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DISSECTING NEW MOLECULAR MECHANISMS IN THYROID DISEASES USING GENETICALLY MODIFIED MOUSE MODELS

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Abstract

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Dissecting New Molecular Mechanisms in Thyroid Diseases Using Genetically Modified Mouse Models

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Turku, 2016

The thyroid gland secretes thyroid hormones (THs) under regulation of thyroid stimulation hormone (TSH) and its receptor (TSHR). THs play a pivotal role in development, growth and metabolism.

An increased secretion of THs causes hyperthyroidism, while a decrease leads to hypothyroidism. Together with thyroid tumors, these thyroid diseases affect more than 10 % of the population. Thus, better understanding of the molecular causes of thyroid diseases is crucial to improve treatment strategies.

In this study, we generated genetically modified mouse models to understand the details of thyroid pathophysiology. First, a *thyroglobulin* promoter-driven, tamoxifen-inducible Cre-mouse line (i*Tg*Cre) was created to enable thyroid-specific gene deletions in a time-dependent manner using the Cre/*loxP* system. Thereafter, this technique was applied to delete the microRNA-processing enzyme *Dicer1*. Knocking out *Dicer1* during development and adulthood revealed that Dicer1, and subsequently miRNAs, are crucial for the maintenance of thyrocyte differentiation and growth. Perinatal miRNA deficiency leads to slowly progressing hypothyroidism, while the deletion of miRNAs in adult mice does not cause acute hypothyroidism.

Furthermore, to understand the development of hyperthyroidism, we generated a knock-in mouse model harboring a constitutively active TSHR mutation D633H. Interestingly, these $TSHR^{D633H}$ mice developed colloid goiter with euthyroidism, subclinical or overt hyperthyroidism depending on sex and age.

In conclusion, we generated new disease models to understand molecular mechanisms in thyroid development, hypo- and hyperthyroidism. These findings revealed a novel role of miRNAs in thyroid growth, development and hypothyroidism. Furthermore, we demonstrated that $TSHR^{D633H}$ mutation causes transient hyperthyroidism and colloid goiter in mice.

Keywords: Thyroid, thyroid stimulating hormone, thyroid stimulating hormone receptor, Cre/*loxP* system, Dicer1, microRNA, constitutively activating mutations, hypothyroidism, hyperthyroidism, goiter

Tiivistelmä

Henriette Undeutsch

Kilpirauhassairauksien Uusien Molekyylimekanismien Tutkiminen Geenimuunnelluilla Hiirimalleilla

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Kilpirauhasen tuottamat hormonit ovat keskeisessä asemassa kehityksen, kasvun ja aineenvaihdunnan säätelyssä. Näiden hormonien erittymistä säädellään kilpirauhasta stimuloivan hormonin (TSH) ja sen reseptorin (TSHR) kautta.

Kilpirauhashormonien ylituotanto johtaa kilpirauhasen liikatoimintaan (hypertyreoosi) ja alituotanto vajaatoimintaan (hypotyreoosi). Näiden kilpirauhassairauksien kilpirauhaskasvaimet mukaan lukien, esiintyvyys väestössä on yli 10 %. Tämän vuoksi kilpirauhassairauksien synnyn molekyylitason tutkimukset ovat ratkaisevan tärkeitä niiden ehkäisyn ja hoitomahdollisuuksien parantamiseksi.

Kilpirauhasen normaalin ja patofysiologisen toiminnan molekyylitason mekanismien tutkimiseksi loimme tässä tutkimuksessa geneettisesti muokattuja hiirimalleja. Työssä tuotettiin tyroglobuliinipromootterin ohjaamia ja tamoksifeenilla aktivoituvia Cre-hiirilinjoja. Kilpirauhasspesifistä *Dicer1*-puutteista hiirilinjaa käytettiin mikroRNA (miRNA) -signaloinnin tutkimisessa. Poistamalla *Dicer1* eri kehityvaiheissa saatiin selvitettyä Dicer1:n ja sen seurauksena mikroRNA:iden merkitys kilpirauhasen solujen erilaistumiselle, toiminnalle ja kasvulle. Tulostemme mukaan mikroRNA:t eivät aiheuta akuuttia hypotyreoosia, jos mikroRNA signallointi poistetaan aikuiselta hiireltä, mutta niiden poisto varhaisessa kehitysvaiheessa johtaa hitaasti kehittyvään kilpirauhasen vajaatoimintaan.

Kilpirauhasen liikatoiminnan tutkimiseksi loimme knock-in -hiirimallin, joka ilmentää konstitutiivisesti aktivoivaa TSHR D633H mutaatiota. Kyseinen mutaatio on alunperin löydetty kilpirauhasen liikatoimintaa sairastavalta potilaalta ja vastaavat mutaatiot ovat tavallisin ei-autoimmuunin hypertyreoosin (NAH) syy. Näiden $TSHR^{D633H}$ hiirten kilpirauhanen on suurentunut molemmilla sukupuolilla hetero- ja homotsygoottisilla eläimillä. Yllättäen, toisin kuin ihmisellä, selvä hypertyreoosi kehittyy ainoastaan homotsygoottisille KI naaraille ja on ohimenevä.

Tässä tutkimuksessa luotujen hiirimallien avulla voidaan selvittää kilpirauhasen kehitykseen, vajaa- ja liikatoimintaan liittyviä molekyylitason mekanismeja. Havaintomme kilpirauhasen mikroRNA–signaloitiin littyen paljastivat uutta tietoa niiden merkityksestä kilpirauhasen kasvuun, kehitykseen ja toimintaan liittyen. Lisäksi TSHR:in aktivoivaa D633H mutaatiota ilmentävä hiirimallimme mahdollistaa ensimmäistä kertaa kilpirauhasen liihatoiminnan synnyn ja kehityksen yksityiskohtaisen selvttämisen.

Avainsanat: Kilpirauhanen, Tyreotropiini, Tyreotropiini reseptori, Cre/*loxP* – menetelmä, Dicer1, microRNA, konstitutiiviset aktivoivat mutaatiot, hypotyreoosi, hypertyreoosi

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Abbreviations

AGO	Argonaute
Ano1	anoctamin
bp	basepair
CAM	constitutively activating mutation
cAMP	cyclic adenosine monophosphate
CH	congenital hypothyroidism
DGCR8	DiGeorge Syndrome Critical Region 8
Dio	deiodinase
DIT	3,5-diiodothyrosine
DTC	differentiated thyroid carcinomas
Duox	dual oxidase
DuoxA	dual oxidase maturation factor A
E	embryonic day
EMT	epithelial-mesenchymal transition
ES cells	embryonic stem cells
EtOH	ethanol
FOXE1	Forkhead Box E1
fT3	free T3
fT4	free T4
G protein	guanine nucleotide binding protein
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HBD	hormone binding domain
HEZ	heterozygous
HHEX	hematopoietically expressed homeobox
HOZ	homozygous
HRP	horseradish peroxidase
IP	inositol phosphate
kb	kilobase
KI	knock-in
KO	knockout
LAT	L-type amino acid transporter
Lrp2	low density lipoprotein receptor-related protein 2, Megalin
MAPK	mitogen-activated protein kinase
miRNA	microRNA
MIT	3-monoiodothyrosine
MNG	multinodular goiter
NAH	non-autoimmune hyperthyroidism
Nis	sodium-iodine symporter
NKX2-1	Nk2 Homeobox 1
nt	nucleotide
PAX8	paired box 8
PBS	phosphate buffered saline
PCR	polymerase chain reaction
piRNA	Piwi-interacting RNA

Pol	DNA polymerase
Pten	phosphate and tensine homolog
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNAse	ribonuclease
rT3	3,3',5'-triiodothyronine, reverse T3
SEM	standard error of mean
siRNA	short interfering RNA
SLCT	Sertoli-Leydig cell tumors
T3	3,3',5-triiodothyronine
T4	thyroxine, 3,3',5,5'-tetraiodithyronine
TF	transcription factor
Tg	thyroglobulin
Tgf-β	transforming growth factor β
Tgfβr	transforming growth factor β receptor
TH	thyroid hormone
TM	transmembrane helix
TPO	thyroid peroxidase
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TRH	thyrotropin releasing hormone
TRHR	thyrotropin releasing hormone receptor
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor, TSH receptor
tT3	total T3
tT4	total T4
WT	wildtype
Zeb	zinc finger E-box binding homeobox

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III:

I. Undeutsch H, Löf C, Offermanns S, Kero J (2014) "A mouse model with tamoxifen-inducible thyrocyte-specific Cre recombinase activity", Genesis 52: 330-40.

II. Undeutsch H, Löf C, Pakarinen P, Poutanen M, Kero J (2015) "Thyrocyte-specific Dicer1 deficiency alters thyroid follicular organisation and prevents goiter development", Endocrinology 156:1590-601.

III. Undeutsch H*, Jaeschke J*, Löf C, Patyra K, Eszlinger M, Zhang F, Poutanen M, Paschke R[#], Kero J[#] "Mice carrying a constitutive active D633H TSHR mutation develop transient hyperthyroidism and colloid goiter", Manuscript. *, [#] Equal contribution.

1 Introduction

Thyroid hormones (THs) regulate energy metabolism, oxygen consumption, and growth, and are crucial for brain development in early childhood. THs are produced by the thyroid gland, an endocrine organ that is controlled by thyroid stimulating hormone (TSH) via its receptor (TSHR) (Kristiansen, 2004, Vassart and Dumont, 1992). An increased secretion of THs causes hyperthyroidism, while a decrease leads to hypothyroidism. These thyroid diseases, together with thyroid tumors, affect over 10 % of the population (Vanderpump, 2011) and have a wide impact on a patient's well-being. Decreased or elevated serum TSH levels can influence the outcome of many diseases, like cardiovascular, bone or metabolic diseases. Furthermore, hyperthyroidism can be life threatening if untreated, and postnatal hypothyroidism can lead to mental and motoric retardation. Thus, better understanding of the molecular causes of thyroid diseases is crucial for improving the prevention and treatment strategies of thyroid diseases.

Genetically modified mouse models are very valuable tools to analyze the role of different genes and pathways in thyroid development and function *in vivo*. Among the different techniques for genetic alterations in the murine genome, the Cre/*loxP* system is the most common method to generate tissue-specific gene knockouts (KOs) in a short period of time. In this method, crossing a mouse line expressing Cre recombinase under a tissue-specific promoter allows for the excising of any gene flanked by two *loxP* sites (floxed) from the genome of all cells targeted by the specific promoter. To be able to delete a floxed gene in a time-dependent manner at any desired time-point during or after development, tamoxifen-inducible Cre mouse lines are used. In these inducible Cre-lines, the Cre recombinase is coupled to a mutated estrogen-receptor hormone binding domain and is only translocated into the nucleus upon tamoxifen binding (Feil et al., 1996, Indra et al., 1999).

In this study, we generated genetically modified mouse models to understand the details of thyroid pathophysiology. First, a thyroglobulin promoter-driven, tamoxifen-inducible Cre mouse line (*Tg*CreER^{T2}) was created to enable thyroid-specific gene deletions in a time-dependent manner using the Cre/*loxP* system. Thereafter, this technic was applied to delete the microRNA-processing enzyme Dicer1.

MicroRNAs (miRNA) are short, non-coding RNAs that engage in post-transcriptional regulation of gene expression, a mechanism known as RNA interference. By binding to their target mRNAs, miRNAs can mediate the degradation or inhibit the translation of the mRNA, and thus influence the protein expression of the targeted gene after the mRNA transcription (Krek et al., 2005, Lim et al., 2005). MiRNAs are deregulated in various tumors, including thyroid cancer. In humans, heterozygous mutations in the miRNA processing ribonuclease (RNAse) DICER1 are linked to the development of different tumors, including differentiated thyroid carcinomas (DTC), as well as multinodular goiter (MNG) (Hill et al., 2009, Slade et al., 2011), pointing to a regulatory role of miRNAs in carcinogenesis and growth. Studies on conditional thyroid-specific Dicer1 KO mice, which develop severe hypothyroidism, suggest that Dicer1 and subsequently miRNAs play a role in the maintenance of thyrocyte differentiation (Frezzetti et al., 2011, Rodriguez et al., 2012). As the deletion of Dicer1 during early embryonic development leads to a more severe phenotype than a loss of Dicer1 later in development, Dicer1 may have a direct role in the control of thyroid development (Rodriguez et al., 2012). However, it remains unclear if the

hypothyroidism is a result of an altered morphology or a direct influence of Dicer1-mediated signaling on TH synthesis. Furthermore, the role of Dicer1 in adult thyroid growth and thyroid function after normal organogenesis is unknown.

To understand the development of hyperthyroidism, we generated a knock-in (KI) mouse model harboring the constitutively active TSHR mutation D633H (Neumann et al., 2001a, Russo et al., 1995). Constitutively activating mutations (CAMs) of the TSHR are the major cause for non-autoimmune hyperthyroidism (NAH). For the TSHR, at least 60 constitutively activating *in vivo* mutations, which lead to the permanent activation of the G-protein $G_{\alpha s}$ and in few cases also to $G_{\alpha q/11}$, have been described (Lublinghoff et al., 2012). To date, functional characterization of these mutations has been exclusively conducted *in vitro,* and the molecular mechanisms leading to manifestation of hyperthyroidism as a result of CAMs have never been shown *in vivo*. An *in vivo* model for a TSHR CAM would also be beneficial to study the physiological effects on energy or bone metabolism, as well as the consequences of homozygote as, thus far, no homozygous CAMs have been identified in patients.

In this study, we have generated new disease models to understand molecular mechanisms in thyroid development, hypothyroidism and hyperthyroidism. The role of miRNAs in thyroid development, function and growth was assessed through tissue-specific deletion of Dicer1. Mice lacking Dicer1 during embryonic development present hypothyroidism, while miRNA signaling during adulthood is required for the maintenance of thyrocyte differentiation and thyroid growth. Further, we demonstrated that the $TSHR^{D633H}$ mutation causes transient hyperthyroidism and colloid goiter in mice.

2 Review of the Literature

2.1 The Thyroid Gland

The thyroid gland is an endocrine organ located in the neck area in mammalian species. It consists of two lobes, in some species connected by the isthmus, and surrounds the trachea (La Perle and Jordan, 2012). The thyroid gland is home to two different hormone-secreting cell types and has dual endocrine functions. The dominant structures of the thyroid gland are the thyroid follicles where thyroid hormone synthesis takes place. A thyroid follicle is formed by a thyrocyte monolayer surrounding the colloid, the storage location for TH precursors (Figure 1). In addition to thyroid follicles, the thyroid gland has parafollicular cells, also known as C-cells, that secrete calcitonin, a hormone that modulates blood calcium levels (Deftos, 1981). Calcitonin can decrease calcium levels in the blood, and protects against hypercalcemia (Kantham et al., 2009, Vaughn and Vaitkevicius, 1974). Calcitonin functions as a counterpart to the parathyroid hormone secreted by the parathyroid glands, a small endocrine organ located under the thyroid capsule (Carter and Schipani, 2006, La Perle and Jordan, 2012).

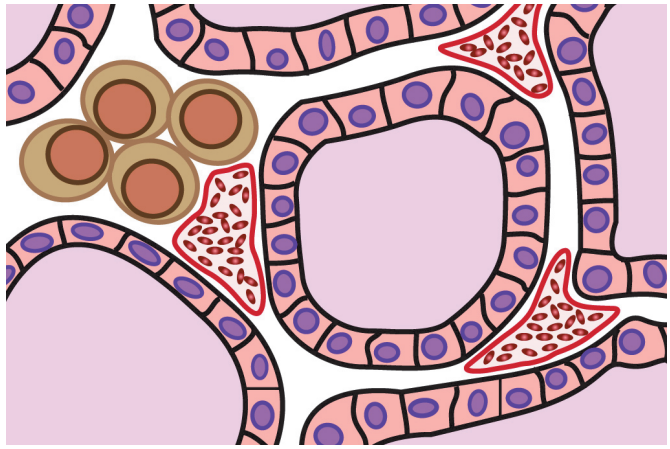

Figure 1: Thyroid morphology

Histologically, the thyroid gland consists of the thyroid follicles where TH synthesis takes place, calcitonin-producing C-cells and a vascular system. The thyroid follicles are formed by a monolayer of thyrocytes (orange cells with purple nucleus) with a colloid-filled lumen (pink). C-cells (brown) are dispersed as cell clusters between the thyroid follicles. The thyroid is highly vascularized and traversed by blood vessels (red structures filled with red blood cells).

Thyrocytes synthesize and secret the THs 3,3',5-triiodothyronine (T3) and 3,3',5,5'-tetraiodithyronine (thyroxine, T4). The synthesis is controlled by thyrotropin (TSH) and thyrotropin-releasing hormone (TRH) (Shupnik et al., 1989, Stathatos, 2012), forming a typical endocrine hypothalamic-pituitary-thyroid feedback system (Chiamolera and Wondisford, 2009, Costa-e-Sousa and Hollenberg, 2012). THs are produced in the colloid in several steps. First, iodine is incorporated into the tyrosyl

residues of thyroglobulin (Tg), then one- or two-fold iodinated tyrosyl residues are coupled to the THs T4 or T3 (Dunn and Dunn, 2001). During the final step, the Tg polypeptide is reabsorbed into the thyrocytes, where T3 and T4 are proteolytically cleaved from the Tg backbone (Dunn and Dunn, 1982). The secreted THs are transported via the circulating bloodstream, where they are mostly bound to serum transport proteins (Hulbert, 2000, Little, 2016, Schreiber et al., 1998). Only a small portion of THs, approximately 0.01 %, is circulating free in the blood stream and can thus enter their target cells via TH transporters (Faix, 2013, Heuer and Visser, 2009, Muller et al., 2014, Schreiber et al., 1998). In the cell, THs activate or repress gene expression by interacting with the nuclear thyroid hormone receptors (TRs) TRα and TRβ (Brent, 2012, Chiamolera and Wondisford, 2009). The THs affect almost every tissue, regulating brain development, growth, cardiovascular function, fertility, and metabolism, such as thermogenesis and oxygen consumption (Bernal, 2000, Brent, 2012, Cheng et al., 2010, Cho, 2015, Kopp, 2002, Vaitkus et al., 2015).

2.1.1 Development of the Thyroid Gland

Thyroid gland development in mice starts around embryonic day 8.5 (E 8.5) during embryonic development, when the two endocrine cell types present in the thyroid gland develop from different progenitor cells. The progenitor cells developing into the calcitonin-expressing C-cells originate from the anterior endoderm, as recently shown by Johansson et al. (Johansson et al., 2015). While it was formerly believed that C-cells originate from the neural crest and migrate to the ultimobranchial bodies, new evidence suggests that the C-cells stem from the same anlage as the ultimobranchial bodies. At E 15.5, C-cells migrate into the thyroid, where they spread between the thyroid follicles (Fontaine, 1979, Johansson et al., 2015, Manley and Capecchi, 1998).

The development of the thyroid follicular cells, originating from the ventral endoderm of the pharyngeal floor, can be separated in three steps. During the first step, the cell fate of thyrocyte progenitor cells is determined. This takes place around E 8.5, when a collective of cells in the pharyngeal floor start expressing the four transcription factors (TFs) Paired Box 8 (PAX8), Nk2 Homeobox 1 (NKX2-1), Forkhead Box E1 (FOXE1) and Hematopoietically Expressed Homeobox (HHEX) (Lazzaro et al., 1991, Plachov et al., 1990, Thomas et al., 1998, Zannini et al., 1997). None of these factors are exclusively expressed in the thyroid gland, but the combination of all four factors can only be seen in thyroid follicular cells and their progenitors (De Felice and Di Lauro, 2004, Fagman and Nilsson, 2010). With exception of FOXE1, these TFs can regulate their own gene expression (di Gennaro et al., 2013, Oguchi and Kimura, 1998, Puppin et al., 2003). PAX8 and NKX2-1 are the key transcription factors that have recently been shown to be sufficient to direct embryonic stem cells (ES-cells) to differentiate to fully functioning thyrocytes *in vitro* (Antonica et al., 2012, Christophe-Hobertus et al., 2012, D'Andrea et al., 2006, Di Palma et al., 2011, Fernandez et al., 2015, Puppin et al., 2004). Around E 9.5, thyrocyte precursor cells start protruding out of the cell collective, forming a bud. From E 10.5 to approximately E 13.5, these budding cells descend caudally to the upper trachea, where they give rise to the two thyroid lobes (De Felice and Di Lauro, 2004, Fagman and Nilsson, 2010, Postiglione et al., 2002). Next, the thyroid follicular cells start expressing Tg around E 14.5 (Milenkovic et al., 2007, Postiglione et al., 2002), followed by TSHR and thyroid peroxidase (TPO) expression around E 15 (Brown et al., 2000, De Felice and Di Lauro, 2004, Lazzaro et al., 1991). At E 16.0, thyrocytes

have fully organized in follicles, and sodium-iodine symporter (NIS) expression can be detected (Fernandez et al., 2015, Postiglione et al., 2002). Finally, from E 16.5 on, the thyroid gland produces THs (De Felice and Di Lauro, 2004, Meunier et al., 2003).

2.1.2 Morphology of the Thyroid Gland

The fully developed thyroid gland consists of thyroid follicles and patches of C-cells between the follicles (De Felice and Di Lauro, 2011). The network of thyroid follicles is traversed by blood vessels (Figure 1), ensuring a sufficient supply of oxygen, iodine and other trace elements crucial for TH synthesis, such as iron and selenium. A strong vascularization of the thyroid gland is further needed for a quick release of THs (De Felice and Di Lauro, 2011, La Perle and Jordan, 2012, Ramsden, 2000, Wang et al., 1998). A thyroid follicle is formed by a monolayer of thyrocytes surrounding the colloid, a hormonal storage, where THs and their precursors, namely 3-monoiodothyrosine (MIT) and 3,5-diiodothyrosine (DIT), are bound to Tg (Mauchamp et al., 1998).

