and manifest as a lack of TH at birth (de Felice & di Lauro, 2004).

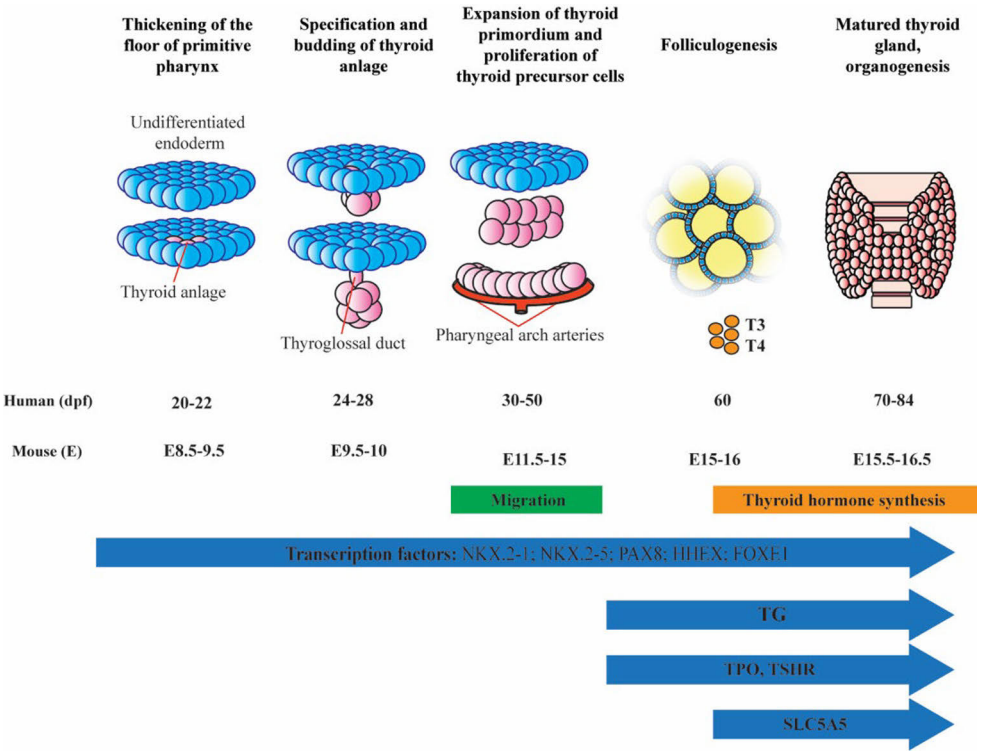


Figure 1. Development of the thyroid gland. Abbreviations: dpf – day post-fertilization; E – embryonic day. Modified from publication (Löf et al., 2018).

2.2 Regulation of the thyroid function

2.2.1 Thyroid-stimulating hormone (TSH)

TSH also known as thyrotropin, is the main thyroid regulator, which mediates its action via its receptor (TSHR) localized on the basolateral membrane of thyrocytes. TSH regulates thyrocyte proliferation, growth, and development, and activates TH synthesis and secretion (Nagayama, 2017). TSH is a 28kDa glycoprotein hormone belonging to cystine-knot growth factor superfamily. It is a heterodimer protein composed of alpha 116 amino acids (aa) and beta (138 aa) subunits, linked together with a non-covalent bond. TSH, luteinizing hormone (LH), follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) all share the same α-subunit, whereas the different ß-subunits create the high specificity of these

hormones. However, in very high concentrations during the beginning of pregnancy hCG can activate TSHR (Rodien et al., 2004).

2.2.2 Hypothalamic-pituitary-thyroid (HPT) axis

TSH is secreted from the anterior pituitary, which together with the hypothalamus form the hypothalamic-pituitary-thyroid (HPT) axis as shown in Figure 2. The HPT axis is an endocrine negative feedback regulation system by which the body can regulate and adapt the TH synthesis and secretion to respond to physiological needs and maintain homeostasis. In this regulation system, the hypothalamus secretes thyrotropin-releasing hormone (TRH) as a 242 aa polypeptide precursor from hypophysiotropic TRH neurons located in the paraventricular nuclei (PVN). TRH precursor proteins undergo processing to biologically active tripeptide (pyroGlu-His-Pro amide) TRH, which binds to its receptor and activates the Ca^{2+} mediated release of TSH from the thyrotroph cells in the anterior pituitary (Fekete & Lechan, 2014; Mendoza & Hollenberg, 2017; Nillni, 2010). Moreover, TRH also stimulates TSHβ expression, and participates in posttranslational maturation of the TSH oligosaccharide chains (Bargi-Souza et al., 2015). The TSH then stimulates its own receptor (TSHR) expressed on the thyrocytes and regulates the TH synthesis and secretion into the bloodstream (Feldt-Rasmussen et al., 2021). The human thyroid gland secretes mainly T_4 hormone, (approximately 80 µg/day) and a lesser amount of the more active metabolite T_3 (2 µg), and an even lesser amount of other TH metabolites. A large part of secreted T_4 is then converted to T_3 in peripheral tissues. Via the negative feedback loop, T_3 inhibits TRH and further TSH expression and secretion (Fekete & Lechan, 2014; Mendoza & Hollenberg, 2017; Segerson et al., 1987). Strict TH concentration in the hypothalamus is controlled via specialized glial cells called tanycytes. The tanycytes are found in ventrolateral walls and floor of the third ventricle, which is located in the brain (Fekete & Lechan, 2014; Lechan & Fekete, 2007). As shown by Müller-Fielitz et al. (2017), activation of TRH receptor 1, increases intracellular Ca^{2+} concentration in the β-tanycytres of median eminence, as a results of $G\alpha_{q/11}$ signaling. Morevoer, activation of $G\alpha_{q/11}$-mediated signaling pathway leads to changes of tanycyte endfeet and induces activity of TRH-degradading ectoenzymes, thus it leads to downregulation of TRH release into pituitary blood vessels (Müller-Fielitz et al., 2017). Interestingly, De Castro et al. (2015) using thyroidectomized rats, have demonstrated that LT-4 induced ubiquitination of Dio2 in the hypothalamus is much more lower than in peripherial tissues (de Castro et al., 2015). TH receptors (TRα1, β1 and β2) play an important role in TRH regulation, TRβ2 is the most important isoform involved in TRH

negative regulation and TSH expression regulation in the pituitary (Abel et al., 1999, 2001; Dupré et al., 2004). Pituitary TSH can also inhibit the TRH and TSH release via shorter feedback loops (Figure 2). In the local or ultra-short loop feedback control, TSH inhibits its own release, by binding to its receptors located in the pituitary, without affecting other pituitary hormones like FSH (Leoni et al., 1971; Solomon & Mckenzie, 1966). The possible role of the ultra-short feedback loop is precise regulation of released hormones by weakening oscillations in their serum levels, by short TSH burst, rather than long prolonged TSH rise till the T_4 concentration returns to normal. Moreover, the ultra-short feedback loop can generate pulsatility of pituitary hormones (Prummel et al., 2004).

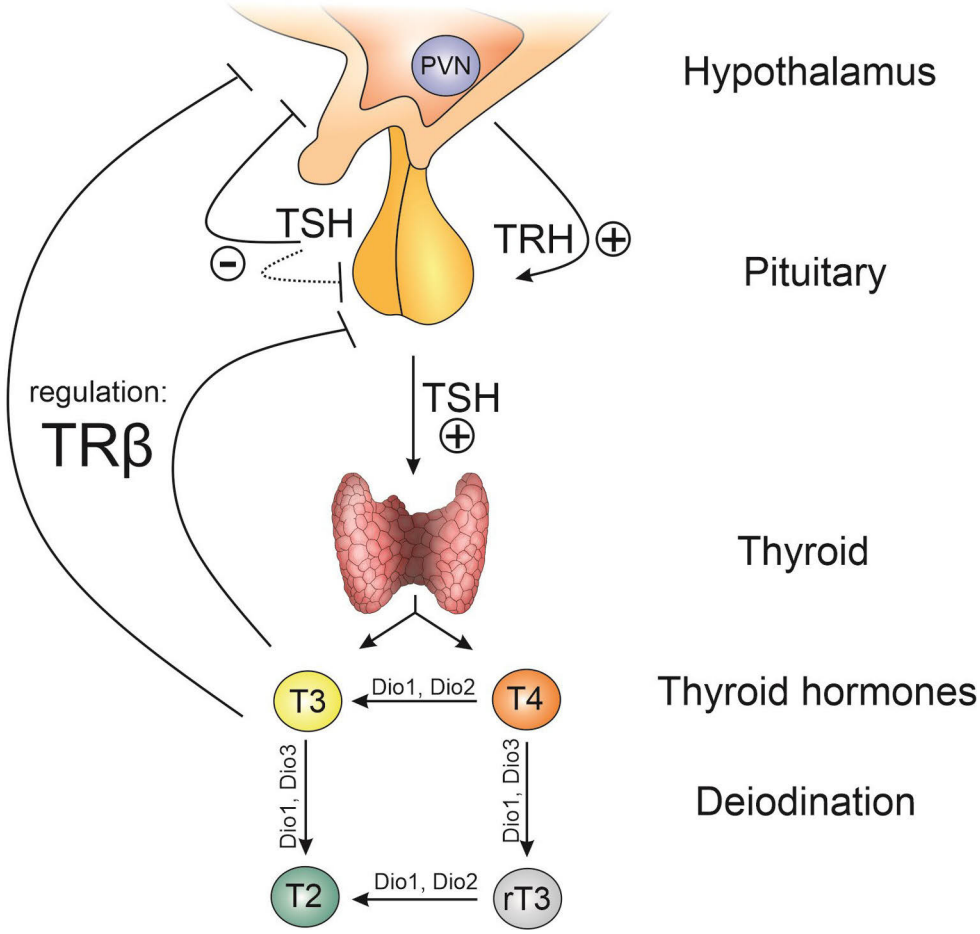


Figure 2. Regulation of hypothalamus-pituitary-thyroid (HPT) axis. The dotted line represents the ultra-short feedback loop. Abbreviations: PVN – Paraventricular nucleus; TSH – Thyroid-stimulating hormone; TRH – Thyrotropin-releasing hormone; T4 – Thyroxine; T3 – Triiodothyronine; rT3 – reverse T3; TRß – Thyroid hormone receptor beta; Dio – deiodinase.

2.2.2.1 Thyroid-stimulating hormone receptor (TSHR) as a member of the G protein coupled receptor (GPCR) family

G protein-coupled receptors (GPCRs) are the largest cell surface protein receptor superfamily involved in the regulation of virtually all physiological functions such as vision, taste, olfaction or cellular response to hormones and neurotransmitters (Rosenbaum et al., 2009; Zhao & Furness, 2019). GPCRs are classified as follows: Class A (Rhodopsin-like), Class B (Secretin receptor), Class C (Metabotropic glutamate), Class D (Fungal mating pheromone receptors), Class E (cAMP receptors) and Class F (Frizzled/Smoothened) (Alexander et al., 2015).

The main function of the GPCRs is to provide communication between the extra- and intracellular environment by triggering a signal transduction and resulting in a specific cell response initiated by extracellular stimuli. Upon binding an agonist, GPCR changes its conformation and activates a specific heterotrimeric G protein(s) and activates multiple signaling pathway(s) (Cabrera-vera et al., 2003). Despite the structural similarity, GPCRs can initiate variable molecular responses due to the fact that they can bind to different G protein subunits and activate unique cellular responses (Rosenbaum et al., 2009). However, GPCRs can also trigger a signal transduction without coupling to the G proteins. For example, beta-arrestins were identified to modulate signaling independently from G proteins leading to receptor desensitization and activating G protein independent signaling pathways (Wilden et al., 1986). The binding of beta-arrestins to GPCRs occur after the phosphorylation of GPCR by G protein-coupled kinases (GRKs), which phosphorylate the receptor (Pitcher et al., 1998). Furthermore, many of the GPCRs have basal activity, which can be modulated by an agonist.

The GPCRs exhibit common seven transmembrane hydrophobic α-helical domains, with an extracellular amino (N-) terminus and an intracellular carboxyl (C-) terminus. GPCRs show a similar structure pattern and mechanisms of signal transduction despite the differences in length and sequence (Lu & Wu, 2016). The N-terminus shows a diversity among receptors and is composed of three extracellular loops (ECL 1-3) which form a binding pocket for a ligand. The intracellular C-terminus is composed of three intercellular loops (IC 1-3) and interacts with G proteins, β-arrestins, GRKs and other downstream signaling proteins (D. Zhang et al., 2015).

2.2.2.2 Thyroid-stimulating hormone receptor (TSHR)

One of the most important regulator of TH synthesis and secretion is the thyroid stimulating hormone and its receptor (TSHR) or thyrotropin receptor, which belongs to the glycoprotein hormone class A (Kleinau et al., 2017). Evolutionary, the TSHR structure resembles a luteinizing hormone receptor (LHR) and a follicle-stimulating hormone receptor (FSHR). However, TSHR has two unique aa sequences, the 8 aa sequence close to the N-terminus and the 50 aa sequence close to the C-terminus (Heckert et al., 1992; Marians et al., 2002). The TSHR undergoes a cleavage into two subunits, extracellular A, and transmembrane-intracellular subunit B, which are subsequently connected with a disulfide bond. Subunit A containing leucine-rich domains interacts directly with the TSH, whereas the intracellular part of the subunit B interacts with signaling proteins (Furmaniak et al., 1987; Vassart & Dumont, 1992). TSHR plays an important role in thyroid cell proliferation and in virtually every step of TH synthesis and secretion. TSH stimulation through TSHR, activates the transcription of genes involved in TH synthesis like sodium-iodide symporter (SLC5A5, NIS) (Riedel et al., 2001) and promotes iodine uptake (Levy et al., 1997), thyroperoxidase (TPO) (Gerard et al., 1988) and thyroglobulin (Tg) (Heuverswyn et al., 1984) synthesis, and can also promote micropinocytosis leading to Tg internalization and TH secretion (Wetzel et al., 1965).

2.2.2.3 Gnas locus and mutations

Gnas is a complex imprinted gene located in humans on chromosome 20 (20q13.32) (Gejman et al., 1990), encoding many proteins: alpha subunit of the $G\alpha_s$, extra-large $G\alpha_s$ (XLαs) (Kehlenbach et al., 1994), neuroendocrine secretory protein 55 (NESP55) (Ischia et al., 1997), antisense GNAS-AS1 (Hayward & Bonthron, 2000) and noncoding A/B (also known as 1A/1A', respectively) (Ishikawa et al., 1990; Swaroop et al., 1991). The $G\alpha_s$ protein is encoded by common exons 2-13 and unique exon 1. In most tissues, Gnas imprinting occurs in both maternal and paternal alleles, however, imprinting can also occur exclusively on one of the alleles. In the case of the maternal Gnas the imprinting is typically present in the renal proximal tubules, the pituitary, the ovary paraventricular nucleus of the hypothalamus and the thyroid, while the paternal allele is silenced in these tissues (Germain-lee et al., 2002; Weinstein et al., 2001). The structure, imprinting and $G\alpha_s$ mutations of the GNAS locus is shown in Figure 3.

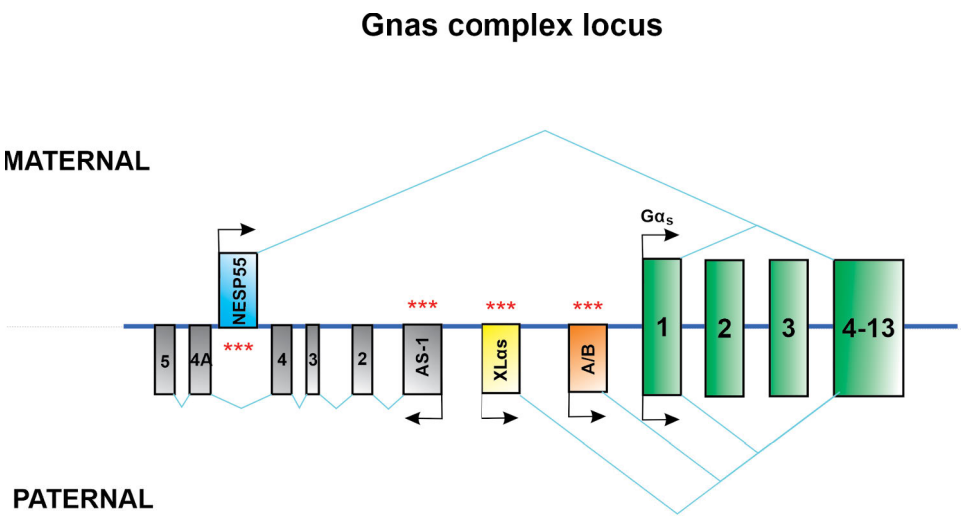


Figure 3. Gnas complex locus and its imprinting. Abbreviations: NESP55 – Neuroendocrine secretory protein-55; AS-1 – Gnas antisense RNA 1; XLαs – Extralarge XL (alpha) s; A/B – Gnas Exon A/B; $G\alpha_s$ – G alpha (s) subunit; *** – methylated promoters. Modified from the (Hartley et al., 2019).

Inactivation or gain-of-function mutations in the GNAS leads to Albright Hereditary Osteodystrophy (AHO) and the McCune-Albright (MAS) syndrome, respectively. AHO was originally described by Fuller Albright and colleagues, as a genetic disorder characterized by short statue with a stocky habitus, round face, brachydactyly and subcutaneous ossification (Albright F. et al., 1942). The AHO disease is caused by heterozygous inactivating mutation in the *GNAS1* gene, which leads to disruption in cAMP production and signaling. The pseudohypoparathyroidism (PHP) is defined as an inadequate response to the parathyroid hormone and is linked to dysfunctional $G\alpha_s$ protein. PHP can be typically classified into Type 1 and Type 2, and the further subtypes 1a, 1b and 1c. Methylation defects in *GNAS* are also involved in PHP etiology (Turan & Bastepe, 2015). There is no specific type of therapy for AHO, however, the treatment aims at normalizing calcium and hormonal concentrations, including PTH and if needed TSH, growing hormone (GH) and gonadotropins (Weinstein et al., 2013).

MAS is a rare complex genetic disorder described originally as a clinical triad of polyostotic fibrous dysplasia of bone (FD), *café-au-lait* skin pigmentation, and precocious puberty (McCune et al., 1937). The disease is caused by early postzygotic gain-of-function somatic mutation in the *GNAS1* gene, leading to increased cAMP production (Boyce & Collins, 2020; Landis et al., 1989). The disease prevalence is between 1:100 000 and 1:1 000 000 (Boyce & Collins, 2020; Dumitrescu & Collins, 2008). Clinical feature of the MAS syndrome can be wide and might include several

endocrine manifestations like hyperthyroidism, acromegaly, and Cushing's syndrome. The disease is diagnosed on a clinical basis. Treatment is targeted at the affected tissues, and endocrinopathies (Boyce & Collins, 2020; Dumitrescu & Collins, 2008).

2.2.2.4 Structure, classifications, and role of G-proteins in the thyroid gland

G-proteins also known as guanine nucleotide-binding proteins, are a superfamily of highly conserved proteins involved in signal transduction between agonist-GPCR and intracellular effectors. Structurally, G-proteins are heterotrimers formed with α, ß and γ subunits. Furthermore, based on the α subunit they can be divided into four main families: $G\alpha_s$, $G\alpha_q$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$. A subunit α family contains 39-52 kDa protein members and is composed of a GTP-binding domain and a helical domain which conceals GTP from the protein. The remaining two subunits ß and γ, form a dimer and increase the Gα subunit's affinity for its receptor and regulates many functions, including the recruitment of GRKs on the cell membrane (Hamm & Annette, 1996). The huge diversity amongst G proteins comes from the fact that in humans each G protein might be composed of one of sixteen different subunits of Gα, five of Gß and thirteen of Gγ; this in practice give rise to many possible combinations, required for connecting specific receptors to signaling pathways (Cabrera-vera et al., 2003; Wootten et al., 2018).

Hu & Shokat (2018) recently showed that the most common cancer-mutation the R201C mutant is not in a persistent GTP-bound state. Specifically, the $G\alpha_s$ R201C mutant in the presence of the βγ subunits can activate adenylyl cyclase (AC) when bound to the GDP. Conversely, the mutant omits GTP binding by direct activation of GDP-bound $G\alpha_s$ by stabilization of an intramolecular hydrogen bond network (H-bond network). This finding may corrupt the canonical roles of GDP and GTP and demonstrate a possible novel regulation mechanism of G-proteins (Q. Hu & Shokat, 2018).

TSHR is a typical GPCR and mainly leads to the activation of $G\alpha_s$-mediated signaling. In earlier studies, using human thyrocytes, TSHR has been shown to couple all G-protein alpha subfamilies (Laugwitz et al., 1996), but a physiological role of other than that of $G\alpha_s$ has been unclear. The schematic G protein signaling in the thyroid is shown in Figure 4. In later studies, the $G_{q/11}$ role has been shown at higher TSH concentrations, and goiter growth in mouse models (Allgeier et al., 1994).

Our previous work on mice suggested that thyrocyte-specific $G_{q/11}$ deficient mice had reduced iodine organification, TH secretion and a normal proliferative TSH response (Kero et al., 2007). Furthermore, in vitro studies have demonstrated the role of $G\alpha_{13}$ proteins in human thyrocytes and shown p44/42 MAPK activation and induction of c-Fos via $G\alpha_{13}$ (Buch et al., 2008). In contrast, in our previous in vivo analysis the thyrocyte-specific $G_{12/13}$ deficient mice had normal TSH and TH concentrations (Löf et al., 2018). However, TSHR has been shown to activate virtually all four G protein families (Laugwitz et al., 1996). In subsequent studies, the Gq/11 role has been shown at higher TSH concentrations and triggering goiter growth in mouse models (Allgeier et al., 1994; Kero et al., 2007). In addition, decreased expression of $G\alpha_{2i}$ was detected in the hyperthyroid gland when compared to normal tissue in cats (Nishida et al., 2000).

