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Molecular Determinants of Congenital Hypothyroidism due to Thyroid Dysgenesis

par

Rasha Abu-khudir

Département de Biochimie et médecine moléculaire

Faculté de Médecine

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Molecular Determinants of Congenital Hypothyroidism due to Thyroid Dysgenesis

Présentée par

Rasha Abu-khudir

a été évaluée par un jury composé des personnes suivantes :

Dre Muriel Aubry, présidente-rapporteuse

Dr Johnny Deladoëy, directeur de recherche

Dre Cheri L. Deal, co-directrice de recherche

Dr Stéphane Roy, membre du jury

Dr Gabor Szinnai, examinateur externe

Dr Janos G. Filep, représentant du doyen de la FES

Résumé

L'hypothyroïdie congénitale par dysgénésie thyroïdienne (HCDT) est la condition endocrinienne néonatale la plus fréquemment rencontrée, avec une incidence d'un cas sur 4000 naissances vivantes. L'HCDT comprend toutes les anomalies du développement de la thyroïde. Parmi ces anomalies, le diagnostic le plus fréquent est l'ectopie thyroïdienne (~ 50% des cas). L'HCDT est fréquemment associée à un déficit sévère en hormones thyroïdiennes (hypothyroïdisme) pouvant conduire à un retard mental sévère si non traitée. Le programme de dépistage néonatal assure un diagnostic et un traitement précoce par hormones thyroïdiennes. Cependant, même avec un traitement précoce (en moyenne à 9 jours de vie), un retard de développement est toujours observé, surtout dans les cas les plus sévères (c.-à-d., perte de 10 points de QI).

Bien que des cas familiaux soient rapportés (2% des cas), l'HCTD est essentiellement considérée comme une entité sporadique. De plus, plus de 92% des jumeaux monozygotiques sont discordants pour les dysgénésies thyroïdiennes et une prédominance féminine est rapportée (spécialement dans le cas d'ectopies thyroïdiennes), ces deux observations étant clairement incompatible avec un mode de transmission héréditaire mendélien. Il est donc cohérent de constater que des mutations germinales dans les facteurs de transcription thyroïdiens connus (NKX2.1, PAX8, FOXE1, and NKX2.5) ont été identifiées dans seulement 3% des cas sporadiques testés et furent, de plus, exclues lors d'analyse d'association dans certaines familles multiplex. Collectivement, ces données suggèrent que des mécanismes non mendéliens sont à l'origine de la majorité des cas de dysgénésie thyroïdienne. Parmi ces mécanismes, nous devons considérer des modifications épigénétiques, des mutations somatiques précoces (au stade du bourgeon thyroïdien lors des premiers stades de l'embryogenèse) ou des défauts développementaux stochastiques (c.-à-d., accumulation aléatoire de mutations germinales ou somatiques). Voilà pourquoi nous proposons un modèle «2 hits » combinant des mutations (épi)génétiques germinales et somatiques; ce modèle étant compatible avec le manque de transmission familial observé dans la majorité des cas d'HCDT.

Dans cette thèse, nous avons déterminé si des variations somatiques (épi)génétiques sont associées à l'HCTD via une **approche génomique** et une approche **gène candidat**. Notre approche **génomique** a révélé que les thyroïdes ectopiques ont un profil d'expression différent

des thyroïdes eutopiques (contrôles) et que ce profil d'expression est enrichi en gènes de la voie de signalisation Wnt. La voie des Wnt est cruciale pour la migration cellulaire et pour le développement de plusieurs organes dérivés de l'endoderme (p.ex. le pancréas). De plus, le rôle de la voie des Wnt dans la morphogénèse thyroïdienne est supporté par de récentes études sur le poisson-zèbre qui montrent des anomalies du développement thyroïdien lors de la perturbation de la voie des Wnt durant différentes étapes de l'organogénèse. Par conséquent, l'implication de la voie des Wnt dans l'étiologie de la dysgénésie thyroïdienne est biologiquement plausible.

Une trouvaille inattendue de notre approche génomique fut de constater que la calcitonine était exprimée autant dans les thyroïdes ectopiques que dans les thyroïdes eutopiques (contrôles). Cette trouvaille remet en doute un dogme de l'embryologie de la thyroïde voulant que les cellules sécrétant la calcitonine (cellules C) proviennent exclusivement d'une structure extrathyroïdienne (les corps ultimobranchiaux) fusionnant seulement avec la thyroïde en fin de développement, lorsque la thyroïde a atteint son emplacement anatomique définitif.

Notre approche **gène candidat** ne démontra aucune différence épigénétique (c.-à-d. de profil de méthylation) entre thyroïdes ectopiques et eutopiques, mais elle révéla la présence d'une région différentiellement méthylée (RDM) entre thyroïdes et leucocytes dans le promoteur de *FOXE1*. Le rôle crucial de *FOXE1* dans la migration thyroïdienne lors du développement est connu et démontré dans le modèle murin. Nous avons démontré *in vivo* et *in vitro* que le statut de méthylation de cette RDM est corrélé avec l'expression de *FOXE1* dans les tissus non tumoraux (c.-à-d., thyroïdes et leucocytes). Fort de ces résultats et sachant que les RDMs sont de potentiels points chauds de variations (épi)génétiques, nous avons lancé une étude cas-contrôles afin de déterminer si des variants génétiques rares localisés dans cette RDM sont associés à la dysgénésie thyroïdienne.

Tous ces résultats générés lors de mes études doctorales ont dévoilé de nouveaux mécanismes pouvant expliquer la pathogenèse de la dysgénésie thyroïdienne, condition dont l'étiologie reste toujours une énigme. Ces résultats ouvrent aussi plusieurs champs de recherche prometteurs et vont aider à mieux comprendre tant les causes des dysgénésies thyroïdiennes que le développement embryonnaire normal de la thyroïde chez l'homme.

Mots-clés: L'hypothyroïdie congénitale, Dysgénésie thyroïdienne, L'ectopie thyroïdienne, Variations somatiques, La voie de signalisation Wnt, La variabilité du nombre de copies, *FOXE1*, Rrégulation épigénétique, Région différentiellement méthylée (RDM).

Abstract

Congenital hypothyroidism from thyroid dysgenesis (CHTD) is the most common congenital endocrine disorder with an incidence of 1 in 4,000 live births. CHTD includes multiple abnormalities in thyroid gland development. Among them, the most common diagnostic category is thyroid ectopy (~ 50 % of cases). CHTD is frequently associated with a severe deficiency in thyroid hormones (hypothyroidism), which can lead to severe mental retardation if left untreated. The newborn biochemical screening program insures the rapid institution of thyroid hormone replacement therapy. Even with early treatment (on average at 9 d), subtle developmental delay is still be observed in severe cases (i.e., IQ loss of 10 points).