The size and shape of thyrocytes and thyroid follicles varies and reflects thyroid activity. A thick follicle epithelium consisting of columnar cells with round nuclei typically indicates an active, TH-producing thyroid gland. This is often accompanied by an increased amount of colloid droplets seen in the thyrocytes. On the contrary, a flattened thyroid epithelium with oval-shaped nuclei and an increased size of the colloid is usually associated with an inactive state of the follicle. With age, the follicle size becomes more variable, and also other factors like sex and diet can influence the appearance of the thyroid follicle epithelium (La Perle and Jordan, 2012). In thyrocytes, cell polarity is crucial for the proper function of the cells. The outer membrane marks the basolateral site, whereas the colloid-facing membrane is the apical site (Mauchamp et al., 1998).

2.1.3 Function of the Thyroid Gland

2.1.3.1 Thyroid Hormone Synthesis

For TH synthesis, the correct location of all factors involved is essential (Figure 2). Pituitary TSH is the main regulator of thyroid function. Its receptor is expressed in the basolateral membrane, where it binds the circulating TSH, activates the secondary messenger system and subsequently facilitates TH production and secretion (Kristiansen, 2004, Vassart and Dumont, 1992). The thyroid gland is the main consumer and storage of iodine, as iodine is a major component of THs. In fact, iodine accounts for 65 % of the molecular weight of T4 and 58 % of T3. Thus, iodide uptake from bloodstream to the thyrocyte is a critical step in TH production. This is mediated by NIS, located in the basolateral membrane, which imports two sodium ions together with one iodide ion against an electrochemical gradient (Dohan et al., 2003). On the apical membrane, Pendrin (Royaux et al., 2000, Scott et al., 1999) and anoctamin-1 (Ano1) (Iosco et al., 2014, Viitanen et al., 2013) regulate the export of iodide into the colloid. Also located on the apical membrane, TPO oxidizes iodide ions to form iodine atoms. The peroxidase activity is facilitated by dual oxidase (Duox) and the dual oxidase maturation factor A (DuoxA), which produce H_2O_2 and co-localize with TPO at the apical side of thyrocytes (Donko et al., 2005).

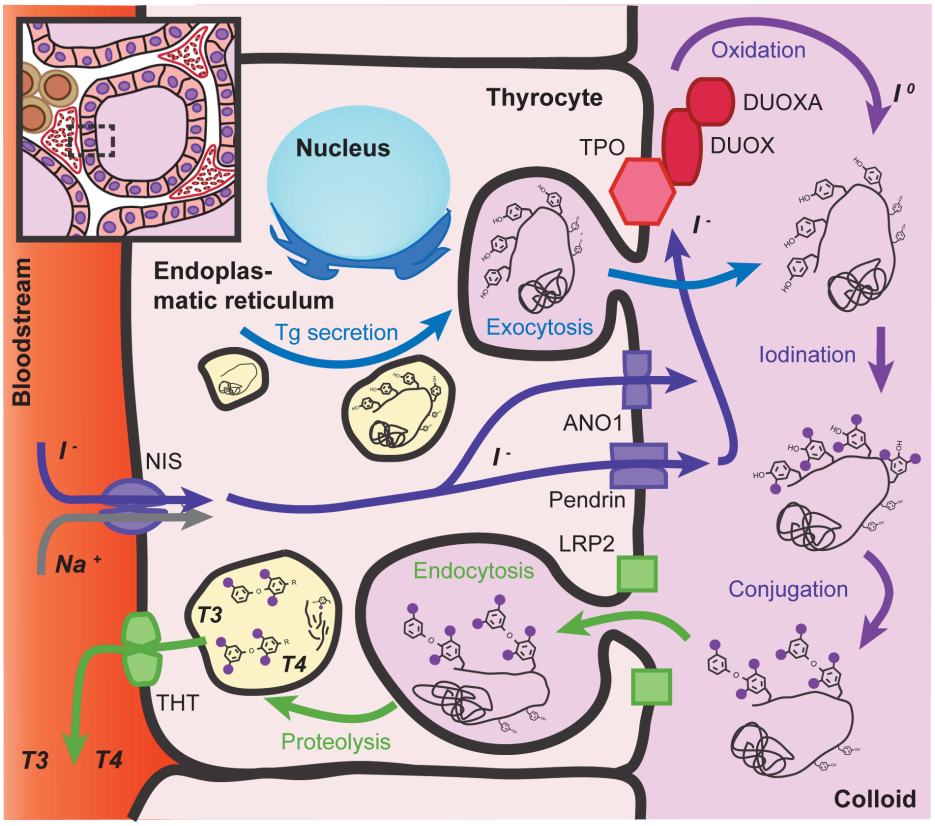

Figure 2: Thyroid hormone synthesis (modified from (Häggström, 2014))

Schematic summary of the thyroid morphology and the TH synthesis in the thyroid gland. The thyroid gland (upper left corner) is composed of thyroid follicles (orange/purple cells), C-cells (brown cells) and blood vessels (red structures). Thyroglobulin (Tg) is expressed in thyroid follicular cells and exported to the follicular lumen by exocytosis (blue pathway). Iodide is taken up into the thyrocytes by NIS on the basolateral site and travels with the intracellular concentration gradient to the apical membrane, where it is exported by Pendrin and ANO1. TPO, bound to the apical membrane, oxidizes the iodide ions. DUOX and DUOXA provide the H_2O_2 for the peroxidase activity of TPO. Iodinated tyrosyl residues of the Tg protein (MIT, DIT) are then covalently linked by TPO, forming Tg-bound T3 and T4 (purple pathway). The iodine-loaded Tg is taken up by endocytosis (partially LRP2-mediated) into the thyrocytes. Next, Tg proteolysis and release of THs is mediated by an endosome-lysosomal system (green pathway). T3 and T4 are released to the blood stream by TH transporters (THT).

The iodine atoms are incorporated into the Tg present in the colloid. Tg is expressed in the thyrocytes, dimerized and glycosylated in the Golgi apparatus, and exported into the colloid via exocytosis (Dunn and Dunn, 2001). TPO catalyzes the iodination of the tyrosyl residues of the thyroglobulin polypeptide, resulting in MIT or DIT. Then, two DIT residues or one DIT and MIT residue are coupled in the presence of TPO, forming the THs T4 or T3, respectively (Dunn and Dunn, 2001, Grasberger et al., 2012, Mansourian, 2011). Iodinated Tg is transported into the thyrocytes through endocytosis. In addition to fluid-phase endocytosis, reabsorption of Tg can be mediated via the low density lipoprotein receptor-related protein 2 (LRP2), also known as megalin, in a calcium-dependent manner (Zheng et al., 1998). Furthermore,

low affinity receptors present on the apical membrane, such as the asialoglycoprotein receptor and other, not yet identified receptors, might also induce endocytosis of Tg (Marino et al., 2001, Zheng et al., 1998). T3 and T4 are then released from Tg by proteolysis via endopeptidases, particularly cathepsin B, D, H and L, and exopeptidases (Dunn et al., 1991, Dunn and Dunn, 1982, Yoshinari and Taurog, 1985). TH transporters, monocarboxylate transporter 8 (MCT8) and MCT10, located on the basolateral membrane, facilitate the secretion of TH (Miot et al., 2000, Muller et al., 2014).

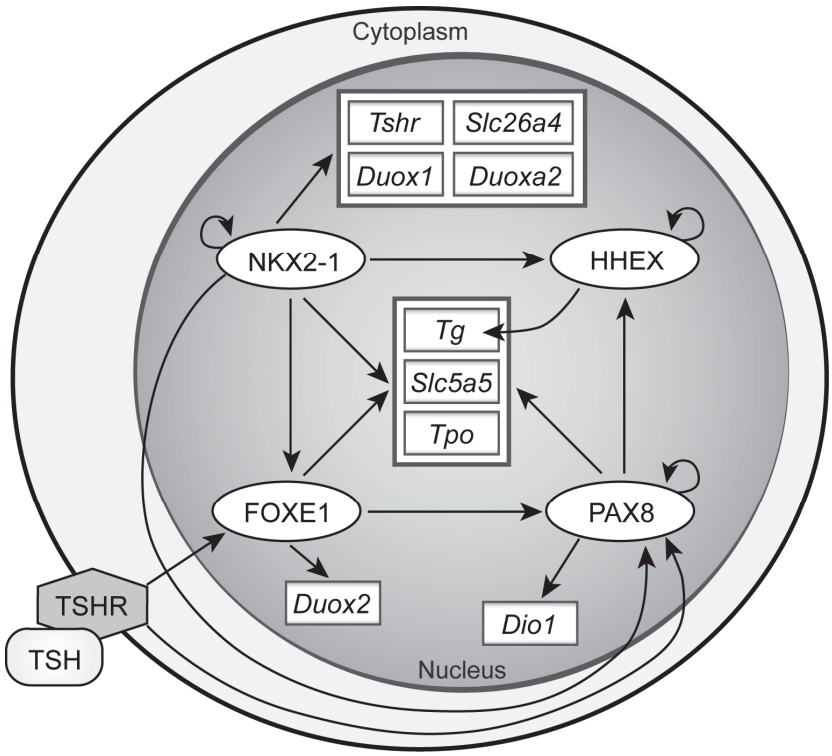

Figure 3: Thyroid-specific gene expression network. Modified from Fernandez *et al.* (Fernandez et al., 2015).

The four main transcription factors PAX8, NKX2-1, FOXE1 and HHEX form a complex gene-regulation network. They are regulating each other as well as the expression of *Tg*. *Nis* (*Scl5a5*), *Tpo*, *Pendrin* (*Slc26A4*), *Duox1*, *Duox2*, *DuoxA2* and *Dio1*. The TFs PAX8 and FOXE1 are controlled by TSHR signaling, and *Tshr* expression is regulated by NKX2-1.

The genes involved in TH synthesis are regulated mainly by the four TFs PAX8, NKX2-1, FOXE1 and HHEX (Figure 3), as summarized by Fernández et al. (Fernandez et al., 2015). Briefly, TSHR signaling stimulates the expression of the TFs *Pax8* and *FoxE1*, as well as *Lrp2*. The four TFs, PAX8, NKX2-1, FOXE1 and HHEX, form a tight control network for the expression of *Tg*, where HHEX and FOXE1 may inhibit the activation of gene expression of *Pax8* and *Nkx2-1* (Pellizzari et al., 2000, Zannini et al., 1997). Similarly, FOXE1, NKX2-1 and PAX8 modulate the expression of *Scl5a5* (coding for NIS) and *Tpo*. Furthermore, NKX2-1 modulates

its own expression, the other three TFs as well as *Tshr*, the Pendrin-coding *Slc26a5*, *Duox1* and *DouxA2*. PAX8 has a self-regulating function and also influences the expression of *FoxE1* and *Hhex*. In addition, PAX8 regulates *Deiodinase 1* (*Dio1*) and might also directly influence *Duox2* expression, which has been shown to be mediated by FOXE1 (Fernandez et al., 2015).

Around 90 % of the THs are secreted by the thyroid gland as the pro-hormone T4. Consequently, T3, which has a ten-fold higher affinity than T4 to the TRs in the target cell, only accounts for approximately 9 % of circulating THs (Golan and Tashjian, 2012, Oetting and Yen, 2007). T4 also possesses a significantly longer half-life (seven days) than T3 (eight hours), underlining its function as a pro-hormone (Saberi and Utiger, 1974). The long half-life of T4 is stabilized by binding to serum transport proteins, such as thyroid-binding globulin, transthyretin or albumin (Hulbert, 2000, Little, 2016, Schreiber et al., 1998). THs have a low solubility in blood due to their hydrophobic properties. Binding to serum transporter proteins allows for the circulation of THs in higher concentrations and ensures a steady distribution of THs in the body. Only 0.01 – 0.02 % of circulating THs are not bound to serum transport proteins, thus referred to as free T3 (fT3) and free T4 (fT4) (Benvenga and Robbins, 1996, Schussler, 2000, Thienpont et al., 2013).

The majority of active T3 is produced from T4 in peripheral tissues by iodothyronine deiodinases (Dio) (Figure 4). Deiodinases are membrane-bound selenoproteins that cleave iodine atoms from the aromatic iodothyronine rings (Schweizer et al., 2014). While Dio1 is capable of cleaving iodine from both the inner and preferably outer tyrosyl rings of THs, Dio2 solely removes iodine atoms from the outer ring under normal conditions (Kurlak et al., 2013, Maia et al., 2011, Moreno et al., 1994). Thus, Dio1 and mainly Dio2 have an activating role in TH processing, as they convert the pro-hormone T4 to active T3 by removing one iodine atom from the outer ring of T4. Furthermore, Dio1 and Dio2 mediate the conversion of 3,3',5'-triiodothyronine (reverse T3) to 3,3'-diiodothyronine (3,3'-T2) (Arrojo and Bianco, 2011, Bianco and Kim, 2006, Gereben et al., 2008). In addition to its TH-activating role, Dio1 may also inactivate active T4 by converting it to reverse T3 (Arrojo and Bianco, 2011, Kohrle, 2000). An additional deiodinase, Dio3, catalytically removes iodine from the inner tyrosyl ring of THs (Kurlak et al., 2013). Through this catalytic activity, Dio3 acts as an inhibiting factor by converting both T4 and T3 to the inactive TH metabolites reverse T3 and 3,3'-T2, respectively. In humans, Dio3 is highly expressed in the placenta and is believed to play a pivotal role in protecting the embryo from elevated maternal THs (Huang et al., 2003, Kurlak et al., 2013).

2.1.3.2 Cellular Action of Thyroid Hormones

The THs T4 and T3 enter their target cells in the periphery through membrane transporters to act on their intracellular target receptors. Besides several unspecific transporter proteins, a few TH-specific transporter proteins have been identified, such as the ubiquitously expressed monocarboxylate transporters (MCT) 8 and MCT10. Additional TH transporters are the L-type amino acid transporter 1 (LAT1) and LAT2, liver sodium/taurocholate co-transporter and the organic anion-transporting polypeptides (Bernal et al., 2015, Jansen et al., 2005). After the conversion of the pro-hormone T4 to the active form T3 in the cytoplasm of target cells, T3 acts via TRs to activate or suppress gene expression. T4 has a ten-fold lower affinity to TRs compared to T3 (Oetting and Yen, 2007). The transfer of THs from the cytoplasm

into the nucleus is not fully understood, but trafficking of T3 bound to TRs is a common model (Zhu et al., 1998). The role of TH transporters like MCT8 in nuclear trafficking has also been speculated on (Heuer and Visser, 2009).

In human and mice, two different genes give rise to two sub-types of TRs: TRα and TRβ. Each gene has two major splice variants, coding for TRα1 and TRα2, or TRβ1 and TRβ2, respectively (Benbrook and Pfahl, 1987, Brent, 2012, Konig and Moura Neto, 2002, Sap et al., 1986, Weinberger et al., 1986). All TRs share a common DNA binding domain with two distinctive zinc finger domains (Rastinejad et al., 1995, Umesono and Evans, 1989). The binding of this DNA binding domain occurs on TH response elements (TREs) in the promoter region of target genes (Umesono and Evans, 1989). The C-terminal region of TRs is responsible for ligand binding and dimerization, and has a high similarity between the two TRβ subtypes (Safer et al., 1997). TRα1 and TRα2 present significant differences in their dimerization and ligand-binding domain due to alternative splicing (Schueler et al., 1990). While ligand binding and dimerization domains allow TRα1, TRβ1 and TRβ2 to engage in homo- and heterodimerization, as well as binding T3, the splice variant TRα2 is unable to dimerize or bind T3 (Brent, 2012, Katz et al., 1992, Koenig et al., 1989, Sinha and Yen, 2000).

TRs can function as monomers, and homo- and heterodimers, formed by two different TR isoforms or one TR dimerized with a different nuclear receptor. The most common heterodimers consist of TR and retinoid X receptor (Barra et al., 2004, Clark et al., 2016). They can interact with various co-repressor and co-activator proteins (Astapova et al., 2008, Chen and Evans, 1995, Liu et al., 2006). Contrary to steroid hormone receptors, TRs are mostly located in the nucleus, where they can bind to TREs independent of their ligand (Zhang and Lazar, 2000). TRs are capable of activating or inhibiting gene expression, depending on co-repressing or co-activating proteins engaging in the complex around the TRE (Brent et al., 1989, Hu and Lazar, 2000). Binding of T3 to TR results in a conformational change, cleaving the interaction of TRs with these cofactors. This subsequently leads to expression of genes positively regulated by THs or repression of negatively regulated genes (Figueira et al., 2011, Yen, 2001).

TRs are differentially expressed during development and in adulthood (Oetting and Yen, 2007). TRα1 and TRβ1 are widely expressed, while TRβ2 is primarily expressed in the hypothalamus and pituitary, but also in the brain, inner ear and, in some species, retina (Bradley et al., 1994, Brent, 2012, Cook et al., 1992, Hodin et al., 1990, Sjoberg et al., 1992, Yen et al., 1992).

2.1.3.3 Neuroendocrine Regulation of Thyroid Function

The synthesis and secretion of THs is stimulated by TSH secreted by the pituitary under the control of TRH released by the hypothalamus in response to the circulating TH. High TH levels inhibit the synthesis and secretion of TRH and TSH. Thus, thyroid function is regulated by a negative feedback loop involving the hypothalamus and pituitary in the so-called hypothalamic-pituitary-thyroid axis (Figure 4) (Costa-e-Sousa and Hollenberg, 2012). In a normally functioning thyroid, this negative feedback regulation maintains the homeostasis of TSH and THs, a status known as euthyroidism.

The tripeptide TRH is produced by hypophysiotropic neurons in the paraventricular nucleus of the hypothalamus (Lechan and Fekete, 2006). As part of the negative feedback regulation, this secretion is inhibited by T3 (Segerson et al., 1987, Sugrue et al., 2010) in the blood circulation as well as locally activated T3 (converted by Dio2) supplied by tanycytes, the cells lining the third ventricle (Fliers et al., 2006, Tu et al., 1997). The T3-dependend regulation of *Trh*-expression is mediated by the binding of T3 to TRβ1 and mainly TRβ2 (Dupre et al., 2004, Guissouma et al., 1998).

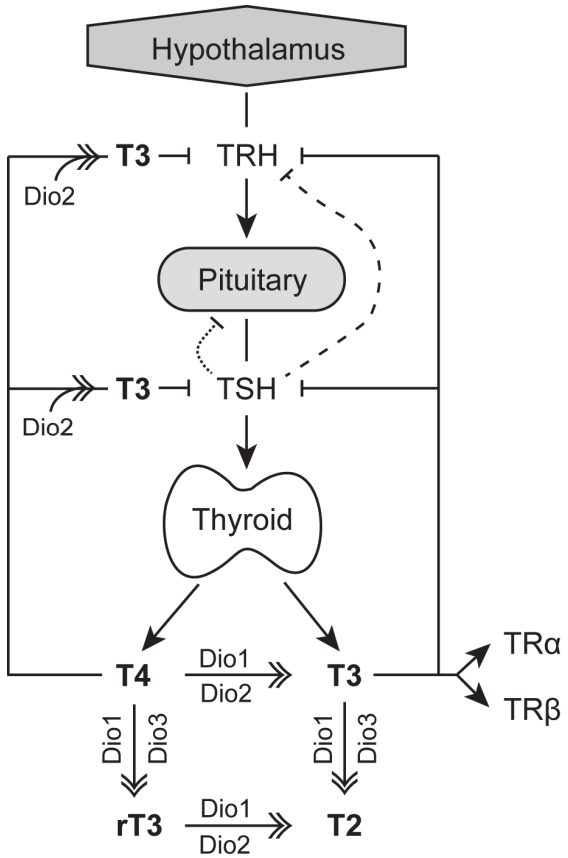

Figure 4: Hypothalamic-pituitary-thyroid axis. (Modified from Hoermann *et al*. (Hoermann et al., 2015)

TH production and release is mediated by TSH and regulated via a network of negative feedback and feedforward loops involving the hypothalamus and pituitary. Empty arrow heads mark enzymatic conversion, filled arrow heads indicate activation, and bar-headed lines symbolize inhibition. In the long negative feedback loop (solid line), T3 (secreted from the thyroid or produced from T4 by Dio1 in peripheral tissues and Dio2 in the hypothalamus and brain) inhibits the TRH production in the hypothalamus. Low levels of THs cause increased TRH secretion, stimulating the TSH expression in the pituitary. TSH then acts on the thyroid gland, inducing synthesis and release of THs, which negatively regulate TRH. Elevated T3 also act on the pituitary, inhibiting TSH production. TSH has an inhibitory function on TRH secretion, leading to the short loop feedback (dashed line). Paracrine suppression of TSH production in the pituitary itself has been described as an ultra-short feedback loop (dotted line). Active THs T4 and T3 can be inactivated by Dio3, producing reverse T3 (rT3) and T2. In target cells, T3 binds to TRα and TRβ to regulate gene expression.

TRH then acts on the pituitary via the TRH receptor (TRHR) in human or TRHR1 in mice (Cao et al., 1998, Hinuma et al., 1994, Straub et al., 1990), inducing the production and release of TSH (Martin et al., 1970, Steinfelder et al., 1991). TSH consists of two subunits, an α-subunit, common to all glycoprotein hormones, and a unique β-subunit, responsible for the specific binding of TSH to its receptor (Pierce and Parsons, 1981). The glycosylation of TSH, also regulated by TRH, influences its bioactivity (Beck-Peccoz et al., 1985, Taylor and Weintraub, 1989). TSH production is restricted to thyrotroph cells in the pituitary (Chin et al., 1981, Stojilkovic et al., 2010). Thyrotrophs not only express TRHR1, but also TRβ2 in a T3-dependent manner (Wood et al., 1991). In fact, T3 influences TSH production directly via TRβ2, and indirectly by inhibiting TRHR1 expression in thyrotrophs (Hinkle and Goh, 1982, Yamada et al., 1992). Circulating T4 can be converted to T3 by Dio2 expressed in thyrotrophs, leading to an indirect regulation of TSH production via T4 (Christoffolete et al., 2006). The secreted TSH can then act on the TSHR in the thyroid gland, stimulating TH synthesis and release.