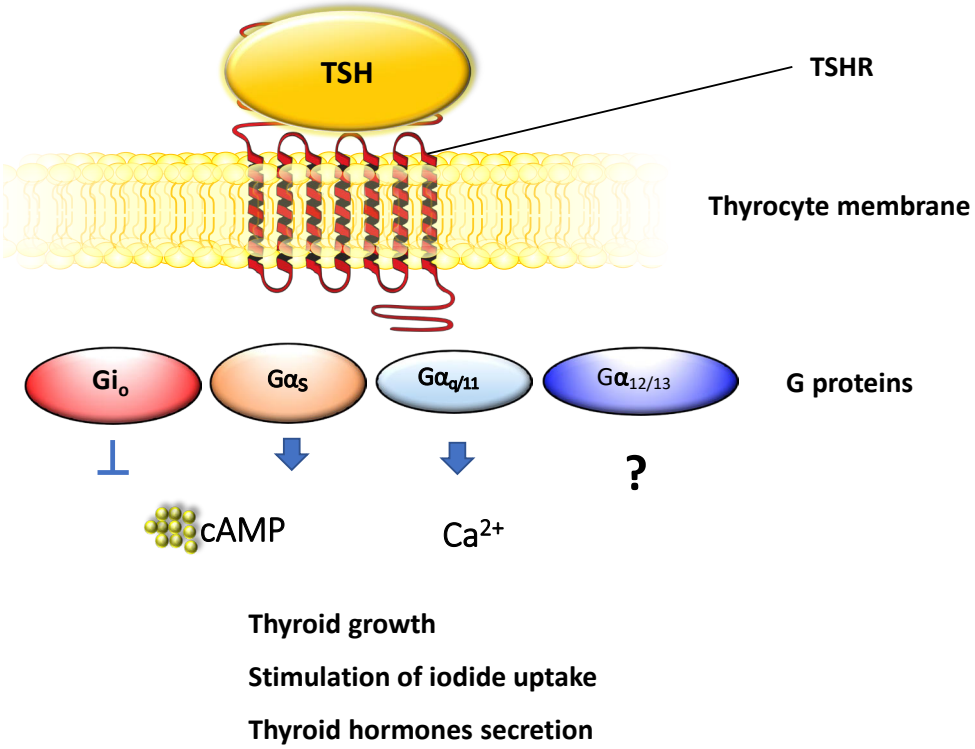


Figure 4. TSH signaling in the thyroid gland. Abbreviations: TSH – Thyroid-stimulating hormone; TSHR – Thyroid-stimulating hormone receptor; Guanine nucleotide-binding protein alpha (Gα): i_o, s, q/11, and 12/13.

The cAMP signaling pathway is the main signaling pathway in the thyrocytes. Upon binding of TSH to its receptor, activates the AC, leading to the production of

secondary messengers i.e. cAMP. Furthermore, it has been shown that high TSH concentrations also activates $G_{q/11}$, leading to the stimulation of phospholipase C, which subsequently leads to an increase in intracellular calcium accumulation and signal transduction (Allgeier et al., 1994; Laurent et al., 1987; Shaver et al., 1993).

Somatic activating mutations in the $G\alpha_s$ protein were previously identified in autonomously hyperfunctioning thyroid adenomas. To date, two hot-spot mutations in exon 8 (codon 201) and exon 9 (codon 227) were found, leading to inhibition of GTPase activity and constitutive activation of adenylyl cyclase. Mutation in codon 201 replaces arginine with histidine (R201H), or cysteine (R201C), whereas mutation in codon 227 leads to the replacement of glutamine with leucine (Q227L), or arginine (Q227R) (M. Murakami et al., 1999).

In vivo studies using a thyroid specific B-Raf proto-oncogene $(BRAF)^{V600E}$ model have shown that inactivating $G\alpha_s$ or TSHR signaling strongly attenuates tumor development, but the deletion of the $G\alpha_s$ does not fully prevent tumor development, suggesting that other TSH-activated pathways may also play a substantial role (Franco et al., 2011).

2.2.3 Thyroid hormone synthesis, storage, and secretion

The human thyroid gland mainly secretes thyroxine (T_4, 3,3',5,5'-tetraiodo-L-thyroxine, approximately 80 µg/day) and lesser amounts of the more active metabolite triiodothyronine (T_3, 3,3',5-triiodo-L-thyronine, approximately 2 µg/day), and even lesser amounts of other TH metabolites like reverse triiodothyronine (rT_3). TH synthesis involves absorption of the ingested iodine and the uptake of iodide by thyrocytes and further iodination of the tyrosyl residues on the precursor protein Tg to synthetise T_3 and T_4 as illustrated in Figure 5. The TH synthesis rate mainly depends on iodine and selenium availability, and TSH-mediated stimulus (Köhrle, 2005). Before the TH synthesis, most of the iodine forms undergo reduction in the gut to iodide (I^-). TH synthesis starts with iodide (I^-) actively transported from the blood via a sodium-iodide symporter (NIS, SLC5A5), which is expressed on the basolateral site of thyrocytes. NIS cotransports two sodium ions along with one I^- using a sodium gradient generated by Na^+/K^+-ATPase (Chung, 2014). At the apical site of thyrocyte, I^- is transported into the follicular lumen via a $Cl^-/HCO3^-$ anion exchanger called pendrin (SLC26A4). However, I^- uptake and efflux can be mediated with SLC26A7 and ANO1, respectively, and the exact mechanisms by which I^- transport via apical membrane occurs is debatable (Cangul

et al., 2018; Iosco et al., 2014; Ishii et al., 2019). Inside the follicle lumen, TPO catalyze two-electron oxidation of I^- to I^+ by H_2O_2.The reaction occurs under high H_2O_2 concentration, produced by the dual oxidase 2 enzyme (DUOX2 or ThOX2). Proper DUOX2 function is maintained by DUOXA2, which plays an essential role in DUOX maturation and activation (Grasberger & Refetoff, 2006). TPO catalyzed electrophilic substitution of active iodine form to the tyrosine residues of the Tg in a so called organification process, results in the formation of monoiodo- (MIT), and diiodotyrosine (DIT) (T. J. Visser, 2018). Further coupling of the DIT with MIT or DIT, forms T_3 and T_4, respectively. Tg also serves as a storage point for TH, when needed, after which it undergoes endocytosis and proteolysis (with cathepsins B, L, D and exopeptidases) in lysosome; this leads to the cleavage of iodinated thyrosine residues into MIT, DIT, T_3 and T_4, which are then released into the bloodstream (Friedrichs et al., 2003; Weber et al., 2017). The mechanism of TH transportation into the bloodstream is unknown, however monocarboxylase transporter 8 (MCT8) was identified as the main TH transporter in mouse thyroids (di Cosmo et al., 2010). The Tg and iodine molecules can be retrieved with DEHAL1 and deiodinases (Dio), respectively, and reused in TH synthesis. Iodotyrosine deiodinase (IYD) retrieves iodine from robustly synthetized T_1 (monoiodothyronine, I-Tyr) and T_2 (diiodothyronine, I_2-Tyr) (Sun et al., 2017). Compared to other organs, the thyroid gland contains the highest selenium concentration, which play very important role in thyroid physiology. Moreover, at least 11 selenoproteins are expressed, especially GPx3 is exclusively localized in thyrocytes and colloid (Schmutzler et al., 2007). Selenium compounds and selenoproteins protect the thyroid gland from H_2O_2 oxidative stress damage, produced during TH synthesis (Köhrle, 2013).

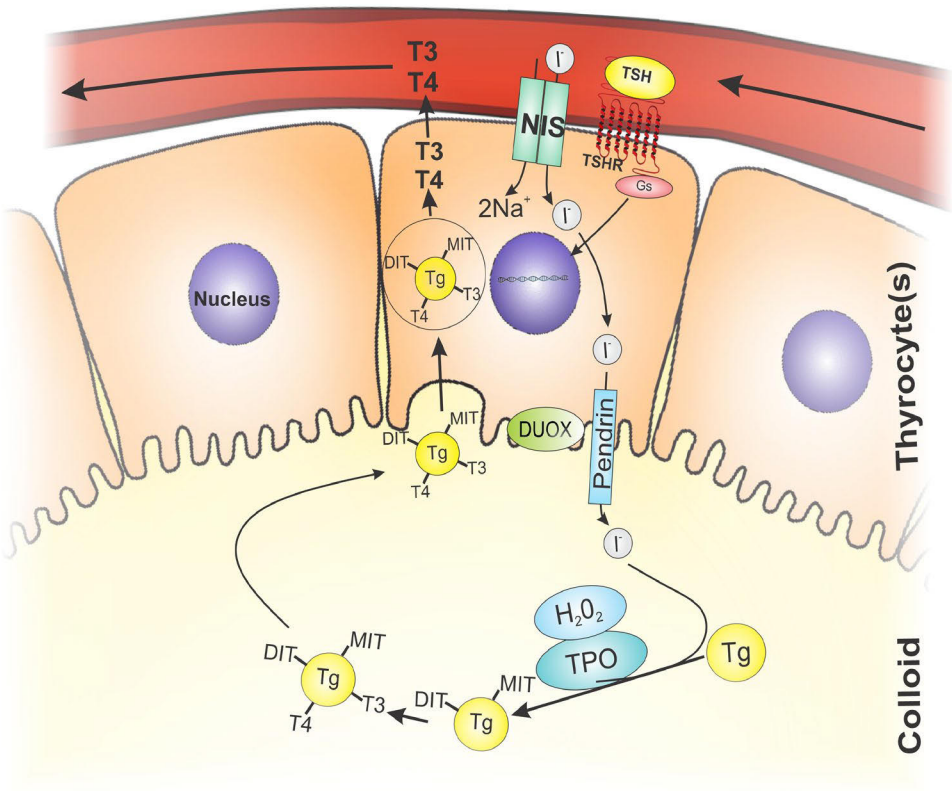


Figure 5. Schematic of thyroid hormone synthesis. Abbreviations: TSH – Thyroid-stimulating hormone; TSHR – Thyroid-stimulating hormone receptor; T3 – Triiodothyronine; T4 – Thyroxine; NIS – Sodium/iodide cotransporter; Gs – Guanine nucleotide-binding protein s; I^- – iodide; Tg – thyroglobulin; MIT – monoidotyrosine; DIT – diiodotyrosine; TPO – thyroidperoxidase.

2.3 Thyroid hormone action

2.3.1 Overview of thyroid hormone action

TH play an important role in many biological processes, including development and metabolism of nearly every organ in the body. Moreover, TH are critical regulators of heat production and reduction in thermogenic tissues (Sentis et al., 2021). Furthermore, they also play a role for example in amphibian metamorphosis (Furlow & Neff, 2006) and thermoregulation during hibernation (Frare et al., 2021). The thyroid gland under TSH regulation produces mostly T_4, and a lesser amount of T_3 (Köhrle, 2019). Small amounts of reverse T_3 and T_2 are also secreted from thyroid (Laurberg, 1978, 1980). Biologically active T_3 is derived from the process of 5'

deiodination by Dio 1 and 2 (Williams & Bassett, 2011). The action of TH can be genomic via specific nuclear thyroid hormone receptors (TRs) which can be classified into type 1 or 2, or non-genomic, which can be classified into type 3 and type 4 (Flamant et al., 2017; Hönes et al., 2017).

TH enters the cell through membrane transport and undergoes Dio-driven modification which varies in different tissues The genomic TH action is triggered via thyroid hormone receptors (TRs), which subsequently bind as a homodimer or heterodimerize with the retinoid X receptor (RXR) to the specific DNA elements called thyroid response elements (TREs) and subsequently to the coactivators or corepressors (O. Bakker, 2004). The TREs are in the promoter region of T_3 target genes. T_3 regulates gene expression by disruption of corepressor binding to the complex and promotion of coactivators binding, what induces polymerase III recruitment and further gene transcription (Brent, 2012). Local ligand availability, transport of TH, expression of TR isoforms, nuclear receptor corepressors and coactivators, sequence and location of thyroid-hormone responsive elements modifies TH action (Brent, 2012).

Non-genomic action of TH can be mediated via integrin αvβ3 on the cell membrane or in the cytoplasm. The integrin αvβ3 can bind TH and subsequently induce gene expression involved in cell proliferation and angiogenesis. Moreover, located in the membrane and cytoplasm is a truncated TRα, named p30 TRα, which binds T_3 and via signal transducing proteins and a nitric oxide system (NOS) system, triggers cellular proliferation. The studies showed that T_3 can activate a sodium-proton exchanger via MAPK1 and MAPK2, and locally activate the sodium pump via phosphatidylinositol 3-kinase (PI3K) /AKT (Davis et al., 2008).

2.3.2 Thyroid hormone transporters

Despite a highly hydrophobic nature, TH cannot simply diffuse through the cell membrane due to the presence of charged amino acids and must be actively transported (Hennemann et al., 2001). TH transporters play an essential role in TH action by delivering them into target tissues and organs across the cell membrane. To date, 16 proteins have been identified as binding TH in humans (Groeneweg et al., 2019). Proteins which can efficiently bind TH include MCT 8 and 10, organic transporting polypeptide (OATP)1C1 and SLC17A4 (Friesema et al., 2003, 2008; Pizzagalli et al., 2002; Teumer et al., 2018). However, the large neutral amino acid transporter (LAT) and sodium/taurocholate co-transporting polypeptide

(SLC10A1/NTCP) also have the ability to bind and transport TH (W. E. Visser et al., 2010; Zevenbergen et al., 2015). TH transporters are differently expressed in each tissue and show different levels of expression as well as affinity towards TH (Bernal et al., 2015).

2.3.3 Thyroid hormone receptor structure and expression

Thyroid hormone receptors (TR) belong to TRs superfamily and can be divided in two different proteins – TRα and TRβ, coded by *THRA* and *THRB* genes, respectively. TR receptors contain three domains: the amino-terminal domain (A-B domain), the central DNA-binding domain and the carboxyl-terminal ligand-binding domain (LBD) and are derived from a single peptide. Each of the proteins can be further divided into two different isoforms (β1-2 and α1-2), which differ in length, TH binding properties, dimerization potency and tissue expression patterns. Among those isoforms, TRα does not binds to T_3 and exact function still remains enigmatic (Hönes et al., 2022). TR isoforms are expressed differently on different tissues. TRα1 is mainly expressed in the central nervous system, bone, heart, skeletal muscle, GI track and cartilage. TRβ1 is abundantly expressed in the liver and kidneys and present in the hypothalamus, pituitary as well as the developing central nervous system (Lazar, 1993). TRβ2 is expressed mainly in the hypothalamus and pituitary however, it is also expressed in the brain, retina and developing ear (Brent, 2012). In peripheral tissues, only low expression of β2 isoform can be detected. Studies performed on the TR-β2 KO mouse model, showed that β2 isoform expression is a critical component of the T_3-mediated negative feedback regulation loop in TRH hypophysiotropic neurons (Abel et al., 1999).

2.3.4 Bioavailability, regulation, and metabolism of thyroid hormones

The concentration of the transcriptionally active hormone T_3 is precisely controlled by Dio catalytic action at a cellular level in different tissues. Dio are small integral membrane proteins composed of a single N-terminal transmembrane segment linked to globular cytosolic domain with selenocysteine in the active center, which can activate or inactivate TH (Gereben et al., 2015). In the process of deiodination, Dio1 and Dio2 can convert T_4 into active T_3 and Dio3 can inactive T_3 through its

conversion into rT_3 or 3',3-T_2 (di Cosmo et al., 2010). Dio1 catalyzes outer- and inner-ring deiodination, which leads to T_3 or inactive rT_3. Dio2 catalyzes only outer-ring deiodination and Dio3 inactivates T_4 and T_3 in inner-ring deiodination process (Gereben et al., 2015). Dio have tissue-specific expression pattern. Dio1 is expressed in the kidney, liver, thyroid and brain. Furthermore, Dio2 is expressed in human brown adipose tissue, brain, pituitary, heart, placenta, and muscle. Placenta, skin, uterus, brain, and fetal tissue express Dio3. Dio3 plays an important role in the placenta and protects the fetus against T_3 excess (Koopdonk-Kool et al., 1996) All Dio for proper function require selenium for catalytic activity (Köhrle et al., 2005). In addition to the aforementioned Dio-driven metabolism, TH can be metabolized in the glucuronidation and sulfation conjugation process, which alters the solubility, distribution, and biological potency of TH (Köhrle, 2004). Glucuronidation of TH increases its solubility in water, which are subsequently rapidly biliary-fecal excreted. Sulfation of TH increases the rate of the Dio1-mediated deiodination process and initiates irreversible TH degradation process. In fetal tissue, due to low Dio1 activity, a sulfation process eliminates excess of TH (Köhrle, 2004).

2.4 Thyroid disorders in humans

2.4.1 Hypothyroidism

Hypothyroidism (underactive thyroid) is defined as insufficient production of TH leading to typical symptoms including fatigue, weight gain, constipation, cold intolerance, dry skin and hair, concentration issues, depression, menstrual irregularities and even manifestation of cardiovascular disease. Hypothyroidism affects up to 5% of the general population and occurs more frequently in women and older people >65 years. It also associates with increased mortality, due to rare complications, like myxydema coma or pulmonary thromboembolism, if untreated (Chaker et al., 2017; Chiovato et al., 2019; Fliers & Wiersinga, 2003; Hostiuc et al., 2015). Hypothyroidism can be classified as a primary, secondary, and tertiary hypothyroidism, caused by defective thyroid gland, pituitary, or hypothalamic function, respectively (Chaker et al., 2017). Hypothyroidism can be also classified as central where a normal thyroid gland is insufficiently stimulated with TSH, or peripheral, where the defect occurs at the level of transport, metabolism or due to resistance to TH (Beck-Peccoz et al., 2017; Rastogi & Lafranchi, 2010). The etiology of the primary hypothyroidism is mostly related to an autoimmune disease process affecting the thyroid gland (Hashimoto's disease), but it can also be caused by gene

mutations, or iodine deficiency or overload. More rarely hypothyroidism can be a consequence of different medications (lithium, antithyroid drugs, etc.), radioiodine or surgical treatment for hyperthyroidism, transient thyroiditis, or infiltrative disease. Central hypothyroidism can be caused by pituitary adenomas, pituitary, or hypothalamus dysfunction because of head trauma, pituitary apoplexy, surgery, radiotherapy, genetic mutations, infiltrative disease, Sheehan's syndrome or in very rare cases by aberrant Dio3 expression in tumor tissue (Chaker et al., 2017).

CH is defined as lack of TH or their action at birth, occurs in 1:2000-4000 of newborns, and if not diagnosed and treated properly, it can lead to mental and growth retardation (Cherella & Wassner, 2017; Chiovato et al., 2019). The study performed by Harris & Pass in 2007, found that the incidence of CH has nearly doubled in the last 20 years in the USA, which can also be a direct effect of changing screening methods (Harris & Pass, 2007). It presents most commonly as a sporadic disease with only 15-20 % of the cases being familial. CH can be caused by defects in thyroid development namely thyroid dysgenesis or by defects in TH synthesis called dyshormonegenesis. TD can lead to agenesis, hypoplasia or hemithyroidism. The most common genes involved in thyroid development and linked to thyroid dysgenesis are *TSHR*, *PAX8*, *NKX2-1* and *FOXE1* (Polak et al., 2004), while mutation in genes involved in TH synthesis (including *TG*, *TPO*, *DUOX2*, *DUOXA2*, *SLC5A5*, *SLC26A4, SLC26A7* and *IYD*) can cause thyroid dyshormongenesis (Cherella & Wassner, 2017; Löf et al., 2016). Overall, there is strong evidence of pathogenic mutations in up to 37 genes which can lead to CH or thyroid agenesis phenotypes (panelapp.genomicsengland.co.uk/panels/31/) Newborn screening tests are an effective way to diagnose CH. Most often CH screening is based on TSH measurements, and occasionally, total T_4 or free T_4 (fT_4) detection at age of 2-5 days measured from heel-prick blood or more rarely cord blood (Rastogi & Lafranchi, 2010). TSH cut-off levels vary among countries (Klosinska et al., 2022). CH is more common among females (approximately 1.5-2 more) than males (Grasso & Hinton, 1991; Lorey & Cunningham, 1992). CH can be associated with other congenital malformations, including spiky hair, cleft palate, neurological abnormalities, genitourinary, lung- or cardiac malformations or higher prevalence of Down syndrome (Kumar et al., 2009; Law et al., 1998; Olivieri et al., 2002; Scavone et al., 2020). The main treatment approach relies on levothyroxine supplementation. CH diagnosis at birth and prompt treatment can prevent the mental and growth retardation (Jonklaas et al., 2014).

2.4.2 Hyperthyroidism

Hyperthyroidism (overactive thyroid) is defined as an excess or overproduction of TH and affects 0.2%-1.3% the worldwide population (Hollowell et al., 2002; Madariaga et al., 2014; Taylor et al., 2018). It is 5-10x more frequent in women. Hyperthyroidism is typically an autoimmune disease. The most common type of hyperthyroidism is Graves' disease, which is driven by loss of immunotolerance and production of thyroid stimulating autoantibodies, which subsequently bind and activate TSHR causing overproduction of TH (Leo et al., 2016). Non-autoimmune hyperthyroidism is rare and can be caused by activating mutations in TSHR or GNAS1 leading to increased TH production. Activating mutations in the GNAS1 can be part of the MAS syndrome (Hébrant et al., 2011). Non-autoimmune hyperthyroidism can be divided into familial non-autoimmune hyperthyroidism, sporadic congenital non-autoimmune hyperthyroidism and autonomous adenomas. For non-autoimmune hyperthyroidism, symptoms are linked to increased metabolism and can span hyperactivity, anxiety, tachycardia, weight loss and exophthalmos (Guerri et al., 2019). In non-autoimmune hyperthyroid cases, the ophthalmopathy and signs of autoimmune disease are usually lacking (Leo et al., 2016; Means & Littlefield, 2016). Treatment options depend on the type of hyperthyroidism or its etiology. Transient hyperthyroidism with mild symptoms can be alleviated with beta-adrenergic antagonist medication. In the case of permanent hyperthyroidism subtotal thyroidectomy, radioactive iodine-131 ablation or antithyroid drug therapy such as thionamide can be used (Biondi et al., 2015; Paschke et al., 2012). In cases of TSHR or Gnas mutation definite therapy is recommended (Mooij et al., 2022).