Although there have been some reports of familial occurrence (in 2% of the cases), CHTD is mainly considered as a sporadic entity. Furthermore, monozygotic (MZ) twins show a high discordance rate (92%) for thyroid dysgenesis and female predominance is observed in thyroid dysgenesis (especially thyroid ectopy), these two observations being incompatible with simple Mendelian inheritance. In addition, germline mutations in the thyroid related transcription factors NKX2.1, PAX8, FOXE1, and NKX2.5 have been identified in only 3% of sporadic cases and linkage analysis has excluded these genes in some multiplex families with CHTD. Collectively, these data point to the involvement of non-Mendelian mechanisms in the etiology of the majority of cases of thyroid dysgenesis. Among the plausible mechanisms are epigenetic modifications, somatic mutations occurring in the thyroid bud early during embryogenesis, or stochastic developmental events. Hence, we proposed a two-hit model combining germline and somatic (epi)genetic variations that can explain the lack of clear familial transmission of CTHD.

In this present thesis, we assessed the role of somatic (epi)genetic variations in the pathogenesis of thyroid dysgenesis via a genome-wide as well as a candidate gene approach. Our genome wide approach revealed that ectopic thyroids show a differential gene expression compared to that of normal thyroids, with enrichment for the Wnt signalling pathway. The Wnt signalling pathway is crucial for cell migration and for the development of several endoderm-derived organs (e.g., pancreas). Moreover, a role of Wnt signalling in thyroid organogenesis was further supported by recent zebrafish studies which showed thyroid abnormalities resulting from the disruption of the Wnt pathway during different steps of organogenesis. Thus, Wnt pathway involvement in the etiology of thyroid ectopy is biologically plausible.

An unexpected finding of our genome-wide gene expression analysis of ectopic thyroids was that they express calcitonin similar to normally located (orthotopic) thyroids. Such a finding, although in contradiction with our current knowledge of the embryological development of the thyroid attributes C cell origins to extrathyroidal structures (ultimobrachial bodies) upon fusion with a fully-formed, normally situated gland.

Using a candidate gene approach, we were unable to demonstrate any differences in the methylation profile between ectopic and eutopic thyroids, but nevertheless we documented the presence of a differentially methylated region (DMR) between thyroids and leukocytes in the promoter of *FOXE1*, a gene encoding the only thyroid related transcription factor known to play a crucial role in regulating the migration of the thyroid precursors during development as shown by animal studies. We demonstrated by *in vivo* and *in vitro* studies that the methylation status of this DMR is correlated with differential expression of *FOXE1* in non-tumoral tissues (thyroids and leukocytes). Knowing that DMRs are hotspots for epi(genetic) variations, its screening among CTHD patients is justifiable in our search for a molecular basis of thyroid dysgenesis, currently underway in a case-control study.

The results generated during my graduate studies represent unique and novel mechanisms underlying the pathogenesis of CHTD, the etiology of which is still an enigma. They also paved the way for many future studies that will aid in better understanding both the normal and pathogenic development of the thyroid gland.

Keywords: Congenital hypothyroidism, Thyroid dysgenesis, Ectopic thyroid, Somatic variations, Wnt signalling pathway, Copy number variants (CNVs), Calcitonin-producing C cells, *FOXE1*, Epigenetic regulation, Differentially methylated region (DMR).

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Abbreviation List

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydoxymethylcytosine
5mC	5-methylcytosine
AAP	American Academy of Pediatrics
AC	Adenylate cyclase
aCGH	Array comparative genomic hybridization
AIS	Androgen insensitivity syndrome
APC	Adenomatous polyposis coli
AR	Androgen receptor
AVE	Anterior visceral endoderm
BAT	Brown adipose tissue
BHC	Benign hereditary chorea
bHLH	Basic helix-loop-helix
BLTS	Triad of brain, lung, thyroid syndrome
BM	Bone maturation
BMPs	Bone morphogenic proteins
BMR	Basal metabolic rate
C cells	Calcitonin-producing cells
Ca^{2+}/NADPH	Calcium- and reduced nicotineamide adenine dinucleotide phosphate
cAMP	Cyclic adenosine monophosphate
CCH	Central congenital hypothyroidism
CDU	Color Doppler ultrasonography
CGIs	CpG islands
CH	Congenital hypothyroidism
CHD	Congenital heart disease
CHI	Congenital hyperinsulinism
CHTD	Congenital hypothyroidism due to thyroid dysgenesis
cKO	Conditional Knockout
CNPs	Copy number polymorphisms
CNS	Central nervous system
CNVs	Copy number variations
CTCF	CCCTC-binding factor
d	Developmental day
D1	Type 1 deiodinase
D2	Type 2 deiodinase
D3	Type 3 deiodinase
DAG	1,2-Diacylglycerol

DEHAL1	Iodotyrosine dehalogenase 1
DIT	Di-iodotyrosine
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic Acid
DNMTs	DNA methyltransferases
DQ	Developmental quotient
Ds	Selenoprotein iodothyronine deiodinases
DUOX2	Dual oxidase type 2
DVL	Disheveled
E	Embryonic day
ECD	Extracellular domain
ECM	Extracellular matrix
ELC	Ether link cleavage
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
EZH2	Enhancer of zeste 2
FGFR	Fibroblast growth factor receptor
FNAH	Familial non-autoimmune hyperthyroidism
fT_3	Free T_3
fT_4	Free T_4
Fzd	Frizzled
GDFs	Growth/differentiation factors
GIT	Gastrointestinal tract
GnRH	Gonadotrophin-releasing hormone
GO	Gene ontology
GPCRs	G protein-coupled receptors
GpHR	Glycoprotein hormone receptor
GSK3β	Glycogen synthase kinase 3β
GW	Gestational weeks
H_2O_2	Hydrogen Peroxide
HAT	Heterodimeric amino acid transporter
HAT activity	Histone acetyl transferase activity
HCP	High CpG-density promoters
HDAC	Histone deacetylase
HMT	Histone methyltransferase
hpf	Hours post-fertilization
HPT axis	Hypothalamic-Pituitary-Thyroid axis
ICP	Intermediate CpG-density promoters
IGF-1	Insulin-like growth factor-1

IHD	Iodohexadecanal
IP3	Inositol 1,4,5 trisphosphate
IQ	Intelligence quotient
IRD	Inner ring deiodination
IRDS	Infant respiratory distress syndrome
ITD	Iodide transport defects
JNK	Jun N-terminal Kinase
KO	Knockout
LATs	L-type amino acid transporters
LBW	Low birth weight
LCP	Low CpG-density promoters
LCRs	Low-copy repeats
LIM	Lin11, Isl-1, and Mec-3
LOF	Loss-of-function
LOH	Loss of heterozygosity
L-T_4	Levothyroxine
MAPK	Mitogen activated protein kinase
MBDs	Methyl-CpG-binding domain (MBD)-containing proteins
MCT	Monocarboxylate transporter
MeCPs	Methyl-CpG-binding proteins
MeDIP	Methylated DNA immunoprecipitation
miRNA	MicroRNA
MIT	Mono-iodotyrosine
MMEJ	Microhomology-mediated end-joining
MNG	Multinodular goitrous
MO	Morpholino antisense oligonucleotide
Na^+/K^+ ATPase	Na^+/K^+-adenosine triphosphatase
NAHR	Non-allelic homologous recombination
NASH	Nonautoimmune subclinical hypothyroidism
NBS	Newborn screening
ncRNAs	Non-coding RNAs
NGS	Next-generation sequencing
NHEJ	Nonhomologous end joining
NIS	Na^+/I^- symporter
NTCP	Na^+/ taurocholate cotransporting polypeptide
NTH	Neonatal transient hypothyroidism
OATP	Organic anion-transporting polypeptide
ORD	Outer ring deiodination
PcG	Polycomb group
PCP	Planar cell polarity