In addition to this long feedback loop involving the hypothalamus, pituitary and the thyroid gland, two shorter feedback loops have been described, affecting only the pituitary and hypothalamus (short loop feedback) or solely the pituitary (ultra-short loop feedback) (Prummel et al., 2004). Briefly, the short feedback loop describes the inhibiting effect of TSH-binding to the TSHR in the hypothalamus, suppressing TRH synthesis and release (Bockmann et al., 1997, Motta et al., 1969, Prummel et al., 2004). The ultra-short feedback loop involves a second cell type in the pituitary, the folliculo-stellate cells expressing the TSHR. Activation of these cells by high levels of TSH leads to a suppression of TSH production in a paracrine manner (Dietrich et al., 2010, Hoermann et al., 2015, Prummel et al., 2004). As a consequence of this hypothalamic-pituitary-thyroid feedback network, an increase in TH secretion results in decreased TRH and TSH production. Insufficient concentration of TH in the circulating blood mediates an increased expression of TRH and TSH.

2.1.4 Thyroid Diseases

2.1.4.1 Hypothyroidism

Insufficient TH production and/or secretion by the thyroid gland is commonly referred to as hypothyroidism. Per definition, hypothyroidism is characterized by decreased TH levels and elevated TSH. Subclinical hypothyroidism refers to a state with mildly increased TSH and normal TH concentrations (Biondi and Cooper, 2008). Hypothyroidism leads to a decreased metabolic rate with weight gain (Laurberg et al., 2012), reduced heart rate (Klein and Ojamaa, 2001, Vargas-Uricoechea et al., 2014) and cold intolerance (Silva, 2003). Hypothyroidism has also been linked to depression (Dayan and Panicker, 2013).

A lack of THs during early development is defined as congenital hypothyroidism (CH), which can cause irreversible mental retardation and delayed growth, requiring early diagnosis and TH replacement (Calza et al., 2015, Hall, 1902, Leger et al., 2014). The incidence for primary CH is approximately 1:2,500. Around 85-90 % of CH are sporadic cases, where defects in the development of the thyroid lead to a partial or complete absence of the thyroid. The remaining 10-15 % of CH cases are

caused by thyroid dyshormonogenesis, mostly caused by inherited genetic defects (Brown, 2000a).
Hashimoto's thyroiditis is an autoimmune disease mainly affecting middle-aged women that can lead to hypothyroidism. Patients with Hashimoto's thyroiditis produce autoantibodies against TPO, TG, and/or the TSHR, in which case the function of the receptor is inhibited. Of these proteins, only TSHR is expressed at the basolateral membrane of thyrocytes and is likely the primary target in the development of Hashimoto's thyroiditis. The antibodies trigger an aggressive immune response, causing destruction of the thyroid tissue, consequently leading to decreased TH production (Akamizu et al., 2000, Radetti, 2014). The antibodies against Tg and TPO can be detected with the progression of the disease and function as a diagnostic marker for Hashimoto's thyroiditis (Akamizu et al., 2000, Khan et al., 2015).

2.1.4.2 Hyperthyroidism

Contrary to hypothyroidism, hyperthyroidism refers to a state of excessive TH secretion by the thyroid gland. It is characterized by elevated TH levels and decreased TSH. A situation with decreased TSH and normal TH levels is known as subclinical or latent hyperthyroidism (Donangelo and Braunstein, 2011, Palmeiro et al., 2013). The physiological consequences of hyperthyroidism include an increased metabolism often associated with weight loss (Laurberg et al., 2012), increased heart rate (Klein and Ojamaa, 2001) and heat intolerance (Silva, 2003). Both hyper- and hypothyroidism are more common in women than men (Wang and Crapo, 1997).

While some physiological consequences are identical, the terms thyrotoxicosis and hyperthyroidism are not interchangeable. Thyrotoxicosis refers to a general excess of TH, regardless of its source (endogenous or exogenous) (Nayak and Burman, 2006). Hyperthyroidism, on the other hand, is defined by a hyper-secretion of TH by the thyroid gland (De Leo et al., 2016).

Autoimmune hyperthyroidism accounts for around 70 % of all cases. Graves' disease, also known as Basedow's disease, is an autoimmune disease that leads to hyperthyroidism through constant activation of the TSHR via TSHR-stimulating antibodies. This disease occurs in 0.5 % of men and 3.0 % of women (Burch and Cooper, 2015, Metso et al., 2008, Nystrom et al., 2013). Hyperthyroidism in absence of TSHR-activating antibodies is commonly referred to as non-autoimmune hyperthyroidism (NAH). Several gain-of-function mutations of the TSHR and the G protein $G_{\alpha s}$ have been reported to cause NAH (Gozu et al., 2010, Paschke, 1996, Vassart et al., 1996, Wonerow et al., 2001). NAH cases where the affecting germline mutation can be detected in family members over a minimum of two generations are categorized as familiar cases of NAH (FNAH). *De novo* germline mutations without a familiar link are considered sporadic cases of NAH (SCNAH) (Gozu et al., 2010, Paschke et al., 1996). Furthermore, functioning thyroid adenoma and TSH-secreting pituitary adenoma can also lead to hyperthyroidism (Brown, 2000b).

2.1.4.3 Goiter

Another common thyroid disorder is goiter, a non-malignant enlargement of the thyroid gland. Goiter growth can be induced by various factors, and patients with goiter can be euthyroid, hypothyroid or hyperthyroid. The most common cause for goiter is iodine deficiency (Brown, 2000b, Maberly, 1998). Besides a lack of iodine

due to dietary factors, inhibited iodine uptake and organification can lead to hypothyroidism and goiter (Reed-Tsur et al., 2008). This state can also be induced by drugs like methimazole and sodium perchlorate, inhibiting the function of TPO or NIS, respectively. Furthermore, reduced TH synthesis and secretion as well as TSH-signaling, are factors promoting benign thyroid growth (Dumont et al., 1992, Medeiros-Neto, 2000, Rakover et al., 2012, Stubner et al., 1987). Goiter caused by hypothyroidism and inhibited iodine metabolism presents a similar histological phenotype, described as dyshormogenetic goiter. In this form of goiter, the follicular lumen contains little to no colloid. The thyrocytes are proliferating and appear active, with columnar shape and round nuclei (Braham et al., 2013, Camargo et al., 1998).

Different morphological changes are seen in euthyroid patients, who develop a colloid goiter. The colloid goiter, also referred to as idiopathic simple goiter, is not associated with neoplasm or inflammation. Rather, the enlargement of the thyroid is caused by an increase of colloid in the thyroid follicles. The molecular mechanisms leading to a euthyroid colloid goiter are poorly understood, but some genetic factors have been linked to this disease (Makarov et al., 1993, Muirhead, 2001).

(Multi)nodular goiter is characterized by one or multiple separate nodules that cause an increase in thyroid size. The thyroid follicles in these nodules can vary considerably in size, shape and iodine uptake. Mutations in genes associated with thyroid function like *TSHR*, *TPO*, *Tg*, *SLC5A5* and *SLC26A4* have been found in nodules from patients (Knobel and Medeiros-Neto, 2003, Medeiros-Neto, 2000).

Graves' disease leads to diffuse thyrotoxic goiter. The histological phenotype of this form of goiter consists of very active, variably sized follicles. The thyrocytes are hypertrophic and protrude into the follicular lumen, resulting in irregularly shaped follicles (Lynch and Woodford, 2014). With the progression of Graves' disease, the follicular lumen diminishes due to increased absorption by the hyperactive thyrocytes. This also leads to an increase in colloid droplets in the thyrocytes. Furthermore, lymphocyte infiltration is visible (Nagayama, 2005, Nagayama et al., 2015).

2.2 Animal Models

While cell-based *in vitro* models are valuable tools to understand intracellular signaling, intercellular networks are difficult to mimic *ex vivo*. As hormones affect the whole organism, *in vivo* models are essential to understand the complex endocrine system. As explained in chapters 2.1.2 and 2.1.3, the three-dimensional structure of a thyroid follicle, as well as the cell polarity of thyrocytes, is essential for TH synthesis (Nunez and Pommier, 1982). Immortalized cell lines derived from thyrocytes often lose their polarity and/or fail to express all genes critical for TH synthesis at a normal level *in vitro* (Kimura et al., 2001). The problem of maintaining cell polarity in culture can be overcome by specific culturing techniques, such as the use of bicameral chambers for primary thyrocytes (Nilsson et al., 1996) or embedding of thyroid tissue in 3D collagen gel (Toda et al., 2002). Yet, culturing one cell type exclusively eliminates all regulatory factors provided by other organs – such as the negative feedback regulation via the hypothalamus and pituitary. Therefore, the endocrine effects of modified thyrocyte function on all peripheral tissues can only be studied *in vivo*.

Due to their short reproduction cycle and high genetic similarity to humans, mouse models have been utilized for medical research for over a century (Bianco et al., 2014). With the development of the first transgenic mouse line 40 years ago (Jaenisch, 1976), an approach wherein additional genetic information is randomly integrated in the genome with one or more copies, the use of mouse models in medical research increased constantly. Since then, new techniques for the generation of genetically modified mice have been developed, such as site-directed genomic modifications via homologous recombination (Alitalo and Pettersson, 1990, Mullins and Ganten, 1990), the Cre/*loxP* system (Orban et al., 1992, Sauer and Henderson, 1990) and Flp/*FRT* system (Fiering et al., 1993, Vooijs et al., 1998) or the CRISPR/Cas9 technique (Cong et al., 2013, Wang et al., 2013).

2.2.1 Mouse Models for Hypothyroidism

Mice had been used for thyroid research already prior to the invention of genetic modifications. To induce hypothyroidism, mice were injected with thyroid-destructive doses of radioactive iodine (Antonica et al., 2012, Grinberg, 1963, Raynaud, 1959) or chemically treated with drugs inhibiting iodine uptake or organification, such as the NIS-inhibiting sodium perchlorate (Connell et al., 1983, Pajer and Kalisnik, 1991) or TPO-inhibiting propylthyouracil (Perez-Delgado et al., 1987, Shoemaker and Dagher, 1979) and methimazole (Sato et al., 1976). The *hyt/hyt* mouse is another model frequently used to study hypothyroidism *in vivo* (Beamer et al., 1981). This mouse line carries a sporadic, inactivating autosomal recessive mutation in the TSHR sequence leading to hypothyroidism when both alleles are affected (Stein et al., 1994). With the availability of genetically modified animals, the role of various genes in thyroid development or function was identified by deleting or mutating these genes. Subsequently, mice lacking genes necessary for thyroid function serve as models for hypothyroidism (De Felice et al., 1998, Mansouri et al., 1998, Marians et al., 2002).

2.2.2 Mouse Models for Hyperthyroidism

Drug-induced thyrotoxicosis in mice can be achieved by feeding them pulverized thyroid gland extract (Horn, 1958, Vacek et al., 1978) or by administering the THs T3 (Bradley and Spink, 1959) or T4 (Hoefig et al., 2016, Stein-Streilein et al., 1987). Autoimmune hyperthyroidism, mimicking Graves' disease, has been induced via genetic immunization against TSHR expressed from recombinant adenovirus or plasmids injected intramuscularly (Costagliola et al., 2000, Nagayama et al., 2002, Nagayama et al., 2015). Very few genetically modified models exist for NAH. A KO of TRβ results in hyperthyroid hormone levels (Forrest et al., 1996), bypassing the control via the hypothalamus-pituitary-thyroid axis. However, the lack of TRβ makes it difficult to interpret the extent of elevated TH levels. In two other studies to model hyperthyroidism, transgenic mouse models were generated expressing the $G_{\alpha s}$-activating cholera toxin A1 subunit (Zeiger et al., 1997) and the adenosine receptor 2a (Ledent et al., 1996, Ledent et al., 1992). In both models, the transgenic increase of cyclic adenosine monophosphate (cAMP) signaling led to early onset of hyperthyroidism at two months of age and premature death. A third transgenic mouse model expressing the $G_{\alpha s}$-activating mutation R201H under the *Tg* promoter (Michiels et al., 1994) developed hyperthyroidism at a later age of eight months.

2.2.3 Tissue-Specific Animal Models

Various genes have vital functions in an organism, and their ubiquitous loss of function results in intrauterine death (Papaioannou and Behringer, 2012). While in some cases heterozygous deletion of the gene of interest might be sufficient to evaluate the role of the suppressed gene (Pilipow et al., 2014, Rantakari et al., 2010, Soto et al., 2016), in many cases a full deletion of the gene of interest is necessary. A tissue-specific deletion or over-expression of genes causing premature death when affected ubiquitously allows to study the role of these genes in specific cell types. Therefore, the promoter region of a gene specifically expressed in the desired cell type is utilized to express a transgene of interest. Tissue-specific promoters are also used to direct the expression of recombinases used to mediate a gene KO into a specific cell type (Bayascas et al., 2006, Sassone-Corsi, 1998).

2.2.3.1 The Cre/loxP System

A common approach to create tissue-specific gene KOs is the Cre/*loxP* mediated recombination (Figure 5). The Cre recombinase is an enzyme derived from bacteriophage P1, which is capable of site-specific cutting and recombination of the DNA sequences (Sternberg and Hamilton, 1981). Cre detects 34 basepair (bp) repeat sequences, termed *loxP*, forms a dimer around each *loxP* site (Hoess and Abremski, 1984, Ringrose et al., 1998) and associates with a second dimer at a second *loxP* site (Ghosh et al., 2007, Hamilton and Abremski, 1984). In this synaptic complex, Cre mediates a double-strand break of the DNA and exchanges the two strands before ligating the DNA at the *loxP* site. Depending on the direction of the two *loxP* sites in relation to each other, this results in the dissection of DNA sequence between both sites and a circular construct of the excised sequence or a reversed insertion of this sequence (Figure 5 A,B). Repeated direction of the *loxP* sites results in excision of the sequence flanked by the *loxP* site, while an inverted direction of the *loxP* site causes an inversion of the flanked DNA sequence (Orban et al., 1992, Van Duyne, 2015).

The simple mechanism that one enzyme catalyzes the deletion or inversion of any DNA fragment flanked by two identical recognition sites functions as a valuable tool to generate various tissue-specific gene modifications. Expressing Cre recombinase under tissue-specific promoters, as a KI or transgenic construct, directs the Cre-mediated genomic recombination into numerous different cell types (Nagy et al., 2009, Ray et al., 2000). Crossing a tissue-specific Cre line with a line where the gene of interest is flanked by *loxP* sites (floxed) allows for studying the role of the same floxed gene in different tissues (Figure 5 C). The number of possible combinations is solely limited by the number of available floxed and cell-type specific Cre recombinase mouse lines (Perkins, 2002, Ray et al., 2000, Van Duyne, 2015).

In order to act on the genomic DNA, Cre recombinase must be present in the nucleus (Indra et al., 1999). The Cre-mediated KO of the gene of interest is timed by the expression onset of the gene regulated by the selected promoter. Once the promoter becomes active, Cre recombinase is constitutively expressed and mediates the recombination of *loxP* sites. Once the *loxP* sites are recombined, all cells originating from a recombined cell will exhibit the recombined genomic information, regardless of further presence of the Cre recombinase (Holzenberger et al., 2000).

To study the role of any floxed gene in adult tissues after a normal organogenesis in the presence of the gene of interest, inducible Cre lines have been established

(Erdmann et al., 2007, Sassmann et al., 2010). This can be achieved either by controlling the *Cre* gene expression via inducible promoters or by coupling the Cre recombinase to the modified hormone-binding domain (HBD) of a nuclear receptor (such as estrogen, progesterone or glucocorticoid receptors), mediating translocation into the nucleus upon ligand binding. An unintended activation of those receptor HBDs through endogenous expression of their ligands is avoided by mutating the HBD to exclusively bind synthetic ligands (Jaisser, 2000, Kellendonk et al., 1999, Kuhn et al., 1995). For the estrogen receptor, a mutated HBD binding tamoxifen has been established, which is often fused to the Cre recombinase to generate tamoxifen-inducible Cre activation in mice. Two different mutations for the estrogen receptor HBD, $CreER^{T}$ and $CreER^{T2}$, differing in their sensitivity towards tamoxifen, are commonly used (Feil et al., 1996, Feil et al., 1997, Indra et al., 1999).

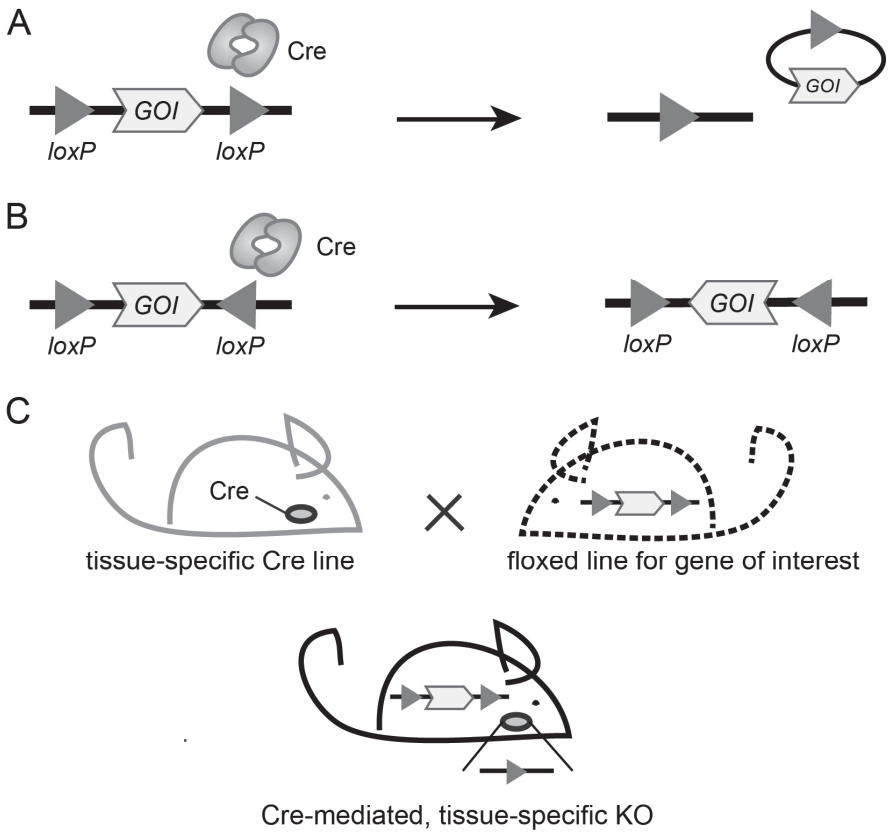

Figure 5: The Cre/*loxP* system

The Cre recombinase recognizes *loxP* sites (grey triagles) and cuts the DNA at these recognition sites. A) A same direction of two *loxP* sites results in the removal of the DNA sequence (gene of interest, GOI) between the *loxP* site as a circular DNA fragment. B) *LoxP* sites with an inverse direction leads to an inversion of the floxed DNA sequence. C) Crossing of a mouse line expressing Cre recombinase under a tissue-specific promoter with a line where the gene of interest is flanked by *loxP* sites generates a tissue-specific KO line for the gene of interest.

2.2.3.2 Thyrocyte-Specific Cre-Expression in Mice

In order to study the effect of gene KOs specifically in thyroid follicles, the promoter region of different thyrocyte-specific genes has been coupled with the *Cre* gene. Currently, four different constitutively active Cre lines for thyrocyte-specific expression are available. Utilizing the *Pax8* promoter, *Cre* expression can be achieved around E 8.5, targeting the early differentiation of the thyroid gland. A KI mouse expressing *Cre* under the endogenous *Pax8* promoter was generated by Bouchard et al. in 2004 (Bouchard et al., 2004), mediating *Cre* expression in thyrocytes but also in tubular and glomeruli cells during kidney development and in renal tubular cells in adult kidneys (Bouchard et al., 2004, Plachov et al., 1990, Traykova-Brauch et al., 2008). Another promoter used to direct Cre recombinase into thyrocytes is the *Nkx2-1* promoter. In the transgenic *Nkx2-1*-Cre line the *Cre* expression can be detected from E 10.5 onwards. Since *Nkx2-1* is not only expressed in the developing thyroid gland but also in the brain, pituitary and lung, the Cre expression is not restricted to thyrocytes (Tiozzo et al., 2012, Xu et al., 2008).

A transgenic mouse line expressing *Cre* under the human *TPO* promoter, generated by Kusakabe et al. in 2004, presents a higher specificity for thyrocytes. This mouse line expresses Cre recombinase with an efficiency of circa 92 % from E 14.5 onwards (Kusakabe et al., 2004). Another transgenic mouse model expressing *Cre* thyrocyte-specific from E 14.5 was established by Kero et al. in 2007. These mice constitutively express Cre recombinase-driven by the murine *Tg* promoter (c*Tg*Cre) with an efficiency close to 100 % (Kero et al., 2007). In these four models, the conditional, thyrocyte-specific expression of Cre recombinase did not alter the development, morphology or function of the thyroid gland (Bouchard et al., 2004, Kero et al., 2007, Kusakabe et al., 2004). However, a second Cre-line expressing *Cre* under the *Tg* promoter, established by Calì et al. in 2007, developed hypothyroidism and presented an altered thyroid morphology likely caused by Cre toxicity (Cali et al., 2007).

In addition to the conditional Cre lines, two thyrocyte-specific, tamoxifen-inducible Cre lines have been described. A KI mouse line with an *Nkx2-1* promoter-driven $CreER^{T}$ construct enables the tamoxifen-induced Cre-mediated recombination of floxed genes in thyrocytes, but also in other *Nkx2-1* expressing cells in the brain, pituitary and lung (Taniguchi et al., 2011). The generation of a tamoxifen-inducible Cre line with an expression exclusively in thyrocytes has not been described in more detail. This line expresses the $CreER^{T2}$ construct under the *Tg* promoter. However, Cre-mediated recombination can be observed in high levels after administration of 1 mg tamoxifen, but already without tamoxifen induction, an effect of leaking Cre activity in the nucleus was detected (Charles et al., 2011).