2.4.3 Thyroid cancer

Thyroid cancer is classified into two major types, follicular carcinoma, which is derived from follicular cells and medullary carcinomas, which originate from parafollicular C cells. Follicular cell-derived cancers can be further classified into 4 types: well-differentiated papillary- (PTC) or follicular- (FTC) thyroid cancer, poorly-differentiated- (PDTC) and anaplastic thyroid cancer (ATC) (Laha et al., 2020). Parafollicular C cell-derived medullary thyroid cancer (MTC) comprises 3-5% of all cancers and is characterized with a prognosis that is worse than well-differentiated cancers, but better than an ATC (Somnay et al., 2013). Among all thyroid tumors, well-differentiated thyroid cancers occur most frequently, especially PTC which comprises 80-85% total cases. FTC accounts for approximately 10-15%,

PDTC<2% and ATC<2% (Laha et al., 2020). The etiology of thyroid cancer is vastly unknown, however, gene mutations, gene translocations and radioactive iodine are identified as the main factors involved in thyroid tumorigenesis. The most common genetic mutations have been described in the BRAF gene; the mutations are typically found in PTCs, and are linked with distinct biological and phenotypical properties, which might play a role in PTCs progression into PDTCs and ATCs (Agrawal et al., 2014; Nikiforova et al., 2003). Other common mutations identified in PTC are found in the *RAS* gene encoding NRAS, HRAS and KRAS, which are involved in signal transduction from tyrosine kinase/ GPCRs to MAPK and PI3K-AKT associated effectors. These mutations were found in thyroid microcarcinomas in codons 12 and 13 which increased affinity for GTP binding and codon 61, which was linked to inhibition of autocatalytic GTP-ase mechanism (Howell et al., 2013). Moreover, RET/PTC, PAX8/PPARγ and NTRK gene translocations were identified as playing a direct role in thyroid tumorigenesis (Valvo & Nucera, 2019). The most common PTC genetic rearrangements especially in radiation-induced tumors are RET/PTC1 and RET/PTC2 which are the results located on chromosome 10 intrachromosomal paracentric inversions (Cyniak-Magierska et al., 2011; Grieco et al., 1990; Melillo et al., 2005). Initial treatment for thyroid cancer varies based on the cancer type and stage, and it can involve partial/total removal of thyroid with/without lymph node and further radioactive iodine administration (to decrease risk of recurrence) or using tyrosine kinase inhibitors when iodine is not taken up by the tumor (Laha et al., 2020; Nguyen et al., 2015).

2.4.4 Generating mouse models for translational studies mimicking thyroid disorders in human

2.4.4.1 Cre-LoxP system

The Cre-LoxP recombination system is a powerful tool for site-specific deletions, insertions, inversions, and translocations in the eucaryotic and procaryotic genome (Akopian & Marshall Stark, 2005; Mullins et al., 1997; Nash, 1999). The recombination can occur in the specific type of cells or can be triggered with external stimuli like a chemical or even light (Kawano et al., 2016). The method became a very attractive tool for researchers to generate tissue- specific KO mouse models in a relatively easy and efficient way. The most used enzyme in the method is bacteriophage P1-derived Cre -recombinase, which catalyzes recombination between two LoxP sites. Cre- recombinase can be expressed under a specific tissue

promotor (eg. Tg, TPO or PAX8 for thyroid specific gene-expression). Furthermore, fusion of Cre-recombinase with mutated ligand-binding domain of human estrogen receptor carrying 3 mutations (ERT2), allows to control CreERT2-induced recombination using the estrogen-like agonist tamoxifen to induce gene KO in a time-specific manner (Feil et al., 1997). The main advantage of mouse models expressing Cre-recombinase in thyrocytes, is spatial and temporal recombination control, leading to inversions and excisions of DNA fragments. The limitation of the method is the expression of the Cre-recombinase under a specific promoter, which selectively targets the cells of interests; for example, the Tg promoter is specific for the thyrocytes and allows to generate thyrocyte-specific mouse models without impacting other organs.

2.4.4.2 CRISPR/Cas9 method

The clustered regularly interspaced short palindromic repeats (CRISPR) are repeated 14bp DNA sequences interspaced with a 32nt sequence, found in bacteria and archaea, which consist of an important part of the antiviral defense system. The CRISPR-associated protein 9 (Cas9) is an endocluease which cleaves DNA-specific fragments, complementary to the CRISPR sequences (Ma et al., 2014). The knowledge of this system has facilitated methods and tools to manipulate and create gene modified disease models. For example, Markossian et al (2018), using CRIPSR/Cas9-method introduced five novel patient-derived germline mutations in the mouse *Thra* gene, including 4 frameshift mutations and N359Y missense mutations. Mice carrying these mutations had a phenotype resembling the phenotype of human patients, including impaired skeletal growth, vulnerability to anemia, mild neurodevelopmental alterations and decreased T_4/T_3 ratio (Markossian et al., 2018). The development of the CRISPR/Cas9 method allowed relatively easy, effective, affordable and precise genome modification. Efficient and robust generation of many point mutations while maintaining the same genetic background, allows different point mutations to be compared in the target gene. The disadvantage of this method is that possible off-targets might occur, thus initial breeding of a few generations is required (Ma et al., 2014; Markossian et al., 2018).

2.4.4.3 Hypothyroidism

Hypothyroidism can be studied using different mouse models, obtained by using radioactive iodine, through treatment with different drugs, or by using genetically modified models (global KOs, or KIs or thyrocyte-specific modifications). Radioactive iodine treatment destroys the thyroid gland, leading to hypothyroidism. The method has been initially described by Hamilton & Lawrence and Herz & Roberts in 1942, who used ^{131}I to treat hyperthyroidism in human patients (Hamilton, 1942; Hertz et al., 1942). The ^{131}I can be administrated to rodents in combination with low-iodine diet and injection of bovine TSH (bTSH) for one week, leading to decreased T_4 and significantly increased TSH concentrations (Schmohl et al., 2015). Drug-treated mice, include drugs which inhibit TH synthesis at different synthesis step, for example TPO is inhibited by propylthiouracil (PTU) and methimazole, Dio1 is inhibited by PTU or NIS transport is inhibited by sodium perchlorate (de Groef et al., 2006; Furman, 2007; Sugawara et al., 1999). Genetically engineered mouse models which mimic hypothyroidism can be divided in global knockouts, point mutations at germline, mice expressing Cre recombinase in thyrocytes and thyrocyte-specific mutants (Löf et al., 2018). Global knockouts, besides exhibiting thyroid phenotype, also carry an additional phenotype due to gene KO in cells other than thyrocytes. For example, transcription factors Nkx2-1 and Pax8 KOs, exhibit not only athyreosis and CH, but also other phenotypes since they are expressed in other organs as well. The Nkx2.1 KO mice have severe brain and lung defects, and the mutation is lethal at birth (S. Kimura et al., 1996). The Pax8 KO mice die shortly after weaning, if not treated with T_4 (Mansouri et al., 1998). One of the first mouse models carrying a point mutation at germline, was spontaneously derived from the Snell's dwarf (DW/J) $TSHR^{(P556L)}$ 'hyt/hyt' mouse model, which develop CH, dwarfism, and brain defects (Green et al., 1988; Stein et al., 1994). The model was used to explore the effect of CH on developmental changes in gene expression.

2.4.4.4 Hyperthyroidism

Hyperthyroidism can be generated in mouse models by chemical induction, immunization or via genetic modification. One of the most common ways to obtain transient hyperthyroidism in rodent models is TH (T_3 or T_4) thyrotoxicosis, provided in drinking water or by i.p. injections. The pharmacologically induced hyperthyroidism e.g in drinking water can be also temporary, however i.p. injections longer than 48h can lead to transient hypothyroidism, as a result of HPT axis inhibition (Engels et al., 2016; M. Zhang et al., 2022). Immunization against the

TSHR leads to production of autoantibodies, which can stimulate the TSHR and mimic Grave's disease. The most feasible way to immunize against TSHR receptor is the use of the extracellular domain (TSHR-289) with plasmid injection or using adenoviruses (C.-R. Chen et al., 2003; Eckstein et al., 2020). Genetically modified mouse models include transgenic mice with constitutively activating mutations (CAM) in TSHR or Gs. For example, somatic R201 mutation in the Gnas1 gene, identified in patients' pituitary tumors, activate alpha chain of $G\alpha_s$ and stimulate adenylyl cyclase (Landis et al., 1989). Michiels et al., demonstrated an oncogenic activity of the $G\alpha_s^{(R201H)}$ mutant expressed under Tg promoter, leading to adenomas and hyperthyroidism development at the age of 8 months (Michiels et al., 1994). Similarly, under the rat Tg promoter, cholera toxin A1 subunit was found to have oncogenic potential in the thyroid follicular cells, leading to increased cAMP intrathyroidal concentrations. Moreover, from three of the mouse lines evaluated, two lines had already developed hyperthyroidism at the age of 2 months, whereas in the third mouse line it developed at the age of 8 months. All mouse lines developed T_4-independent thyroid hyperplasia (Zeiger et al., 1997). More recently $TSHR^{(D633H)}$ KI model carrying a patient-derived TSHR CAM mutation was demonstrated to lead to thyroid adenomas, sex-, age- and gender-dependent transient hyperthyroidism. Furthermore, homozygous (HOM) KI mice developed papillary thyroid carcinomas at the age of 12 months (Jaeschke et al., 2018).

2.4.5 Extrathyroidal TSHR expression and function in different organs

The TSHR is the most critical protein needed for proper thyrocyte growth and thyroid function. Interestingly, the TSHR is not exclusively expressed in the thyroid gland, but its expression has also been reported in other organs, like adipose tissue, adrenal gland, kidneys, liver, endometrium, erythrocytes, extra-ocular muscle, lymphocytes, pituitary, thymus, and vascular smooth muscle (Aghajanova et al., 2011; Bahn, 2004; Balzan et al., 2007; Bell et al., 2000; Chabaud & Lissitzky, 1977; Dutton et al., 1997; M. Murakami et al., 1996; Prummel et al., 2000; Tian et al., 2014; W. Zhang et al., 2009). The exact physiological function of this extrathyroidal expression of TSHR is unclear, however, the most classical known pathogenic function is described by Robert Graves, who identified an autoimmune disease leading to hyperthyroidism, where TSHR is activated by autoantibodies leading to orbital adipose tissue expansion (Bahn, 2004; Ellis, 2006). In the liver and kidneys, the functionality of the TSHR was experimentally confirmed in vitro using the hepatocyte L-02 cell line, human neonatal primary kidney cells and the green monkey kidney-derived MA-104 cell line, where bTSH induced cAMP production

(Sellitti et al., 2000; W. Zhang et al., 2009). However, bTSH can also include impurities as it is a tissue preparation. However, in vitro stimulation with recombinant human TSH using human orbital preadipocyte fibroblasts and human erythrocytes can also lead to increased to cAMP and Na^+/K^+ ATPase activity, respectively, suggesting functionality of TSHR in these cells (Balzan et al., 2007; Valyasevi et al., 1999). Tian et al., using vascular smooth muscle cells, found that TSH stimulation leads to cAMP production and promotes cell proliferation (Tian et al., 2014). The TSHR expression and functionality has also been shown in human hair follicles (Bodo et al., 2009).

2.5 The adrenal gland

2.5.1 Adrenal gland function, histology, and steroidogenesis

Adrenal glands produce steroid hormones, which play a crucial role in regulation of blood pressure, electrolyte balance, carbohydrate metabolism, and stress response. Histologically, the adrenal glands consist of the cortex and medulla. The cortex is organized of functionally and histologically different zonas: glomerulosa (ZG) and fasciculata (ZF) secreting mainly aldosterone and cortisol, respectively. The inner layer zona reticularis (ZR) is present only in higher primates and secrets weak androgens mainly dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S). The adrenal medulla consists of chromaffin cells producing catecholamines. Adrenal steroidogenesis provides a major and continuous source of steroid hormones, which are synthetized from cholesterol through actions of cytochromes P450 (CYPs) and hydroxysteroid dehydrogenases (HSDs). The cholesterol biosynthesis rate is controlled by expression of hydroxymethylglutaryl coenzyme A reductase in the mevalonate pathway. In the last step of cholesterol synthesis, lanosterol is converted into cholesterol with lanosterol 14α-demethylase (Cyp51). In adult adrenals, cholesterol is transported from the outer to inner mitochondrial membrane with a steroid acute regulatory protein (StAR), where it is subsequently converted into pregnenolone by the CYP11A1 enzyme. After further diffusion into the ER, pregnenolone is converted into progesterone and subsequently into 11-deoxycorticosterone (11-DOC) with HSD3B2 and CYP21A2 enzymes, respectively. Finally, 11-DOC is converted in the mitochondria into corticosterone (ZF) and aldosterone (ZG) with CYP11B2. In ZF, pregnenolone and progesterone can be also converted into 17α-hydroxy- pregnenolone/ and progesterone respectively with CYP17A1 enzyme. In the last steps of adrenal steroidogenesis, 17α-hydroxyprogesterone is converted into 11-deoxycortisol and to its final product

cortisol, with the action of CYP21A2 and CYP11B1, respectively. In mice, corticosterone is the main glucorticoid and cortisol is not produced. Steroidogenesis is depicted in Figure 6.

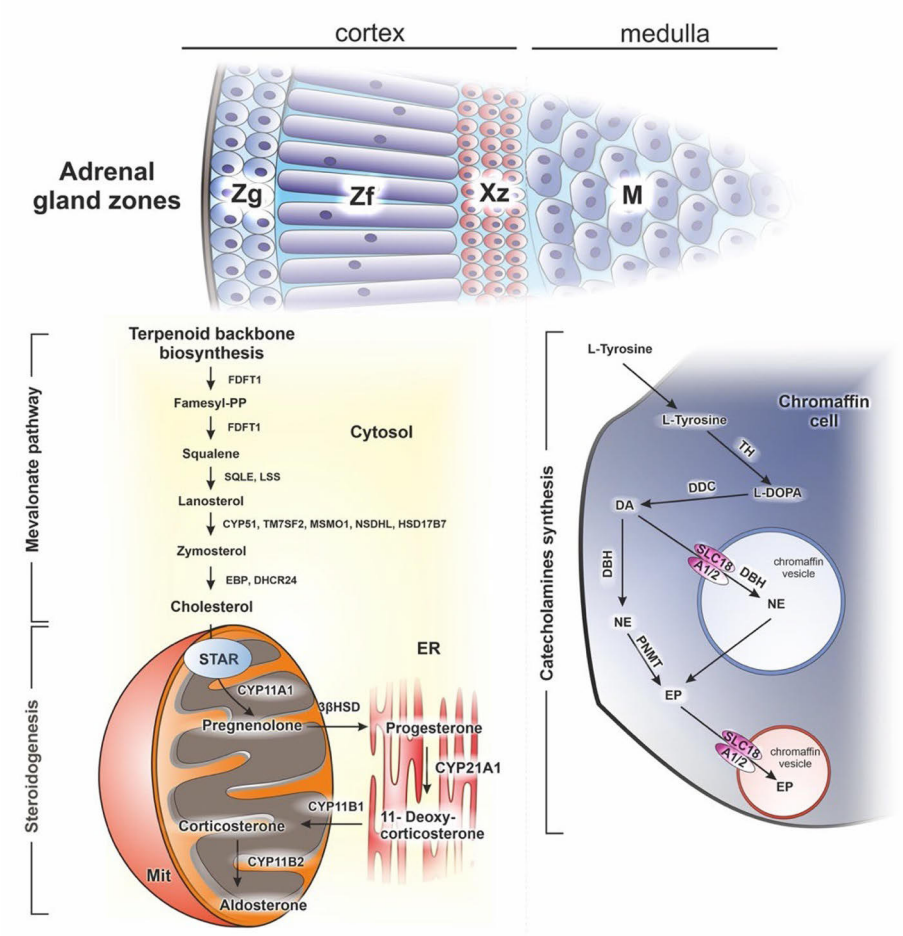


Figure 6. Mouse adrenal gland zonation, steroidogenesis and catecholamines synthesis. Abbreviations: Zg – zona glomerulosa; Zf – zona fasciculata; Xz – X-zone; M – medulla; Cortex (mevalonate pathway and steroidogenesis): FDFT1 - Farnesyl-Diphosphate Farnesyl/transferase 1; SQLE – Squalene epoxidase; LSS - Lanosterol synthase; CYP51 – Sterol 14α-demethylase; TM7SF2 - Delta(14)- sterol reductase; MSMO1- Methylsterol Monooxygenase 1; NSDHL - NAD(P) Dependent Steroid Dehydrogenase-Like; HSD17B7 - Hydroxysteroid 17-Beta Dehydrogenase 7; EBP – EBP Cholestenol Delta-Isomerase; DHCR24 - 24-Dehydrocholesterol Reductase; STAR - Steroidogenic Acute Regulatory Protein; CYP11A1 - Cytochrome P450 Family 11 Subfamily A Member 1; 3βHSD - 3β-Hydroxysteroid dehydrogenase; CYP21A2 - Cytochrome P450 Family 21 Subfamily A Member 2; CYP11B1 - Cytochrome P450 Family 11 Subfamily B Member 1; CYP11B2 - Cytochrome P450 Family 11 Subfamily B Member 2; Medulla (catecholamines synthesis): TH - Tyrosine Hydroxylase; DDC – Dopa decarboxylase ; DBH - Dopamine Beta-Hydroxylase; PNMT – Phenylethanolamine N-Methyltransferase; NE – norepinephrine; EP – epinephrine; SLC18A1/2 - Solute Carrier Family 18 Member A1/2. Modified from the (Patyra et al., 2022).

2.5.2 The link between the thyroid and the adrenal gland

2.5.2.1 Lessons from case studies and mouse models

Up to the present time, the connection between the thyroid and adrenal gland has not been well investigated, however, some earlier studies and observations have shown that adrenal androgen synthesis is depressed in patients with hypothyroidism, and that adrenal function can be altered with TH (Foldes et al., 1983). A case report from De Luca et al. (1986), described two young patients with precocious gonadarche, complete absence of sexual hair development and severe longstanding primary hypothyroidism. After the T_4 replacement therapy and restoration of euthyroidism, the physiological DHEA-S hormonal concentrations were rapidly increased together with the presence of pubic hairs, suggesting a connection between the thyroid and adrenal glands (de Luca et al., 1986). In addition, Galanou et al. (2019) have shown the important role of TH in adrenal maturation and remodeling in humans. Specifically, they identified in neonates with diagnosed severe CH significantly higher concentrations of adrenal steroids like 17-OHP, DHEA-S, Δ4-androstenedione and testosterone comparing to the euthyroid neonates. In the CH group, TSH values were positively correlated with 17-OH-progesterone, DHEAS, Δ4-androstendione, testosterone and cortisol, whereas negative correlation was observed between fT_4 was negatively correlated with same steroids except 17-OH-progesterone. After the T_4 treatment, the steroid hormone concentrations returned to normal values (Galanou et al., 2019).

Many mouse studies have shown a link between TH and adrenals suggesting possible impact on development of a secondary disease related to abnormal adrenal steroidogenesis. In thyroidectomized rats, Murakami et al. observed decreased plasma and pituitary ACTH concentration, which possibly resulted in decreased corticosterone production (N. Murakami et al., 1984). In a hypothyroid hyt/hyt ($TSHR^{P556L}$) mouse model, adrenal weight was significantly decreased, and X-zone was poorly developed or almost absent (Stein et al., 1994). Studies in hyperthyroid animal models show that adrenal weight was increased (D'Angelo S.A. & Grodin J.M., 1964; Johnson et al., 2005). Recent mouse studies have demonstrated that 20αHSD expression partially overlaps with TRβ1 expression. Furthermore, *Thrb*$^{-/-}$ mice treated with T_3 did not have enlarged X-zone compared to treated WT. This X-zone 'rescue' experiment showed that TH cannot restore the adrenal X-zone in mice lacking TRβ, suggesting that TRβ in the X-zone is a direct target for TH in neonatal adrenal (Huang et al., 2015). Moreover, more extensive studies showed that adrenal gland in T_3 treated prepubertal mice exhibit sexual dimorphism at the transcriptome level and increased and decreased expression of genes involved in cholesterol and

catecholamine synthesis, respectively (Lyu et al., 2020). Interestingly, thyrostimulin expression was also detected in the adrenal gland, however its exact role is still unclear (Li et al., 2005; Okada et al., 2006).

3 Aims

1. To establish efficient screening system for patients with suspected familial thyroid disorders.

2. To investigate the genetic etiology of congenital thyroid disorders in Finland.

3. To investigate the impact of $G\alpha_s$ KO on the thyroid, and its consequences for metabolism using $iTG\alpha_sKO$ mouse model.

4. To study the impact of thyroid hormones on adrenal development using hypo- ($TSHR^{(V488fs31)}$ KO, TSHR KO*), and hyperthyroid ($TSHR^{(D633H)}$ KI) mouse models and human patients with premature adrenarche and congenital hypothyroids diseases.

4 Materials and Methods

4.1 Study design and rationale

In the first study, we aimed to investigate the genetic factors leading to TH disorders in newborns. To identify already known and novel mutations in the Finnish population, a next generation sequencing method was applied due to its complexity and robustness. Our designed gene panel aimed to span all genes (known at that time) involved in TH biosynthesis, secretion, and metabolism. Each identified mutation was confirmed with Sanger-sequencing method. Moreover, novel pathogenic mutations were tested *in vitro* and *in silico* models.

In the second study, we generated a tamoxifen-inducible thyrocyte-specific $G\alpha_s$ KO mouse model to study a lack of $G\alpha_s$ protein on thyroid physiology and the metabolic linked consequences of hypothyroidism. In this model, Cre recombinase is expressed under a Tg promoter, providing the specificity of KO without affecting the functionality of other important organs.

Mice were injected i.p. with 100µl of tamoxifen solution (1 mg), diluted in rapeseed oil or rapeseed oil (vehicle) for 5 consecutive days. After the tamoxifen KO induction at the age of 1 month, the mouse phenotype was followed-up for the next 5 months to study the impact of growth and metabolism on hypothyroidism. Mice were sacrificed at the age of 2 months to study the thyroid and adipose tissue histology. In addition to the 2-month-old group, an additional 6-month-old group was established, to analyze possible changes in thyroid histology, linked to G-protein signaling and progression of hypothyroidism.

In the third study, we evaluated the impact of TH on the mouse adrenal gland development and physiology. We used our hyperthyroid $TSHR^{(D633H)}$ KI mice with a mutation previously identified in patients with toxic thyroid adenoma (Parma et al., 1997) and the hypothyroid $TSHR^{(V488fs31)}$ KO mouse model, due to the altered TH concentrations and prominent changes in adrenal weight. We analyzed the histology, transcriptome, and hormonal levels in those animals to obtain insight into the TH action in these organs. Moreover, we analyzed TSH, TH and steroid concentrations in human patients diagnosed with PA and CH to discover the possible correlations and translation of our animal study to the human situation.