PD	Paired-box DNA binding domain
PI3K	Phosphatidyl Inositol 3 Kinase
PIOD	Partial iodide organification defect
PKA	Protein Kinase A
PLC	Phospholipase C
PRC2	Polycomb repressive complex 2
pTFCs	Precursors of thyroid follicular cells
PTMs	Post-translational modifications
PU	Phenylthiourea
RA	Retinoic acid
rhTSH	Recombinant human thyroid-stimulating hormone
RTHs	Resistance to thyroid hormones
RXRs	Retinoid X receptors
SDs	Segmental duplications
SET	Su(var), Enhancer of zest, and Trithorax
SFRPs	Secreted frizzled-related proteins
SNP	Single nucleotide polymorphisms
T_3	Tri-iodothyronine
T_3S	Sulfated T_3
T_4	Thyroxine
TAMs	Thyronamines
TBG	Thyroxine-binding globulin
TBII	Thyrotropin-binding inhibitor immunoglobulin
TD	Thyroid dysgenesis
TDA	Thyroid developmental anomalies
TDHG	Thyroid dyshormonogenesis
T-DMR	Tissue-specific differentially methylated region
TEs	Transposable elements
TET	Ten-eleven translocation
TFCs	Thyroid follicular cells
TFs	Transcription factors
Tg	Thyroglobulin
TGFβ	Transforming growth factor beta
THCMTD	Thyroid hormone cell membrane transport defects
THMD	Thyroid hormone metabolism defects
THOX2	Thyroid oxidase 2
THs	Thyroid hormones
TIOD	Total iodide organification defects
TMD	Transmembrane domain
TPO	Thyroid peroxidase

TRAPs	Thyroid receptor auxiliary proteins
TRB-Ab	Thyrotropin receptor blocking antibody
TRE	TH response elements
TRH	Thyrotropin-releasing hormone
TRs	Thyroid receptors
TRα	Thyroid receptor alpha
TRβ	Thyroid receptor beta
TSH	Thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
UBB	Ultimobranchial bodies
UPD	Uniparental isodisomy
US	Ultrasound
WES	Whole-exome sequencing
Wnt	wingless-int-1
WT	Wild-type

To my family

“Your assumptions are your windows on the world.Scrub them off every once in a while, or the light won't come in”

Isaac Asimov

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CHAPTER 1: INTRODUCTION

1. The thyroid gland

In humans, the thyroid gland is considered to be one of the largest endocrine glands in the body, weighing 15-20 grams in normal adults. It synthesizes, stores, and secretes thyroid hormones (THs), thyroxine (T_4) and tri-iodothyronine (T_3). The production and secretion of THs is under the regulation of the hypothalamic-pituitary-thyroid (HPT) axis. Mainly through the nuclear thyroid hormone receptors (TRs), TRα and TRβ, THs exert various physiological responses: they are responsible for the maintenance of the basal metabolic rate as well as controlling pre- and post-natal growth and differentiation of numerous tissues, notably the brain, through their effects on protein, lipid and carbohydrate metabolism. In addition to THs, the thyroid gland secretes the serum calcium-lowering hormone, calcitonin (Kirsten, 2000).

1.1. Embryology and development

1.1.1. Normal thyroid development

In vertebrates, similar to other endodermal-derived organs, the morphogenesis of the thyroid gland begins in the floor of the primitive pharynx with a specification event during which a monolayer of endodermal cells, precursors of thyroid follicular cells or pTFCs, are committed to attaining the thyroid fate. Subsequently, thickening of the monolayer of committed endodermal cells representing the thyroid anlage results in the formation of a multilayered structure, the thyroid placode, by embryonic day (E) 9-9.5 in mice and E22 in humans (De Felice and Di Lauro, 2011). The formation of the thyroid placode is followed by its expansion into the underlining mesenchyme resulting in the formation of the thyroid bud (median thyroid primordium). By E10.5 in mice, the thyroid bud is recognized as a narrow necked flask-like structure that promptly becomes a diverticulum (De Felice and Di Lauro, 2004). The thyroid bud begins to migrate caudally along the anterior neck region while it is connected by a narrow channel (thyroglossal duct) to a small hole (foramen cecum) at its site of origin in the floor of the pharynx. Consequent to the atrophy of the thyroglossal duct (E11.5 in mice and E30-40 in humans), the median primordium detaches from the pharyngeal floor and starts its lateral expansion by E12 in mice (De Felice and Di Lauro, 2004). At E13.5 in mice and E45-50 in

humans, the median thyroid primordium reaches its final destination (Van Vliet, 2003; De Felice and Di Lauro, 2004). The mechanisms underlying the translocation of the thyroid primordium have not yet been clarified and different ones have been proposed among which are active transport (De Felice *et al.*, 1998), involvment of cardiac mesoderm and major blood vessels (Alt *et al.*, 2006b; Fagman *et al.*, 2006; Opitz *et al.*, 2012), or relocalization secondary to the differential growth of the whole embryo (Gasser, 2006).

Upon reaching its final location in front of the trachea, fusion between both the median thyroid primordium and the ultimobranchial bodies (UBB) or the lateral primordia occurs by E14 in mice and E60 in humans (De Felice and Di Lauro, 2004). The UBB are a pair of transient embryonic structures derived from the fourth and fifth (caudal) pharyngeal pouches in mouse and human, respectively to which the precursors of the parafollicular or C cells, cells devoted to the production of calcitonin, have migrated from the neural crest and become localized (Pearse and Carvalheira, 1967; Fontaine, 1979; Fagman and Nilsson, 2010). In contrast to the established neuronal origin of the progenitors of the ultimobranchial C cells in avian species (Kameda, 1995), that of mammals has been recently challenged (Kameda *et al.*, 2007b). In this regard, Kameda *et al.* have shown that the murine ultimobranchial cells, that ultimately differentiate into thyroid C cells, are endodermally derived. Moreover, they have also indicated that the UBB were not occupied by neural crest-derived cells at any developmental stage as well as that the thyroid C cells exhibited no expression of the neural crest markers (Kameda *et al.*, 2007b). Recently, calcitonin-producing C cells have been reported in human ectopic lingual thyroids, thus suggesting that UBB are not the sole source of C cells in humans and that the interactions between TFCs and calcitonin-producing cells occur early during embryonic development than previously known (Vandernoot *et al.*, 2012).