2.2.3.3 Knock-In Mouse Models

The copy number and integration site of transgenic constructs in the mouse genome can vary and eventually lead to mosaic expression or unintended loss of function for the gene locus of the random integration site (Wolf et al., 2000). KI mouse models offer a method for site-specific gene modifications, overcoming these common problems of transgenic animal models. A gene KI can be achieved either via CRISPR/Cas9 technology (Cong et al., 2013, Wang et al., 2013) or gene targeting via homologous recombination (Alitalo and Pettersson, 1990, Doyle et al., 2012, Mullins and Ganten, 1990, Robbins, 1993).

Gene targeting by homologous recombination utilizes the mechanism wherein foreign DNA material may be inserted into the genomic DNA of a cell at sites of high sequence similarity. Thus, when flanking the desired genetic information with homologous regions matching the targeted gene locus, so-called homologic arms, one can direct the integration into the desired locus (Bouabe and Okkenhaug, 2013, Capecchi, 1989). Therefore, a vector with a targeting construct containing the desired KI sequence flanked by 5'- and 3'-homologic arms and a selection marker is generated (Bouabe and Okkenhaug, 2013, Robertson, 1991). ES cells are then transfected with this vector, and the selection marker is often excised from the genome after successful selection of the targeted ES cells. The targeted ES cells are injected into blastocysts and implanted into a pseudopregnant surrogate mother. Germline transmission of the targeted gene in the chimeric offspring is then controlled by the coat color of the first filial generation obtained from breeding chimeras with wild-type mice of a recessive coat color (Auerbach et al., 2000, Pettitt et al., 2009). Finally, sequencing and genomic polymerase chain reactions (PCRs) are used to confirm the presence of the desired genetic modification at the targeted site (Bouabe and Okkenhaug, 2013, International Mouse Knockout et al., 2007).

2.3 RNA Interference

In the past two decades, the broadly conserved mechanism of RNA interference as a post-translational regulator of gene expression has been discovered and thoroughly characterized in various species. Small, noncoding RNA molecules, namely short or small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) have been found to bind to their target mRNAs, leading to mRNA degradation or repressed translation of the targeted mRNAs (Fire et al., 1998, Olsen and Ambros, 1999, Pasquinelli et al., 2000, Ratcliff et al., 1997, Svobodova et al., 2016).

2.3.1 Small Non-Coding RNAs

The three classes of small non-coding RNAs differ in terms of origin, processing and cell abundance. While miRNAs and siRNAs are ubiquitously expressed in viruses, microorganisms, plants, and vertebrates as well as invertebrates (Golden et al., 2008, Kooter et al., 1999, Marques and Carthew, 2007, Pandya et al., 2014, Voinnet, 2002), piRNA expression has been detected in invertebrates and vertebrates. In mammals, piRNAs are exclusively expressed in germ cells of mammalian species. The 26–31-nucleotide (nt)-long piRNAs are thought to regulate germ cell development and maturation by suppressing mRNA expression levels (Aravin et al., 2007, Pfeifer and Lehmann, 2010, Zhang et al., 2015). SiRNAs are 21–22-nt-long RNA molecules characteristically cleaved to double-stranded RNA duplexes with a 2-nt-long 3' overhang during siRNA processing. They are considered to be a defense mechanism against invading pathogens such as viruses (Marques and Carthew, 2007, Mittal, 2004, Montgomery and Fire, 1998, Nykanen et al., 2001). Similar to siRNAs, miRNAs are involved in both suppression of mRNA translation as well as degradation of their target mRNAs. The 21–24-nt-long miRNAs possess a seed sequence spanning nt 2–8 at the 3' end, which is responsible for the specific binding to their target mRNAs. Typically, miRNAs do not bind their target mRNAs fully complementarily, but with a central mismatch. The shorter seed sequence and

incomplete homology allows one miRNA to bind to up to 200 different mRNA targets specifically (Krek et al., 2005, Lim et al., 2005).

2.3.2 The Mechanism of RNA Interference

Whether the targeted mRNA is degraded or suppressed depends on the sequence homology of the small RNA molecule with its target mRNA (Carthew and Sontheimer, 2009). Complete sequence homology of siRNA or miRNA directs the double-stranded short-RNA-mRNA complex to the RNA-induced silencing complex (RISC), where the mRNA is degraded by the endonuclease Argonaute 2 (AGO 2) (Broderick et al., 2011, Hutvagner and Zamore, 2002, Meister et al., 2004). While a complete match of short RNA and mRNA sequence leads to degradation, a central mismatch in the sequence homology will lead to translational repression of the targeted mRNA by any of the four members of the mammalian Argonaute protein family (AGO1-4), which are essential components of the RISC complex and loaded with mature miRNA and siRNA molecules. (Broderick et al., 2011, Ghosh and Adhya, 2016, Wu et al., 2006, Yekta et al., 2004).

2.3.3 Processing of Small Non-Coding RNAs

The processing of those small non-coding RNAs involved in RNA interference differs in their initial phase, but in their final maturation step both miRNAs and siRNAs undergo Dicer1-mediated cleavage (Bernstein et al., 2001, Caudy et al., 2003, Tam et al., 2008, Watanabe et al., 2008).

The maturation of miRNAs can be divided in two phases, the nuclear phase and the cytoplasmic phase (Figure 6). In the nucleus, several-kilobases (kb)-long primary miRNAs (pri-miRNAs) with a characteristic hairpin structure are transcribed by DNA polymerase II (Pol II), and, in rare cases, Pol III from the genome (Borchert et al., 2006, Lee et al., 2004). The pri-miRNAs are then cleaved to 60–70-nt-long hairpin-structured precursor miRNAs (pre-miRNAs) with a 2-nt 3’ overhang by the microprocessor, consisting of the RNAse III Drosha and its co-enzyme DiGeorge Syndrome Critical Region 8 (DGCR8) (Gregory et al., 2004, Han et al., 2004, Lee et al., 2003).

Next, pre-miRNAs are exported to the cytoplasm by the RanGTP-dependent nuclear receptor exportin 5 (Lund et al., 2004, Yi et al., 2003), where mature miRNA duplexes are generated by cleavage of the stem loop by Dicer1 (Grishok et al., 2001, Hutvagner et al., 2001). In this process, Dicer1 functions as a molecular ruler. The Piwi/Argonaute/Zwille (PAZ) domain of the Dicer1 protein recognizes and binds the 3’ end from the pre-miRNA, and the cleavage center processes the pre-miRNA in a defined distance of 55–70 Å. This 55–70 Å distance differs between species and translates to a length of 22–25 nt. Cleavage by the two RNAse III domains, which form the cleavage center of Dicer1, results in mature miRNA duplexes with a 3’ overhang of both strands (Lau et al., 2012, Macrae et al., 2006, Takeshita et al., 2007). The leading strand of the miRNA duplex is then loaded onto an AGO protein and thus integrated into RISC, while the other strand is degraded by RISC (Gregory et al., 2005, Ohrt et al., 2008, Okamura et al., 2004, Siomi and Siomi, 2009). The processing of siRNA is comparable to the cytoplasmic stage of miRNA processing. However, the turnover rate for pre-siRNA cleavage by Dicer1 is 100-fold lower than the maximum cleavage rate of pre-miRNAs (Chakravarthy et al., 2010).

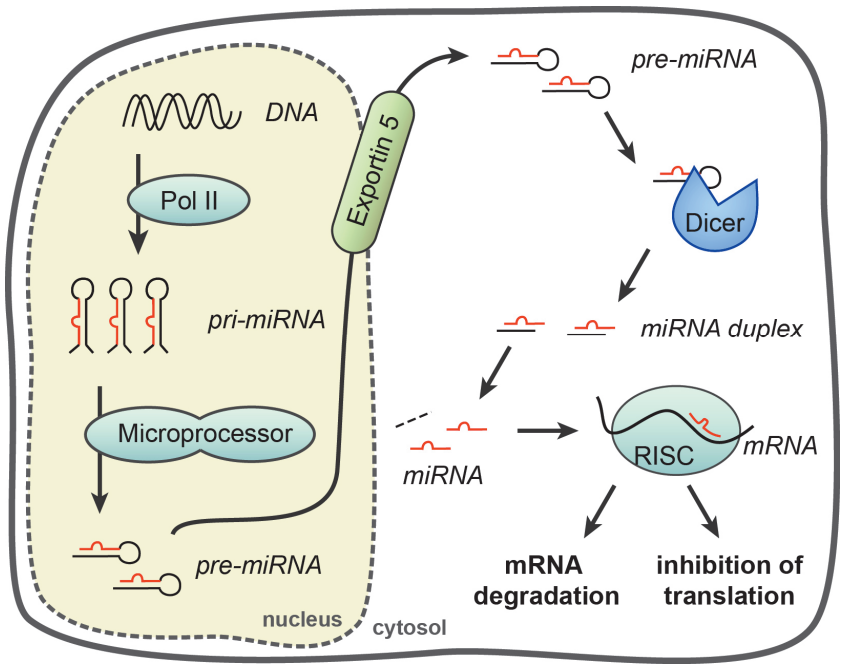

Figure 6: miRNA processing

The maturation of miRNAs is a multi-step process, where pri-miRNA molecules are transcribed in the nucleus by polymerase II (Pol II) and then cleaved to pre-miRNAs by the microprocessor. After the export of the pre-miRNAs into the cytosol by exportin 5, Dicer1 cleaves the stem loop, producing mature miRNA duplexes. The miRNA duplexes dissociate, and while one strand is degradaded, the leading strand is integrated into the RISC complex, where it engages in the suppression of the translation or degradation of mRNA.

2.3.4 Dicer1 in Murine Thyroid Gland

2.3.4.1 Dicer1 During Early Thyroid Development

Dicer1 and subsequently the RNA interference pathways are crucial for embryogenesis, and a full KO of Dicer1 is embryonically lethal (Bernstein et al., 2003). Several tissue-specific Dicer1 KO models revealed the role of Dicer1 in the proper differentiation of e.g. the liver (Chen and Verfaillie, 2014, Sekine et al., 2009), pancreatic islet cells (Lynn et al., 2007), male germ cells (Maatouk et al., 2008), or intestines (McKenna et al., 2010). To assess the developmental effects of Dicer1 and miRNA regulation during early development of the thyroid gland, two independent Dicer1 KO lines with a constitutive KO of *Dicer1* during early development (E 8.5) have been generated. Both mouse lines utilize a *Pax8* promoter-driven Cre recombinase, but two different *Dicer1*-floxed mouse lines (Frezzetti et al., 2011, Rodriguez et al., 2012). *Pax8* has been shown to be strongly expressed in thyroid epithelial cells, but also in glomeruli and tubular cells during kidney development and in renal tubular cells in adult kidneys (Bouchard et al., 2004, Plachov et al., 1990, Traykova-Brauch et al., 2008). In both models, loss of Dicer1 causes severe hypothyroidism with delayed growth and premature death, either between the age of

6–12 weeks (Frezzetti et al., 2011) or shortly after weaning (Rodriguez et al., 2012). Unexpectedly, T4 substitution in the animals that died after weaning could have prolonged their life span for up to two weeks, but could not have prevented the mice from dying. These findings were surprising, as *hyt/hyt* hypothyroid mice and *Pax8* KO mice presenting severe hypothyroidism could be rescued with T4 substitution (Ansari et al., 1997, Beamer et al., 1981, Biesiada et al., 1996, Christ et al., 2004). This indicates that the lethality of a *Pax8*-driven Dicer1 KO might not solely be caused by hypothyroidism.

During embryonic development, at E 15.5, thyroid histology and size appears normal in *Pax8*-Cre-driven Dicer1 KO mice with unaltered protein expression of PAX8, NKX2.1 and Tg. However, within the first five postnatal days, the first morphological changes are present, and at one month of age the thyroid size of Dicer1 KO mice is smaller and shows severe loss of follicular architecture. In both *Pax8*-Cre-driven Dicer1 KO models, at four weeks of age, NIS gene and protein expression is severely decreased. Additionally, *Tshr* and *Tpo* gene expression, as well as Tg protein expression, are downregulated in these Dicer1 KO mice. While PAX8 and NKX2.1 protein levels are unaltered in one model (Frezzetti et al., 2011), those thyroid-related TFs were decreased on gene expression and protein levels in the second model (Rodriguez et al., 2012). The model by Rodriguez *et al.* presents a slightly more severe phenotype, with decreased *Nis* and *Tg* gene and protein expression already at five days of age. This progressive loss of gene and protein expression of thyroid TFs and key regulators of thyroid function, together with the loss of follicular organization at one month of age after normal development, reveals that a lack of Dicer1 leads to dedifferentiation of thyrocytes over time.

In a recent study, another factor in the miRNA maturation cascade, the Microprocessor-component DGCR8, was knocked-out in *Pax8*-expressing cells. This DGCR8-deficient mouse model confirms that the hypothyroidism, growth delay, decreased thyroid weight and the lacking follicular architecture are consequences of the disabled miRNA synthesis (Bartram et al., 2016). However, DGCR8 KO mice still express *Nis* at one month of age, which is strongly decreased in Dicer1 KO mice already at five days of age. Furthermore, DGCR8 KO mice showed an increase in apoptosis and proliferation, whereas Dicer1 KO mice had an eight-fold higher proliferation, but no changes in the apoptosis rate (Frezzetti et al., 2011). Interestingly, as PAX8 is also expressed in kidney epithelia, the DGCR8 KO mice present a severe kidney phenotype. Mice with a *Pax8*-driven DGCR8 KO suffer from end-stage renal disease and die at the age of 4–6 weeks. The kidney-phenotype of DGCR8 mice might explain why thyroxine-substitution was not sufficient to rescue Dicer1 KO mice (Bartram et al., 2016).

2.3.4.2 Dicer1 in Late Thyroid Development

While a *Pax8*-Cre line leads to a KO of Dicer1 during early embryonic development, when thyroid differentiation is not fully completed, a *Tg* promoter-driven Cre line causes Dicer1 KO around E 14.5 during the final steps of thyroid differentiation. Mice lacking Dicer1 from E 14.5 can be divided in two sub-groups according to their phenotype. A severe phenotype is comparable to the *Pax8*-Cre-driven Dicer1 KO described in chapter 2.3.4.1, with 88 % decreased T4 serum concentrations and premature death shortly after weaning. The second subgroup presents a moderate, non-lethal phenotype with 40 % decreased T4 concentrations and a partially residual

follicular architecture of the thyroid at three weeks of age. Contrary to the early KO of Dicer1 (E 8.5), where NIS gene and protein expression is strongly diminished from an early age, *Nis* gene expression is unaltered in five-day-old *Tg*Cre-driven Dicer1 KO mice. However, in the severe subgroup NIS protein expression was undetectable in immunohistochemical staining. Already at five days of age, the mRNA levels of the TFs *Pax8* and *FoxE1*, as well as *Tg*, were decreased. Additionally, at three weeks of age, the gene expression for *Nis*, *Tpo*, *Tshr and Nkx2.1* was decreased in *Tg*Cre-driven DicerKO mice. Immunohistochemical analysis of the severe phenotype confirmed the decrease of transcribed proteins for all aforementioned genes. In the mild phenotype sub-group, NIS appears to be the only protein that was downregulated. Despite no changes in thyroid size, an 18-fold increased proliferation has been reported (Rodriguez et al., 2012).

2.3.5 Dicer1 Syndrome in Human Patients

In humans, germline mutations in the *DICER1* gene are associated with DICER1 syndrome, also known as *DICER1*-pleuropulmonary blastoma familial tumor predisposition syndrome (Hill et al., 2009, Slade et al., 2011). Carriers of heterozygous *DICER1* mutations have an increased risk of developing carcinoma. However, only 20 % of male and 50 % of female patients with heterozygous *DICER1* mutations develop tumors (Foulkes et al., 2014), suggesting the existence of additional factors, such as somatic mutations in the second *DICER1* allele (Rutter et al., 2016). The most common tumors associated with DICER1 syndrome are familial pleuropulmonary blastoma and Sertoli-Leydig cell tumors (SLCT). However, MNG, DTC, as well as cystic nephroma have also been frequently linked to this syndrome (Cross et al., 2010, Foulkes et al., 2014, Hill et al., 2009, Priest et al., 2009, Rutter et al., 2016). Furthermore, patients with DICER1 syndrome might develop juvenile hamartomatous intestinal polyps (Priest et al., 2009), nasal chondromesenchymal hamartoma (Priest et al., 2010), ciliary body medulloepithelioma (Priest et al., 2011), cerebral medulloepithelioma, and childhood embryonal rhabdomyosarcomas (Cross et al., 2010). With such a variety of cancer types linked to DICER1 syndrome, co-occurrence of two tumor types in one patient has been described several times (Durieux et al., 2016, Rio Frio et al., 2011, Rutter et al., 2016).

2.4 G Protein-Coupled Receptor Signaling

G protein-coupled receptors (GPCRs) form a large family of plasma membrane receptors, which are coupling to guanine nucleotide-binding proteins (G proteins). GPCRs are characterized by seven transmembrane helixes (TMs), which are connected via intra- and extracellular loops of variable sizes. The size and shape of the N-terminal, extracellular domain and the intracellular C-terminal region also differ vastly between different members of the GPCR family (Kristiansen, 2004, Trzaskowski et al., 2012).

The intracellular signaling of GPCRs is mediated by G proteins. Without coupling to a GPCR, G protein complexes are inactive, forming a heterodimeric complex consisting of G_α, G_β and G_γ subunits. In this inactive state, the G_α subunit, which possesses a GTPase activity, is bound to a guanosine diphosphate (GDP). The G_β and G_γ subunits are capable of forming heterodimeric $G_{\beta\gamma}$ complexes (Digby et al., 2006,

Hurowitz et al., 2000). Upon ligand binding, a conformational change of the TM helices creates a binding domain for the G_α subunit. After coupling of the G protein complex to this binding domain of the GPCR, the G_α subunit is activated, resulting in the exchange of the bound GDP with cytoplasmic guanosine triphosphate (GTP) (Kristiansen, 2004, Van Eps et al., 2011). This exchange initiates the dissociation of the G_α subunit from the heterotrimeric complex. After dissociation, both the G_α subunit and the $G_{\beta\gamma}$ complex can activate different downstream signaling cascades (Kristiansen, 2004, Oldham and Hamm, 2008). Until the GPCR is inactivated (ligand dissociation, internalization, desensitization) the GPCR is able to activate further G proteins. The G_α subunit deactivates itself via the GTPase activity by dephosphorylation of GTP to GDP and re-couples to the $G_{\beta\gamma}$ subunit (Digby et al., 2006, Ross and Wilkie, 2000, Yuen et al., 2010).

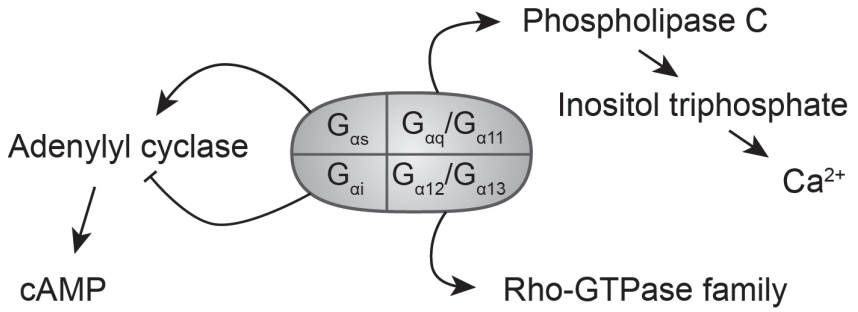

Figure 7: Overview of G_α-mediated signaling cascades.

While $G_{\alpha s}$ leads to activation of adenylyl cyclase, catalyzing the production of cAMP, the stimulation of $G_{\alpha i/0}$ inhibits the adenylyl cyclase activity. $G_{\alpha q/11}$ signaling activates phospholipase C, which mediates inositol triphosphate production, leading to an increase of Ca^{2+}. $G_{\alpha 12/13}$ activates Rho-GTPase-regulated signaling cascades.

In mammals, four G_α protein families are described: $G_{\alpha s}$, $G_{\alpha i/0}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ (Milligan and Kostenis, 2006). These different G_α subunits activate different signaling cascades (Figure 7). $G_{\alpha s}$ leads to increased production of the second messenger cAMP by stimulation of adenylyl cyclases, which catalyze the conversion of adenosine triphosphate (ATP) to cAMP (Weinstein et al., 2004). $G_{\alpha i/0}$ inhibits the conversion of ATP to cAMP by adenylyl cyclase, and can thus suppress the $G_{\alpha s}$-mediated signaling (Birnbaumer, 2007). $G_{\alpha q/11}$ mediates the production of the second messenger Ca^{2+} as a result of phospholipase C activation and subsequent stimulation of inositol triphosphate (IP3) release (Homma, 1994). An activation of $G_{\alpha 12/13}$ causes activation of RhoA GTPase by coupling to Rho Guanine-nucleotide-exchange factors (Buhl et al., 1995, Worzfeld et al., 2008).

2.4.1 GPCR Signaling in the Thyroid Gland

The TSHR belongs to the family of glycoprotein hormone receptors (Hsu and Hsueh, 2000). The TSHR has been shown to couple to all known G protein families *in vitro*. However, the TSH concentrations needed for coupling differs vastly between the different G protein family members (Laugwitz et al., 1996). Thus far, a physiological

role of TSHR coupling has only been described for $G_{\alpha s}$ (Vassart and Dumont, 1992) and $G_{\alpha q/11}$ (Kero et al., 2007). The $G_{\alpha s}$–stimulated signaling cascade is crucial for TH synthesis, TH secretion, iodide uptake and thyrocyte proliferation (Vassart and Dumont, 1992). Coupling and signaling via $G_{\alpha q/11}$ is important for iodine organification, TH secretion and goiter development (Kero et al., 2007). To date, the effects of $G_{\alpha 12/13}$ coupling has only been studied *in vitro* using human follicular carcinoma cells. These *in vitro* studies indicate that activation of p44/42 mitogen-activated protein kinase-activation (MAPK) is $G_{\alpha 13}$–dependent (Buch et al., 2008). The influence of p44/42 MAPK on various cell functions such as proliferation, mitosis, differentiation or apoptosis is well known (Buch et al., 2008, Rossomando et al., 1989, Yoon and Seger, 2006), and suggests a role of $G_{\alpha 12/13}$ in thyroid growth and differentiation.