4.2 Next generation sequencing (NGS) strategy and data analysis

The goal of the sequencing was to cover all the areas (exons and exon-intron) linked to CH using the Haloplex targeting system (Agilent, Santa Clara, CA, USA) with custom primers designed with Sure Design software (Agilent). Samples were sequenced using a Illumina MiSeq instrument (Illumina, San Diego, CA). The sequencing gene panel included 13 genes (*DUOX1*; and *2*, *IYD*, *NKX2-5*, *PAX8*, *SLC26A4*; and *5A5*, *TG*, *TPO*, *TRH*, *TRHR*, *TSHB* and *TSHR*) with minimal coverage of 95.82%. The adapter removal and read trimming step was performed using Cutadapt software (code.google.com) and reads shorter than 20 bp were removed from the analysis. A BWA-MEM algorithm (sourceforge.net) was applied to map all the reads to the hg19 reference genome. Variants calling was performed using GATK HaplotypeCaller (broadinstitute.org), Atlas2 (sourceforge.net), Platypus (well.ox.ac.uk), and SAMTools-mpileup (sourceforge.net). Subsequently, variants were annotated with SnpEff (sourceforge.net), ANNOVAR (openbioinformatics.org), and databases such as ExAC (currently gnomAD: broadinstitute.org),1000genomes(internationalgenome.org), dbSNP (ncbi.nlm.nih.gov) and ClinVar (ncbi.nlm.nih.gov). Only variants located in the exon or splicing region, with high or moderate impact, and with <1% allele frequency in 1000 genome database, were selected for further analysis. Variants were additionally visually inspected with an Integrative Genomic Viewer tool (broadinstitute.org) and further annotated using Condel (bg.upf.edu). Acommercially available Sanger sequencing service was used to confirm all the variants. Primers were designed using NCBI primer tool and PCR was performed using Taq DNA polymerase (ThermoFischer) according to manufacturer's protocol. Finally, array based CGH and MLPA analyses for *NKX2.1* gene deletion and duplication were performed at the CeGAT laboratories (Tübingen, Germany).

4.2.1 Molecular modeling

Molecular modeling was performed for PAX8 (R31C) and TPO (R438H) mutations as described below. The PyMOL (DeLano WL, version 1.5, San Carlos, CA, USA) software was used to generate the pictures.

4.2.2 PAX8 (R31C)

The PAX8 (R31C) model fragments were obtained by using backbone from the crystalized PAX5 structure (PDB ID: 1K78) (Garvie et al., 2001) due to its high sequence identity of the Gly12-Lys135 region.

4.2.3 TPO (R438H)

The TPO (R438H) model was obtained from a homologous and dimer-structure model of the myeloperoxidase (MPO, PDB ID: 3F9P) (Capena et al., 2009). The Sybyl X2.0 (Certara, Princeton, NJ, USA) software was used to perform molecular modeling, including structural changes, energy minimizations and molecular dynamic simulations. For the energy minimization (until convergence at a termination gradient of 0.05 kcal / (mol x Å) and dynamics (2ns), the AMBER F99 force field was applied.

4.3 Mouse models

4.3.1 Generation of mouse models

4.3.1.1 $G\alpha_s$

Tamoxifen-inducible thyrocyte-specific $G\alpha_s$-deficient mice (iTGα_sKO) were generated by crossing Tg-CreERT2 mice (Undeutsch et al., 2014) with Gnas Exon1$^{(fl/fl)}$ mice (M. Chen et al., 2005). The study was performed for the F3-F4 mixed background generation, and main control group were mice treated with tamoxifen Cre$^{(-/-)}$ $G\alpha_s$ Exon 1$^{(fl/fl)}$. All mice were HOM for floxed $G\alpha_s$ Exon 1$^{(fl/fl)}$, and heterozygous (HET) for Cre$^{(+/-)}$. Moreover, Cre$^{(+/-)}$ males were bred with Cre$^{(-/-)}$ females. The generation of the mouse model for the study is depicted in Figure 7.

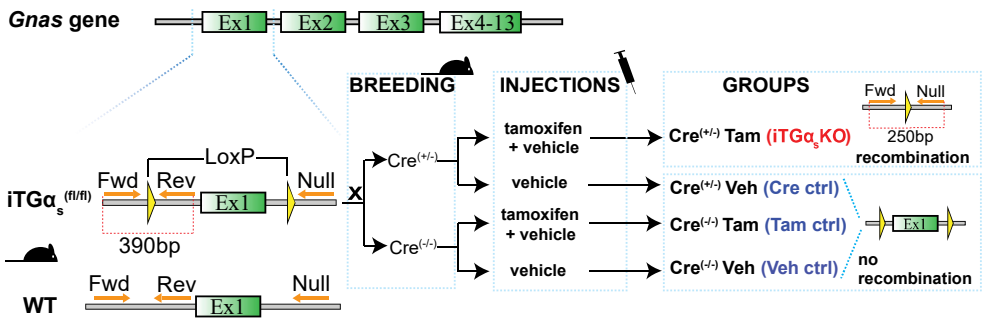


Figure 7. Generation of iTGαsKO mouse model. Abbreviations: Ex – exon; fl/fl – floxed/floxed; Fwd – forward genotyping primer; Rev – revere genotyping primer; Null – reverse genotyping primer behind the exon 1; LoxP - locus of X (cross)-over in P1; Tam – tamoxifen; Veh – vehicle.

4.3.1.2 TSHR$^{(D633H)}$ KI mouse model

TSHR$^{(D633H)}$ KI mouse model was generated by replacing the GAC nucleotide sequence (codon 633) with CAC sequence using a homologous recombination as described previously (Jaeschke et al., 2018). For phenotypical characterization of the adrenal gland mixed and pure C57BL/6N the genetic background mice were used. For the control group, WT littermate mice were used.

4.3.1.3 TSHR$^{(V448Afs31)}$ mouse model

TSHR$^{(V448Afs31)}$ KO (further referred to as TSHR KO*) mouse model carrying the inactivating mutation in TSHR was generated using the CRISPR-Cas9 method. Mouse exon 10 was targeted with Alt-R crispr RNA (crRNA). Alt-R trans-activating crispr RNA (tracrRNA), and the Alt-R 3xNLS Cas9 were purchased from IDT (Coralville, IA, USA). RNA oligonucleotides were dissolved in the IDT duplex buffer containing 30nM HEPES, 100mM Potassium Acetate with pH 7.5. In the next step, crRNA and trackrRNA were mixed in equal molar concentration and heated for 5 min at 95 °C, then slowly cooled town to RT. Guide RNA complex was diluted to a concentration of 50 ng/µl in the TE buffer containing 10 mM Tris-Cl, 0.1mM EDTA with pH 7.5. Finally, 25 ng/µl Alt-R 3xNLS Cas9 nuclease was mixed with the guide RNA complex. The mouse C57BL/6N zygote was microinjected and obtained founders were backcrossed 2-5 times for a pure C57BL/6N background.

4.3.2 Husbandry, diet, and breeding strategy

All the animals were kept in IVCR cages under controlled condition (12h day/night, 21±1°C) at the Central Animal Laboratory (CAL), University of Turku in Finland. Preliminary analyses were performed using mixed genetic background mice and further repeated in those with a backcrossed (2-5 times) pure C57BL/6N genetic background. The mice had ad libitum access to RM3 (E) pellet (Special Diet Services, Essex, UK), iodine content: 1.4 mg/kg) and tap water. The mouse were bred only between litters originated from different parents. Backcrossing was performed using pure WT C57BL/6N animals.

4.3.3 Data collection from growth, body weight/composition, temperature, activity, and food intake

For $G\alpha_s$ mice, body weight, length and tail length data were collected every 2 weeks for the following 5 months, starting at 2 months after 4-weeks of tamoxifen injections for $n \geq 5$ per each group.
At the age of 6 months, body composition was estimated using an EchoMRI-700 instrument (EchoMRI, Houston, TX, USA). The total water, lean and fat mass values were normalized to the weight of the mouse.
Inner body temperature was measured using a rectal probe (Physitemp Instruments, Inc., Clifton, NJ, USA).
The activity of 2-month-old single caged mice mice (n = 6-9/per each group) was measured using wireless running wheels ENV-047 (MedAssociates Inc., Fairfax, VT, USA) for 72h, after 2 days of an adaptation period. The total counts per each group were assessed.
Food intake was estimated for 9 mice per each group at the age of 7 weeks. One week before measurements, the mice were divided into 2 or 3 animals per cage to reduce stress. Every 4 days, consumption of the chow pellets was checked.

4.4 Genotyping

4.4.1 Sample collection, DNA isolation and genotyping PCR

Ear samples were collected at the age of 2 weeks and DNA was isolated using an In-house method. Ear samples were lysed with 500µl lysis solution containing tail lysis buffer, 20% SDS and Proteinase K and subsequently incubated o/n at 56ºC. The following day, the lysed solution was mixed with 150 µl NaCl, vortexed thoroughly

and centrifuged for 20 min at 13 000 rpms at 4°C. The sample was carefully transferred to 1ml 99,6% EtOH, mixed well and centrifuged at 13 000 rpms at 4°C. The supernatant was discarded and the pellet was washed with 70% EtOH, dried at 70°C and dissolved in 20°C dH_2O. PCR was performed using DreamTaq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The genotyping primers of iTGα_sKO, TSHR$^{(D633H)}$ KI and TSHR$^{(V448Afs31)}$ KO mouse lines are listed in Table 1.

Table 1. Mouse line genotyping primers.

Mouse line	Gene (Exon)	Forward primer (5'>3')	Reverse primer (5'>3')	Product size (bp)
iTGα_sKO	*Gnas* (E1 fl)	GAGAGCGAGAGGAAGACAGC	TCGGGCCTCTGGCGGAGCTT	WT: 330 HOM: 390
	Gnas (E1 KO)		AGCCCTACTCTGTCGCAGTC	KO: 250
	Cre	AGTCCCTCACATCCTCAGGTT	ATGCCAACCTCACATTTCTTG	WT: n.p. HET: 280
TSHR$^{(D633H)}$ KI	*TSHR* (E10)	AAGCCACCTGCACATGAAAGCATAT	TCACCCTTGATCCCCTTGACCCTTG	WT: 361 HOM: 475
TSHR$^{(V448Afs31)}$ KO	*TSHR* (E10)	ATGACTACACGGTGTGTGGG	AGTCGATGGCGTGGTTGTAG	WT: 309 KO: 304

Abbreviations: fl- floxed; WT- wildtype; HOM - homozygous**;** KO – knockout; KI - knock-In; HET – heterozygous; n.p.- No product.

4.4.2 Tumor genotyping

Selected PTC-like structures were cut out using the Palm MicroBeam laser capture microscope (Carl Zeiss, Germany). DNA was isolated from cut samples using the protocol from the 4.3.5.1 section, with minor modification (5x less volumes for all the reagents). The Braf (c. 1799 c>t, V600) codon was amplified using PCR. The primers used for PCR were: F1: 5'-TTCATGAAGACCTCACGGTAAAA-3' and R1: 5'-GTGAGTAGTGGGAACTGTGAAAG-3'. The PCR products were separated in 1.5% agarose gel in 1x Tris-buffered saline for 30 min at 120 V. Finally, 5 µM of the F1 primer was mixed with 20 ng/µl PCR product and sequenced using a commercial Sanger sequencing service (Eurofinns Genomics, Ebersberg, Germany). Data were analyzed using FinchTV 1.4.0 software (Perkin Elmer).

4.5 Histological analysis

4.5.1 Fixation methods

For HE, IHC, RNAscope and ISH staining, samples were fixed with 10% neutral buffered formalin (Oy FF-Chemicals Ab, Haukipudas, Finland) for a minimum 24h for thyroids and 3 days for larger tissues. Samples were transferred into cossets and initial dehydration was performed in 50% EtOH for 1h at RT, then samples were transferred to 70% for o/n dehydratation at +4ºC and further embedded in paraffin. Samples were cut with a Leica microtome (Leica, Wetzlar, Germany) into 4 µm sections, dried o/n and stored at +4ºC for further analysis.
For Oil Red O staining, fresh samples were embedded in a Tissue-Tek OCT compound (Sakura Finetek, Japan) and placed for 5-10s in ice-cold 2-methylbutane (Sigma-Aldrich, Saint Louis, MO, USA). After this, the samples were covered with aluminum foil and stored at -80 ºC**.** Samples were cut using cryotome into 10 µm sections and stored at -20 ºC for further analysis.

4.5.2 Hematoxylin-eosin (HE) staining

Samples were deparaffinized, re-hydrated and dipped three times in dH_2O. In the next step, samples were stained for 15 min in Hematoxylin (Delafield) and washed. Then the samples were placed for 1 min in 70% EtOH/1% HCl, washed as in previous steps and stained with eosin for 2 min. After Eosin, the samples were washed twice in EtOH 99.6%, dehydrated in xylene and mounted with Pertex.

4.5.2.1 Immunohistochemistry (IHC)

Samples (4 µm thick) were re-hydrated and heated in a pressure cooker with 10mM sodium citrate (pH: 6.0). After the antigen retrieval step, samples were twice washed in 1xTBST and then once in TBS for 5 min (all next washing steps) and blocked for 30 min with 3% BSA. After blocking, the samples were incubated in H_2O_2 for 10 mins in order to quench endogenous peroxidases. Samples were again washed, and primary antibodies were added for o/n incubation at +4ºC. The following day, the samples were washed, and secondary antibodies were added for 2h and incubated at RT. Samples were developed with Dako's DAB kit for a previously optimize time and counterstained with Mayer's hematoxylin for 20 s and washed for 10 min in running tap water. Finally, samples were dehydrated and mounted with Pertex. The primary antibodies used are listed in Table 2.

Table 2. **IHC antibodies.**

Antigen	Isotype / Clonality (clone)	Manufacturer (Cat. No.)	Dilution	Project
$G\alpha_s$	Rabbit IgG / PC (-)	Abcam (83735)	1:150	II
KI67	Rat IgG2a / MC (SolA15)	eBioscience (14-5698-80)	1:2000	II
NKX2.1	Mouse / MC (8G7G3/1)	Dako (M3575)	1:200	II
ERK1/2	Rabbit IgG / MC (137F5)	Cell signaling Technology, Inc (4695)	1:250	II
Phospho-ERK1/2	Rabbit IgG/ MC (D13.14.4E)	Cell signaling Technology, Inc (4370)	1:400	II
20αHSD	Rabbit / PC (-)	Antibody Research Corporation (591009)	1:2400	III
CgA	Rabbit / PC (-)	Abcam (ab15160)	1:2500	III
CYP51	Rabbit / PC (-)	Abcam (ab210792)	1:30	III
CYP11A1	Rabbit / PC (-)	Sigma Aldrich (HPA016436)	1:300	III
HSD3B2	Mouse / MC (1E8)	Sigma Aldrich (SAB1402232)	1:2000	III
CYP11B1	Rabbit / PC (-)	Abcam (ab197908)	1:25	III
CYP11B2	Rabbit / PC (-)	LifeSpan Bioscience (LS-C167202)	1:100	III

Abbreviations: $G\alpha_s$ – G alpha s; KI67 – Marker of proliferation Ki67; NKX2.1 – NK2 Homeobox 1; ERK1/2 – Extracellular signal-regulated kinase 1/2; 20αHSD – 20alpha-hydroxysteroid dehydrogenase; CgA – chromogranin A; CYP51 – Cytochrome P450 14alpha-sterol demethylase; CYP11A1 – Cytochrome P450 Family 11 Subfamily A Member 1; HSD3B2 – 3 beta-hydroxysteroid dehydrogenase; CYP11B1/B2 – Cytochrome P450 Family 11 Subfamily B Member 1/2; PC – polyclonal; MC – monoclonal.

4.5.2.2 RNAscope In Situ (ISH) hybridization

In Situ hybridization of *RNA Polymerase II subunit A* (*Polr2a)*, *Thyroid hormone receptor beta* (*Thrb*), thyroid stimulating hormone (*Tshr*) and *4-Hydroxy-tetrahydrodipicolinate reductase* (*DapB*) was performed as a part of Project III, using commercially available RNAscope® 2.5 HD Reagent Kit – Brown (Advanced Cell Diagnostics, Newark, CA, USA) according to the manufacturer's protocol. The hybridization process was carried out in an HybEZ oven (Advanced Cell Diagnostics). The probes used in the experiment are listed in Table 3.

Table 3: RNAScope *In Situ* hybridization probes.

Probe	Catalog number	Accession number	Base pairs	Target region	Project
Positive Control Mm-Polr2a	312471	NM_009089.2	20	2802 - 3678	III
Mm-Thrb	451441	NM_00111347.1	20	783-1993	III
Mm-Tshr	547611	NM_011648.5	20	2 - 1036	III
Negative Control-DapB	310043	EF191515	10	414-862	III

Abbreviations: *Polr2a* – RNA Polymearse II subunit A; *Thrb* – Thyroid hormone receptor beta; *Tshr* – thyroid-stimulating hormone receptor; *DapB* – 4-Hydroxy-tetrahydrodipicolinate reductase.

4.5.2.3 Oil Red O (ORO) staining

Frozen sections were cut into 10 μm using Cryotome. Samples were briefly fixed in formalin for 5 min and subsequently washed with running tap water for 3 min. In next step, samples were rinsed in 60% isopropanol for 1 min, stained with an Oil red O working solution for 10 min, and rinsed three times again for 1 min in isopropanol. The nuclei were stained in Mayer's hematoxylin for 10s and samples were washed with running tap water for 2 min. Finally, samples were mounted with an aqueous mounting medium and dried for 2 weeks at RT.

4.5.3 Morphometric analysis of thyroid follicles and PTC classification guidelines

All the slides were scanned using a Panoramic Scanner 250 Flash or P1000 (3DHistech Ltd. Budapest, Hungary). Panoramic Viewer software ver. 1.15.4 (3DHistech Ltd.) was used to calculate adjacent thyrocytes thickness and follicle lumen area. Abnormal areas in the thyroid gland were classified as PTC-like when one or more characteristic features for PTC were found: enlarged, overlapping, irregular nuclei, or nuclei with grooves or a chromatin clearing.

4.6 Gene expression analysis

4.6.1 RNA isolation, DNase I treatment and reverse transcription (RT)

Snap frozen adrenals were homogenized with stainless metal beads (QIAGEN, Hilden, Germany) at 50 rpms for 2 min in 200 µl TRIsure (Bioline, London, UK). Homogenized samples were transferred into 600 µl TRIsure reagent and incubated for 5 min. Subsequently chloroform was added, samples were mixed vigorously for 15 min and incubated for 3 min at RT. After the incubation, samples were centrifuged for 12 000 g for 15 min at +4 ºC. The RNA-containing phase was transferred to new tubes containing 400 µl of cold isopropanol, and subsequently briefly vortexed. After the precipitation step, samples were centrifuged as before, and RNA was washed twice with 75% EtOH in DEPC H_2O. RNA was air-dried for 40 min and dissolved in 20 µl nuclease free water (Sigma Aldrich). The concentration of RNA was measured using NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at -80 ºC. Total RNA (1 µg) was used in DNase I treatment (Sigma-Aldrich) and a further cDNA synthesis using SensiFAST™ cDNA Synthesis Kit (Bioline) was used according to the manufacturer's protocols.

4.6.2 Quantitative PCR (qPCR)

The cDNA was diluted 1:100 in sterile water (Braun GmbH, Kronberg im Taunus, Germany) and qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. Gene expression values were calculated using a qBase MS Excel VBA applet (Hellemans et al., 2008) for Project III and the Pfaffl method (Pfaffl, 2001) for Project II. Cyclophilin A (Ppia) and ribosomal protein L19 were used as reference genes. Relative gene expression from each group (n = 5) was compared with WT values. Each qPCR product was sequenced and the sequence was confirmed. The used primers are listed in Table 4.

Table 4. qPCR primers.

Gene symbol	Forward primer (5' > 3')	Reverse primer (5' > 3')	Study
Tshr	CCATCTCCTTCTATGCGCTGTCG	GCTGAGCAGGATGAACACGTCC	II, III
Foxe1	GACTCTGGGCGGCATCTACAAGTT	TTGAGGAAGCAGTCGTTGAGGGT	II
Hhex	CGGACGGTGAACGACTACAC	CGTTGGAGAACCTCACTTGAC	II
Pax8	CCTGCTGAGTTCTCCATATTATTAC	CCTTTGTGTGACTCTCTGGG	II
Nkx2.1	CAGCCGACGCCGAATCAT	CTGGCCCTGTCTGTACGC	II
Tg	CTTATGGGAGGCTCTGCAC	CACAGCCAGGAGCTTGGTC	II
Tpo	CAAAGGCTGGAACCCTAATTTCT	AACTTGAATGAGGTGCCTTGTCA	II
Nis	GGGATGCACCAATGCCTCTG	GTAGCTGATGAGAGCACCACA	II
Dio1	GGGCAGGATCTGCTACAAGG	CGTGTCTAGGTGGAGTGCAA	II
Srxn1	CCCAGGGTGGCGACTACTA	GTGGACCTCACGAGCTTGG	II
Sod3	CCTTCTTGTTCTACGGCTTGC	TCGCCTATCTTCTCAACCAGG	II
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT	II, III
Rpl19	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC	II, III
Akr1b7	GCCACCCTTATCTCACCCAG	TCTCCATTACTACGGGGTCTTC	III
Akr1c18	TCGTCCAGAGTTGGTCAGAC	CTTTAGGCAAAAGCTCATTCCCT	III
CgA	CCAAGGTGATGAAGTGCGTC	GGTGTCGCAGGATAGAGAGGA	III
Sf1	TCATCCTCTTCAGCCTCGAT	GCACAATAGCAACTGCTGGA	III
Gata4	AAGACGCCAGCAGGTCCTG	AGTACTGAATGTCTGGGACATGGAG	III
Gata6	CAAAAGCTTGCTCCGGTAAC	TGTAGAGGCCGTCTTGACCT	III
Thra 1/2	GGTCACCAGATGGAAAGCGAA	CCTTGTCCCCACACACGAC	III
Thrb 1/2	TTGTGAGCTGCCATGTGAAGA	ATCTGAAACCACCCCAAGGC	III
Mc2r	ACACCGCAAGAAATAACTCCG	AGGAGGACAATCAAGTTCTCCA	III
Cyp51	GACAGGAGGCAACTTGCTTTC	GTGGACTTTTCGCTCCAGC	III
Star	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG	III
Cyp11a1	ACTTCCGGTACTTGGGCTTT	GGATGAGGCTGAACTTGGTC	III
Hsd3b1	TGCAGACAAAGACCAAGGTG	TGACATCAATGACAGCAGCA	III
Cyp21a1	CTCCGGCTATGACATCCCTA	ACAGCCAAAGGATGGTGTTC	III
Cyp11b1	CGGAGGATGTTGAGAAGCTG	TGCAGTCGGTTGAAGTACCA	III
Cyp11b2	AGGAAAAGGAGTTTCCAGCA	GTGTCTCCACCTGGAAGGTT	III
Mki67	AAAGGCGAAGTGGAGCTTCT	TTGGAGTGCTGGGTCTTCTT	III
Ppib	AGCCTTAGCTACAGGAGAGAAAGGA	ATGCTCTTTCCTCCTGTGCCA	III
Hmbs	AAATCATTGCTATGTCCACCACG	GCCAGGAGGTAGTATGGTAGGCA	III

Abbreviations: *Tshr – Thyroid-stimulating hormone receptor; Foxe1 – Forkhead box protein E1; Hhex - Hematopoietically expressed homeobox; Pax8 – Paired box 8; NKX2.1 – NK2 homeobox 1; Tg – Thyroglobulin; Tpo – Thyroid peroxidase; Nis – Sodium/iodide symporter; Dio1 – Deiodinase 1; Srxn1 - Sulfiredoxin-1; Sod3 – Superoxide dismutase 3; Ppia - Peptidylprolyl Isomerase A; Rpl19 – Ribosomal protein L19; Akr1b7 - Aldo-keto reductase family 1, member B7; Akr1c18 - Aldo-keto reductase family 1 member C18 (20alpha-hydroxysteroid dehydrogenase); CgA – Chromogranin A; Sf1 – Steroidogenic factor-1; Gata4; -6 – GATA binding protein 4, -6; Thra – Thyroid hormone receptor alpha; Thrb – Thyroid hormone*

receptor beta; Mc2r – Melanocortin 2 receptor; CYP51 – Cytochrome P450 14alpha-sterol demethylase; Star - Steroidogenic acute regulatory protein; CYP11A1 – Cytochrome P450 family 11 subfamily A member 1; HSD3B1 – 3 Beta-hydroxysteroid dehydrogenase,3 beta; Cyp21a1 - Cytochrome P450, family 21, subfamily a, polypeptide 1; CYP11B1*;* -B2 *– Cytochrome P450 family 11 subfamily B member 1; -2; KI67 – Marker of proliferation Ki67; Ppib - Peptidylprolyl Isomerase B; Hmbs - Hydroxymethylbilane synthase.*

4.6.3 RNA sequencing

RNA sequencing was performed using the Novogene sequencing service (Novogene Co. Ltd, Beijing, China) using a NovaSeq 6000 PE150 sequencer (Illumina, Inc., San Diego, CA, USA) as briefly described in manuscript number 3 (Patyra et al. 2021). RNA was isolated as described in section 4.6.2 and quality and quantity analysis was performed using a Bioanalyzer instrument (Agilent, Santa Clara, CA, USA).