At E15 the bilobed shape of the gland is attained and by E15.5 (around E70 in humans), the final steps of thyroid morphogenesis are detected by the appearance of follicular organization. Subsequently, activation of the terminal or functional differentiation takes place whereby the thyroid follicular cells (TFCs) ultimately exhibit the expression of the set of proteins known to be involved in the biosynthesis of THs, including thyroglobulin (Tg), thyroid peroxidase (TPO), Na^+/I^- symporter (NIS), and the thyroid-stimulating hormone (TSH) receptor (TSHR) (De Felice and Di Lauro, 2004; 2011).

Currently, knowledge concerning the signals inducing the process of commitment or specification of a group of multipotent cells to the thyroid fate is still limited. However, studies carried out in animal models have pointed to the plausible role of extrinsic (acting outside the pTFCs) genetic factors such as Nodal-related signals as well as a number of mesenchymal inductive signals in the specification of the thyroid (Fagman and Nilsson, 2010; De Felice and Di Lauro, 2011; Fagman and Nilsson, 2011).

On the other hand, intrinsic genetic factors (acting inside the pTFCs) involved in subsequent morphogenetic steps and their functional interactions have been identified (Figure 1) (De Felice and Di Lauro, 2011). The pTFCs are differentiated from other cells in the primitive pharynx by the coexpression of four transcription factors: thyroid transcription factor 1 (TTF-1 or NKX2.1), paired homeobox-8 (PAX8), thyroid transcription factor 2 (TTF-2 or FOXE1), and hematopoietically expressed homeobox (HHEX) (De Felice and Di Lauro, 2004; 2011). Based on their early expression in the thyroid anlage, it has been suggested that these transcription factors collaborate to drive the budding, migration, survival, and growth of the pTFCs (De Felice and Di Lauro, 2004; Santisteban and Bernal, 2005; De Felice and Di Lauro, 2011). In addition, they are also involved in controlling the terminal differentiation of the TFCs via the regulation of thyroid-specific gene expression as well as the maintenance of the differentiated phenotype of the mature TFCs (Figure 2) (Damante *et al.*, 2001; De Felice and Di Lauro, 2004; Santisteban and Bernal, 2005; De Felice and Di Lauro, 2011). Animal models deficient in thyroid transcription factors pointed to their crucial role in thyroid development: In the absence of either *Nkx2-1*, *Pax8*, *Foxe1*, or *Hhex*, a proper specification of the thyroid anlage occurs but subsequent morphogenesis of the thyroid is severely impaired (Wendl *et al.*, 2002; Elsalini *et al.*, 2003; Parlato *et al.*, 2004).

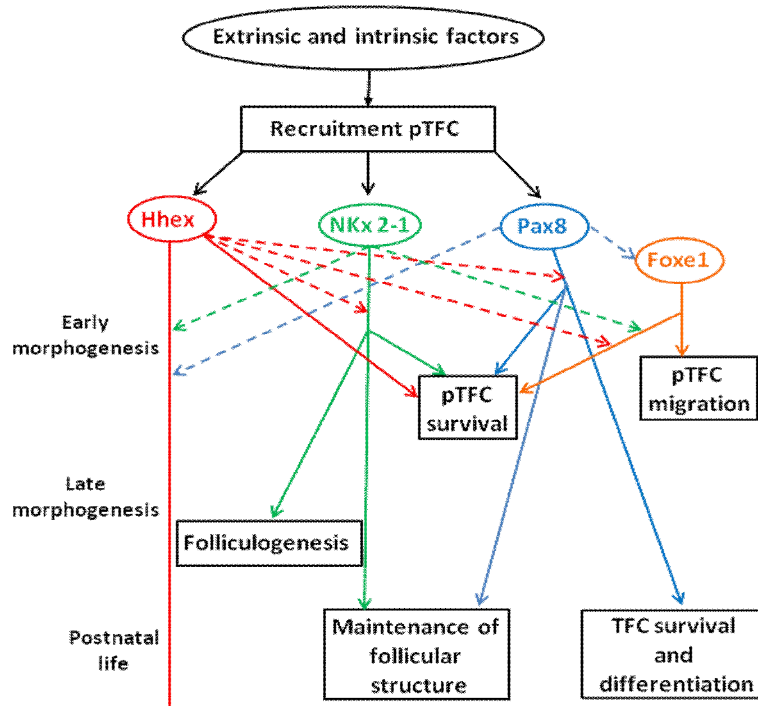

Figure 1: Roles and functional interactions of intrinsic genetic factors involved in thyroid morphogenesis (adapted From De Felice and Di Lauro, 2011).
Dashed arrows: involvement in the initiation or maintenance of other factors.
Solid arrows: controlling crucial steps of thyroid morphogenesis and physiology.

	Budding	Migration	Survival/proliferation of precursor cells	Functional differentiation	Expansion of differentiated cells
	Floor of primitive pharynx		cell precursors	Thyroid follicle	
Hhex					
Titf1					
Foxe1					
Pax8					
TSHR					
Tg, TPO, NIS					

Figure 2: Developmental stages of the thyroid and the list of transcription factors and thyroid-specific genes involved in cell fate determination and differentiation (modified from Santisteban and Bernal, 2005).

In addition to the above mentioned thyroid-enriched transcription factors, several recent studies in animal models have shed light on novel regulators of thyroid development and differentiation. Among the new genetic factors are those involved in early stages of thyroid morphogenesis including those encoding the basic helix-loop-helix (bHLH) transcription factor Mash1 (Kameda *et al.*, 2007a), the LIM homeodomain transcription factor Isl1 (Westerlund *et al.*, 2008), and the bHLH transcriptional repressor Hes1, a known target of the Notch signalling pathway (Carré *et al.*, 2011). In addition, genes implicated in late morphogenetic stages were also identified among which are the *Hox* genes, *Hoxa-3* and its paralogs *Hoxb-3* and *Hoxd-3* (Manley and Capecchi, 1995; 1998), *TSHR*, the gene encoding for the TSH receptor (Postiglione *et al.*, 2002), and the *Eyes absent* (*Eya*) gene *Eya1* (Xu *et al.*, 2002). Recently, it has been shown that mouse models deficient in the microRNA-processing enzyme Dicer exhibit impaired postnatal thyroid function, thus indicating the role of an intact microRNA processing machinery in maintaining thyroid homeostasis (Frezzetti *et al.*, 2011; Rodriguez *et al.*, 2012). It has been shown that genes expressed in the foregut endoderm known to play crucial roles in pharyngeal development are involved in the developmental process of the thyroid as well, among which are genes involved in fibroblast growth factor receptor (FGFR) signalling (Celli *et al.*, 1998; Ohuchi *et al.*, 2000; Revest *et al.*, 2001; De Felice and Di Lauro, 2004; Lania *et al.*, 2009), the *Nkx.2* class homeobox genes *Nkx2.5* and *Nkx2*.6 (Tanaka *et al.*, 2001), the gene encoding Hoxa5 (Meunier *et al.*, 2003), the candidate gene of the 22q11 deletion syndrome, *Tbx1* (Fagman *et al.*, 2007; Lania *et al.*, 2009), as well as the gene encoding the novel regulator of thyroid development, the tyrosine kinase receptor EphA4 (Andersson *et al.*, 2011). The role of the above mentioned genes in the development of the thyroid gland will be discussed in detail later in this chapter.