2.4.2 Constitutively Activating Mutations of GPCRs

Gain-of-function mutations are mutations leading to a constitutive activation of an enzyme or receptor. CAMs in GPCRs cause a permanent conformational change of the transmembrane helices and/or intracellular domain towards the active conformation. Thus, the receptor activates G protein-mediated signaling regardless of ligand binding, elevating the basal levels of second messengers involved in GPCR signaling. CAMs have been identified in a variety of GPCRs (Thompson et al., 2014, Thompson et al., 2008), and are often linked to a conformation change in the sixth transmembrane helix (TM6) (Tsukamoto and Farrens, 2013). In humans, a heterozygous genotype is sufficient to cause a clinically relevant, autosomal dominant phenotype (Fuhrer et al., 1996, Nordhoff et al., 1999).

2.4.3 Constitutively Activating Mutations in TSHR Signaling

To date, over 60 CAMs of the TSHR are identified from human patients suffering from hyperthyroidism, autonomously functioning thyroid nodules or differentiated thyroid carcinomas (Fuhrer et al., 1996, Lueblinghoff et al., 2011). All TSHR CAMs identified in humans to date are heterozygous mutations (Fuhrer et al., 1996). While *de novo* mutations without familiar links are categorized as sporadic cases of NAH, germline mutations causing NAH that can be observed in relatives over a minimum of two generations are considered to be familiar cases of NAH (Gozu et al., 2010, Paschke et al., 1996). Most CAMs in the TSHR sequence activate $G_{\alpha s}$–dependent signaling, while a few of them can also activate $G_{\alpha q/11}$ (Lueblinghoff et al., 2011). One mutation leading to simultaneous $G_{\alpha s}$ and $G_{\alpha q/11}$ activation is D633H (Neumann et al., 2001a) located in TM6, which has been identified in several patients with thyroid adenomas (Russo et al., 1995) (http://endokrinologie.uniklinikum-leipzig.de/tsh/) and carcinoma (Neumann et al., 2001a, Russo et al., 1997). The exchange of aspartic acid to histidine *in vitro* results in an approximately five-fold increase of basal cAMP levels and two-fold elevated IP levels with unaltered TSHR surface expression when compared to cells transfected with wildtype (WT) TSHR (Lueblinghoff et al., 2011, Neumann et al., 2001a).

3 Aims of the Study

This study aimed to investigate the molecular mechanisms of thyroid function and diseases. For this purpose, we generated an inducible mouse model allowing Cre/*loxP*-mediated thyroid-specific gene deletion at any desired time point after E 14.5. After successful characterization and validation of this model, we applied this tamoxifen-inducible Cre mouse line to investigate the role of the miRNA-processing enzyme Dicer1 in thyroid physiology. In addition, in order to understand the molecular mechanisms leading to non-autoimmune hyperthyroidism *in vivo*, we generated another mouse model. In this project we introduced a constitutively activating TSHR mutation into the murine TSHR sequence as a gene KI. This KI mouse model was generated as a tool to investigate the development of hyperthyroidism and as a potential model for drug testing.

1. To generate and evaluate a thyrocyte-specific, tamoxifen-inducible $CreER^{T2}$ mouse model.
2. To determine the role of Dicer1 in thyroid development and function by generating a conditional and an inducible thyroid-specific *Dicer1* KO mouse model.
3. To study the molecular mechanisms of hyperthyroidism by generating a KI mouse model carrying the constitutively activating TSHR mutation D633H.

4 Materials and Methods

4.1 Experimental Animals (I-III)

4.1.1 Animal Housing (I-III)

All animal experiments were approved by the Finnish Animal Experiment Board (I-III) or Regierungspräsidium Karlsruhe (I) and the procedures were carried out in accordance with the institutional animal care policies of the University of Turku. The mice were housed in the animal facilities of the University of Turku under controlled conditions (12 hours light/12 hours dark, 21 °C ± 2 °C, humidity 55 % ± 15 %). All animals were provided water and pelleted chow RM3 (E) (Special Diets Services) *ad libitum*. Ear markings were set between the ages of two to six weeks and used for genotyping.

4.1.2 Generation of Genetically Modified Animal Models (I-III)

4.1.2.1 Tamoxifen-Inducible TgCreER^{T2} Mouse Line (I, II)

To generate a mouse line with tamoxifen-inducible thyrocyte-specific Cre recombinase expression, a mouse *Tg* EST probe was used to isolate a 100 kb PAC containing the murine *Tg* locus from the mouse genomic RPCI21 library (Kero et al., 2007, Vente et al., 1999). Next, 5' and 3' homologous arms were added by PCR to a pCreER^{T2} plasmid containing the bovine growth hormone polyadenylation signal and an *frt*-site flanked ampicillin resistance cassette (I: Fig. 1a). When designing the 5' homologous arm, the start codon ATC of the CreER^{T2} sequence was aligned to replace the start codon for *Tg*. The two fragments coding for the homologous arms were then cloned into the *Kpn*I, *Eco*RV and *Nhe*I restriction sites of the CreER^{T2} plasmid. Next, the construct was cloned into the *Tg* PAC by homologous recombination. After removal of the ampicillin resistance cassette by Flp-mediated recombination, the construct was confirmed by Southern blot, PCR, and sequencing and the linearized construct was injected into the pronucleus of fertilized oocytes. To confirm germline transmission of the desired transgenic CreER^{T2} construct, mice positive for CreER^{T2}, as confirmed by Southern blotting and genomic PCR, were bred with C57Bl/6 mice. The mice were later backcrossed with C57bl/6J mice to achieve a pure genetic background. A Gt(ROSA)26Sor reporter mouse line (Soriano, 1999) was used to analyze the Cre recombinase activity of the *Tg*CreER^{T2} mice.

The genotype of *Tg*CreER^{T2} mice was determined by genomic PCR from DNA obtained from ear pieces, using the following primers at an annealing temperature of 59 °C, resulting in a amplicon of 480 base pairs (bp) when the Cre recombinase sequence is present: TgProm_S 5'-ATGCCAACCTCACATTTCTTG-3' and *Tg*Cre_AS: 5'-AGTCCCTCACATCCTCAGGTT-3'. The genotype of Gt(ROSA)26Sor mice was determined with genomic PCR at 65 °C annealing temperature using three primers: Rosa26_F 5'-GCGAAGAGTTTGTCCTCAACC-3', Rosa26_wt_R 5'-GGAGCGGGAGAAATGGATATG-3' and Rosa26_mut_R 5'-AAAGTCGCTCTGAGTTGTTAT-3' amplifying a 650 bp long fragment from WT alleles and a 340 bp amplicon from mutated alleles.

4.1.2.2 Dicer1 Knockout Mouse Line (II)

Two different thyrocyte-specific Dicer1 KO lines were generated using the Cre/*loxP* system. A constitutive KO (c*Tg*DcrKO) was generated using a mouse line expressing Cre recombinase constitutively under the *Tg* promoter (Kero 2007), while the aforementioned tamoxifen-inducible thyrocyte-specific Cre mouse line was utilized to generate inducible Dicer KO mice (i*Tg*DcrKO). Both Cre lines were crossed with a Dicer1 floxed mouse line (Harfe et al 2005), which contained two *loxP* sites flanking Exon 24, encoding for the RNAse III domain of Dicer1. All obtained mice had a mixed genetic background of C67Bl/6N (Cre lines) mixed with C57Bl/6N and 129SvEv (Dicer-floxed line). Cre-negative littermates of c*Tg*DcrKO mice serves as controls for this strain, while three different controls were used for the tamoxifen-inducible i*Tg*DcrKO mice, specifically cre-negative, vehicle treated littermates ($Dcr^{fl/fl}$) cre-negative, tamoxifen treated ($Dcr^{fl/fl}$Tam) and Cre-expressing, vehicle treated littermates (i*Tg*$Dcr^{fl/fl}$).

The presence of Cre recombinase was analyzed by genomic PCR as described in chapter 4.1.2.1, and the genotype for the Dicer1 gene was determined by PCR at 59 °C annealing temperature (Dicer_F: 5'-CCTGACAGTGACGGTCCAAAG-3' and Dicer_R: 5'-CATGACTCTTCAACTCAAACT-3'). The primers used produced a 351 bp amplicon for Dicer1 WT alleles and a 420 bp amplicon for floxed Dicer1 alleles. The Cre-mediated recombination of the Dicer1 gene was tested by PCR with genomic DNA extracted from thyroid glands, using the Dicer_F primer together with the primer Dicer_Null 5'- CCTGAGTAAGGCAAGTCATTC-3' at 59 °C annealing temperature. The PCR resulted in a 600 bp amplicon when the *loxP* sites flanking part of the Dicer1 gene is recombined.

4.1.2.3 $TSHR^{D633H}$ Mouse Line (III)

A mouse line carrying the D633H mutation in the murine TSHR sequence was generated using homologous recombination (III: Fig. 1a). First, an 8,100 bp sequence containing exon 9, exon 10, intron 9 and the 3'-UTR of the *Tshr* gene was subcloned from BAC clones carrying the mouse *Tshr* gene (ENSMUSG00000020963, Children's Hospital Oakland Research Institute, USA) into the pACYCY177 vector (New Endgland Biolabs, USA) by Red/ET recombination following the manufacturer's protocol (Gene Bridges GmbH, Germany). To generate the desired point mutation from GAC coding for D633 to CAC in exon 10, a DNA fragment containing exon 10 and partial intron 9, amplified by PCR, was first inserted into a pGEM-4Z vector digested with *Bam*HI and *Hind*III (Promega, USA). Then, site directed mutagenesis was carried out using the QuikChange Site-directed Mutagenesis kit (Stratagene, USA) and the following primers: TshrMUTse 5'-CTGTGTTGATCTTCACTCACTTCATGTGCATGGCGC-3' and TshrMUTas 5'-GCGCCATGCACATGAAGTGAGTGAAGATCAACACAG-3'. A Neo resistant gene with two flanking *loxP* sites was introduced into intron 9 using a *loxP*-PGK-nt5-Neo-*loxP* cassette (Gene bridges, Germany) containing *Tshr* homology arms amplified by PCR. Finally, the desired DNA construct of 3,983 bp, containing exon 10 with the selected point mutation and the Neo cassette was excised from pGEM-4Z with *Bam*HI and *Hind*III restriction. Red/ET recombination was applied to replace the WT exon 10 region in paCYC177 plasmid with this construct. The exchange was validated by restriction enzyme digestion and sequencing.

For the gene targeting in ES cells, G4 ES cells (derived from mouse strains 129S6/C57Bl/6Ncr) were cultured on neomycin-resistant primary embryonic fibroblast feeder layers. Next, 10^6 cells were electroporated with 30 µg of linearized targeting construct and plated on 100 mm culture dishes in presence of 300 µl/ml antibiotic G418 (Sigma-Aldrich, USA) for seven to nine days. Then, targeted ES cell colonies were picked, cultured in a 96 well plate, and electroporated with a pCAGGS-Cre plasmid to excise the Neo cassette gene. Again, the cells were first grown on 100 mm culture dishes for three to five days before colonies were picked up and grown on 96 well plates. The colonies were screened for the correct targeting construct by PCR and further confirmed by sequencing. ES cells carrying the correct targeting construct with Neo deletion were injected into C57Bl/N6 mouse blastocysts (Charles River Laboratories, USA). The generated male chimeric mice were crossbred with C57Bl/N6 females to achieve germline transmission. The offspring were first screened by coat color and the presence of the mutation was confirmed by genotyping PCR and sequencing (III: Fig 2b, c). Genomic DNA extracted from ear samples was used for genotyping with an annealing temperature of 63 °C using the following primers: TshrMutKI_F 5'-AAGCCACCTGCACATGAAAGCATAT-3' and TshrMutKI_R 5'-TCACCCTTGATCCCCTTGACCCTTG-3. In this PCR, while the amplicon from the WT allele was 361 bp, mutated alleles resulted in a 475 bp amplicon.

4.1.3 Tissue and Serum Sampling (I-III)

Mice were sacrificed with CO_2 and blood was sampled by cardiac puncture. Bodyweight (I-III), body length (I-III) and tail length (III) were recorded. The thyroid gland was surgically excised without trachea and weighted prior to further processing of the tissue. Various control tissues such as brown and white adipose tissue, gonads, adrenals, kidney, liver, spleen, lung, heart, pituitary, hypothalamus or the whole brain were collected. For gene and protein expression analysis via quantitative PCR (qPCR) and Western blot, the tissues were snap frozen in liquid nitrogen and stored at -80 °C. For histological examinations, the tissues were fixed in 10 % formalin over night. Tissues for Oil Red O staining were rapidly frozen in Tissue-Tek O.C.T (Sakura Finetek, The Netherlands) at -60 °C. Blood for serum collection of living animals was obtained from the lateral saphenous vein. All blood samples were incubated at room temperature for two hours or 4 °C over night, then centrifuged with 2,000 x g before the serum was transferred to a new tube and stored at -20 °C until measuring the hormone concentrations.

4.1.4 Treatments

4.1.4.1 Tamoxifen Induction (I, II)

To activate the thyrocyte-specific Cre recombinase expressed in *Tg*CreER^{T2} mice, the animals were injected with 1 mg tamoxifen in 5 % ethanol (EtOH) in rapeseed oil for one (I), three (I) or five (I, II) consecutive days. While intraperitoneal (i.p.) injection (I, II) was favored, subcutaneous injections were performed for initial tests and pregnant females (I). To control the effect of the vehicle substances, a control group was treated with 5 % EtOH in rapeseed oil for the same extent of time (I, II).

4.1.4.2 Goiter Induction (I, II)

Mice were treated with 5 g/l sodium perchlorate (Sigma-Aldrich, USA) and 0.05 g/l methimazole (Sigma-Aldrich, USA) in drinking water to chemically induce hypothyroidism and goiter growth. The animals were kept on this goitrogenic treatment for up to eight weeks and closely monitored to exclude any health problems due to the developing hypothyroidism.

4.1.4.3 TSH Stimulation (II)

In order to determine the response to increased TSH concentration, a group of animals was stimulated with bovine TSH. Prior to the stimulation, 200 µl of blood was sampled to measure the basal TH levels. Next, the animals were injected i.p. with 50 mlU TSH per 10 g body weight and sacrificed six hours post-injection. Cardiac blood was collected after euthanasia for hormone measurements.

4.2 Histological Analysis (I-III)

After fixation of the tissue with 10 % formalin, tissues were dehydrated with 50 % followed by 70 % EtOH washes and embedded in paraffin. For histological examinations, tissues were sectioned at 4 µm thickness using a microtome. O.C.T-embedded tissues were cut into 10 µm thick sections in a cryomicrotome and fixed in 10 % formalin for 5 min before staining.

4.2.1 Histological Stainings (I-III)

After sectioning, tissue samples were rehydrated with serial washes in xylene, 100 %, 96 %, and 70 % EtOH and distillated water. Afterwards, the tissue sections were stained with either hematoxylin and eosin (HE) (I, III) or periodic acid Schiff's reagent (PAS) (II) for basic histological examination of the thyroid gland. To determine fibrotic tissue, a van Gieson staining (II) was performed according to standard protocols. The β-galactosidase activity of the recombined *LacZ* gene in *Tg*CreER^{T2}-Rosa26 mice was visualized by X-gal staining (I). Tissues were fixed in 0.2 % glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in PBS for 30 min, washed and then stained in 2 mM $MgCl_2$, 0.01 % NaDeoxycholate, 0.02 % Tergitol-type NP-40, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 1 mg/ml X-gal in PBS for 60 min in 37 °C. After macroscopic imaging of the samples with an Olympus SZ61 stereo microscope (Olympus, Japan) coupled to an Invenio-3S microscope camera (DeltaPix, Denmark), tissues were paraffin-embedded, sectioned and counter-stained with hematoxylin (I). To visualize fat tissue, Oil Red O staining (II) was performed from cryosections without rehydration of the tissue sections. After rinsing the formalin fixed tissue sections with tap water for 3 min and for 1 min in 60 % isopropanol, the slides were stained with filtered 0.3 % Oil Red O solution for 10 min. Destaining followed two times for 1 min in 60 % isopropanol and 1 min in distilled water. The sections were counterstained with Meyer's hematoxylin for 10 min and rinsed with tap water, before mounting the slides with Aqua-Mount (Thermo Fisher Scientific, USA). All histology sections were imaged with a Leica DMRBE microscope (I, II) or a Pannoramic Slidescanner (3D Histech, Hungary) (III).

4.2.2 Immunohistochemistry (I-III)

Paraffin sections were rehydrated as described in chapter 4.2.1, and antigen retrieval was performed by boiling tissue sections in 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker. After inhibiting the endogenous peroxidase activity with 3 % H_2O_2, the sections were blocked with 3 % BSA in phosphate buffered saline (PBS) with a pH of 7.4. Incubation with primary antibodies was performed at 4 °C over night or in room temperature for 60 min. To visualize the formed antigen-antibody complexes the slides were incubated with corresponding horseradish peroxidase (HRP)-labeled secondary antibodies for 30 min at room temperature and subsequently stained with DAB+ chromogen system (Dako, Denmark). The sections were then counter-stained with hematoxylin, dehydrated and mounted. All antibodies were diluted in 1 % BSA in PBS with the concentrations mentioned in Table 2.

4.2.3 Terminal Uridine Deoxynucleotidyl Nick End Labeling (II)

Terminal uridine deoxynucleotidyl nick end labeling (TUNEL) staining was used to visualize apoptotic cells. Firstly, rehydration and heat-mediated antigen retrieval with 10 mM sodium citrate buffer (pH 6.0) was performed as previously described in chapter 4.2.1 and 4.2.2. Subsequently, the sections were incubated with terminal transferase and Biotin-16-dUTP (Roche, Switzerland) for 60 min at 37 °C. The biotin-labeled dUTP, which was incorporated into the fragmented DNA by terminal transferase, is detected by the HRP-labeled ExtrAvidin®-Peroxidase (Sigma-Aldrich, USA), used at a 1:200 dilution, and DAB+ reagent (Dako, Denmark).

4.3 Hormone Analysis (I-III)

4.3.1 Thyroid Hormone Determination (I-III)

Commercial ELISA kits were used to analyze total T3 (III), total T4 (I, III), and free T4 (II, III) according to manufacturer's instructions (Novatec, Germany). Based on the manufacturer's information, the detection limit for tT3 is 0.16 ng/ml, tT4 has a sensitivity of 0.04 µg/ml and fT4 kits have a detection limit of 5 ng/l. All kits have intra- and inter-assay variation below 10 %.

4.3.2 TSH Determination (I-III)

Serum TSH levels were determined in different ways, as no suitable commercial kits were available for TSH measurements at the time of performing the experiments.

4.3.2.1 TSH Bioactivity (II)

TSH bioactivity was determined using JP26 and JP02 cell lines (Perret J, Ludgate M 1990), two chinese hamster ovary cell lines stably transfected with recombinant human TSH receptor and pSLV neo vector respectively. The cells were seeded in 24 well plates (II) or 96 well plates (III) and cultured at 37 °C and 5 % CO_2 in DMEM/F12 medium (Sigma-Aldrich, USA) with 10 % fetal calf serum (FCS) and

0.4 mg/ml G418 (Roche, Switzerland). At 85-95 % confluency, cells were washed twice with PBS, incubated with FCS-free DMEM/F12 with 1 % BSA for 60 min and then stimulated with the serum sample [10 % serum in DMEM/F12 with 1 % BSA and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma-Aldrich, USA)] for four hours at 37 °C and 5 % CO_2. The cells were harvested and cAMP concentrations were measured from cell lysate using a colorimetric ELISA (Cell Biolabs Inc, CA) (II) or RIA as described in chapter 4.3.2.2.

4.3.2.2 Radioimmunoassays (I-III)

The cAMP radioimmunoassay (RIA) was done according to the method described by Harper and Brooker (Harper and Brooker, 1975). First, samples were mixed 1:1 with 2 mM theophylline, incubated for 5 min at 95 °C and chilled on ice. Next, samples were acetylated with 0.03 volumes of riethylamine/acetic anhydride mix (ratio 2:1). 100 µl sample or standard (ranging from 10,000 – 9.75 fmol/100 µl) in PBS with 0.1 % BSA were incubated together with 100 µl cAMP antiserum and 100 µl I^{125}-cAMP (14,000 cpm/100 µl) at 4 °C over night. The blank contained 100 µl PBS with 0.1 % BSA and 100 µl cAMP antiserum. As total reference, 100 µl I^{125}-cAMP were used. The incubated samples, standards and blank (but not total) were mixed with 200 µl globulin (0.5 %) in PBS and 2 ml of 16 % polyethylene glycol 6,000 in PBS (both Sigma Aldirch, Steinheim, Germany). After a 30-min incubation at 4 °C, the samples were centrifuged with 15,000 x g for 20 min at 4 °C. The supernatant was discarded and the tried pellet was analyzed by γ-counter (Wallac 1470 Wizard Gamma Counter, Perkin Elmer, Turku, Finland).

Two different RIAs were performed for TSH measurements following a similar procedure. For one assay, the TSH RIA reagents used for serum TSH measurements were obtained from Dr. A. F. Parlow (National Hormone / Peptide Program, Harbor-UCLA Medical Center, Torrance, USA) (I, II). For the other assay, frozen serum samples were shipped to Dr. S. Refetoff, Endocrinology Laboratory, University of Chicago, Chicago USA, who performed a TSH RIA as previously described (Pohlenz et al., 1999).

4.4 RNA Extraction and cDNA Synthesis (I-III)

Total RNA was extracted from snap-frozen tissues using TRIsure™ reagent (Bioline, UK) as described by the manufacturer. The quantity of RNA was determined by absorbance spectrometry and RNA integrity was analyzed via agarose gel electrophoresis. For RT-PCR, 0.5–1 µg of total RNA was treated with desoxyribonuclease I (DNaseI, Amplification Grade, Invitrogen, USA) to remove DNA contamination and 0.67 µg (I, II) or 0.95 µg (III) of RNA was reverse-transcribed using DyNAmo cDNA synthesis kit (Thermo Fisher Scientific, USA) (I, II) or SensiFAST cDNA synthesis kit (Bioline, UK) (III) following the manufacturers instructions and diluted 1:50 or 1:100 for gene expression analysis via quantitative PCR.