4.7 Hormone measurements

4.7.1 TSH and total T4 in mice

TSH from the serum was measured using a commercially available Mouse Pituitary Magnetic Bead Panel Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Fluorescence was measured with a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA) and data concentrations were calculated using Luminex Xponent software v. 3.1.
Total T_4 was measured using a total T4 ELISA kit (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany) according to the manufacturer's protocol. A Wallac Victor X4 plate reader (Perkin Elmer, Waltham, MA, USA) was used to measure absorbance and WorkOut Plus software (v. build 0600, Dazdaq Solutions Ltd., Brighton, UK) to calculate the concentrations.

4.7.2 Steroid hormone measurements in mice

A panel of steroid hormones, including estrogen, androstenedione, progesterone, dihydrotestosterone, testosterone, and estradiol were measured using GC-mass spectrometry as described previously (M. E. Nilsson et al., 2015).
Mouse serum corticosterone concentration was measured using a Corticosterone ELISA Kit (Enzo Life Sciences, Inc., New York, NY, USA) according to the manufacturer's protocol. Absorbance (405 nm) was measured with a Wallac Victor

X4 plate reader (Perkin Elmer, Waltham, MA, USA) and concentrations were calculated using WorkOut Plus software (v. build 0600, Dazdaq Solutions Ltd., Brighton, UK).
Mouse serum aldosterone concentration was measured with an Aldosterone ELISA kit (ab136933, Abcam, Cambridge, UK) according to the manufacturer's protocol. Plate absorbance was read using an EnSight instrument (PerkinElmer) at 405 nm.

4.7.3 Serum sodium concentration in mice

VetScan® Critical Care Plus Reagent Rotor kit (Abaxis, Inc., CA, Union City) was used to measure serum sodium concentration using the VetScan VS2 Chemistry Analyzer 1200-1001 (Abaxis, Inc.) according to the manufacturer's protocols.

4.7.4 Human TSH, T4 and 17-hydroxyprogesterone

Umbilical serum TSH (uS-TSH) is measured in Finland routinely at birth as a part of the screening program for newborns. Furthermore, at the age of 2-5 days an additional blood spot sample is collected on filter paper as described previously (Löf et al., 2016).

Umbilical serum (uS-) TSH and uS-T_4 were measured using a time-resolved immunofluorometric assay (TR-IFMA) using an AutoDelfia instrument (PerkinElmer), according to the manufacturer's protocol. A time-resolved fluoroimmunoassay was also used to measure TSH and 17-OHP using DELFIA Neonatal or GSP Neonatal kits, based on the manufacturer's protocols (PerkinElmer). The fT_4 concentrations were measured using the ECLIA method with a COBAS 8000 instrument (Roche Diagnostics, Mannheim, Germany).

4.8 cAMP measurements with radioimmunoassay (RIA)

For the cAMP from thyrocyte primary cells, concentrations were measured using a radioimmunoassay in house method as described previously (Harper & Brooker, 1975).

For the cAMP from adrenal primary cell culture, concentrations were measured with the RIA cAMP kit (RIA-5517, DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's protocol.
Radioactivity was measured with the 2470 WIZARD Automatic Gamma Counter instrument (PerkinElmer, Waltham, MA, USA). cAMP concentration was calculated using WorkOut software v. 0561 (Dazdaq Solutions Ltd., Brighton, England).

4.9 Plasmid construct generation and in vitro testing

4.9.1 Construct generation

Site-directed PCR mutagenesis was used to generate NKX 2.1 c313G>A, G267-269 and PAX8 c.91C>T mutation. PAX8 and NKX2.1 were cloned into pcDNA3 and pcDNA3.1, respectively. The mutagenesis primers are listed in Table 5.

Table 5. Mutagenesis primers.

Gene	Mutation	Amino acid change	Forward primer (5'>3')	Reverse primer (5'>3')
NKX2.1	*c.313G>A*	V105M	CACATGACGGCGGCG GGGATGCCCCAGCTC TCGCACTC	GAGTGCGAGAGCTG GGGCATCCCCGCCG CCGTCATGTG
NKX2.1	c.793-801del	ΔG267-269	CAGGACAGCGGCGGC G*GCGGGGGCACCGG GTGCCCG	CACCCGGTGCCCCC GC*CGCCGCCGCTG TCCTGCTGC
PAX8	*c.91C>T*	R31C	CCGGAAGTGGTCTGC CAGCGCATCG	CGATGCGCTGGCAG ACCACTTCCGG

Abbreviations: *NKX2.1* – NK2 Homeobox 1; *PAX8* – Paired Box 8.

4.9.2 Cell culture and luciferase assay

Generated plasmid constructs with novel mutations (4.9.1) were subsequently tested in luciferase assay using the HeLa cell line.
The cells were grown in a DMEM/F12 (Sigma-Aldrich, St. Louis, MO, USA) media mixed with 10% FCS (Biowest, Nuaillé, France), 2nM L-glutamine (Thermo Fisher

Scientific, Waltham, MA, USA) and 50 U/ml penicillin and 50 µg/ml (Sigma-Aldrich). Cells were plated on the 96-well plate in concentrations of 10.000 cells per well. The next day, the cells were transfected with a pcDNA3 vector and constructs: NKX2.1 (WT, V105M, ΔG267-269) and PAX8 (WT and R31C) with pGL4-pTG1084 and pRL-SV40 Renilla plasmids (Promega, Madison, WI). A TG promoter was amplified with PCR using primers 5'-GCGGGGTACCACCTGGCTGGATTGGTCTTC-3' (forward primer) and 5'-GGCGAAGCTTTTTCCTGGCCCTTCCTGGG-3' (reverse primer) and subsequently cloned into the pGL4.14 plasmid using KpnI and HindIII restriction sites. Renilla luciferase luminescence intensity was used for normalization of the variations in the transfection efficiency of the pRL-SV40 Renilla control plasmid. The cells were transfected with the Turbofect transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Dual-Glo Luciferase Assay System (Promega) was applied to measure luciferase activity according to manufacturer's instructions. Luminescence intensity was measured using the EnSight Multimode Plate Reader (PerkinElmer, Waltham, MA, USA).

4.10 Mouse primary cell cultures

4.10.1 Thyroid

For the thyroid primary cell culture, the method described by Jeker et al. (1999) was used with small modifications (Jeker et al., 1999). At the age of 3 months, thyroids were dissected into sterile DMEM (MilliporeSigma, Burlington, MA, USA) containing 10% calf serum (FCS; Biowest, Nuaille, France) and subsequently cut into tiny pieces and digested with collagenase (225 U/ml), hyaluronidase (75 U/ml) and dispase (3 U/ml) for 30 min at 37ºC using a mixer. After centrifuging at 1500 rpms for 5 min, samples were washed with PBS and resuspended in a DMEM culture media and then a 96-well plate. The culture media was composed of 10% FCS, 10 µg/ml insulin, 5 µg/ml transferrin, 3.5 ng/ml hydrocortisone, 10 ng/ml somatostatin, and 2 ng/ml Gly-His-Lys acetate (MilliporeSigma). After 3-4 days, the cells were washed three times with PBS, and 200 µl of the culture media without hormones and FCS, but with 1 mM 3-isobutyl-1- methylxanthine (MilliporeSigma) was added. The cells were stimulated with 10 mU/ml bTSH (MilliporeSigma) for 1h, and media containing extracellular cAMP was collected. For intracellular cAMP, a solution containing 0.1 M HCl was added to the wells and collected after 30 min incubation on ice.

4.10.2 Adrenals

Adrenals were dissected, cleaned from adipose tissue, and placed in the tube containing DMEM F12 (Sigma Aldrich, Saint Louis, MO, USA). Each adrenal gland was cut into 4 pieces using a scalpel followed by digestion in a buffer containing DMEM F12, hyaluronidase (1 mg/ml), collagenase type IA (2 mg/ml) and recombinant DNase (0.1 mg/ml). Digestion of adrenals was carried out for 15 min in the incubator at 37 ºC, and every 5 min samples were mixed by pipetting. Cells suspended in digestion buffer were transferred into 5 ml DMEM F12 medium containing 20 % iFCS (Biowest, Nuaillé, France) and 0.5 % of streptomycin/penicillin (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and centrifuged 300 g for 10 min at +4 ºC. The supernatant was discarded and cells were washed twice with 5 ml PBS. In the last step, the cells were mixed well and resuspended evenly on a 24-well plate. After one day, the cells were washed, and the medium was replaced with one containing 10% iFCS instead of 20%. After two days, the cells were washed with PBS and incubated with pure DMEM F12 premixed with 1 mM iBMX for basal activity, forskolin (0.01 mU) for positive control or recombinant human TSH (Thyrogen, Sanofi, Paris, France) with final concentrations of 1, 5 and 10 mU/ml. After 1h of stimulation, the medium was collected for cAMP measurement and the plate was used for protein concentration measurement with a BCA kit (ThermoFisher Scientific) for normalization of values.

4.11 Statistical analyses

Two tailed t-test and a non-parametric Mann-Whitney U test was used for samples with homogenous and non-homogenous variances, respectively. ANOVA with a Duncan's post-hoc test was applied to analyze more than one group. A Pearson correlation and t-test were applied to calculate the correlation between TSH or fT4 and 17-OHP. All statistical tests were performed and visualized with GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA).

4.12 Study approval and ethical license

Animal studies were performed based on institutional guidelines and were approved by the National Animal Experiment Board (License no: 10266). Human studies were approved by the Ethics Committee of Hospital District of Southwest Finland (108/180/2010). The study protocol from the third study was previously approved by the Research Ethics Committee of Kuopio University Hospital. Moreover, written consent was obtained from the parents and their children.

5 Results

5.1 Next generation sequencing screening of patients with congenital hypothyroidism

We have developed a next-generation sequencing (NGS) panel including exon and exon-intron gaps of 13 genes known to cause CH using a Haloplex targeting system. The aim was to generate the panel with high total coverage for selected genes: *DUOX1* (95.83%), *DUOX2* (95.82%), *SLC26A4* (99.71%), *SLC5A5* (99.85%), *IYD*, *NKX2-5*, *PAX8*, *TG*, *TPO*, *TRH*, *TRHR*, *TSHB* and *TSHR* (100%). We applied this panel to the cohort of CH patients and their parents to analyze genetic etiology and screen for novel and known mutations.

5.1.1 Mutations associated with congenital hypothyroidism

5.1.1.1 Sporadic mutations and clinical profile

The NGS of 7 patients with CH identified 9 previously known (likely pathogenic or with unknown functional effect) point mutations in 7 genes, including TRH (c.212C>T), TSHR (c.106G>C), SLC26A4 (c.1363A>T, 2326C>T, 1790T>C), DUOX1 (c.656C>T), TG (c.353C>T, 5921T>C) and DUOX2 (c.908C>T). Furthermore, among the 8 CH cases, 10 novel point mutations (pathogenic, likely pathogenic or with unknown functional effect) were found in 6 genes, including NKX2.1 (c.313G>A), SLC26A4 (c.1796C>T), NKX2.5 (c.428G>A), TG (c.1963C>T, 4378G>A, 6130C>T, 3416C>T) and DUOX2 (c.518A>G). Moreover, a small 9bp hemi allelic deletion was identified in the NKX2.1 gene. Mutation in the TG c.1963 (Q655*) leading to stop codon and deletion in the NKX2.1 was most likely linked to CH. The novel and known sporadic mutations are listed in Table 6.

Table 6. Sporadic mutations.

#	Gene	GeneBank ID (NM)	Base change	Protein change	ExAc MAF	Read depth	Classification
39	*TRH*	007117.4	c.212C>T	A71V	0.00039	997	C*
39	*TSHR*	000369.2	c.106G>C	D36H	0.00602	966	C*
40	***NKX2.1***	**001079668**	**c.313G>A**	**V105M**	—	—	**C***
42	***SLC26A4***	**000441.1**	**c.1796C>T**	**A599V**	**0.00019**	**915**	**B**
42	***NKX2.5***	**001166176.1**	**c.428G>A**	**R143Q**	**0.00082**	**994**	**C**
55	*SLC26A4*	000441.1	c.1363A>T	I455F	—	640	C
56	*DUOX1*	017434	c.656C>T	P219L	0.00010	989	C
57	*TG*	003235.4	c.353C>T	P118L	0.01471	510	C
57	*DUOX2*	014080.4	c.908C>G	P303R	0.01067	564	B
59	*SLC26A4*	000441.1	c.2326C>T	R776C	0.00200	496	C
71	***NKX2.1***	**001079668**	**c.793_801del**	**G264-266del**	—	—	**C***
73	***NKX2.1***	**001079668**	**hemidel**	**—**	—	—	**A**
77	***TG***	**003235.4**	**c.1963C>T**	**Q655***	**0.00013**	**329**	**B**
77	***TG***	**003235.4**	**c.4378G>A**	**V1460I**	**0.00020**	**182**	**B**
90	***DUOX2***	**014080.4**	**c.518A>G**	**A173S**	—	**997**	**C**
99	***TG***	**003235.4**	**c.6130C>T**	**R2044C**	**0.00005**	**996**	**B**
99	*TG*	003235.4	c.5921T>C	M1974T	0.08417	999	C*
103	*SLC26A4*	000441.1	c.1790T>C	L597S	0.00826	988	B
103	***TG***	**003235.4**	**c.3416C>T**	**S1139L**	**0.00030**	**719**	**B**

Novel mutations are bolded. **Abbreviations:** ExAc MAF — Exome aggregation consortium minor allele frequency; Classifications: A — pathogenic; B — likely pathogenic; C — functional effect unclear; * — stop codon. Table modified from the (Löf et al., 2016), with obtained permission from the Thyroid®, Mary Ann Liebert Inc., publisher.

The thyroid of patients carrying sporadic mutations, was either normal, hypoplastic or not developed. Furthermore, for three of the CH patients, heart issues were diagnosed, and the diagnoses included a ventricular septal defect (VSD), persistent foramen oval (PFO) and a ventricular septal defect (VSD). Four CH patients carrying sporadic mutation were diagnosed at birth with mild respiratory problems or respiratory distress syndrome (RDS). No developmental problems were noticed in

the remaining diagnosed patients. In summary, screening of the sporadic cases identified the genetic cause of CH in 13 patients. Clinical data of patients carrying sporadic mutations is shown in Table 7.

Table 7: Clinical data of patients with sporadic mutations.

#	uTSH / uT4 / fT4	TSH (at day)	fT4	TG	Disease	Age	Sex	Other diagnosis in family members
39	9 -	9.1	16.1	-	HyperTSH	5mo	F	Mild developmental delay
40	16 -	8.3 (5y)	15.4 (5y)	-	HyperTSH , hemi- genesis	5yrs	M	-; M: AIH
42	240 -	270 (3d)	10.3 (3d)	-	CH	0	F	RDS
54	373 -	>100 (3d)	6.0 (3d)	-	CH	0	M	-
55	880 4 -	>100 (3d)	<3.0 (3d)	-	CH	0	M	Hyperbilirubinemia
56	530 -	550 (4d)	8.2 (4d)	1.86	CH	0	F	-
57	44 108 13.8	23 (3d)	25.1 (3d)	9.66	CH	0	M	Transient tachypnea, hypoglycemia
59	83 -	64 (3d)	20.4 (3d)	4.8	CH	0	F	VSD, M: abnormal TFTs w/ anomalic thyroid
70	111 -	ND	-	-	CH, hypoplasia	0	M	-
71	520 -	258 (3d)	14.4 (3d)	-	CH, agenesis	0	F	Prematurity (36+0)
73	400 -	170 (3d)	<5.2 (3d)	-	CH	0	M	RDS, PH, interstitial lung disease, mental and retardation
77	84 -	78 (3d)	19.5 (3d)	<0.0 5	CH, normal	0	F	PDA, PFO
78	88 113 10.8	53 (3d)	15.8 (3d)	0.89	CH	0	M	-
90	250 83 -	375 (8d)	-	-	CH, agenesis	0	F	VSD, M: thyroid carcinoma
92	12 -	13 (13d)	14.8 (13d)	-	CH, hypoplasia	27d	M	Gemini
99	315 67 7.8	380.9 (2d)	7.5 (2d)	2.19	CH, normal	0	F	Asthma
103	670 116 12	250 (3d)	16.3 (3d)	4.99	CH	0	M	-

uTSH/uT4/fT4 = hormone levels measured at birth from umbilical cord; TSH and fT4 = confirmation of the positive screening values (age). **Ref. values:** uTSH/uT4/fT4: 120 nM/L, >10 pmol/L); control TSH and fT4 at 3 days old: 0.4–6 mIU/L and 9–19 pmol/ L, TG: 3.5–56 lg/L; **Abbreviations:** uTSH – umbilical thyroid-stimulating hormone (TSH); uT4 – umbilical thyroxine (T4); fT4 – free thyroxine (T4); TG – thyroglobulin; RDS – respiratory distress syndrome; VSD – ventricular septal defect; TFTs – thyroid function tests; PH – pulmonary hypertension; PDA – persistent ductus arteriosis; PFO – persistent foramen ovale; M – mother; AIH – autoimmune thyroid disease. Table modified from the (Löf et al., 2016), with obtained permission from the Thyroid®, Mary Ann Liebert Inc., publisher.

5.1.1.2 Familial mutations and clinical profile

Among the 11 families affected with CH, 4 previously described pathogenic mutations were identified, including insertions in TPO (c.1182_1183insCGGC) and point mutations in TSHR (c.1555C>T) and PAX8 (c.91C>T). Moreover, 2 novel monoallelic familial mutations were found in TPO (c.1212G>A) and TG (c.1963C>T).

All cases carrying the TPO mutations were biallelic, except in one case where the person had an additional monoallelic novel TPO c.1313G>A mutation. Moreover, in family #11, with two children, a PAX8 c.91C>T (p.R31C) mutation was found, leading to a loss of functionality (Table 8). Furthermore, in family #6, a rare novel heterozygous TG c.1963C>T (Q655*) mutation was found, leading to premature termination of translation at position 655. This mutation was present in the healthy father but was not present in the other sibling, thus it does not segregate with the phenotype. Identified familial mutations are listed in Table 8.

Table 8. Familial mutations.

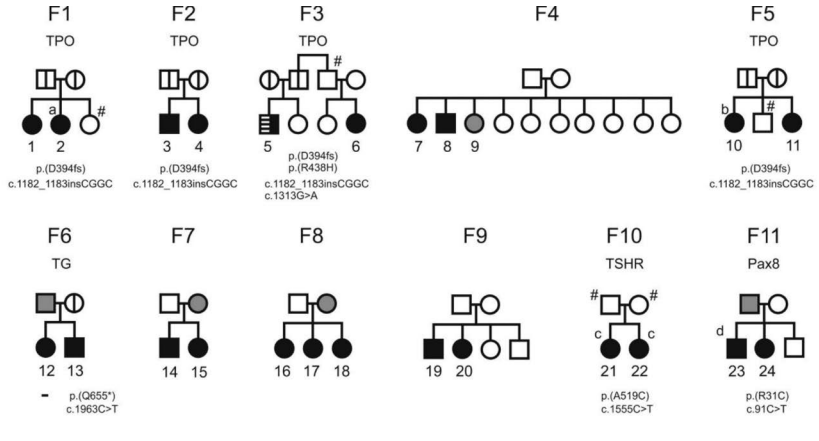
Gene	GeneBank ID	Base change	Protein change	ExAc MAF	Mono-/ Biallelic	RD	CI
TPO	000547.5	c.1182_1183 insCGGC	D394fs	—	Biallelic	11	A
TPO	000547.5	c.1182_1183 insCGGC	D394fs	—	Biallelic	3	A
TPO	000547.5	c.1182_1183 insCGGC	D394fs	—	Monoallelic	4	A
TPO	000547.5	**c.1313G>A**	**R438H**	—	**Monoallelic**	**997**	**B**
TPO	000547.5	c.1182_1183 insCGGC	D394fs	—	Biallelic	8	A
TG	**003235.4**	**c.1963C>T**	**Q655***	**0.0001**	**Monoallelic**	**887**	**B**
TSHR	000369.2	c.1555C>T	R519C	0.00004	Monoallelic	997	A
PAX8	003466.3	c.91C>T	R31C	—	Monoallelic	992	A

Novel mutations are bolded. **Abbreviations:** ExAc MAF ExAc MAF — Exome aggregation consortium minor allele frequency; RD – Read depth; CI - classifications: A — pathogenic; B — likely pathogenic; C — functional effect unclear; * — stop codon. Table modified from the (Löf et al., 2016), with obtained permission from the Thyroid®, Mary Ann Liebert Inc., publisher.

Among patients affected with familial CH, and TPO mutations the thyroid gland was normal or enlarged and no goiter was observed.