1.1.2. Abnormal thyroid development

Abnormalities in thyroid gland development, designated thyroid dysgenesis (TD), include a thyroid gland that is either ectopically located (thyroid ectopy), completely absent (athyreosis), or severely reduced in size (thyroid hypoplasia) (Van Vliet, 2003; De Felice and Di Lauro, 2004). The ectopic thyroid tissue occurs consequent to the aberrant migration of all or part of the thyroid precursor cells. Although it is commonly located at the base of the tongue (lingual position), the ectopic gland can be detected in other locations along the normal migratory route

followed by the thyroid precursors from the foramen cecum to the neck (Van Vliet, 2003; De Felice and Di Lauro, 2004). Rarely, ectopic thyroid tissue has been detected in several other sites either within the head and neck region (e.g. submandibular, trachea, iris, and pituitary) or distantly (e.g. heart, lung, duodenum, gall bladder, adrenal gland, ovary and uterus). The presence of ectopic thyroid tissue in or near the heart could be attributed to developmental disturbances of organs sharing a common embryological origin. In subdiaphragmatic positions (e.g. duodenum, gall bladder, and adrenal gland), heterotropic differentiation of unspecified endodermal cells might be the underlying etiology. Moreover, it has been reported that thyroid tissue located in the ovary can arise within a teratoma, an encapsulated tumor containing more than one differentiated tissue (Ibrahim and Fadeyibi, 2011; Noussios *et al.*, 2011).

Thyroid ectopy is the most common cause of permanent primary congenital hypothyroidism due to thyroid dysgenesis (CHTD), where it represents ~ 50 % of the cases (Van Vliet and Deladoëy, 2012). In the majority of cases, the ectopic thyroid is the only detectable thyroid tissue (Batsakis *et al.*, 1996). Ectopic thyroids exhibit both normal histological organization and normal capacity to trap and organify iodine and hence to produce THs and Tg (Leger *et al.*, 1988; Gallo *et al.*, 2001; Toso *et al.*, 2009). Consequently, the congenital hypothyroidism observed in most of the patients with thyroid ectopy is likely attributed to the smaller amount of tissue (due to absence of lateral lobes) and from a limited TSH-dependent compensatory growth (Stoppa-Vaucher *et al.*, 2010). Of note, some euthyroid individuals (with normal thyroid function) have been found to have thyroid ectopy (Castanet *et al.*, 2010a; Stoppa-Vaucher *et al.*, 2011). In spite of being generally limited, the thyroid hormone producing capability of ectopic thyroids remains stable over time, thus reflecting a normal postnatal survival of the ectopic cells (Grant *et al.*, 1989; Léger and Czernichow, 1990).

In addition to thyroid ectopy, absence of TFCs or athyreosis is the second common variant of TD (Van Vliet, 2003; De Felice and Di Lauro, 2004; Castanet *et al.*, 2010a). Although the specification of the thyroid bud occurs, subsequent abnormalities leading to defects in either survival and/or proliferation of the precursors of TFCs (pTFCs) is the underlying cause resulting in absence of TFCs. Lack of differentiation of TFCs or shifting to another fate has been suggested as alternative mechanisms leading to the disappearance of TFCs (Van Vliet, 2003; De Felice and Di Lauro, 2004). A rare variant of TD is hypoplasia of an orthotopic (normally located) bilobed thyroid gland. Finally, absence of one of the two lobes of the thyroid, mainly the

left one, is a rare variant of TD referred to as hemiagenesis (Van Vliet, 2003; De Felice and Di Lauro, 2004).

Thyroid dysgenesis (TD) is the most common cause of congenital hypothyroidism (CH). The characteristics of congenital hypothyroidism from thyroid dysgenesis (CHTD) and its underlying pathogenesis will be discussed in later sections.

1.2. Anatomy and Histology

1.2.1. Anatomy

The thyroid is a butterfly-like shaped gland located in the anterior portion of the neck, just below the larynx on opposite sides of, and anterior to, the trachea. In mammals and in some reptiles, it consists of two lobes joined by the isthmus in the midline (Figure 3) (Stathatos, 2006). Occasionally, a pyramidal lobe exists ascending from the isthmus towards the hyoid bone. The thyroid is surrounded by a fibrous capsule, the extension of which into the body of the gland leads to the formation of septae, thus producing an irregular and incomplete lobulation (Stathatos, 2006).

Major differences in the anatomy of the thyroid are observed among classes of vertebrates: In birds and amphibians, the thyroid consists of two unconnected lobes. Moreover, in cartilaginous fish, the thyroid follicles are arranged in the form of a compact gland surrounded by a capsule (Gorbman, 1986). On the other hand, absence of glandular organization is observed in the majority of bony fish (teleosts) where the thyroid follicles are non-encapsulated and loosely distributed (Gorbman, 1986), as is observed in zebrafish where the thyroid follicles are loosely dispersed along the ventral aorta in the lower jaw region and hence no bilateral lobes are formed (Wendl *et al.*, 2002). In contrast to what is observed in mouse and humans concerning the composite structure of the thyroid gland resulting from the fusion of the median and lateral primordia (Fagman *et al.*, 2006), the two embryonic structures remain separated in chicken (Kameda, 1995) and zebrafish (Alt *et al.*, 2006a).

In addition to differences in thyroid anatomy, species-specific timing of crucial embryonic developmental steps do exist as well (Table I) (Deladoëy, 2012).

Table I: Timing of morphogenetic events during thyroid embryonic development in different species (modified from Deladoëy, 2012).

Species	Specification	Budding	Migration	Follicle formation
Zebrafish (Alt *et al.*, 2006a; Opitz *et al.*, 2011)	24 hpf	36–46 hpf	48–55 hpf	55 hpf
Mouse (Fagman and Nilsson, 2010)	E8.5	E10	E10.5–13.5	E15.5
Human (De Felice and Di Lauro, 2004)	E20–22	E24	E25–50	E70

hpf: hours post-fertilization; **E**: embryonic day

The thyroid gland is highly vascularized (Figure 3) (Stathatos, 2006). Its arterial supply is provided by two superior and two inferior arteries that originate from the external carotid arteries and thyrocervical trunks, respectively. The thyroid *ima* artery, a branch of either the brachiocephalic or the aorta, contributes infrequently to the blood supply of the thyroid. Three pairs of veins, superior, middle and inferior, are responsible for the venous drainage of the thyroid gland. Both the superior and middle veins drain into the internal jugular vein while the inferior ones drain into the brachiocephalic and subclavian veins (Stathatos, 2006).