Expression of miRNA was determined using a TaqMan miRNA kit (Applied Biosystems, USA). Reverse transcription of miRNA was performed on 10 ng total RNA with TaqMan miRNA Reverse transcription kit (Applied Biosystems, USA) and miRNA-specific primers supplied by the kit.

4.5 Quantitative PCR (I-III)

Gene expression was analyzed by qRT-PCR using the DyNAmo Flash SYBR Green qPCR kit (Thermo Fisher Scientific, USA) and a DNA engine thermal cycler with a Chromo4 detector (Bio-Rad Laboratories, USA). The primer sequences, annealing temperature and amplicon size for all genes investigated are listed in Table 1. In addition to the genes involved in thyroid development, TH synthesis and release, the expression of *transforming growth factor β* (*Tgf-β*) *1*, *Tgf-β2* and *Tgf-β3*, its receptors (*Tgfβr*), *phosphate and tensine homolog* (*Pten*) and *zinc finger E-box binding homeobox* (*Zeb*) *1* and *Zeb2* was quantified for study II. For c*Tg*DcrKO and i*Tg*DcrKO mice at three to four months of age were analyzed. For $TSHR^{D633H}$ mice, qRT-PCR was performed for samples from two and six months old animals. For every gene analysed, a standard curve with cDNA dilutions of 0.1, 0.2, 0.01, 0.02, 0.001, 0.0001 and 0.00001 was generated to determine the PCR efficiency. All samples were measured in duplicates and normalized to the geometrical mean of the expression levels for *ribosomal protein L19* (*Rlp19*) and *gluceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) (I, II) or *Rlp19* and *peptidylprolul isomerase A* (*Ppia*) (III). The relative gene expression compared to control animals was calculated using the PCR efficiency corrected Pfaffl method (Pfaffl, 2004). For c*Tg*DcrKO and i*Tg*DcrKO mice miRNA expression was measured at three month of age. MiRNA-specific qPCR for miR-30d, miR-141, miR-200a, mir-205 and miR-871 was performed using the TaqMan miRNA kit (Applied Biosystems, USA) and TaqMan fast advance mastermix (Applied Biosystems, USA). The relative miRNA expression was normalized to the geometrical mean of *Sno234* and *U6* snRNA expression by cycle threshold analysis (Livak and Schmittgen, 2001).

Table 1: qPCR primers used in the studies (I-III).

Gene	Sequence	Product [bp]	Ta [°C]	Study
Ano1	5'-CCGTGCCAGTCACCTTTTTG-3' 5'-CCTTGACAGCTTCCTCCTCC -3'	161	59	III
Dicer1	5'-CTTGACTGACTTGCGCTCTG-3' 5'-AATGGCACCAGCAAGAGACT-3'	361	60	I, II
Dio1	5'-GGGCAGGATCTGCTACAAGG-3' 5'-CGTGTCTAGGTGGAGTGCAA-3'	98	60	III
Dio2	5'-TCCTAGATGCCTACAAACAGGTTA-3' 5'-GTCAGGTGGCTGAACCAAAG-3'	92	60	III
Gapdh	5'-AACGACCCCTTCATTGAC-3' 5'-TTCACGACATACTCAGCAC-3'	191	60	I, II
Lrp2	5'-TGAACTCCCCATCACCATGC-3' 5'-AAGAGCCATTGTCGTCCCTG-3'	166	59	III
Mct8	5'-CTCCTTCACCAGCTCCCTAAG-3' 5'-ACTTCCAGCAGATACCACACC-3'	154	58.3	III
Nkx2-1	5'-CAGCCGACGCCGAATCAT-3' 5'-CTGGCCCTGTCTGTACGC-3'	151	59	II, III
Pax8	5'-CCTGCTGAGTTCTCCATATTATTAC-3' 5'-CCTTTGTGTGACTCTCTGGG -3'	164	58	II, III
Ppia	5'-CATCCTAAAGCATACAGGTCCTG-3' 5'-TCCATGGCTTCCACAATGTT-3'	165	60	III
Pten	5'-TCTGCCATCTCTCTCCTCCTT-3' 5'-CTTTCTGCAGGAAATCCCATAGC-3'	168	60	II

Rlp19	5'-CTGAAGGTCAAAGGGAATGTG-3' 5'-GGACAGAGTCTTGATGATCTC-3'	195	60	I, II, III
Slc5a5 (Nis)	5'-GGGATGCACCAATGCCTCTG-3' 5'-GTAGCTGATGAGAGCACCACA-3'	187	60	II, III
Tg	5'-CTTATGGGAGGCTCTGCAC-3' 5'-CACAGCCAGGAGCTTGGTC-3'	183	58	II, III
Tgf-β1	5'-ACGTCAGACATTCGGGAAGC-3' 5'-CACTCAGGCGTATCAGTGGG-3'	176	58	II
Tgf-β2	5'-TGGAGTCACAACAGTCCAGC-3' 5'-CCCCAGCACAGAAGTTAGCA-3'	174	61	II
Tgf-β3	5'-GATCACCACAACCCACACCT-3' 5'-AGGTTCGTGGACCCATTTCC-3'	195	60	II
Tgfβr1	5'-CTCTGTCCACAGCAAGTGGT-3' 5'-GGGCCATGTACCTTTTAGTGC-3'	225	61	II
Tgfβr2	5'-AACGTGGAGTCGTTCAAGCA-3' 5'-CTCGGTCTCTCAGCACACTG-3'	175	61	II
Tgfβr3	5'-AGAAGATAGGATCCCCCGGC-3' 5'-CGAGTAGCCATTGGTCTGGAA-3'	178	61	II
TRβ	5'-TTGTGAGCTGCCATGTGAAGA-3' 5'-ATCTGAAACCACCCCAAGGC-3'	175	59	II
Tpo	5'-CAAAGGCTGGAACCCTAATTTCT-3' 5'-AACTTGAATGAGGTGCCTTGTCA-3'	82	59	II, III
Tshr	5'-TCCCTGAAAACGCATTCCA-3' 5'-GCATCCAGCTTTGTTCCATTG-3'	112	60	II, III
Tshβ	5'-TCCGTGCTTTTTGCTCTTGC-3' 5'-TGCCATTGATATCCCGTGTCAT-3'	154	60	II
Zeb1	5'-TGCTCACCTGCCCGTATTGTGATA-3' 5'-AGTGCACTTGAACTTGCGGTTTCC-3'	224	60	II
Zeb2	5'-ATTGCACATCAGACTTTGAGGAA-3' 5'-GTTCCAGGTGGCAGGTCATT-3'	193	60	II

4.6 miRNA Expression Profiling (II)

The Illumina small RNA sequencing method was used to study the miRNA expression pattern in Dicer1 KO mice compared to controls. Therefore, total RNA was extracted from three pooled thyroid samples per group as described previously in chapter 4.4 and the quality of the RNA was controlled with RNA nano-chips using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). A small RNA library was generated from the isolated RNA with a small RNA library preparation kit (Illumina, USA). The Agilent bioanalyser was used for the validation of this library. Using Illumina's MiSeq machine, miRNA was sequenced for all samples and the sequencing results were obtained by the Ilumina/Solexa 1G genome analyzer. The detected miRNAs were mapped to the mouse reference genome (m37 assembly; National Center for Biotechnology Information, Bethesda, MD). The edgeR method (R/Bioconductor software) was used for normalization as described previously (Robinson et al., 2010). For further analysis, miRNAs with an expression level below 100 counts in control samples were excluded, as well as samples below a threshold set at 1.5-fold change in expression between controls and Dicer1 KO.

4.7 Western Blotting (II, III)

To determine protein expression levels by Western blot, protein was extracted from snap-frozen tissues. Initially, tissues were homogenized in lysis buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 % Nonidet P-40; 0.5 % Na-deoxycholate; 0.05 % sodium dodecyl sulfate; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol; cOmplete protease inhibitor mix (Roche, Switzerland)], incubated on ice for 30 min and centrifuged at 12,000 x g, 4 °C for 10 min to eliminate the cell debris. The protein concentration was determined with the Pierce BCA protein kit (Thermo Fisher Scientific, USA), and equal concentrations of protein were loaded on 6 % (II), 7 % (III) or 12 % (III) sodium dodecyl sulfate-polyacrylamide gel for separation. After the electrophoresis, the proteins were blotted onto polyvilylidene fluoride membranes (Amersham, UK), and incubated with primary antibodies (specified in Table 2) and a corresponding HRP-linked secondary antibody. The specific protein bands were detected using Western Lightning Plus enhanced chemiluminescence substrate (Perkin Elmer, USA). For protein normalization, the membranes were stripped and blotted with rabbit anti-mouse tubulin antibody. The program ImageJ was used to quantify the band intensities (Schneider et al., 2012).

Table 2: List of antibodies used in these studies

Antibody	Antigen	Manufacturer	Dilution	Study
Anti-phospho-histone H3 (Ser10)	Phospho-histone H3 (Ser10)	Millipore, USA	1:1,5000	II
Anti-TTF-1	Rat-TTF1 (NKX2-1)	Dako, Denmark	1:200	II
Goat anti-mouse IgG	Anti-mouse IgG	Millipore, USA	1:1,000	II
Nis Antibody (S-16)	NIS	SantaCruz, USA	1:1,000	II
Pax8 Antibody	PAX8	SantaCruz, USA	1:500	II
Thyroglobulin (N-15)	Thyroglobulin	SantaCruz, USA	1:1,000	II, III
TSHR Antibody (N-19)	TSHR	SantaCruz, USA	1:1,000	II, III
Tubulin-α AB-2	Alpha-Tubulin	Thermo Fisher Scientific, USA	1:3,000	II, III
Zymax Rabbit Anti-Goat IgG	Anti-Goat IgG	Invitrogen, USA	1:1,000	II, III

4.8 Statistical Analysis (I-III)

GraphPad Prism 5.0 was used to plot the data and perform statistical analyses. Data are shown as bar diagrams presenting mean values ± standard error of mean (SEM) (I, II) or whiskers box plots (III). An unpaired *t*-test (I, II) was performed for data sets with two experimental groups to determine statistical significance. Data sets with more than two experimental groups were analyzed using one-way ANOVA with Dunnett's post hoc analysis (II, III). For data sets without equal variance, the non-parametric Kruskal-Wallis test was performed. A p-value < 0.05 was assigned as threshold for statistical significance where * indicates $p < 0.05$, ** marks $p < 0.01$ and *** represents $p < 0.001$.

5 Results

Main Results of Studies I-III

I. A thyrocyte-specific, inducible Cre-recombinase-expressing mouse line allows tamoxifen-inducible thyrocyte-specific gene deletions of any gene of interest in a time-dependent manner.

II. Dicer1-mediated microRNA signaling is necessary for thyroid homeostasis, thyrocyte differentiation and goiter growth.

III. The constitutive activating $TSHR^{D633H}$ mutation leads to colloid goiter and transient hyperthyroidism.

5.1 Generation of a $TgCreER^{T2}$ Mouse Model (I)

In order to study the role of various genes in the fully developed thyroid, an inducible, thyrocyte-specific Cre line was developed. This Cre line allows for the deletion of any floxed gene in thyrocytes, starting after induction with tamoxifen, which avoids developmental defects caused by the gene KO. For the generation of the *Tg*$CreER^{T2}$ mouse line, a targeting construct with a large *Tg* fragment fused with the $CreER^{T2}$ sequence was created using the BAC- or PAC-based ET recombination technique. The construct was confirmed by Southern blot (Manuscript I: Fig 1 B, C) and injected into the pro-nucleus of fertilized oocytes. Of the 35 obtained pups, five showed Cre expression detected by PCR (I: Fig. 1 D) and were therefore selected for further breeding.

5.1.1 Thyrocyte-Specific Expression of Cre Recombinase in $TgCreER^{T2}$ Mice (I)

The mice expressing $CreER^{T2}$ under the *Tg* promoter were crossed with a Gt(ROSA)26Sor reporter mouse line resulting in a *Tg*$CreER^{T2}$-Rosa26 line, which allows the visualization of the presence of Cre recombinase by X-gal staining. Cre-expressing mice were treated with 1 mg tamoxifen for five consecutive days to induce Cre-mediated recombination and compared with tamoxifen-treated Cre-negative mice and vehicle-treated Cre-expressing mice. The mice were sacrificed at the age of two months, one month after the induction. Different tissues, including thyroid with trachea, lung, liver, white adipose tissue, pituitary and ovaries, were collected for X-gal staining (I: Fig. 1E). One founder line showed extrathyroidal β-galactosidase activity, while the other four founder lines only showed X-gal staining in the thyroid gland of Cre-expressing, tamoxifen-induced mice. The founder line with the strongest homogenous expression of Cre recombinase was picked for further analysis.

5.1.2 The Efficiency of Tamoxifen-Induced Cre-Mediated Recombination is Dosage Dependent (I)

The tamoxifen inducibility of *Tg*CreER^{T2} mice was tested with different doses (total of 1, 3 and 5 mg) of tamoxifen injections. In macroscopic and microscopic evaluation with X-gal, dosages of 3 and 5 mg appeared more efficient than a single dose of 1 mg tamoxifen (I: Fig. 2A-D). The microscopic analyses reveal a strong correlation of Cre-mediated recombination and the dosage used for induction. A tamoxifen dose of 1 mg results in a recombination efficiency of ca. 30 %, while the efficiency increases to approximately 75 % for 3 mg up to an efficiency of over 90 % for 5 mg tamoxifen (I: Fig. 2 E-H, Fig 3A-C). Using the Gt(ROSA)26Sor reporter line, we observed Cre-mediated recombination in approximately 2 % of thyrocytes of vehicle-treated animals (I: Fig. 1E, Fig. 2A, E, I and Fig. 3A). The thyrocyte-specific Cre recombinase expression was also confirmed by immunohistochemical staining for Cre recombinase (I: Fig. 3D, E).

In order to test the Cre-mediated recombination before birth, pregnant mice were treated with 1 mg tamoxifen injected subcutaneously from embryonic day E 14 to E 19 - around the starting point of *Tg* expression in the pups. Newborn mice were then dissected at post-natal day 2 and genotyped and macroscopically analyzed after X-gal staining. While Cre-negative pups did not show any X-gal staining in thyroids, Cre-expressing pups presented clear X-gal staining after induction *in utero* (I: Fig. 2 I-J). Unspecific X-gal staining of intestines could be observed in all pups, serving as a positive staining control. Furthermore, when administering tamoxifen, subcutaneous encapsulation of the tamoxifen-oil-emersion could be detected in several female mice after dissection.

5.1.3 Inducible TgCreER^{T2}-Expression Has No Impact on Thyroid Physiology (I)

After assessing the induction efficiency, various body parameters were analyzed to evaluate if the transgenic CreER^{T2} expression in thyrocytes causes any physiological effects. In both females and males, body weight appeared normal after induction (I: Fig. 4A, B), and the sex and genotype of the pups followed Mendelian distribution. Serum TSH and total T4 levels were unaltered in tamoxifen-induced 2–3-month-old *Tg*CreER^{T2} mice (I: Fig. 4C, D). Thyroid weights and histology were comparable between tamoxifen-induced *Tg*CreER^{T2} and vehicle-treated control mice (I: Fig. 3A-C, Fig 4E).

5.1.4 Influence of Cre-Expression on Goiter Growth (I, Unpublished Data)

Goiter growth in tamoxifen-treated *Tg*CreER^{T2} mice could be induced with a goitrogenic diet to the same extent as in vehicle-treated Cre-negative littermates and vehicle-treated *Tg*CreER^{T2} mice (I: Fig. 4F). On the contrary, in mice expressing Cre constitutively under the *Tg* promoter (*Tg*Cre (Kero et al., 2007)), the thyroid weight of Cre-expressing animals did not increase significantly under goitrogenic conditions, while Cre-negative controls responded normally to the treatment with goiter-inducing drugs (Figure 8). However, TSH serum concentrations were strongly increased in

Cre-expressing and Cre-negative mice from both lines constitutive *Tg*Cre and inducible *Tg*CreER^{T2}. These elevated TSH levels caused hypertrophy of the thyrocytes and a decrease in colloid amount on a microscopical level (data not shown).

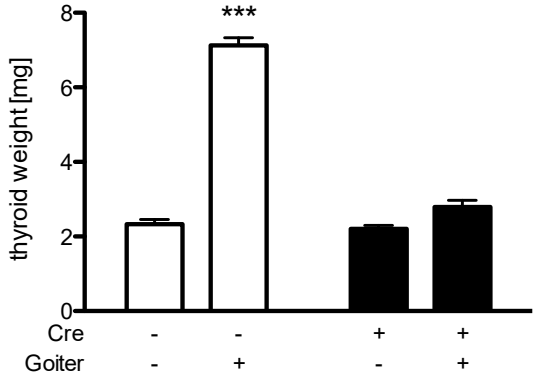

Figure 8: Response of *Tg*Cre mice to goitrogenic diet.

The thyroid weight in Cre-negative littermates is 3.5-fold increased after four weeks on a goitrogenic diet, while *Tg*Cre-mice expressing Cre recombinase constitutively do not show a significant increase in thyroid weight after goiter-inducing drug treatment. Bars and error bars represent mean ± SEM, white bars resemble Cre-negative controls, black bars refer to Cre-expressing *Tg*Cre mice. *** = $p<0.001$, n=3 for untreated mice, n = 9 for goiter treated mice.

5.2 Dicer1 Deletion in Thyrocytes Leads to a Decrease in miRNA Expression (II)

We generated two thyroid-specific Dicer1 deficient mouse lines: a constitutive Dicer1 KO (c*Tg*DcrKO) starting with *Tg* expression at E 14.5, and a tamoxifen-inducible Dicer1 KO model (i*Tg*DcrKO). As no significant differences in the measured parameters (TSH and TH concentrations, body weight, length, thyroid weight, goiter-growth and gene expression) were observed between the control groups (Cre-negative, vehicle-treated littermates (Dcr$^{fl/fl}$); Cre-negative, tamoxifen-treated (Dcr$^{fl/fl}$Tam) littermates; and Cre-expressing, vehicle-treated littermates (i*Tg*Dcr$^{fl/fl}$)), results are only shown for one control group.

For both mouse lines, the Cre-mediated recombination of the floxed Dicer1 gene was detected from genomic DNA of the thyroid gland, but not from control tissues (kidney, liver, spleen, cartilage, adrenal and gonads) in Cre-expressing animals where the Cre recombinase was directed to the nucleus either by constitutive expression (c*Tg*DcrKO) or tamoxifen induction (i*Tg*DcrKO) (II: Fig 1B and data not shown). In addition, qPCR for Dicer1 using whole thyroid RNA (including C-cells, endothelial cells, fibroblasts and parathyroid (Dumont et al., 1992)) demonstrated a 50 % decrease in both c*Tg*DcrKO as well as tamoxifen-treated i*Tg*DcrKO mice (II: Fig. 1C). Consequently, in miRNA sequencing the majority of miRNAs were

downregulated in c*Tg*DcrKO mice compared to controls (II: Fig. 1D and Figure 9). A decrease in miRNA expression was also confirmed by miRNA-specific qPCR for thyroids from c*Tg*DcrKO (II: Fig. 1E).

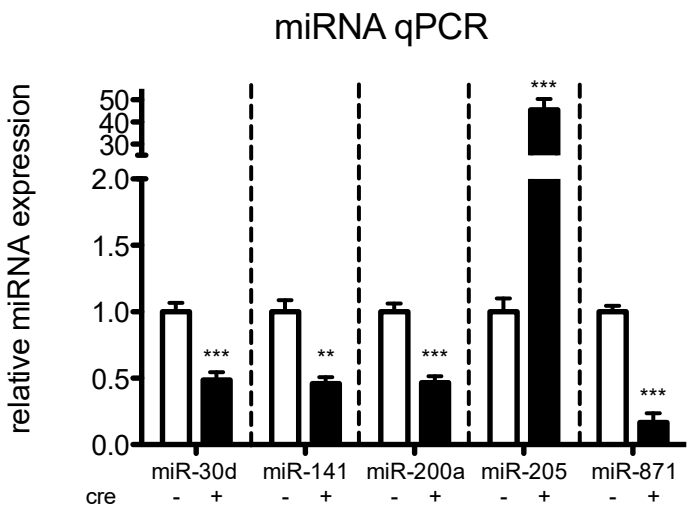

Figure 9: Expression of selected miRNA in c*Tg*DcrKO mouse thyroids using qPCR.

The miRNA expression of miR-30d, miR-141, miR-200a and miR-871 is significantly decreased in c*Tg*DcrKO mice compared to controls. MiR-205 expression is 45-fold upregulated in c*Tg*DcrKO mice. Bars and error bars represent mean ± SEM, whereas white bars refer to control mice and black bars represent c*Tg*DcrKO mice. ** = $p<0.01$; *** = $p<0.001$; n=3 per genotype.

5.2.1 Constitutive Dicer1 KO Mice Develop Hypothyroidism (II)

A constitutive deletion of Dicer1 during late embryonic development resulted in slowly progressive hypothyroidism. In c*Tg*DcrKO mice, TSH levels were six-fold higher than in Cre-negative littermates (II: Fig. 2A) and free T4 levels were 30 % to 40 % decreased in c*Tg*DcrKO animals at three months of age. The serum TH levels in c*Tg*DcrKO mice gradually decreased from 40 % to 65 % at seven months of age when compared with controls (II: Fig 2B). While the embryonic deletion of Dicer1 led to the development of hypothyroidism, no significant changes in serum TSH or fT4 concentrations were detected in inducible Dicer1 KO mice two months after induction (II: Fig 2C, D).