No disease-causing mutations in TG were found in 11 families, however, two children were carrying heterozygous TG variant and were affected with goitrous CH, although it was not observed to segregate with the phenotype. In two siblings carrying the PAX8 c.91C>T (p.R31C) mutation and affected with CH, the thyroid

gland was hypoplastic and kidney agenesis was visible in one of the siblings. The clinical data and pedigrees of the pateints are shown in Figure 8.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Ref. values
uTSH mU/l	300	256	261	221	407	250	184	300	n	470	530	60	270	633	292	240	>100	288	540	660	16	17	390	310	0.35-40 mU/l
TSH mU/l	420	287	324	209	454	>60	288	100	11	758	813	277	426	300	361	144	>100	60	557	173	29	48	239	334	0.35-40 mU/l
fT4 pmol/l	5.3	5.2	3.3	4.5	3.9	NA	5.2	NA	8.4	3.2	2.9	7.7	3.8	6.7	8.0	25	14	34.6	3.4	6.2	14.2	24.7	13.9	12.8	9-19 pmol/l
*TG µg/l	12.2	2.8	14	78	10.4	NA	33	NA	153	11	246	NA	NA	NA	NA	NA	NA	NA	<.05	0.9	NA	NA	NA	NA	3.5-56 µg/l
Age (yr)	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	.2	.6	0	0	
Thyroid size	n*	n	n*	n*	n*	n*	n	+	+	n	n*	+	n	a	a	n	n/h	n	n	n	n	n	h	h	

Figure 8. Genetic and clinical summary of the familial congenital hypothyroidism cases. Pedigrees: rectangular – male; circle – female. Colors: Black – affected patients with CH; symbols with midline – asymptomatic heterozygous carriers; gray – adult-onset hypothyroidism; white – no thyroid disease. Lined box – TPO R438H mutation. Letters above symbols: a – mild hypotonia, abnormal hearing response, delay of speech and motor development; b – abnormal hearing; c – born in week 29 + 3; d – renal agenesis. **Clinical data:** The clinical data below pedigrees shows hormonal values (uTSH, TSH and fT4) measured at age of 3 days or during the recruitment to the study. Thyroglobulin (Tg) concentration was measured after recruitment to the study. Thyroid size evaluated by thyroid USG: n – normal thyroid size and location; + – goiter; a – athyreosis/no thyroid gland; h – hypoplastic or small thyroid; n* – no clinical signs of goiter during follow-up; # – no DNA available. Original figure is reprinted from the (Löf et al., 2016), with obtained permission from the Thyroid®, Mary Ann Liebert Inc., publisher.

5.1.2 In vitro validation of novel PAX8 R31C mutation

In Vitro validation of novel PAX8 c.91C>T, R31C mutation identified in the family #11, was performed to study the impact of mutations on protein activity. Protein modeling and in vitro test showed that c.91C>T and R31 mutations showed about 1.5-fold decreased luciferase activity, compared to WT PAX8, thus leading to complete inactivation of the PAX8 transcription factor (n=15, ****$p \leq 0.0001$).

5.1.3 In vitro validation of novel NKX2.1 (c.313G>A, V105 and c.793_801del, G237-G239del) mutations

In vitro validation of the NKX2.1 mutation identified in sporadic cases, was performed to study the impact of mutations on protein activity. In vitro tests showed no difference in NKX2.1 c.313G>A, V105 and c.793_801del, G237-G239del activity, comparing to WT NKX2.1.

5.2 The role of G-proteins in the thyroid

5.2.1 Characterization of the inducible thyrocyte-specific G alpha s Knock-Out (iTGα_sKO) mouse model

At the age of 4 weeks, mice were injected with tamoxifen solution which triggered transition of CreERT2 into the nucleus and induced a thyrocyte-specific recombination, leading to excision of the *Gnas* exon 1 and generation of the iTGα_sKO mice. The Vitro bTSH stimulation test, performed on isolated thyrocytes, showed significant decreased basal and stimulated cAMP with bTSH in KO thyrocytes (red color), compared to thyrocytes isolated from the $Cre^{(-/-)}$ Tam-treated mice (blue color, figure 9A). Moreover, IHC staining performed on the thyroid sections showed decreased Gα_s staining in KO thyrocytes (right panel), compared to $Cre^{(-/-)}$ Tam-treated control mouse thyrocytes (left panel, Figure 9B)

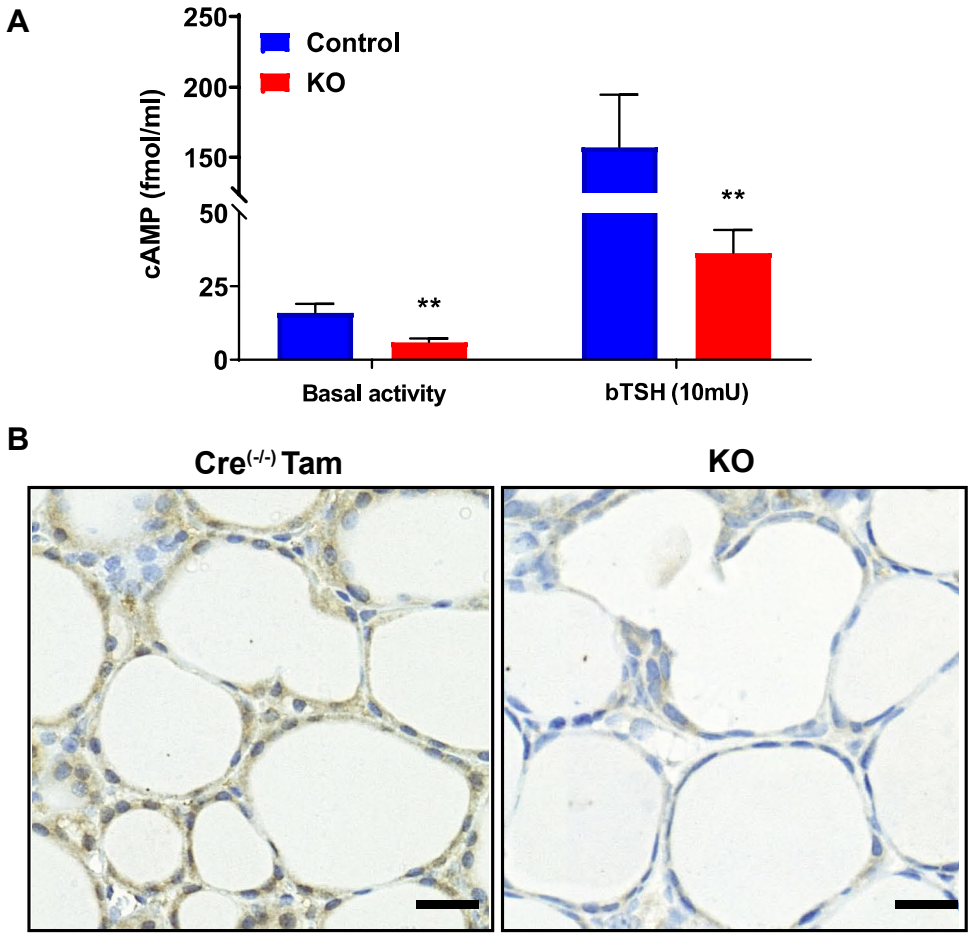


Figure 9. iTGα$_s$KO mouse model KO confirmation. (A) *In Vitro* cAMP production functionality test in control and iTGα$_s$KO thyrocytes. Significance levels: **p = 0.01. The whiskers represent mean with SD values. (B) Immunohistochemistry (anti-G alpha s) of Cre$^{(-/-)}$ Tam control (left panel) and iTGα$_s$KO (KO) thyroids (right panel). Scale bars: 50 μm.

5.2.1.1.1 Hormone concentrations, thyroid, and pituitary weight

One month after the tamoxifen injection, at the age of 2 months, the iTGα$_s$KO mice had significantly increased serum TSH (Figure 10A) and decreased or undetectable serum total T_4 concentrations (Figure 10B) compared to the vehicle (Veh) Cre$^{(+/-)}$ or Cre$^{(-/-)}$ and tamoxifen (Tam) treated Cre$^{(-/-)}$ controls. Similarly, at the age of 6 months, the same changes in TSH (Figure 10C) and total T_4 (Figure 10D) concentrations were observed.

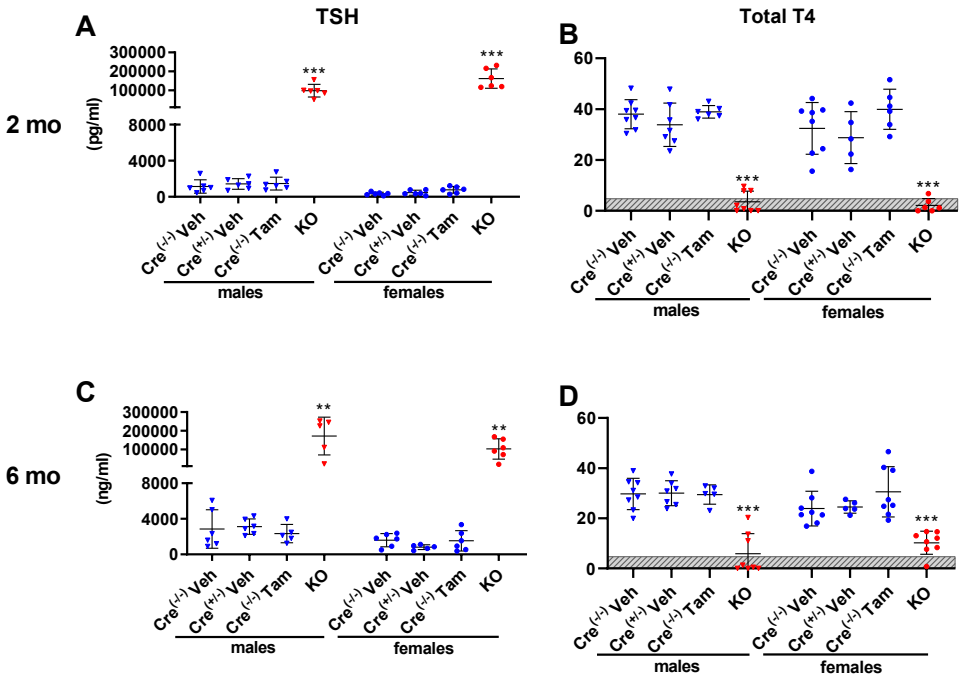


Figure 10. TSH and total T4 concentrations. TSH (A and C) and total T4 (B and D) concentrations in the vehicle (Veh) Cre expressing ($Cre^{(+/-)}$) or Cre negative ($Cre^{(-/-)}$) and tamoxifen (Tam) treated $Cre^{(-/-)}$ controls and $Cre^{(+/-)}$ Tam treated iTGα_sKO (KO) mice at the age of 2- and 6 months. Significance levels: **p = 0.01; ***p = 0.001. The statistical analysis was performed between $Cre^{(-/-)}$ Tam and KO groups. The whiskers represent mean with SD values. The grey area shows undetectable total T4 concentration. Abbreviations: TSH – Thyroid-stimulating hormone; T4 – total thyroxine; $Cre^{(-/-)}$ – Cre recombinase non-expressing mice; Veh – Vehicle treated mice; $Cre^{(+/-)}$ – Cre (+/-) recombinase expressing mice; Tam – tamoxifen treated mice; KO – tamoxifen-inducible thyrocyte-specific Gα_s KO mice (iTGα_sKO). Modified from (Patyra et al., 2018).

Moreover, the weights of the thyroids (Figure 11A and C) and pituitary (Figure 11B and D) of the iTGα_sKO were reduced and increased, respectively, compared to the controls at 2 and 6 months of age (1 month and 5 months after the tamoxifen injections).

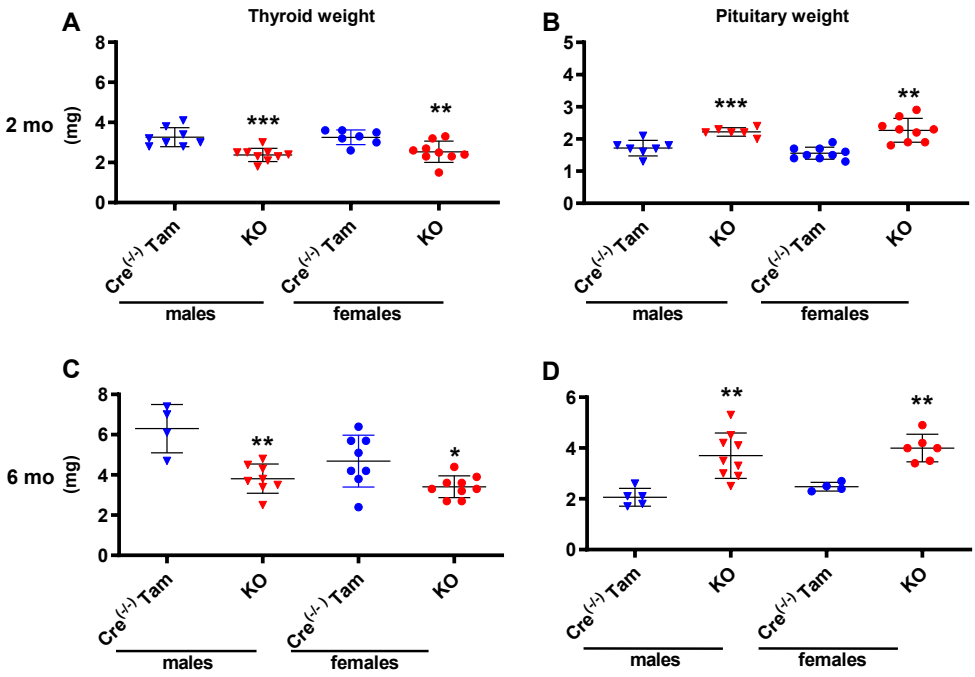


Figure 11. Thyroids and pituitary weights. Thyroid (A and C) and pituitary (B and D) weights in the Cre negative (Cre$^{(-/-)}$) tamoxifen (Tam) treated controls and Cre expressing (Cre$^{(+/-)}$) Tam treated iTGα$_s$KO (KO) mice at the age of 2- and 6 months. Significance levels: *p = 0.05; **p = 0.01; ***p = 0.001. The whiskers represent mean with SD values. Modified from (Patyra et al., 2018).

5.2.1.2 Thyroid histology and gene expression

Thyroid HE histology analysis demonstrated that iTGα$_s$KO mice had decreased thyrocyte thickness and much more smaller thyroid follicles, compared to the Cre$^{(-/-)}$ Tam-treated control mouse thyroids at the age of 2 months in both males and females (Figure 12A). Moreover, at the age of 6 months, the same reduction in thyrocyte thickness and thyroid follicle size was observed, however, hyperplastic, and neoplastic regions were occasionally visible in both males and females (Figure 12B).

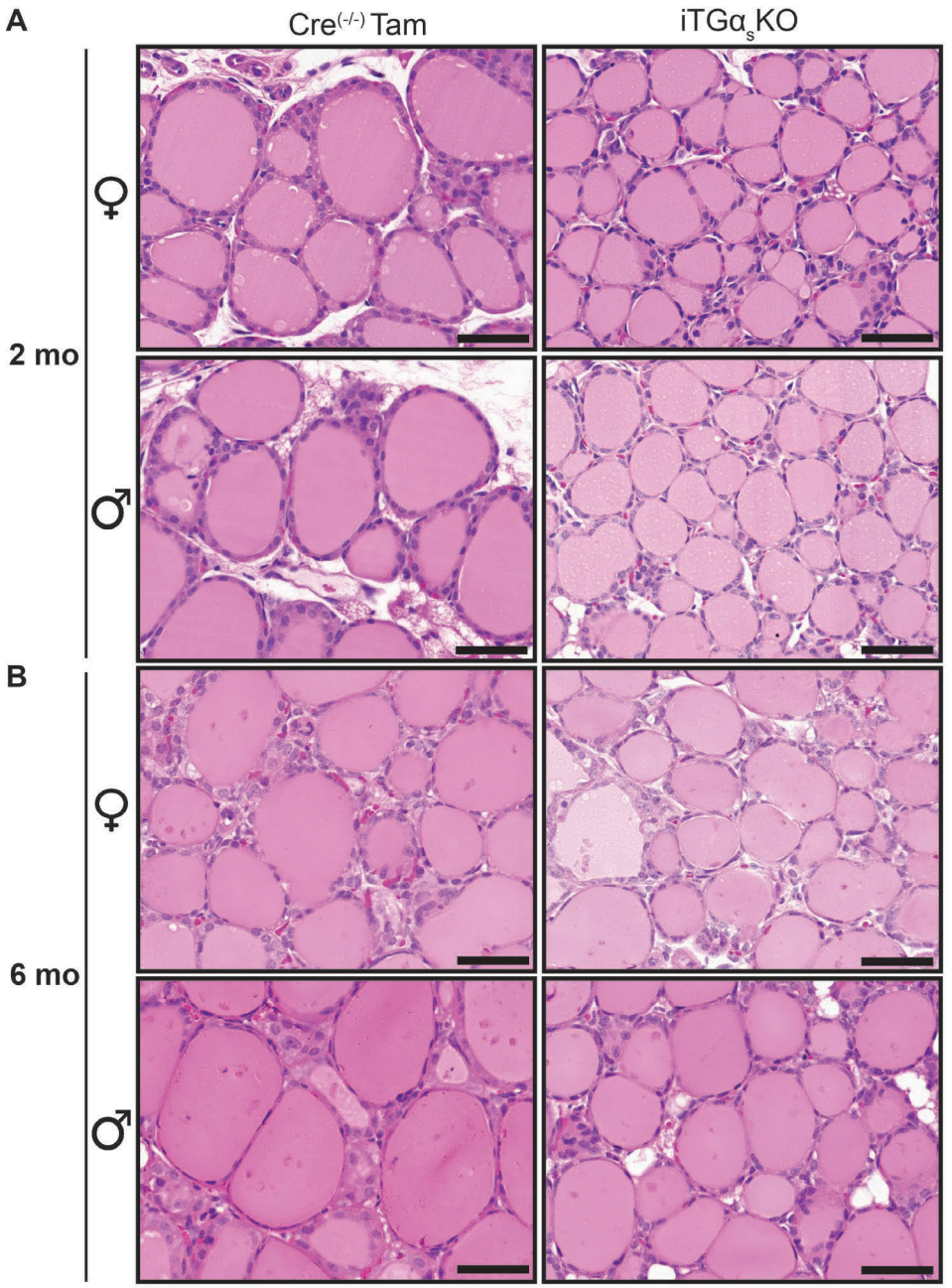


Figure 12. Thyroid histology and morphometric analysis in the iTGα$_s$KO (KO) and control (Cre$^{(-/-)}$ Tam) mouse thyroids. Hematoxylin-eosin staining of male and female control (Cre$^{(-/-)}$ Tam) and the iTGα$_s$KO (KO) mouse thyroids at the age of 2- (A) and 6-months (B). Scale bar: 50 μm. Abbreviations: Tam – tamoxifen treated. Modified from (Patyra et al., 2018).

Morphometric analysis (Figure 13A and B) of the iTGα_sKO thyroid gland revealed significant reduction of adjacent thyrocytes thickness in the 2 month (Figure 13B) and 6-month-old mice (Figure 13D), compared to the WT controls. Furthermore, the follicle lumen area was significantly reduced in both 2- (Figure 13C) and 6 months (Figure 13E) old iTgα_sKO mice (red color), compared to euthyroid Cre$^{(-/-)}$ Tam-treated control thyroids (blue color).

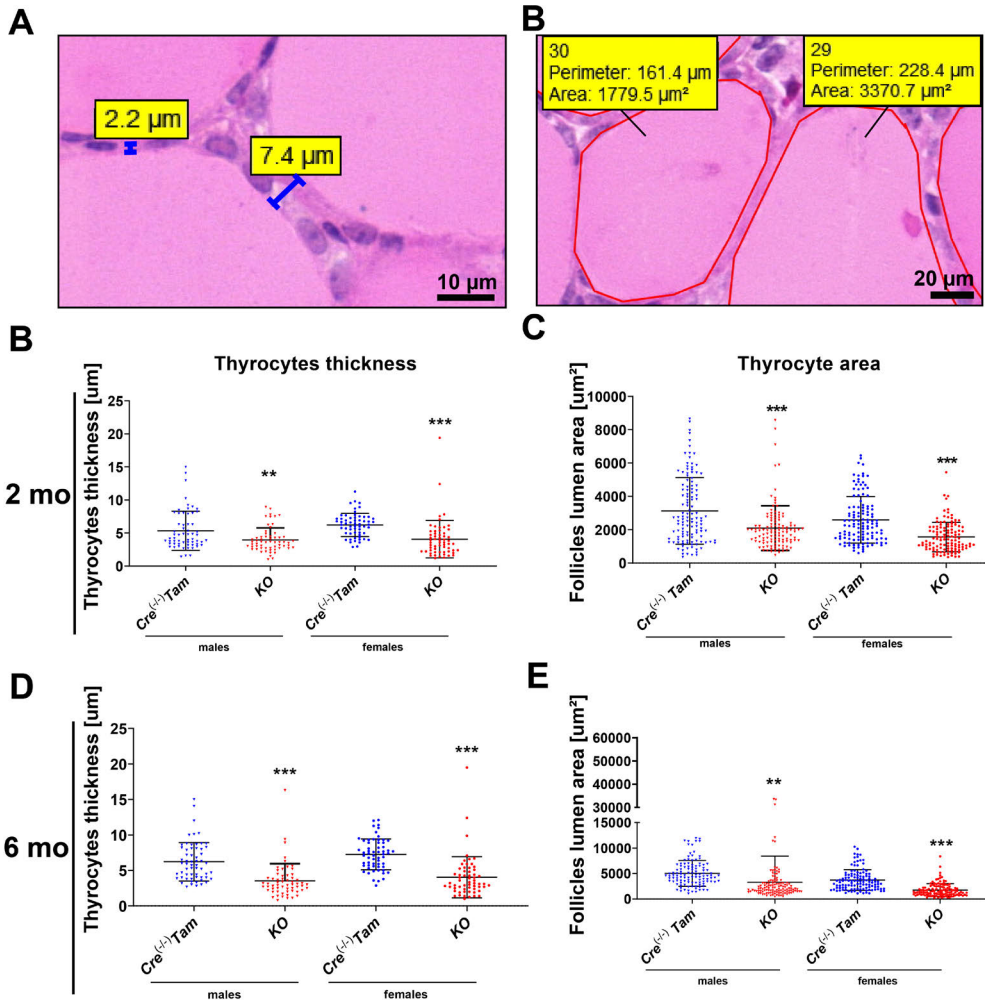


Figure 13. Morphometric analysis of thyrocytes' thickness and follicle lumen area in the iTGα_sKO and control (Cre$^{(-/-)}$ Tam) thyroids. Illustration of quantification method of the thyrocytes' thickness (A) and follicle lumen area (B) in the 2- (B and C) and 6- (D and E) months old iTGα_sKO (red color) and Cre$^{(-/-)}$ Tam-treated control (blue color) thyroids. Significance levels: *p = 0.05; **p = 0.01; ***p = 0.001. The whiskers represent mean with SD values. Modified from (Patyra et al., 2018).