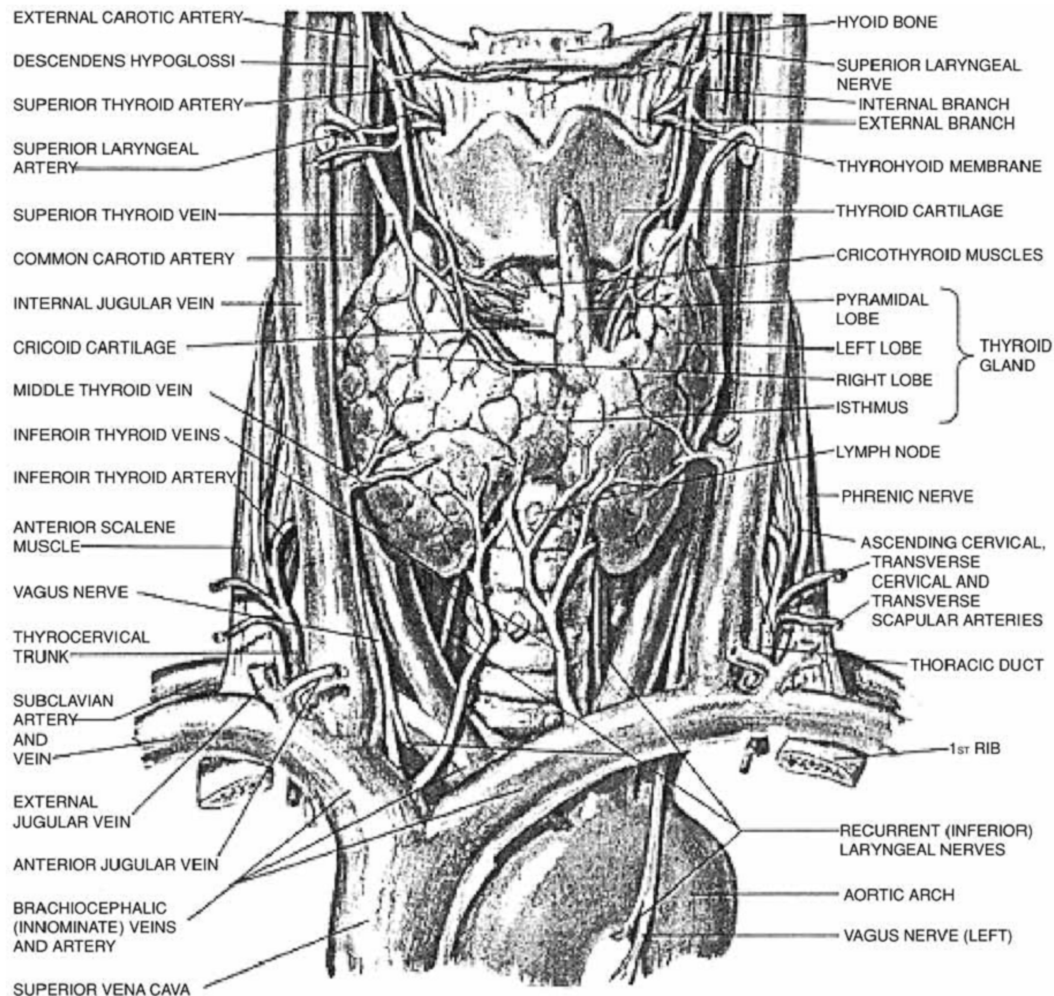

Figure 3: Anatomy of the thyroid gland and surrounding structures (From Stathatos, 2006).

1.2.2. Histology

The thyroid follicle is the main histological and functional unit of the thyroid gland (Figure 4) (Stathatos, 2012). Each follicle consists of a single layer of secretory epithelial cells known as the thyroid follicular cells (TFCs) or thyrocytes surrounded by a basement membrane. The lumen of each thyroid follicle is filled with a homogenous colloid, mostly formed of thyroglobulin (Tg), a macromolecular glycoprotein that functions as a scaffold for the synthesis of THs. A significant variation in the size of follicles within the same thyroid gland is attributed to the variability of the colloid content within the different follicles. In addition to TFCs, the parafollicular or C cells (calcitonin-producing cells which participate in calcium regulation) are found in between the follicles (Stathatos, 2006; 2012).

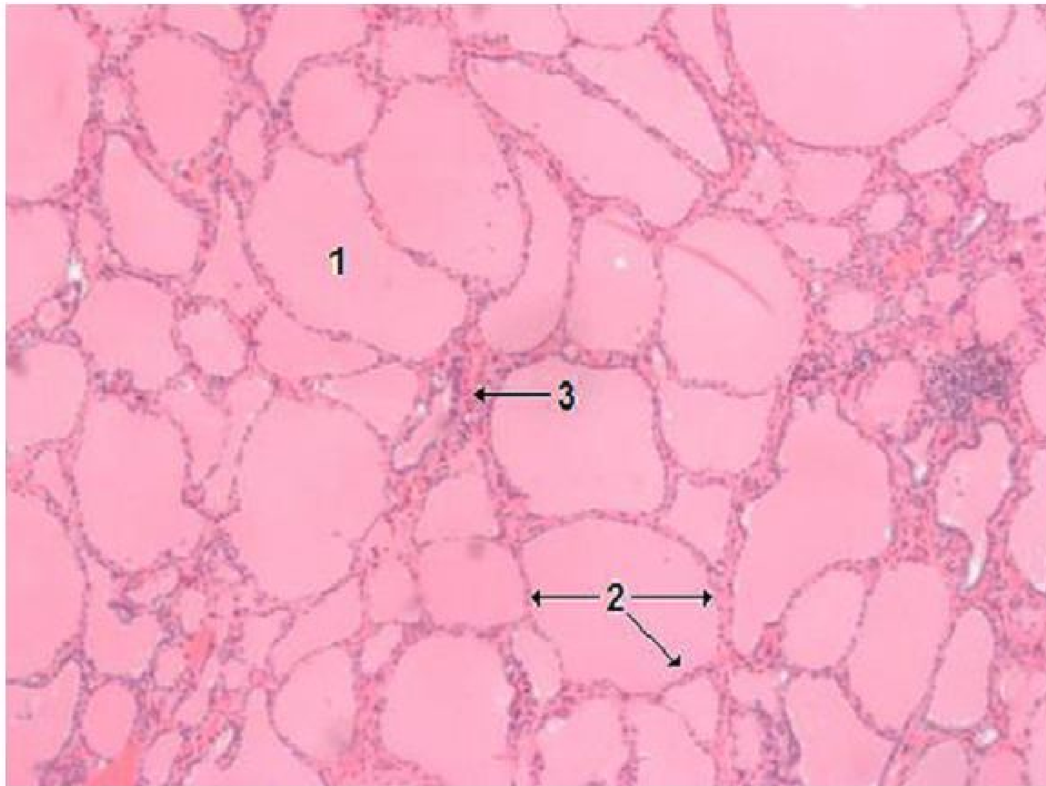

Figure 4: Histology of thyroid gland; (1) Thyroid follicle, (2) Follicular cells, (3) Parafollicular (C cells) (From Stathatos, 2012).