Despite the increased TSH values in c*Tg*DcrKO mice, which were confirmed by TSH bioactivity and qPCR analysis for *Tshβ* (II: Suppl. Fig. 1A, B), we did not observe an increase in macropinocytosis (II: Suppl. Fig 2A). As expected, inducible Dicer1 KO mice, which did not present altered TSH or fT4 levels, also did not present changes in pinocytosis upon TSH stimulation (II: Fig 2B). However, stimulation with TSH did result in significantly lower T4 release in i*Tg*DcrKO mice when compared to Cre-negative littermates (II: Suppl. Fig. 2C).

5.2.2 Deletion of Dicer1 in Thyrocytes Causes Loss of Follicular Organization (II)

Similar histological alterations were observed in both c*Tg*DcrKO and i*Tg*DcrKO mice thyroids, while hypothyroidism was present only in c*Tg*DcrKO mice. When Dicer1 was deleted during development, a significantly lower amount of colloid, together with a partial loss of follicular structure, could be observed already at an early age of one month and progressed to a more severe phenotype at an older age (II: Fig 3). Immunohistological staining revealed that the cells in areas with disorganized follicular structure had a lower expression of the thyrocyte-specific TF NKX2-1 (II: Fig 4C). Those areas also presented an increased amount of fibrosis based on van Gieson's staining (II: Fig 5B). In addition, c*Tg*DcrKO mice presented a significant increase of intrathyroidal adipocytes when compared to control mice (II: Fig 5A).

Histological changes in the thyroids of tamoxifen-inducible Dicer1 KO mice were progressive and resembled the histology of c*Tg*DcrKO mice (II: Fig 3). While the follicular organization is not clearly altered at one month after induction, the amount of colloid is already decreased at this time point. However, at two months after induction, some areas of the thyroid gland lack a normal follicular organization. Those areas also show a decrease of NKX2-1 staining (II: Fig. 4C) and increase of fibrosis (II: Fig. 5B), consistent with the observed alterations in c*Tg*DcrKO mice.

5.2.3 Gene and Protein Expression for Thyroid Markers is Altered in Dicer1 Deficient Mice (II)

The gene expression of various thyrocyte-specific genes was analyzed by qPCR for c*Tg*DcrKO mice compared to control mice. On the mRNA level, the expression of *Tg*, *Tpo*, and *Nkx2-1* was approximately 50 % reduced in mice lacking Dicer1 when compared to control animals (II: Fig. 4A). Those three genes showed a similar decrease in mRNA expression in i*Tg*DcrKO mice two months after tamoxifen induction (II: Fig. 4B). Consistent with the mRNA expression levels in c*Tg*DcrKO mice, the protein levels of NKX2-1 and Tg were 80 % and 50 % decreased (II: Fig 4D).

Interestingly, while the mRNA expression of *Tshr*, *Nis* and *Pax8* was unaltered in c*Tg*DcrKO mice, the protein expression from these three genes was altered. Specifically, TSHR protein levels showed a 90 % decrease in c*Tg*DcrKO mice compared to Cre-negative controls. PAX8 protein levels were 50 % and NIS 20 % decreased in c*Tg*DcrKO mice compared to controls (II: Fig 4D).

5.2.4 Deletion of Dicer1 in Adult Mice Inhibits Drug-Induced Thyroid Growth (II)

Under normal conditions, no changes in the thyroid weight of c*Tg*DcrKO mice or i*Tg*DcrKO mice when compared with control mice were observed. Despite the unaltered thyroid weight, an increased histone H3 and KI-67 expression was found in both constitutive and inducible Dicer1 KO mice, indicating an increased proliferation (II: Fig. 6D, E, and data not shown). However, when treated with a goitrogenic diet for eight weeks, the thyroid weight of i*Tg*DcrKO mice did not increase similarly as in the controls. While approximately a four-fold increase of thyroid weight was noted

for all controls after goiter-inducing drug treatment, the thyroid weight of goiter-induced i*Tg*DcrKO mice was comparable to untreated mice (II: Fig. 6A, B). As expected, treatment with a goitrogenic diet resulted in strongly elevated serum TSH levels (II: Suppl. Fig 2D) with undetectable fT4 concentrations in all groups.

Despite the inhibited growth of the thyroid gland in response to a goitrogenic diet, i*Tg*DcrKO mice showed similar histological changes to the control mice (II: Fig 6C). In detail, all treated mice presented hyperplastic thyrocytes, increased vascularization, and the colloid had vanished below 0.5 % in both the controls and also the induced Dicer1 KO mice (II: Fig. 6C). The apoptosis rate determined by TUNEL staining or caspase-3 Western blot was not altered in i*Tg*DcrKO mice when compared to control mice (data not shown). While histone H3 expression was increased in untreated i*Tg*DcrKO mice compared to controls, the histone H3 expression did not differ between goiter-induced i*Tg*DcrKO mice and goiter-induced controls. Consistent with the TSH levels (II: Suppl. Fig 2D) and histological alteration (II: Fig. 6C) due to the goitrogenic diet, the proliferation rate detected by histone H3 expression was significantly higher in both, control mice (2.3 % ± 0.5 %) as well as i*Tg*DcrKO mice (1.9 % ± 0.2 %) after goiter-inducing drug treatment when compared to mice, which were not treated with goitrogenic drugs (II: Fig. 6E).

5.2.5 TGF-β Signaling is Altered in Dicer1 Deficient Mice (II)

Several miRNAs, which were decreased in c*Tg*DcrKO mice compared with controls in miRNA sequencing, (namely the let-7 family, miR-30d/e, miR-204, and the miR-200 family members miR-141 and miR-200a), are known to regulate genes involved in TGF-β signaling pathways (Braun et al., 2010, Tzur et al., 2009, Wang et al., 2011, Wang et al., 2010). Thus, qPCR analysis for *Tgf-β*-signaling genes was performed in both c*Tg*DcrKO as well as i*Tg*DcrKO mice. In c*Tg*DcrKO mice, mRNA expression was significantly increased for *Tgf-β1* and *Tgf-β2*, the *Tgf-β* receptors *Tgfβr1*, *Tgfβr2* as well as the *Tgf-β* induced TF *Zeb2*. The expression levels of *Tgf-β3*, *Tgfβr3*, *Zeb1*, *Pten* and *Thrβ* were not significantly altered in c*Tg*DcrKO mice compared to littermate controls (II: Fig 7A, C, D, Suppl. Fig 3). Of all the *Tgf-β*-signaling genes, which appeared to be differentially expressed in c*Tg*DcrKO mice, only *Tgf-β2* was significantly upregulated in i*Tg*DcrKO mice.

5.3 Characterization of a Mouse Line Expressing the Constitutively Active TSHR^{D633H} Mutation (III)

5.3.1 TSHR^{D633H} Mutation Leads to Constitutive Activation of Human and Murine TSHR Signaling in vitro (III)

Characterization of the human and mouse TSHR variant D633H revealed in all functional assays a comparable phenotype. Expression of these constructs and the respective WT in HEK293GT cells showed a cell surface expression of 80-90 % for the human and mouse variant when compared with the WT receptors (set as 100 %) (III: Supplementary Fig. 1A).

Dose response curves with recombinant human TSH confirmed the previous observation that the TSHR^{D633H} variant could still be activated by TSH (Neumann et

al., 2001a). Most importantly, the same mutation in different species showed a five-fold increase in constitutive cAMP activity (III: Supplementary Fig. 1B). A weaker but significant increase of basal $G_{\alpha q/11}$ activation was also measured for the human and murine $TSHR^{D633H}$ as indicated by a comparable two-fold IP accumulation. Both variants responded well to the recombinant human TSH stimulation, with the human and mouse $TSHR^{D633H}$ variant reaching similar cAMP and IP concentrations as the WT receptors (III: Supplementary Fig. 1C).

5.3.2 Mice Substituted with the $TSHR^{D633H}$ Mutation are Vital and Fertile (III)

We generated a KI mouse line carrying the D633H mutation in the murine TSHR sequence. Therefore, the nucleotide sequence GAG coding for aspartate 633 was exchanged with the sequence CAC coding for histidine by homologous recombination (III: Fig 1A). After injecting the blastocysts with the targeted ES cells, chimeric mice were obtained from two different ES cell clones. Germline transmission of the $TSHR^{D633H}$ variant was detected in four chimeric males (two from each ES cell clone), presenting normal Mendelian distribution of genotype (WT:heterozygote = 1:0.83) and sex (female:male = 1:0.90) and average litter size of 6.1 ± 2.4 pups/litter in the first generation. The presence of the $TSHR^{D633H}$ variant was determined via genomic PCR, resulting in an amplicon sized of 361 bp for WT allele and 457 bp for the mutated allele (III: Fig. 1B). Sequencing of the targeted region of the murine TSHR confirmed the presence of the mutation from guanine to cytosine in heterozygote (HEZ) and homozygote (HOZ) $TSHR^{D633H}$ mice (III: Fig 1C). Thus, HEZ animals present one allele with the WT TSHR sequence and one allele carrying the D633H substitution, while in HOZ mice both alleles for the TSHR are substituted with the $TSHR^{D633H}$ variant. The HEZ and HOZ $TSHR^{D633H}$ mice did not present differences in body weight, length or tail length or fertility (III: Table 1) compared to WT controls matched for age and sex.

5.3.3 Basal cAMP Accumulation is Increased in Thyrocytes of $TSHR^{D633H}$ Mice (III)

Introducing the D633H mutation into the murine TSHR sequence caused an increase in basal cAMP production compared to cells with the WT receptor *in vivo*. In primary cell culture from six-week-old mice (n=3), the basal cAMP level was significantly 1.6-fold increased in HOZ mice (III: Fig. 2A). A similar pattern of basal cAMP levels can be seen for intrathyroidal cAMP detected in thyroid homogenate (III: Fig 2B), where HOZ mice showed a 1.6-fold increase in cAMP levels (six weeks of age, n=2). Cells from all three genotypes responded in a similar manner to stimulation with bovine TSH, indicating a functional expression of the TSHR (III: Fig. 2A). A comparable expression of TSHR was confirmed by Western blot at two and six months (III: Fig 2C).

5.3.4 $TSHR^{D633H}$ Mice Develop Colloid Goiter (III)

Already at two months of age, HOZ mice present a 2.8- (female) and 2.3-fold (male) increase in thyroid weight when compared with WT controls (III: Fig 4A). At six

months of age, the thyroid weight of HEZ mice was increased 2.0- and 2.2-fold in females and males, respectively. In HOZ mice, the weight further increased to a 3.7- (female) and 2.5-fold (male) difference when compared with WT littermates (III: Fig 4B). At two months of age, HEZ mice do not show clear histological alteration, while HOZ animals present areas with increased thyroid follicle size and flattened thyrocyte epithelium (III: Fig 4C, upper panel; Table 2). In two-month-old mice, the intrafollicular area, defined as the lumen of the follicle, was significantly enlarged in HEZ females (1.6 fold), HOZ males (2.7 fold) and HOZ females (2.9 fold) when compared to WT controls (III: Table 1).

At the age of six months, HEZ and HOZ TSHR^{D633H} mice presented enlarged follicles and flattened thyrocyte epithelium and nuclei in nearly all follicles. This histological phenotype was more pronounced in HOZ than HEZ mice (III: Fig 4C, middle panel, Table 1). At this age, the intrafollicular area was significantly increased in both HEZ and HOZ mice. Female mice showed 2.2- (HEZ) and 3.9-fold (HOZ) enlarged colloid area per follicle. In male mice, the intrafollicular area was 1.7 times bigger in HEZ and 3.2-fold enlarged in HOZ. As visible in histological images (III: Fig. 4C, middle panel), the thyrocytes were significantly flattened in TSHR^{D633H} mice. The HEZ mice showed 31 % (females) and 36 % (males) thinner thyroid epithelium as determined by measuring the thyrocyte thickness per two follicles, and in the HOZ mice the thyrocyte thickness was reduced by 37 % and 45 % in females and males, respectively (III: Table 1).

At nine months of age, HOZ TSHR^{D633H} mice have microadenomas. These microadenomas are identified as regions of hypertrophic thyrocytes and infolding of thyrocytes into the colloid and can be detected between areas of colloid goiter, which consist of enlarged follicles surrounded by flattened thyroid epithelium (III: Fig 4, lower panel).

5.3.5 TSHR^{D633H} Mice Develop Transient Hyperthyroidism (III)

Thyroid function tests were performed at two and six months of age (III: Table 2) to determine the physiological impact of the TSHR^{D633H} mutation. At two months of age, serum TSH was strongly diminished in HEZ or HOZ females and in HOZ males (III: Fig. 3A). Interestingly, only HOZ females had significant changes in tT3, tT4 and fT4, while these TH levels appeared unaltered in HEZ females and males as well as HOZ males (III: Fig. 3B-D). Surprisingly, the hyperthyroid state of HOZ female mice did not persist throughout adulthood. At six months of age, TSH levels were below the detection limit in three out of six HOZ females (III: Fig. 3E) and the TH levels significantly decreased when compared with WT mice (III: Fig. 3F-H). In six-month-old males there was no significant difference in TSH or serum TH levels between WT, HEZ or HOZ mice (III: Fig. 4E-H).

5.3.6 Gene And Protein Expression of TSHR^{D633H} Mice (III)

Consistent with the TSH values below the detection limit in HOZ mice of both sexes (III: Fig. 3A) and the increased TH concentrations in female mice at two months of age (Fig. 3 B-D), the *Tshr* gene expression was 1.7-fold increased in HOZ mice when compared with WT (III: Fig. 5A). However, the expression levels of the TSHR protein were not changed in two- or six-month-old HEZ or HOZ mice (Fig. 2C).

Additionally, increased *Pax8* expression could be observed in female and male HOZ animals at two months. At this age, the gene expression levels of *Nis*, *Tpo*, *Ano1*, *Lrp2* and *Dio1* were also elevated (III: Fig. 5B). At six months of age, only *Tg* gene expression was altered in both female and male HOZ mice, with a 0.4-fold downregulation compared to WT controls (III: Fig. 5C, D). Despite the decreased *Tg* gene expression and an increased colloid amount based on the significantly enlarged intrafollicular area (III: Table 1), no changes in Tg protein expression were detected in Western blot analysis of two- (III: Fig. 2D, upper panel) or six-month-old animals (Fig. 2D, lower panel) in either genotype.

6 Discussion

6.1 Generation of a TgCreER^{T2} Mouse Model (I)

6.1.1 Advantages Over Existing Thyrocyte-Specific Cre Lines (I)

We have successfully generated a mouse line expressing the tamoxifen-inducible Cre recombinase construct (CreER^{T2}) under the murine thyroglobulin promoter (Indra et al., 1999). This mouse line is a valuable tool to recombine and thus knock out or invert any gene flanked by two *loxP* sites in an inducible manner. Induction of four-week-old *Tg*CreER^{T2}-positive mice with 5 mg tamoxifen resulted in a 92 % recombination in thyrocytes, based on X-gal staining using ROSA26LacZ reporter mice. This recombination efficiency of our *Tg*CreER^{T2} mice was comparable to the mouse line expressing Cre recombinase under the *TPO* promoter (Kusakabe et al., 2004). In parallel with the development of our model, another tamoxifen-inducible *Tg* promoter-driven Cre line was created (Charles et al., 2011). In contrary to this report, we observed a very low leakage of the Cre recombinase into the nucleus without tamoxifen treatment. In our analysis, the Cre-mediated recombination in the absence of tamoxifen was detected by X-gal staining in approximately 2 % of thyrocytes. Furthermore, Cre activity was not observed by genomic PCR or qPCR for the recombined alleles of Dicer1 floxed mice crossed with our *Tg*CreER^{T2} mice. While this leakage did not cause any phenotypical changes in our studies, we cannot rule out that non-induced Cre activity can lead to a phenotype with other floxed genes. For gene alterations leading to a Cre-mediated loss of function, it is unlikely that a loss of 2 % activity leads to significant phenotypical changes. However, when causing a gain-of-function alteration using the *Tg*CreER^{T2} line, the leakage of Cre might result in a phenotype without induction.

As expected, the *Tg*CreER^{T2} mice developed normally. Their TH levels and thyroid morphology were unaltered at least two months after induction. No extrathyroidal β-galactosidase activity was detected in *Tg*CreER^{T2}-ROSA26LacZ mice, proving that the *Tg* promoter restricts the Cre expression with high specificity to the thyrocytes. Moreover, we showed that Cre-mediated recombination could be achieved during embryonic development by tamoxifen treatment of pregnant females around day 14–19 of pregnancy, during the onset of *Tg* expression in embryogenesis.

When treating pregnant female mice with tamoxifen, the negative influence of the drug on the fertility of the dam has to be taken into consideration for the breeding strategy (Bloxham et al., 1977, Llarena et al., 2015). Yet, the generated *Tg*CreER^{T2} mouse line can be used to study the effect of a thyrocyte-specific KO of any floxed gene during late stages of fetal thyroid development. Hence, the *Tg*CreER^{T2} mouse line is an ideal model to combine both Cre-mediated recombination during adulthood, after the normal development of the thyroid gland, as well as Cre-mediated recombination during late embryonic development, similar to the existing constitutive Cre lines under *Tg* and *TPO* promoter (Kero et al., 2007, Kusakabe et al., 2004).

6.1.2 Thyroid Growth Appears Unaltered in TgCreER^{T2} Mice (I, Unpublished Data)

Cre activity has been reported to cause apoptosis and cell degeneration in different mouse models expressing Cre recombinase under tissue-specific promoters (Cali et al., 2007, Jimeno et al., 2006, Naiche and Papaioannou, 2007). A cytotoxic effect of Cre recombinase expressed in cells without *loxP* sites *in vitro* has also been linked to chromosomal aberrations and decreased cell growth (Silver and Livingston, 2001). To investigate the putative harmful effect of Cre recombinase on thyroid growth, we compared the thyroid weights of tamoxifen- and vehicle-treated *Tg*CreER^{T2} mice as well as WT littermates after eight weeks of chemical induction of goiter growth. In addition, the c*Tg*Cre mice (Kero et al., 2007) were included in the test to compare the effects of constitutive, potentially stronger Cre expression with the tamoxifen-inducible model. Unexpectedly, an inhibition of goiter growth was observed in the c*Tg*Cre line. Therefore, the c*Tg*Cre mice should not be used for studies concerning thyroid growth. Yet, as no histological or hormonal changes have been described for this Cre line, it can be utilized for basic, functional characterization. However, no impact of Cre expression and tamoxifen-induced translocation of the Cre recombinase into the nucleus on goiter growth was observed in *Tg*CreER^{T2} mice. Thus, the *Tg*CreER^{T2} mouse line is suitable for studying the role of floxed genes also on thyroid growth. The effect of constitutive Cre expression on thyroid growth was unexpected and emphasizes the importance of a careful characterization of the Cre-expressing mouse lines and inclusion of Cre-expressing and negative controls to the experiments.

6.2 Role of Dicer1 in Thyrocytes (II)

Two different Dicer1 KO mouse lines, a constitutive Dicer1 KO starting from E 14.5 as well as a tamoxifen-inducible Dicer1 KO line, were generated. Both mouse lines showed 50 % decreased *Dicer1* mRNA and protein expression in thyroids where the Cre recombinase was located in the nucleus (either constitutively or after tamoxifen treatment). Both RNA for gene expression as well as protein were extracted from the whole thyroid glands, which included C-cells, endothelial cells, fibroblasts and parathyroid glands in addition to the thyrocytes (Dumont et al., 1992). The Dicer1 expression in other cell types in the thyroid most likely explains the residual 50 % Dicer1 expression detected in both models. Thus, the target gene expression changes observed in the whole thyroid also depend on whether the gene of interest is expressed specifically in thyrocytes or in several cell types present in the thyroid gland. In the two previously described thyroid-specific Dicer1 KO models and a *Pax8* promoter-driven DCGR8 KO model, the recombination efficiency was not quantified (Bartram et al., 2016, Frezzetti et al., 2011, Rodriguez et al., 2012). As a consequence of the lack of Dicer1 in thyrocytes, the majority of mature miRNAs processed by Dicer1 showed a decreased expression in thyroids from c*Tg*DcrKO mice compared to the control littermates.

6.2.1 Lack of Dicer1 Leads to Thyrocyte Dedifferentiation (II)

In both previously described Dicer1 KO models mediated by *Pax8* and *Tg* promoter-driven Cre lines, as well as the *Pax8*-driven DGCR8 KO, inhibition of miRNA

maturation leads to disorganization of thyroid follicles at a young age. The lack of follicular organization was more prominent in mice with early deletion of Dicer1 and DCGR8 using a *Pax8*-Cre line (Bartram et al., 2016, Frezzetti et al., 2011, Rodriguez et al., 2012). At one month of age, the histological phenotype of our c*Tg*DcrKO mice was comparable to the mild phenotype of the previously published *Tg*Cre-driven Dicer1 KO mice (Rodriguez et al., 2012). In the inducible i*Tg*DcrKO mice, we observed changes in thyroid morphology already four weeks after induction, and the severity of the follicular disorganization progressed over time. At for weeks after induction, the thyroid follicular lumen was smaller in size, and the colloid was partially absent from the lumen. After eight weeks post-induction, the thyrocytes were not organized as follicles anymore, but resembled the histological phenotype observed in *Tg* promoter-driven constitutive Dicer1 KO mice. The cells in the disorganized regions of the thyroid gland partially lacked the expression of thyrocyte-specific genes, as shown with immunohistochemistry for NKX2-1. The loss of cell type-specific TF expression is a strong indicator for dedifferentiation. As described by Rodriguez *et al.*, *Pax8*-driven Dicer1 KO mice presented normal follicular architecture at five days of age, but a disorganized follicular architecture at the one month of age (Rodriguez et al., 2012). This clearly implies a post-natal dedifferentiation, similar to our observations in the i*Tg*DcrKO mice. Our results corroborate the previous work showing that Dicer1 and subsequent miRNA signaling is needed for the maintenance of thyrocyte differentiation.