Gene expression analysis of 6-month-old males, showed reduced expression of *Hhex* and *Pax8* transcription factors, and TH synthesis/conversion-related genes – *Tg*, *Tpo*, *Nis* and *Dio1* (Figure 14A). In 6-month-old females, expression of *Hhex*, *Nkx2.1*, *Tg* and *Dio1* was decreased, compared to WT controls (Figure 14B). No changes in the genes involved in antioxidation (*Srxn1* and *Sod3*) was detected.

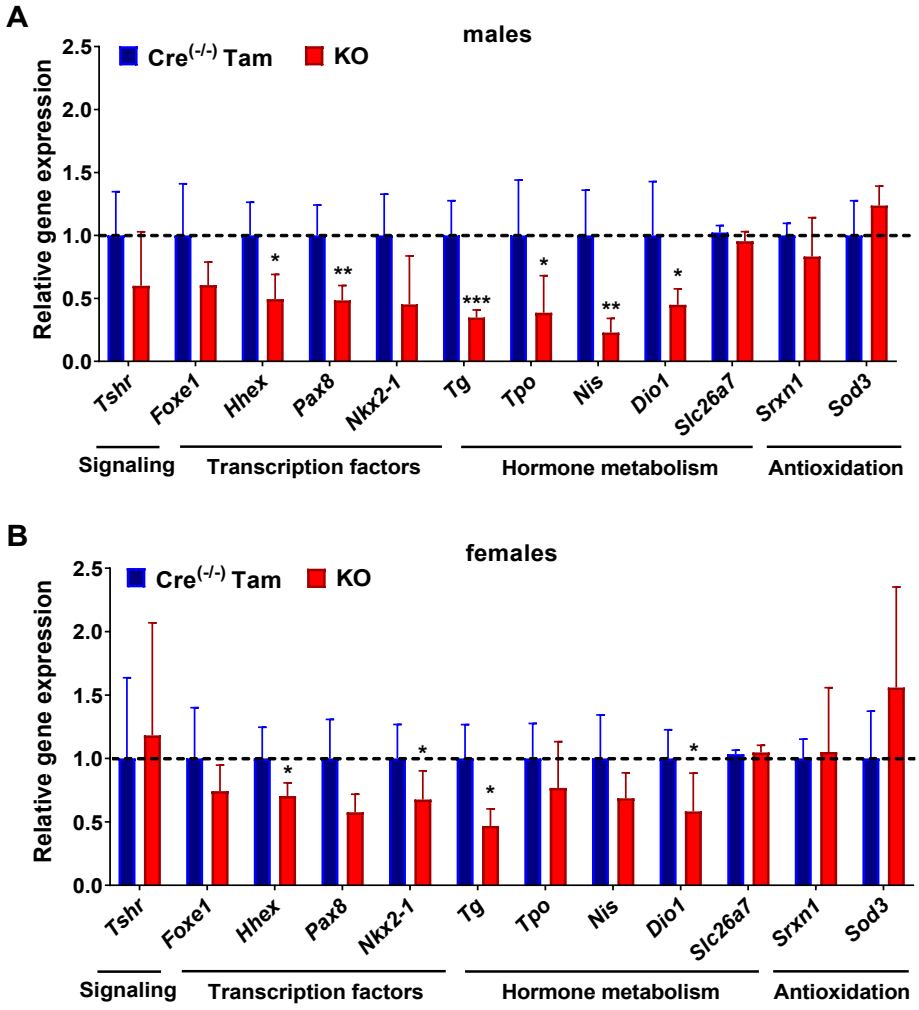


Figure 14. Gene expression analysis in the iTGα$_s$KO mouse thyroid. Relative expression of genes involved in signaling, transcription, hormone metabolism, and antioxidation in the control (Cre$^{(-/-)}$ Tam) and iTGα$_s$KO (KO) mouse thyroids at the age of the 6 months in males (A) and females (B). Abbreviations: *Tshr* – thyroid stimulating hormone receptor; *Foxe1* – Forkhead box protein E1; *Hhex* – Hematopoietically-expressed homeobox protein; *Pax8* - Paired box gene 8; *Nkx2-1* – NK2 homeobox 1; *Tg* – thyroglobulin; TPO – Thyroid peroxidase; *NIS* – Sodium/iodide cotransporter; *Dio1* – Iodothyronine deiodinase 1; *Srxn1* – Sulfiredoxin-1; *Sod1* – Superoxide dismutase 1. Significance levels: *p = 0.05; **p = 0.01; ***p = 0.001. The whiskers represent SD values. Modified from (Patyra et al., 2018).

5.2.2 Metabolic consequences

As expected, the presence of tamoxifen-induced hypothyroidism at the age of 2 months in the iTGα_sKO mice impacted their metabolic parameters. Body weight was significantly decreased in the iTGα_sKO male mice with age, compared to the euthyroid Cre$^{(-/-)}$ Tam controls (**II:** Figure 3A, left panel). Interestingly, female KO mice exhibited no body weight changes. Furthermore, tail lengths in both genders were unchanged (**II:** Figure 3A, right panel).
At the age of 2 months, body temperature was reduced in the hypothyroid males and females compared to the euthyroid Cre$^{(-/-)}$ Tam controls (**II:** Figure 3B, right panel). In parallel with a reduced body temperature, the iTGα_sKO mice consumed less food, both the males and the females (**II:** Figure 3B, left panel).
At the age of 6 months (4 months of hypothyroidism), reduction of the adipose tissue percentage only affected the hypothyroid males, whereas the hypothyroid females were unaffected (**II:** Figure 3C**)**.
Interestingly, night activity from 9 pm till 1 am was lower in both female and males iTGα_sKO mice, compared to the euthyroid Cre$^{(-/-)}$ Tam controls (**II:** Figure 3D).

5.2.2.1 Development of thyroid tumors

At 6 months of age, the histological analysis of iTGα_sKO thyroids besides exhibiting reduced follicle lumen area and thyrocyte thickness, revealed visible hyperplastic and PTC-like structures, containing PTC features such as elongated, overlapping or enlarged nuclei, and grooves (Figure 15).

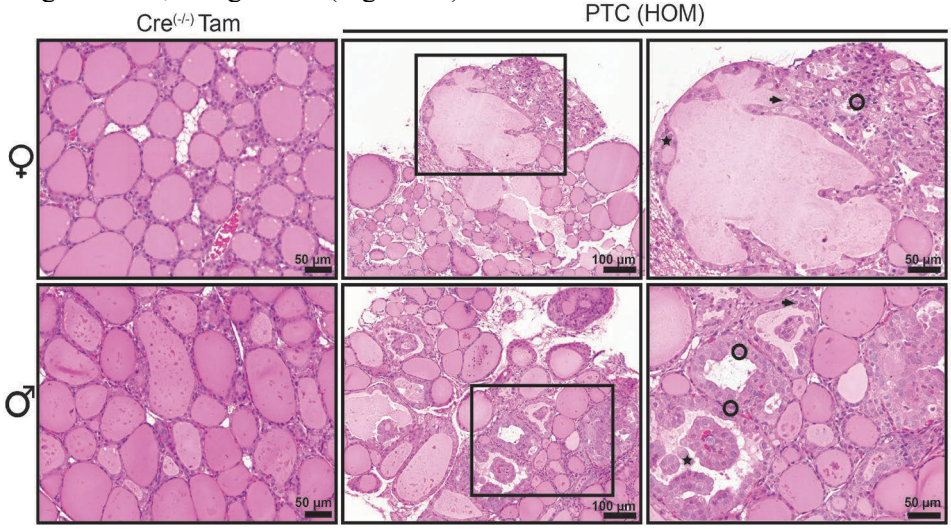


Figure 15. Papillary thyroid carcinoma (PTC)-like structures in the iTGα_sKO mouse thyroids. Papillary thyroid carcinoma-like lesions in the HOM iTGα_sKO mouse thyroids. The symbols show PTC features: circle - enlarged and elongated nuclei with crowding and overlap; star – cytoplasm clearance; arrowhead – nuclear pseudoinclusions.

Immunohistochemical staining of the iTGα_sKO mouse thyroids showed that the hypothyroid (KO) areas, where recombination occurred were not stained with Gα_s and phospho-ERK1/2 (pErk1/2), but only with Nkx2.1 and occasionally with Ki67. Furthermore, the PTC-like structures were stained with Gα_s, Nkx2.1, Ki67 and pErk1/2 (Figure 16A-D).

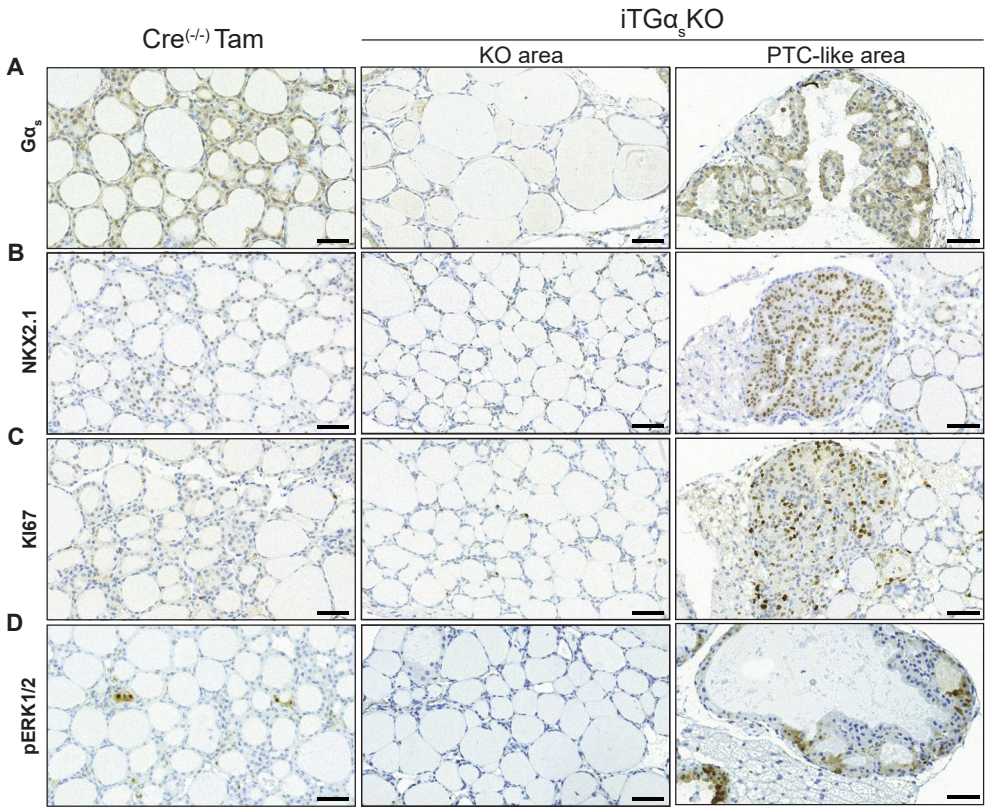


Figure 16. IHC thyrocyte staining, proliferation, and carcinogenesis markers in the iTGα_sKO thyroids. IHC staining of the Gα_s (A), NKX2.1 (B), KI67 (C) and pERK1/2 (D) IHC staining in the Cre$^{(-/-)}$ tam controls and iTGα_sKOs (KO and PTC-like areas) thyroids. Scale bar: 50 µm.

5.3 Impact of thyroid hormones on adrenal development and function in the TSHR$^{(D633H)}$ KI and TSHR KO* mouse models

5.3.1 Validation of TSHR(D663H) KI and TSHR KO* mouse models and thyroid function test

5.3.1.1 TSHR$^{(D633H)}$ KI

After generation, the TSHR$^{(D633H)}$ KI model was further validated by sanger sequencing, a primary cell culture bTSH stimulation test and by measurement of TSH and TH in the follow-up groups (Jaeschke et al., 2018).
TSH concentrations in females were decreased at the age of 1, 2, 4, 6, 9, and 12 months in the HOM TSHR$^{(D633H)}$ KI females, compared to, 2-, 4-, 6-, 9- and 12-month-old WT littermates, respectively (**III:** Figure S1).

Homozygous KI female mice exhibit transient hyperthyroidism at the age of 1, 2 and 4 months of age, compared to euthyroid WT controls. From the age of 6 to 9 months, no changes were observed in total T_4 concentrations. At the age of 12 months, HOM KI females again became hyperthyroid, compared to their 1-, 2-, 4-, and 12-months old WT littermates, respectively **(III:** Figure 1A).

5.3.1.2 TSHR KO*

After generation, the TSHR KO* model was further validated by Sanger sequencing and measurement of TSH and TH at the age of 1 month (**III**: Figure S3A and B) (Patyra et al., 2022).
TSHR KO* mice had significantly elevated TSH and an undetectable total T_4, compared to WT littermates (**III:** Figure S3E and F). Histological analysis was not possible due to the fact that HOM mice were lacking thyroid glands, compared to their HET and WT littermates (**III:** Figure S3D). In addition to the lack of thyroid, TSHR KO* (HOM) mice were much smaller and weaker, compared to their HET and WT littermates (**III:** Figure S3C).

5.3.1.3 Other pituitary hormones and ACTH concentrations in both hyper- and hypothyroid mouse models

Pituitary hormones, including LH, PRL, FSH, and GH were measured at the age of 1, 2, 4, 6 and 12 months and ACTH and BDNF at the age of 1, and 2 months, to study the impact of hyperthyroidism on pituitary function in the $TSHR^{(D633H)}$ KI female mice. At the age of 2 months, LH and PRL concentrations were significantly decreased in HOM KI females compared to WT littermates **(III:** Figure S8A and B). Moreover, at the age of 4 months, FSH and PRL were increased and decreased in the HOM KI females when compared to the WT controls, respectively (**III:** Figure S8B and C). No ACTH changes were observed in the HOM KI females (**III:** Figure S8D).

5.3.2 Adrenal weight and histology

$TSHR^{(D633H)}$ KI HOM males had significantly elevated TH concentrations at the age of 1-2 months and HOM females at the age of 1-4 months, compared to WT littermates. Unexpectedly, adrenal gland weight was increased in HOM females compared to the WT controls (Figure 17A-F, left panel). No change in adrenal weight was observed in the HET females, as well as the $TSHR^{(D633H)}$ KI HET and HOM males (**III:** Figure S2A-F). Histological analysis of HOM female adrenals showed an enlarged X-zone area and a further delay in its vacuolization and involution, compared to the WT C57BL6/N adrenals (Figure 17A-F, right panel). Furthermore, TSHR KO* HOM adrenal weight was significantly decreased, compared to the WTs (Figure 17A). Interestingly, TH hormone concentration was positively correlated with the mean female adrenal weight ($r=0.54$, $***p<0.001$, $n=264$).

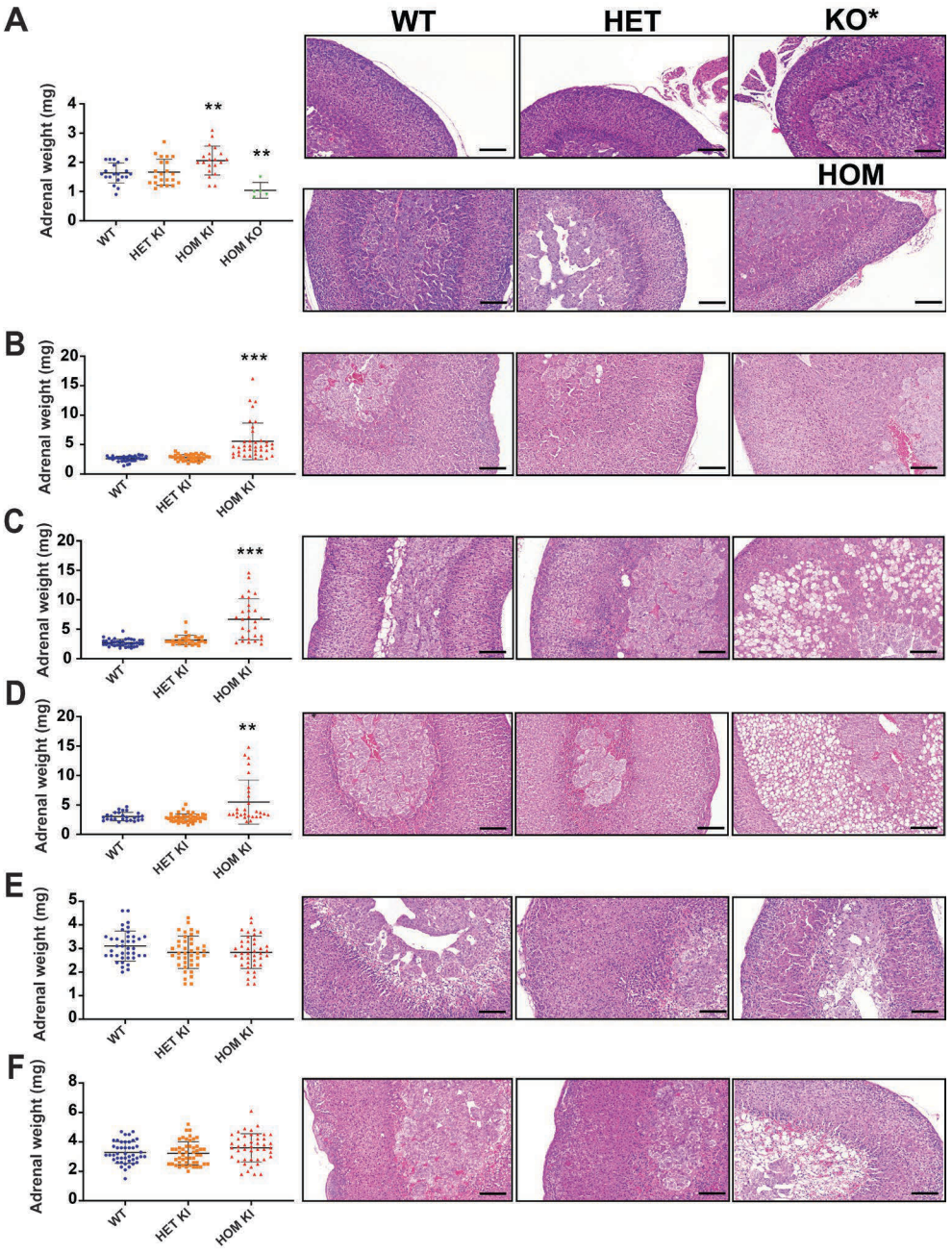


Figure 17. Adrenal weight and histology in the TSHR$^{(D633H)}$ KI and TSHR KO* HOM female mice. Adrenal weight and histology in the 1-month-old TSHR KO* and TSHR$^{(D633H)}$ KI (A) and 2 (B), 4 (C), 6 (D), 9 (E) and 12 (F) -month-old TSHR$^{(D633H)}$ KI female and WT controls. Significance levels: **p = 0.01; ***p = 0.001. The whiskers represent mean with SD values. Scale bars: 100 µm. Modified from (Patyra et al., 2022).

5.3.3 Localization of steroidogenic enzymes and Oil Red O (ORO) staining

IHC staining of CYP51, CYP11A1, HSD3B2, CYP11B1 and CYP11B2 was performed to detect enzyme distribution inside the adrenal gland, including altered X-zone areas. IHC staining showed that the cortex was stained with all enzymes listed above. Unlike the altered X-zone area, HSD3B2 staining was not visible in the altered area. None of the enzyme was present in the medulla area (**III**: Fig. 4A). Oil Red O (ORO) was performed to detect lipid accumulation in the adrenal gland. ORO staining showed intensive staining within the cortex of TSHR$^{(D633H)}$ KI HOM mice, compared to age matched WT littermates (**III:** Figure S4A-C). Moreover, ORO staining in TSHR KO* adrenals was reduced, compared to WT littermates (**III:** Figure S4D). No visible changes were observed in the adrenal medulla in the TSHR$^{(D633H)}$ KI mouse model (**III**: Figure S4). Interestingly, the adrenal medulla was located inside the cortex area in some of the TSHR KO* mice, however that could also be the reason for the expanded medulla area into the adrenal cortex.

5.3.4 RNAScope In Situ hybridization

Thrb and *Tshr* transcripts were localized using an RNAScope method to explore the location of transcripts involved in the TH action within different adrenal zones. The *Thrb* transcript was abundantly stained in the inner cortex of the TSHR KI adrenals (**III:** Figure S6B). Furthermore, the inner TSHR KO* adrenal cortex *Thrb* was almost absent (**III:** Figure S6E). No *Tshr* staining was detected in both TSHR$^{(D633H)}$ KI and TSHR KO* HOM adrenal glands **(III:** Figure S6C and F).

5.3.5 Adrenal gene expression panel

RNA sequencing was performed in the 2 months old TSHR$^{(D633H)}$ KI and TSHR KO* HOM females and WT controls, to explore alterations in gene expression induced by congenital hyper- and hypothyroidism. The gene expression analysis identified 186 upregulated and 26 downregulated genes in the TSHR$^{(D633H)}$ KI adrenals. Furthermore, in the hypothyroid TSHR KO* adrenals, 327 genes were up- and 34 were down-regulated. Adrenal progenitor genes, such as *Fgf9*, *Lef1*, *Mc2r*, *Wnt4* and *Rspo3* were down-regulated, whereas *Ptch2* and *Mrap* were up-regulated in the hyperthyroidism (Figure 18A). The most altered genes included those involved in cholesterol metabolism, including *Fdft1*, *Sqle*, *Lss*, *Tm7sf2*, *Msmo1*, *Nsdhl*, *Ebp*, *Dhcr24* and *Cyp51*, which were up-regulated in the hyperthyroid TSHR$^{(D633H)}$ KI and

down-regulated in the hypothyroid TSHR KO* adrenals (Figure 18B). Steroidogenesis involved genes, namely S*tar*, *Cyp11a1*, *Hsd3b1*, *Cyp21a1*, *Cyp11b1*, *Cyp11b2* were down-regulated in both hypo- and hyperthyroid mouse models (Figure 18C). Genes involved in catecholamine synthesis, including *Slc18: a1*/a2, *Th*, *Ddc, Dbh* and *Pnmt*, were down- and up-regulated in the KI and KO adrenals, respectively (Figure 18D). Finally, TH signaling linked genes, including *Slc16a10*, *Slc16a2* and *Thrb* were up-regulated in TSHR$^{(D633H)}$ KI and down-regulated and TSHR KO* mouse adrenals. However, the *Thra* gene (isoform 1 and 2) was up-regulated in the TSHR KO* adrenals (Figure 18E).