1.3. Physiology

1.3.1. Synthesis and secretion of THs

Synthesis of thyroid hormones (THs) takes place in the thyroid follicles. It requires a normally developed thyroid gland, sufficient dietary iodide intake, as well as a set of successive biochemical steps (Figure 5) (Bizhanova and Kopp, 2009). To be able to synthesize THs, thyrocytes have to trap iodide from the blood, at their basolateral membrane, in an active process against a concentration gradient. Such an energy consuming process is mediated by the Na^+/I^- symporter (NIS), which transports two Na^+ and one I^- down the Na^+ ion gradient generated from the activity of Na^+/K^+-adenosine triphosphatase (Na^+/K^+ ATPase). The NIS-mediated iodide transport increases its concentration in thyrocytes 20- to 40-fold compared to that in serum (Stathatos, 2012). The release of the trapped iodide in the lumen across the apical membrane is mediated by an iodide channel, the candidate of which is the anion transporter pendrin (Song *et al.*, 2007). However, the elaborated function of pendrin and its role as an apical iodide

transporter is still a matter of debate and the reported data are controversial (Bizhanova and Kopp, 2011; Twyffels *et al.*, 2011).

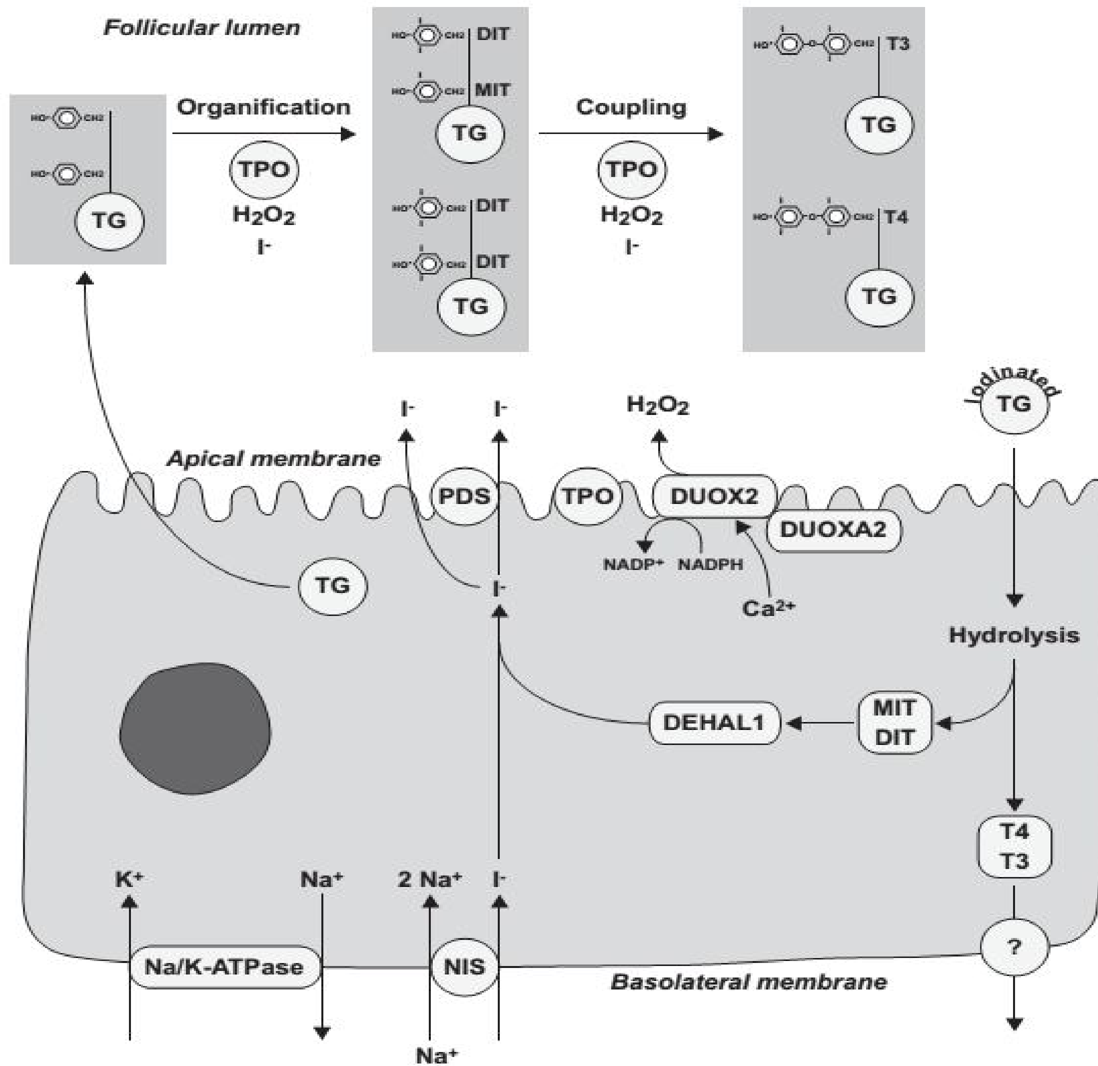

Figure 5: Thyroid hormone synthesis in thyrocytes (From Bizhanova and Kopp, 2009).

At the cell-lumen boundary and in the presence of hydrogen peroxide (H_2O_2), thyroid peroxidase (TPO) catalyzes the organification of iodide via its coupling to selective tyrosyl residues in thyroglobulin (Tg) leading to the formation of mono- and diiodotyrosines (MIT and DIT). Coupled to TPO at the apical membrane is the dual oxidase type 2 (DUOX2), originally known as thyroid oxidase 2 (THOX2), a calcium- and reduced nicotineamide adenine dinucleotide phosphate (Ca^{2+}/NADPH)-dependent oxidase, required for the production of H_2O_2.