In addition to the thyrocyte dedifferentiation, constitutive and inducible *Tg*DcrKO mice showed an increased intrathyroidal adipogenesis and fibrosis. TGF-β is a key regulator involved in thyroid differentiation, growth and epithelial-mesenchymal transition (EMT), causing fibrosis and adipogenesis (Colletta et al., 1989, Pisarev et al., 2009, Yu et al., 2010). Different miRNAs, mainly the miR200 family, have been shown to regulate *Tgf-β* (Wang et al., 2011). In fact, various genes involved in *Tgf-β* signaling were upregulated in c*Tg*DcrKO mice, whereas miR-200a and miR-141, both members of the miR-200 family, were significantly decreased. An altered TGF-β signaling might not only explain the findings of increased fibrosis and adipogenesis. TGF-β is also a driving factor in EMT, a process in which epithelial cells lose their characteristic features like cell-cell connections and polarity and acquire mesenchymal characteristics such as increased mobility and resistance to apoptosis (Kalluri and Weinberg, 2009). Several studies have shown that TGF-β can induce EMT in thyrocytes (Grande et al., 2002, Nilsson et al., 1995, Toda et al., 1997). Hence, TGF-β-mediated EMT may be one underlying mechanism of the thyrocyte dedifferentiation observed in thyrocyte-specific Dicer1 KO models.

6.2.2 Thyroid Hormone Synthesis is Not Directly Affected by Dicer1 (II)

The lack of thyrocyte differentiation is likely the cause for the hypothyroidism in constitutive Dicer1 KO mice. As seen in the previously described models as well as in our c*Tg*DcrKO mice, gene and protein expression for various thyrocyte TFs and genes necessary to maintain TH synthesis are downregulated when Dicer1 is knocked out. Immunohistochemical analysis revealed a residual protein expression in the remaining peripheral thyroid follicles, while in the disorganized areas the expression of the thyrocyte-specific markers such as NKX2-1 (II: Fig. 4C), PAX8, FOXE1, TPO, NIS, and Tg (Rodriguez et al., 2012) was absent. All constitutive Dicer1 KO mouse lines

developed hypothyroidism, with correlating severity between thyroid histology and TH levels. The *Pax8*-driven Dicer1 KO mice, which show a decreased thyroid weight (Frezzetti et al., 2011) and have very few colloid-storing follicles in the periphery, have almost undetectable TH levels and strongly elevated TSH serum concentration (Frezzetti et al., 2011, Rodriguez et al., 2012). With a higher number of normally formed thyroid follicles in *Tg*Cre-driven Dicer1 KO mice, the TH levels appear to be less decreased. Mice presenting with a mild histological phenotype have approximately 50 % decreased T4 concentrations, consistent with the phenotype observed in our c*Tg*DcrKO mice (Rodriguez et al., 2012). However, i*Tg*DcrKO mice did not develop hypothyroidism within the first two months after tamoxifen induction. Despite the partial loss of follicular architecture eight weeks after tamoxifen treatment, TSH appears to be only slightly but not significantly elevated, while fT4 shows a mild decrease. The remaining thyroid follicles, mainly in the periphery, of i*Tg*DcrKO mice could be sufficient to maintain TH levels in the normal range. Thus, given more time, i*Tg*DcrKO mice might develop hypothyroidism as a secondary effect from the altered thyroid histology.

6.2.3 Lethality of Thyrocyte-Specific Dicer1-Deficient Mice (II)

In *Pax8*-Cre-driven Dicer1 KO mice, a high lethality was observed, with 80 % of Dicer1-deficient mice dying before the age of 12 weeks. Surprisingly, these mice could not be rescued by T4 substitution. The premature death could be explained due to the *Pax8* expression in renal tubular cells and consequent deletion of miRNAs in the kidney. It was shown later that *Pax8*-Cre-driven miRNA deficiency due to DGCR8 KO can also lead to end-stage kidney disease (Bartram et al., 2016, Frezzetti et al., 2011, Rodriguez et al., 2012). However, a premature death was also observed in the severe subgroup of *Tg*Cre-driven Dicer1 KO mice. Nevertheless, we did not detect a correspondingly severe phenotype when using our *Tg*Cre-driven constitutive or inducible Dicer1 KO mice. All lines used in thyrocyte-specific Dicer1 KO studies had a mixed genetic background. Different strains can have substantial variation in the TH levels. A slight shift in the genetic background towards one of the founder strains might influence the severity of hypothyroidism (Pohlenz et al., 1999). It is also possible that our breeding strategy and the fact that we performed our experiments on mice of the third to fifth filial generation caused an out-breeding of the severe phenotype, as mice in the first filial generation presenting this phenotype would not have survived until sexual maturity.

Furthermore, it could be speculated that diet or other housing conditions might impact the severity of hypothyroidism and the survival rate of Dicer1 KO mice. It is known that, for example, iodine content in the diet can impact the onset of hypo- and hyperthyroidism (Han et al., 2012, Leung and Braverman, 2014, Rose et al., 2002).

6.2.4 Dicer1 is Necessary for Goiter Growth (II)

It is not surprising that mutations in the *DICER1* gene leading to partial or total loss of function can promote cancer development in humans, as miRNAs are differentially expressed in various tumors. In tumors, miRNAs have been shown to modify expression of both tumor-promoting and -suppressing genes (Jansson and Lund, 2012, Taucher et al., 2016, Voorhoeve and Agami, 2007, Yoruker et al., 2016). Moreover, in some species, DICER1 has been shown to have miRNA-independent functions, for

example in inflammatory processes, apoptotic DNA degradation and the regulation of Polymerase II (Burger and Gullerova, 2015, Johanson et al., 2013, Kurzynska-Kokorniak et al., 2015). *DICER1* mutations in humans are also linked to multinodular goiter, pointing to an inhibiting role of DICER1-mediated signaling pathways in growth regulation. However, our mouse model suggests a growth-promoting role of Dicer1, as i*Tg*DcrKO mice did not show goiter growth after administration of a goitrogenic diet. While the size and weight of the thyroid gland did not increase in i*Tg*DcrKO mice treated with goitrogenic drugs, histological and hormonal alterations corresponded to the changes in control mice, and proliferation markers were increased. The surprising finding of increased expression of proliferation markers without tissue growth or changes in the apoptosis rate was also shown for the *Pax8*-Cre-driven Dicer1 KO mouse line (Frezzetti et al., 2011). One potential explanation might be that the lack of a majority of miRNAs causes a cell-cycle arrest of thyrocytes and additionally blocks the induction of apoptosis (Lin et al., 2016, Tagscherer et al., 2016).

The discrepancies between the human and murine phenotypes could be partially caused by the additional roles of Dicer1 (Burger and Gullerova, 2015, Johanson et al., 2013, Kurzynska-Kokorniak et al., 2015). Further, the human DICER1 syndrome is caused by a germline mutation affecting every cell, while our mouse model has a thyrocyte-specific in-frame deletion of the RNAse III domain. Also, the nature of the mutations in patients, such as missense mutations and splice variants, differs from the in-frame deletion of the RNAse III domain in mice. These two factors could further influence how the genetic alterations in the *DICER1* gene modulate the Dicer1-mediated regulation of gene expression.

As mentioned in chapter 6.2.1, several genes involved in TGF-β signaling were upregulated in cTgDcrKO mice, and in iTgDcrKO mice the expression of *Tgf-β2* was elevated. The multifaceted role of TGF-β, including the regulation of thyroid function and growth (Pisarev et al., 2009), might partially explain the inhibition of goiter growth in the Dicer1 KO mice. To validate this hypothesis, overexpression of TGF-β under a thyrocyte-specific promoter could be applied. Yet, it is likely that additional signaling pathways are involved in the thyroid growth arrest of Dicer1 KO mice. Deleting the miRNA-processing enzyme Dicer1 leads to downregulation of the majority of miRNAs, likely affecting the majority of signaling cascades, since a single miRNA is capable of specifically binding up to 200 different mRNA targets (Krek et al., 2005, Lim et al., 2005). Thyrocyte-specific deletion of single miRNAs or miRNA families could be a suitable tool to determine single pathways affecting thyroid growth or maintenance of thyrocyte differentiation.

6.3 TSHR^{D633H} Mice Develop Transient Hyperthyroidism and Colloid Goiter (III)

While it has been known for the past 20 years that hyperthyroidism can be caused by CAMs of the TSHR, their molecular role in the development of hyperthyroidism has only been studied *in vitro* (Fuhrer et al., 1996, Lublinghoff et al., 2012, Vassart et al., 1996, Wonerow et al., 2001). We have successfully generated a mouse model harboring the constitutively activating TSHR mutation D633H (Neumann et al., 2001b), which enables us to study the role of a TSHR CAM in the development of hyperthyroidism *in vivo*. These TSHR^{D633H} mice develop colloid goiter with signs of

microadenoma at an older age, and also transient hyperthyroidism. Furthermore, we observed a clear sex difference in TSH and TH levels (III: Table 2).

6.3.1 Comparison of TSHR^{D633H} Signaling in vitro and in vivo (III)

The TSHR is known to have a substantial basal activity without ligand binding (Duprez et al., 1994, Parma et al., 1993). As already shown by Neumann and co-workers (Neumann et al., 2001b), murine variants of TSHR CAMs derived from patients show comparable capabilities to increase basal cAMP activation *in vitro*. Thus, they concluded that introducing activating mutations into the murine TSHR sequence is a possible way to induce NAH in mice. The functional *in vitro* characterization of the selected D633H mutation confirmed the previous study by Neumann et al., showing that there were no differences between murine and human TSHR^{D633H}. *In vitro*, human and mouse TSHR^{D633H} showed a strong, six- to eight-fold increase in basal cAMP accumulation when compared with the WT receptor.

In contrast, measurements of the cAMP content in thyroid homogenate and primary cell culture revealed only moderate increases in basal cAMP accumulation. These marked differences between the *in vitro* and *in vivo* capability of the TSHR^{D633H} mutation to permanently activate $G_{\alpha s}$ have also been observed in thyroid homogenates from autonomously functioning thyroid nodules obtained from patients (Persani et al., 2000). As the TSHR is overexpressed in cell lines, the receptor number is highly increased in *in vitro* studies. In contrast, our TSHR^{D633H} mice have a substitution of the endogenous TSHR. The lower receptor number in murine thyrocytes might cause the smaller increase in cAMP *in vivo* compared to *in vitro*. Another explanation might be the degradation of cAMP by phosphodiesterases, which are partially expressed in a cAMP-dependent manner (Takahashi et al., 2001). Furthermore, the set of phosphodiesterases expressed in different cells varies based on the species, developmental stage, cell type and environment. Thus, a different expression pattern of phosphodiesterases in murine thyrocytes *in vivo* and heterologous cell systems used *in vitro*, could additionally influence the detectable cAMP concentrations (Omori and Kotera, 2007).

6.3.2 Early Onset of Colloid Goiter Growth in TSHR^{D633H} Mice (III)

Since patients with NAH are only examined when they develop symptoms of overt hyperthyroidism, the onset and natural history of this disease has never been systemically analyzed. Therefore, the histological phenotype and hormonal status of the time period before manifestation of overt hyperthyroidism due to a TSHR CAM is unknown. In familiar and sporadic cases of non-autoimmune hyperthyroidism, the thyroid histology has been described as a heterogeneous mixture of large follicles surrounded by flattened thyrocytes and clusters of small, hyperactive follicles (Hebrant et al., 2011). Furthermore, patents with NAH often develop goiter (Gozu et al., 2010). In our TSHR^{D633H} model, the thyroid weight gradually increases from two to nine months of age and does not appear to reach a plateau. At two months of age, the histological alterations are mild with only slightly enlarged follicles despite the significant increase in thyroid weight. Surprisingly, at six months of age, HEZ and HOZ mice of both sexes have significantly larger thyroid follicles with flattened thyroid epithelium. These characteristics are often associated with an inactive state of the thyroid (La Perle and Jordan, 2012). In addition to these findings, at nine months

of age, areas resembling microadenomas with hypertrophic thyrocytes can be detected. This histology reflects the morphology described in patients with NAH (Hebrant et al., 2011) and is also consistent with the observations in the transgenic mouse models with increase $G_{\alpha s}$ signaling in the thyroid (Ledent et al., 1992, Michiels et al., 1994, Zeiger et al., 1997). Further examinations are needed to assess if those lesions resembling hyperactive follicles lead to hyperthyroidism.

6.3.3 Impact of the TSHR^{D633H} Variant on TSH and Thyroid Hormone Concentrations in Mice (III)

At two months of age, the serum TSH levels were below the detection limit in HEZ and HOZ female TSHR^{D633H} mice. Like HEZ females, HOZ males show latent hyperthyroidism with partially undetectable TSH and normal TH levels. HEZ females remain latent hyperthyroid until the age of six months, while male HEZ and HOZ mice were euthyroid at this age. Latent hyperthyroidism due to a TSHR mutation has also been described in cases of familial NAH (Nishihara et al., 2010), but the majority of cases are overt hyperthyroid. In our mouse model, overt hyperthyroidism occurs only in HOZ females at two months of age, displaying a genetic status not identified in humans so far. Surprisingly, the thyroid function appears to be decreased in HOZ females at six months of age, as they present decreased TH levels and decreased TSH. However, this is in line with the inactive thyroid histology observed in six-month-old mice. The more prominent changes in serum TSH levels of female mice could explain the stronger increase in thyroid weight, as thyroid growth is regulated via TSHR signaling (Dumont et al., 1992).

The decreased TH levels together with low or normal TSH concentration in TSHR^{D633H} mice at six months of age is potentially the result of a compensatory response to hyperthyroidism at an early age. In mice recovering from induced thyrotoxicosis, it has been shown that THs, specifically T3, can drop below the control levels before reaching the normal baseline. A similar over-compensation with a decline below baseline has been shown for the heart rate in this model (Hoefig et al., 2016). An alternative explanation for the decreased TH levels detected at six months of age could be an excessive conversion of active THs to their inactive metabolites by deiodinases (Kurlak et al., 2013, Peeters et al., 2013). However, the gene expression of *Dio1* and *Dio2* in the thyroid was not altered at the age of six months.

6.3.4 Manifestation of Hyperthyroidism in TSHR^{D633H} Mice Might Occur at Old Age (III)

Due to the considerably mild increase in basal activity *in vivo*, the development of stable hyperthyroidism and histological changes similar to the alterations observed in hyperthyroidism in humans (Hebrant et al., 2011) could require more time than the six months during which we analyzed our mouse model. This hypothesis is also supported by the preliminary histological data from animals at nine months of age.

Late development of hyperthyroidism in TSHR^{D633H} mice would be consistent with the previously shown late onset of hyperthyroidism caused by a constitutive activating mutation of the $G_{\alpha s}$ subunit. These mice with a transgenic expression of a $G_{\alpha s}$-activating mutation show an increase of TH and decrease of TSH serum levels at eight months of age (Michiels et al., 1994). On the other hand, the onset of

hyperthyroidism in mice overexpressing adenosine receptor a2 (Ledent et al., 1996, Ledent et al., 1992) or cholera-toxin subunit A (Zeiger et al., 1997) occurs at two months of age. It could be speculated that the age at onset of hyperthyroidism is dependent on the intensity of the intrathyroidal cAMP signal. While our $TSHR^{D633H}$ mice have 1.3- to 1.6-fold elevated basal cAMP levels, transgenic expression of cholera-toxin subunit A leads to a 12- to 20-fold increase in cAMP levels, depending on the clone. It is likely that this strong increase in cAMP is sufficient to cause excessive, not compensable TH synthesis and release, regardless of serum TSH levels, while the mild cAMP increase in our mice can be compensated by various mechanisms, resulting in the transient hyperthyroid state at two months of age.

Determination of TH concentrations at a late age of nine and 12 months is needed to confirm a late onset of hyperthyroidism in $TSHR^{D633H}$ mice, similar to the late-onset phenotype of the mouse line expressing a constitutively active $G_{\alpha s}$ mutation (Michiels et al., 1994). Furthermore, the nature of the observed hyperactive lesions in thyroid histology sections of HOZ $TSHR^{D633H}$ has to be analyzed. Similar to mice with a $G_{\alpha s}$-activating mutation, our $TSHR^{D633H}$ mice could develop thyroid adenomas at a late age.

6.3.5 Mild Alterations in Gene Expression in $TSHR^{D633H}$ Mice (III)

Overall, the increase of expression for genes involved in TH synthesis and regulation in our $TSHR^{D633H}$ mouse model might indicate an activated state of the thyroid gland of HOZ mice at two months of age. Consistent with the (latent) hyperthyroidism and increased *Tshr* gene expression at two months of age, an increased expression of the transcription factor *Pax8* was observed (Medina et al., 2000, Van Renterghem et al., 1996). Furthermore, in male mice at two months of age, the PAX8-regulated genes *Nis* (Ohno et al., 1999), *Tpo* (Zannini et al., 1992) and *Dio1* (Ruiz-Llorente et al., 2012) are also significantly elevated. In female mice, the increased *Pax8* expression does not seem to have a significant influence on the expression of its target genes.

At six months of age, the expression of thyroid-specific genes appears to be unaltered, pointing to a normal thyroid function. Only *Tg* expression is significantly decreased by 40 %. This is consistent with the mostly euthyroid TH status detected at six months of age. The decrease in *Tg* expression also fits with the histological phenotype at six months of age, presenting mostly inactive follicles.

6.4 Prospects

6.4.1 Assessing the Role of Dicer1 in Cancer Growth (II)

The contrary findings of thyroid growth inhibition in Dicer1 KO mice and the development of MNG and DTC in patients with Dicer1 mutations raise the question of the impact of Dicer1 in thyroid tumor growth. Several mutations in oncogenes, such as B-Raf(V600E) (Nucera et al., 2009, Pritchard et al., 2007), or proto-oncogene expression, for example the RET/PTC1 proto-oncogene (Castellone and Santoro, 2008, Cho, 2015), lead to aggressive cancer growth. Furthermore, several studies aim to assess the role of miRNAs in thyroid cancer (Chruscik and Lam, 2015, Forte et al., 2015). Knocking out Dicer1 in murine thyrocytes of mice developing thyroid cancer

due to oncogene mutations or proto-oncogene expression would be a promising approach to evaluate the role of miRNA signaling in thyroid cancer growth. This model could give further insight into the mechanism of cancer growth and the role of miRNAs therein. However, the effect of Dicer1 KO is widespread, affecting all signaling pathways mediated by Dicer1-dependent miRNA and siRNA. Therefore, it might be difficult to assess the results, and further, miRNA-specific KO models should be utilized.

6.4.2 Utilizing TSHR^{D633H} Mice as a Model for Drug Screening (III)

We believe that our TSHR^{D633H} mouse line serves as a new disease model to investigate the development of NAH. As patients carrying a somatic TSHR mutation usually present themselves with clinical symptoms at the final point of the development of hyperthyroidism, the cellular events leading to the clinical phenotype are unknown. Since the preliminary histology for nine-month-old mice suggests a late onset of hyperthyroidism, the TSHR^{D633H} mice offer an interesting opportunity to study the molecular events preceding hyperthyroidism. A detailed understanding of the development of NAH would be beneficial for the development of novel treatment strategies for NAH. To our surprise, the KI of a TSHR CAM into the murine *Tshr* gene did not result in overt hyperthyroidism in HEZ mice. Judging from the collected data, mice seem to have a compensatory mechanism to manage a mild increase of basal activity of the TSHR. A detailed examination of these compensatory mechanisms could uncover novel drug target candidates. By mimicking and boosting the compensation observed in mice with mildly increased cAMP signaling, it might be possible to also manage stronger basal activation of cAMP in patients. Furthermore, HOZ female mice could serve as a model for mild hyperthyroidism at a young age, providing a potential tool for screening of TSHR-antagonizing agents.

6.4.3 Evaluation of Sex Differences in the Development of Thyroid Diseases (III)

It is well known that thyroid disorders are more prevalent in women than in men (Wang and Crapo, 1997). In mice, it has also been shown that TSH levels in females are significantly lower than in male mice of various strains. Furthermore, a sex difference in fT4 but not tT4 has been described (McLachlan et al., 2014). The role of sex hormones in the regulation of thyroid function and onset of thyroid diseases is often speculated upon, but to date the underlying mechanisms remain unclear. The TSHR^{D633H} mice present a clear sex difference in the manifestation of hyperthyroidism, resembling the human situation with higher incidence of all thyroid diseases in women than in men. Recently, the role of elevated leptin levels in female mice has been suggested as a cause for the higher prevalence of thyroid autoimmunity (Merrill and Mu, 2015). Yet, as sex differences are found in both autoimmune and non-autoimmune thyroid diseases, this can only partially serve as an explanation. Our mouse model, presenting stable sex differences, could serve as a tool to assess the role of sex steroids on the onset of hyperthyroidism. In addition to the determination of endogenous steroid hormone levels, manipulating testosterone or estrogen production will further provide a better understanding if and how sex hormones influence the manifestation of thyroid diseases.

7 Summary and Conclusion

The molecular mechanisms leading to hypo- and hyperthyroidism are versatile and not fully understood. Here we have demonstrated an essential role of Dicer1 and miRNAs in the development of hypothyroidism and maintenance of thyrocyte differentiation. In this study, we investigated the role of Dicer1 in thyroid function and growth in adult mice. Furthermore, we have successfully created a model for NAH using the constitutively active $TSHR^{D633H}$ variant identified from human hyperthyroid patients. To our knowledge, this is the first *in vivo* model of hyperthyroidism caused by a CAM of the TSHR. The main conclusions of these studies are:

1. The *Tg*$CreER^{T2}$ mouse model can be applied to study the role of floxed genes in thyrocytes in a tamoxifen-inducible manner.
2. Dicer1 is crucial for proper thyroid development and the maintenance of thyrocyte differentiation. Loss of Dicer1 during development causes loss of follicular architecture and subsequently hypothyroidism. Loss of Dicer1 in adulthood leads to progressive dedifferentiation of thyrocytes but not to acute hypothyroidism.
3. Dicer1 plays a crucial role in goiter growth. Deletion of Dicer1 in adult mice inhibits thyroid growth when treated with a goitrogenic diet.
4. A constitutively activating TSHR mutation causes transient hyperthyroidism and colloid goiter development in mice.

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