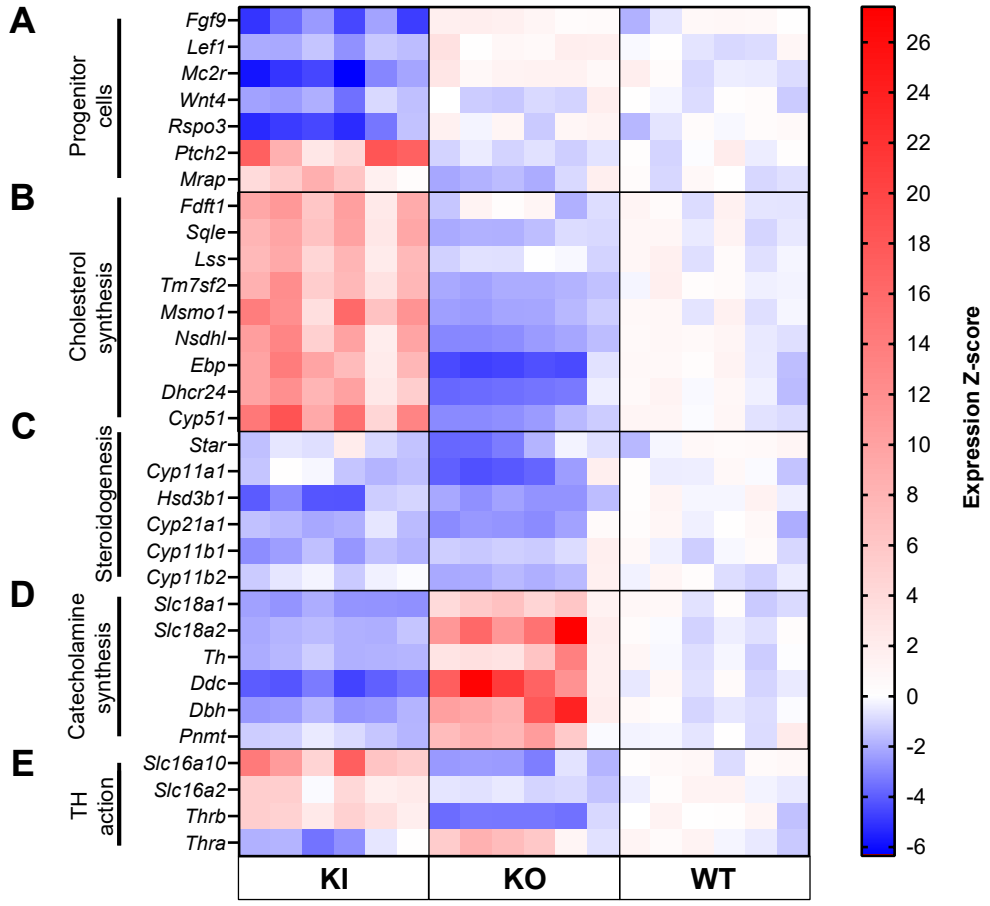


Figure 18. Adrenal gene expression analysis of selected genes in the TSHR$^{(D633H)}$ KI and TSHR KO* female mice. Adrenal gene expression of selected genes, including progenitor cells (A) and genes involved in the cholesterol synthesis (B), steroidogenesis (C), catecholamine synthesis (D) and thyroid hormone (TH) action (E) in the TSHR$^{(D633H)}$ KI (KI), TSHR KO* (KO) and WT female mice. Gene abbreviations: *Fgf9* – Fibroblast Growth Factor 2; *Lef1* – Lymphoid Enhaner Binding Factor 1; *Mc2r* – Melanocortin 2 Receptor; *Wnt4* – Wnt Family Member 4; *Rspo3* – R-Spondin 3; *Ptch2* – Patched 2; *Mrap* – Melanocortin 2 Receptor Accessory Protein; *Fdft1* – Farnesyl-Diphosphate Farnesyl/transferase 1; *Sqle* – Squalene epoxidase; *Lss* – Lanosterol synthase; *Tm7sf2* – Delta (14)-sterol reductase; *Msmo1* – Methylsterol Monooxygenase 1; *Nsdhl* – NAD(P) Dependent Steroid Dehydrogenase-Like; *Ebp* – EBP Cholestenol Delta-Isomerase; *Dhcr24* – 24-Dehydrocholesterol Reductase; *Cyp51* – Sterol 14α-demethylase; *Star* – Steroidogenic Acute Regulatory Protein; *Cyp11a1* – Cytochrome P450 Family 11 Subfamily A Member 1; *Hsd3β1* – 3β-Hydroxysteroid dehydrogenase; *Cyp21a1* – Cytochrome P450 Family 21 Subfamily A Member 1; *Cyp11b1/b2* – Cytochrome P450 Family 11 Subfamily B Member 1/2; *Th* – Tyrosine Hydroxylase; *Ddc* – Dopa decarboxylase; *Dbh* – Dopamine Beta-Hydroxylase; *Pnmt* – Phenylethanolamine N-Methyltransferase; *Slc18a1/2* – Solute Carrier Family 18 Member A1/2; *Slc16a10* – Solute Carrier Family 16 Member 10; *Slc16a2* – Solute Carrier Family 16 Member 2; *Thrb* – Thyroid Hormone Receptor Beta; *Thra* – Thyroid Hormone Receptor Alpha. Modified from (Patyra et al., 2022).

5.3.6 Intra-adrenal steroid hormone concentration, aldosterone, corticosterone, and sodium serum concentration

Sodium serum concentrations were elevated in the TSHR$^{(D633H)}$ KI female mice compared to WT littermates (**III:** Figure 6A). No changes in concentration were found in the TSHR KO* serum (**III:** Figure 6B).
Steroid analysis from the adrenal homogenates collected from the TSHR$^{(D633H)}$ KI female mice at the age of 4 months, showed decreased progesterone concentration in HOM KI female mice, compared to WT littermates (**III:** Figure 6E). No changes were found in the estradiol, estrogen, androstenedione, dihydrotestosterone, and testosterone concentrations (**III:** Figure 6E). Furthermore, no changes were observed in corticosterone and aldosterone concentrations in both mouse models (**III:** Figure 6C and D).

5.4 Steroid hormone concentrations in newborns diagnosed with premature adrenarche and congenital hypothyroidism

The umbilical serum thyroid stimulating hormone (uS-TSH) and 17-hydroxyprogesteronne (17-OHP) concentrations were measured in newborns diagnosed with PA to test any possible correlations between PA and CH (**III:** Figure 7A-C). Among the 33 confirmed CH cases, a weak correlation (***P=0.0001, r=0.51) between uS-TSH and 17-OHP was found **(III:** Figure 7D). Serum TSH concentrations in children with PA correlated positively with DHEA and DHEA-S. No correlation was found between serum TSH and androstenedione and testosterone. Among healthy controls, no correlations were found between TSH and DHEA, DHEA-S, androstenedione, and testosterone (**III:** Table 1).

6 Discussion

6.1 Screening of genetic variations in congenital hypothyroidism in Finland

CH is one of the most common preventable causes of mental retardation affecting every 1:2 000-3 000 newborns worldwide. The incidence of CH has been reported to be increasing, but the etiology is largely unknown. Genetic defects comprise only 10-20% of all CH cases, with few familial cases remaining unsolved. The genetics of CH has not been studied in Finland until now. In this thesis work we aimed to establish and test the next generation sequencing panel for the screening of genetic etiology of CH in a Finnish patient cohort. At the time when our first results were published (Löf et al., 2016) the NGS screening methods were being rapidly developed into the screening of genetic variations of candidate genes for multiple diseases from a research basis or in clinical practice. A common practice in the past when establishing genetic etiology of familial CH cases was to select the candidate gene based on the possible clinical signs using different algorithms and sequencing the gene using Sanger-sequencing. This was a challenge as over 15 causative CH gene existed, the possible clinical signs were subtle and variable (goiter, hypoplasia) and detailed clinical tests were not often performed (thyroid ultrasonography, iodine uptake or discharge tests). Regarding these problems the NGS panel offered a general, rapid, reliable, and cost-effective method to screen the suspected candidate genes. Our first panel consisted of exome coding regions of 13 candidate genes and showed good sequencing depth and validity among the control samples (Löf et al., 2016). In this analysis, we could identify the pathogenic candidate gene mutation in 6 of 11 screened families. Furthermore, screening of the cohort of apparently sporadic CH cases revealed several pathogenic and likely pathogenic mutations in several CH genes. Interestingly, despite the fact that Finland has a rather unique genetic makeup due to its strong genetic isolation and the historically small number of founder individuals, the genetic etiology of familial CH cases does not seem to differ largely. In our screenings the most common variant in familial cases was the *TPO*fs mutation which is the same as described previously and shown to be the most

prevalent cause for thyroid dyshormogenesis in several populations (Abramowicz et al., 1992; B. Bakker et al., 2000; Cangul et al., 2013). This mutation is "prevalent" in the Finnish South Ostrobothnia area where families with thyroid dyshormogenesis were known to exist (Virtanen et al., 1984). In addition, mutations in other "common" CH genes *PAX8* (R31C), *TSHR* (R519C), were also prevalent in Finnish CH families (Löf et al., 2016; Macchia et al., 1998). Due to the high sequencing and depth of coverage, our results suggested that it would be unlikely that we would have missed a pathogenic mutation in the exon coding areas of the selected genes in the 5 families without pathogenic variants in the screening panel. However, our screening could not detect larger gene deletions which are typical among some CH candidate genes such as *NKX2.1* and known as a common pitfall in NGS screening (Thorwarth et al., 2014). Thus, in addition to the NGS screening strategies, other more conventional techniques are still needed to detect e.g. chromosomal aberrations or larger genetic deletions. On the other hand, the NGS panel screening allowed us to select those 5 families with no new mutations in the candidate genes for exome-sequencing, which later led to the discovery of a novel CH gene, *SLC26A7* among the families (Cangul et al., 2018). In addition to the common *TPO*fs mutation this *SLC26A7* F631Lfs*8 variant seems to be more common in the Finnish population (Cangul et al., 2018). NGS techniques have developed rapidly and specifically the costs and time needed for an analysis has significantly decreased allowing e.g. the whole exome or whole genome sequencing to be performed in a more cost-effective way (Paulsson et al., 2021). However, interpreting the WES or WGS (intronic variant, oligogenic, incidental findings) is still a more challenging process than interpreting the specific panels. Perhaps, "virtual panels" from WES or WGS data allowing possible further analysis would be the most cost-effective method at the present time (Morris et al., 2021).

6.1.1 Insights provided by TSHR signaling: the role of inducible thyrocyte specific $G\alpha_s$ deletion in thyroid growth and function

TSH is the main thyroid regulator and by binding to its G protein-coupled receptor TSHR potentially activates all the G protein families, mainly the G_s- and $G_{q/11}$-mediated signaling cascades. Altered TSH concentrations, different autoantibodies or different TSHR mutations can lead to variations in the activation of G protein-mediated signaling, but there is still a knowledge gap concerning the role of the individual G protein cascades in thyroid pathophysiology. In this study, we generated inducible thyrocyte-specific $G\alpha_s$ deficient mice (iT$G\alpha_s$KO) to examine the impact of

$G\alpha_s$ on thyroid physiology. Validation of the model by analysis of Cre-mediated thyrocyte-specific $G\alpha_s$ recombination, $G\alpha_s$ protein expression and function, revealed that $G\alpha_s$ -mediated signaling was effectivley, but not entirely reduced in iTGα_sKO mouse thyrocytes. As expected, based on the essential role of $G\alpha_s$ in TH synthesis, the iTGα_sKO mice developed rapid onset hypothyroidism, with high TSH and low or undetectable total T_4 concentrations. This was in contrast to our previously developed mouse models with impaired $G_{q/11}$ or $G_{12/13}$ signaling, where only subclinical hypothyroidism or euthyroidism was observed, respectively (Kero et al., 2007; Löf et al., 2018). In line with the hypothyroidism at the age of 2 months, the thyrocyte thickness was significantly reduced in the iTGα_sKOs compared to the controls. However, unexpectedly at the age of 6 months, beside the thinner thyrocytes, some of the thyroid follicles in the mutant mice presented hyperplastic PTC-like lesions. Further immunohistochemical analysis indicated that those altered hyperplastic areas showed significant $G\alpha_s$ staining and were also NKX2.1-positive, suggesting that prolonged elevated TSH concentration led to the overgrowth of these cells. Previous studies have shown that high TSH or gene mutations leading to $G\alpha_s$ activation can lead to tumor growth (Xing, 2013). Furthermore, epidemiological studies have demonstrated an association between elevated TSH levels and an increased risk of developing thyroid cancer (Fiore & Vitti, 2012). In our model, strong staining of $G\alpha_s$ was observed in the hyperplastic and tumor areas suggesting that the tumors originate from the non-recombined thyrocytes expressing the intact $G\alpha_s$. These data indicate that $G\alpha_s$ is required for thyroid tumor development. On the other hand, high TSH concentration and intact Gα, were also present in mice lacking $G_{q/11}$, but they did not develop tumors (Kero et al., 2007), which suggests that simultaneous activation of both G_s and $G_{q/11}$ pathways are needed for tumorigenesis. However, the generalization of our findings should be evaluated critically due to differences in TSH-mediated signaling among various species (T. Kimura et al., 2001; Vassart & Dumont, 1992).

6.1.2 iTGα_sKO mice have impaired metabolic functions

After induction the iTGα_sKO mice developed rapid onset hypothyroidism, with high serum TSH and low or undetectable total T_4 concentrations. Hypothyroidism in this model can be considered to be comparable to that seen in primary hypothyroidism and it can be induced at any timepoint. However, the gene deletion does not occur in 100% of the thyrocytes and theoretically, a similar situation, partial $G\alpha_s$ deficiency could be seen in AHO type 1A cases, where the maternal transmission of the inactive $G\alpha_s$ mutant leads to an osteodystrophy phenotype associated with resistance to

several hormones (PTH, TSH and gonadotropins) and for example, hypothyroidism (Weinstein et al., 2005). In contrast to the clinical situations with a loss of G_s signaling in the thyroid, we speculate that the chronic TSH stimulation in the non-recombined thyrocytes in our model resembles the pathogenesis in patients with a constitutive active somatic TSHR/G_s mutation; this is typically associated with autonomously functioning thyroid adenomas and rarely with thyroid carcinomas (Parma et al., 1997; Pazaitou-Panayiotou et al., 2012).

Our hypothyroid iTGα_sKO mice had variations in several gender-specific metabolic parameters, including activity, body weight, temperature, food consumption, and amount of adipose tissue. Body weight was significantly decreased in male mice, comparing to their WT littermates. No significant change in body weight was found in females what can be linked to possibly lower recombination rate leading to milder phenotype. Moreover, food consumption and body temperature were decreased in both KO males and females, compared to the WT controls. In males, the total percentage of adipose tissue was significantly decreased when compared to WT controls. No changes were observed in KO females. Both males and females showed reduced activity at night, comparing to WT controls. Similar observations were found in a previous study, where MMI/$ClO4^-$/LoI-treated mice had a decreased body weight, food intake and body temperature, which are classical signs of hypothyroidism in mice (Rakov et al., 2017). Warner et al. (2013), using TRα1+m mice, found that hypothyroidism cause a defect in vasoconstriction, heat dissipation and conservation (Warner et al., 2013). Interestingly, recent studies identify an endocrine loop in which leptin and HPT axis play an important role in stabilizing body temperature in hypothyroid mice (Weiner et al., 2021). The weight loss in rodent models is a consequence of reduced food intake as well as adaptive thermogenesis to maintain body temperature (Kaspari et al., 2020). Gender-specific differences between males and females can be explained by different energy expenditure, sex-specific changes in Dio activity, different types of energy expenditure or by a reduced growth hormone concentration caused by hypothyroidism (Hervas et al., 1975). Furthermore, Rakov et al. (2017) in recent study, explored sex differences in TD between male and female mice, finding that total T4 concentrations were higher in hyperthyroid female mice than male mice, probably due to higher binding capacity of thyroxine-binding globulin by estrogens (Rakov et al., 2017). Independently from thyroid hormone status, estrogens are known to increase body temperature in female mice (Z. Zhang et al., 2021). Behavioral tests performed on hyperthyroid female mice showed that muscle strength was decreased but muscles were more resistant to fatigue than in males. Moreover, females showed higher locomotor activity and better motor coordination (Rakov et al., 2016, 2017). Unlike in rodents, human hypothyroidism is manifested with weight gain (Chaker et al., 2017).

Taking all aspects into consideration, our thyrocyte specific inducible $G\alpha_s$ deficient model demonstrates the essential role of $G\alpha_s$ in TH synthesis and shows that chronically elevated TSH can lead to the development of PTC-like tumors. This model is a useful tool to study adaptation mechanisms in hypothyroidism and G_s-mediated tumor development.

6.2 Thyroid hormones impact adrenal cortex in the hyper- and hypothyroid mouse models

In this study, we found that thyroid function directly impacts adrenal histology and gene-expression. We generated two contrary mouse models, $TSHR^{(D633H)}$ KI which carried an activating mutation and developed transient hyperthyroidism which peaked at the age of 2-4 months in females, and TSHR KO*, which developed severe CH in both sexes. To our knowledge this was the first report of a hyperthyroid model carrying a patient derived TSHR CAM leading to an adrenal phenotype. However, previous findings in pharmacologically induced hyperthyroid rodent models have indicated that TH supplementation can lead to enlargement of the adrenal gland (D'Angelo S.A. & Grodin J.M., 1964; Johnson et al., 2005), while CH in rodents decreased the weight of the adrenal gland (J. G. M. Shire and W. G. Beamer, 1984). Shire and Beamer, in their study, found that hypothyroid hyt/hyt young female mice (TSHR inactivating mutation) had a poorly developed X-zone, while treatment with thyroid powder resulted in a well-developed X-zone in those mice (J. G. M. Shire and & W. G. Beamer, 1984). The X-zone is a transient fetal zone which slowly disappear in nulliparous female mice or rapidly disappeard after the first pregnancy (Holmes & Dickson, 1971). In our $TSHR^{(D633H)}$ KI HOM female mice, the adrenal cortex showed extensive and prolonged X-zone involution, with extensive lipid accumulation, confirmed by ORO staining, when compared to WT female adrenal. Interestingly, we found that TH concentration correlated positively with adrenal weight in our mouse models. No changes in adrenal gland weight were observed in $TSHR^{(D633H)}$ KI male mice older than 4 weeks. In males, the X-zone normally disappears after puberty, which can be caused by androgen receptor signaling and testosterone action (Gannon et al., 2019; Jones, 1952). Huang et al., discovered that X-zone is a direct target for TH via TRβ1 (Huang et al., 2015). Similarly, the thyroid gland of our hyperthyroid $TSHR^{(D633H)}$ KI HOM female mice had increased TRβ and no TSHR staining within the X-zone area, suggesting that enlargement of the adrenal

phenotype is caused rather by TH action than directly by activating the D633H mutation in the TSHR. This is in contrast to a previous study suggesting that TSHR expression in the adrenal gland uses IHC (Dutton et al., 1997). No visible changes were observed in the TSHR$^{(D633H)}$ KI and TSHR KO* adrenal medulla, however CgA-positive cells and upregulation of medullary genes were observed within the TSHR KO* adrenal cortex, suggesting possible medulla overgrowth.
In summary, our hypo and hyperthyroid models show that altered thyroid function in early life has a direct impact on adrenal development and gene-expression.

6.2.1 Correlation between premature adrenarche and congenital hypothyroidism

A recent study performed by Galanou et al., found significantly higher concentrations of 17-OHP in neonates with severe CH (Galanou et al., 2019). Prompted by this work and our own findings with hypo-and hyperthyroid models, we recruited patients diagnosed with premature adrenarche (PA) and CH in order to study the possible link between adrenal and thyroid function. We found that in CH patients the concentrations of serum 17-OHP and TSH were positively correlated. Moreover, a weak positive correlation was found between serum TSH and DHEA/ DHEA-S in patients diagnosed with PA. Unlike our congenital hypo- (TSHR KO*), and hyperthyroid (TSHR$^{(D633H)}$ KI) mouse models, we did not see any adrenal phenotype in our induced hypothyroid iTGα_sKO mouse model (Patyra et al., 2018). Therefore, in human CH patients diagnosed with PA, a positive correlation between TSH and adrenal steroids would be contrary to observations in CH mice, where the fetal X-zone was greatly reduced. The difference between mouse and human findings can be related to the structural and functional changes between the human fetal zone and the mouse X-zone. The exact mechanism of initiations of adrenarche is not well known, however, during adrenarche growth of the zona reticularis and up-regulation of steroidogenic enzymes was found (Nakamura et al., 2009), and changes in the adrenal cortex probably occurred in early infancy, rather than at the time of diagnosis of PA and CH (Liimatta et al., 2020; Witchel et al., 2020).

6.3 Novelty of findings and prospects

The sequencing screening panel developed in the first project of this PhD thesis helped to identify the genetic etiology of patients with congenital thyroid disease. It was also useful in the selection of families for further analysis.

In addition to its implementation in the treatment of thyroid disease, genetic screening for novel and known mutations would help to investigate the genetic etiology and allowing some unsolved cases to be selected for future studies; this would aid the identification of novel mechanisms in thyroid diseases. In the second project, we studied the role of $G\alpha_s$ in the thyroid gland. We found that $G\alpha_s$ is an indispensable part of the TH synthesis mechanism and cannot be compensated for any other mechanism. Moreover, the unexpected finding of hyperplastic PTC-like lesions demonstrated that intensive and prolonged TSH stimulation led to overgrowth of thyrocytes, however, it is not sufficient to induce tumorigenesis. This model is useful when studying the development and sex-specific differences of primary hypothyroidism as well as TSH induced tumorigenesis.

In the third project, we discovered the link between congenital hypo-, and hyperthyroidism and adrenal development and function in our mouse models. Moreover, we found a functional connection in newborns between PA and CH patients. These findings point out the need for further analysis of TH and adrenals, for example, in patients with congenital thyroid disease or TSHR or TR mutations.

7 Summary/Conclusions

TH are indispensable biomolecules which impact the development and metabolism of virtually every organ in the body. In this thesis, we generated a genetic screening panel for thyroid diseases and demonstrated novel genetic mutations in Finnish patients with congenital thyroid disorders. Furthermore, we created new models to study more details of the TSHR-signaling in thyroid pathophysiology. Based on these studies and models the main conclusions are as follows:

1. Next generation sequencing provides an effective way to detect known and novel genetic variants in patients with CH and thus improve our knowledge of the management of these patients.

2. $G\alpha_s$ protein plays an essential role in the TH synthesis and thyrocyte growth, and prolonged TSH stimulation can lead to overgrowth and development of PTC-like lesions in mouse models.

3. Congenital TH concentrations play an important role in adrenal development in mice and might also play a role in patients with congenital thyroid diseases or adrenache.

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