DUOXA2 is a specific maturation factor required by DUOX2 for the proper translocation of the DUOX2/DUOX2A complex from the endoplasmic reticulum (ER) to the apical plasma membrane of thyrocytes where H_2O_2 is produced (Ohye and Sugawara, 2010). In humans and many other species, generation of H_2O_2 as well as binding of oxidized iodide to tyrosine residues of Tg and formation of THs are activated by the TSH-dependent phospholipase C-Ca^{2+}-diacylglycerol (DAG) pathway and inhibited by cyclic adenosine monophosphate (cAMP) signalling cascades (Song *et al.*, 2007). In addition, the generation of H_2O_2 is inhibited at high concentrations of iodide (the Wolff-Chaikoff effect) (Wolff and Chaikoff, 1948) via the iodinated lipid, 2-iodohexadecanal (IHDA) (Corvilain *et al.*, 1988; Panneels *et al.*, 1994). In many vertebrate systems, H_2O_2 activates signal transduction pathways downstream of insulin and growth factors (Rhee *et al.*, 2005) and it enhances proliferation in various mammalian cells at physiological levels (Stone, 2004). However, a signalling role of H_2O_2 in thyrocytes has not been yet elucidated, but it can be deduced from studies carried on other cells (Song *et al.*, 2007). In comparison with the amounts of iodide incorporated into proteins, H_2O_2 is largely produced in excess within the thyroid cells (Song *et al.*, 2007). Similar to what is observed in other cell types, H_2O_2 exerts toxic effects on thyroid cells *in vitro* that are potentially mutagenic if not properly repaired: at concentrations lower and higher than 0.1 mm, H_2O_2 induces DNA single- and double-strand breaks, respectively (Mondello *et al.*, 2002; Chico Galdo *et al.*, 2006). Moreover, apoptosis and necrosis are among the effects of high H_2O_2 levels on thyrocytes (Riou *et al.*, 1999; Demelash *et al.*, 2004). *In vivo*, an H_2O_2-induced mutagenesis in human thyroid cells is substantially supported (Maier *et al.*, 2006; Song *et al.*, 2007). Consequently, the thyroid cells exhibit a number of defense mechanisms against the generated H_2O_2 among which is the stringent separation of both the iodination system, located at the apical membrane of the cell, and the interior of the cell (Song *et al.*, 2007).

In addition to the formation of MIT and DIT, TPO catalyzes the coupling of two residues of DIT or one DIT and MIT to form either T_4 or T_3, respectively. Iodinated Tg is stored in the follicular lumen until needed (Bizhanova and Kopp, 2009). For THs to be released, iodinated Tg is reabsorbed into follicular cells by pinocytosis. Subsequent lysosomal digestion leads to the release of T_4 and T_3 via the basolateral membrane into the bloodstream through an unknown mechanism. Deiodination of the unused MIT and DIT is mediated via iodotyrosine dehalogenase

1 (DEHAL1) and the released iodide is recycled for further synthesis of THs (Bizhanova and Kopp, 2009).

1.3.2. Transport of THs

The majority (99 %) of the T_4 and T_3 released into the plasma remains inactive by being bound to carrier proteins, mainly thyroxine-binding globulin (TBG; 70-80 %) and what is left to thyroid-binding prealbumin (transthyretin) and albumin. Hence, less than 1 % of the total plasma THs are in a free state. The effects of THs on peripheral tissues are exerted by these biologically active free T_4 (fT_4) and T_3 (fT_3) hormones. Several other serum proteins, in particular high density lipoproteins, bind T_4 and T_3 (Stathatos, 2012).

In order to be peripherally metabolized and to exert their intracellular genomic actions, THs have to be firstly transported across the plasma membrane (Visser *et al.*, 2011). Earlier, it had been presumed that THs are transferred across the phospholipid bilayer by simple diffusion owing to their lipophilic structure. However, several experimental lines of evidence have pointed out the role of specific transporters in the process of THs' uptake by target cells (Krenning *et al.*, 1981; Hennemann *et al.*, 1986; Krenning *et al.*, 1988), among which are those studies showing that, in certain cells, the cellular uptake of THs is energy- and Na^+-dependent (Hennemann *et al.*, 2001). Such observations have been reinforced by the identification of the foremost THs transporters, the organic anion transporter subtypes oatp2 and oatp3 (Abe *et al.*, 1998). Later, various transporters of iodothyronine derivatives have been identified, including the Na^+/ taurocholate cotransporting polypeptide (NTCP) (Friesema *et al.*, 1999; Visser *et al.*, 2010), members of the organic anion-transporting polypeptide (OATP) family including the specific THs transporter OATP1C1 (Friesema *et al.*, 1999; Hagenbuch and Meier, 2003; Hagenbuch, 2007; van der Deure *et al.*, 2010), the L-type amino acid transporters (LATs) 1 (LAT1) and 2 (LAT2) which belong to the heterodimeric amino acid transporter (HAT) family (Friesema *et al.*, 2001; Jansen *et al.*, 2005), as well as various members of the monocarboxylate transporter (MCT) family among which is the specific THs transporter MCT8 and the aromatic amino acid transporter MCT10 (Friesema *et al.*, 2003; Visser *et al.*, 2007; van der Deure *et al.*, 2010). While a number of plasma membrane transporters involved in the intracellular uptake of THs have been identified, those incorporated in the translocation of THs across the membranes surrounding

either the mitochondria or the nucleus (if required), where the biologically active TH (T_3) mediates its effects on transcription, are yet unidentified (Visser *et al.*, 2011).

1.3.3. Peripheral metabolism of THs

The major TH secreted by the thyroid gland is the prohormone thyroxine (T_4), with only a minor amount of T_3 (less than 10% of the total blood TH) being secreted under normal conditions. The majority of T_4 is converted to the biologically active 3,5,3′-triiodothyronine (T_3). Hence, the majority of circulating T_3 is derived from the secreted T_4 instead of from the thyroid itself (Kirsten, 2000).

The conversion of the long-lived less active T_4 to the short-lived more active T_3 occurs via the outer (phenolic) ring 5′-deiodination (ORD) of T_4, a process catalyzed by two selenoprotein iodothyronine deiodinases (Ds) types 1 (D1) and 2 (D2) (Darras and Van Herck, 2012) (Figure 6). The deiodinase D1 is expressed in the thyroid, liver, and kidney and is known to be responsible for the conversion of the majority of T_4 to T_3 in circulation (Gereben *et al.*, 2008a; Mebis and van den Berghe, 2009). D2 is expressed in brain, pituitary, brown adipose tissue (BAT), skeletal muscle, and thyroid and is responsible for the local conversion of T_4 to T_3 (Gereben *et al.*, 2008a; Mebis and van den Berghe, 2009).

On the other hand, type 3 deiodinase (D3), expressed mainly in placenta, brain, skin, and several fetal tissues, is considered to be the main T_3 and T_4 inactivating deiodinase (Gereben *et al.*, 2008a; Mebis and van den Berghe, 2009). Through inner (tyrosyl) ring 5-deiodination (IRD), it catalyzes the transition of both T_3 and T_4 to their inactive metabolites 3,3′-diiodothyronine (T_2) and reverse T_3 (rT_3), respectively (Figure 6) (Darras and Van Herck, 2012). Owing to its broader substrate specificity, D1 is also capable of terminating the action of T_3 and prohibiting the activation of T_4 by converting them into their inactive metabolites via IRD (Darras and Van Herck, 2012).

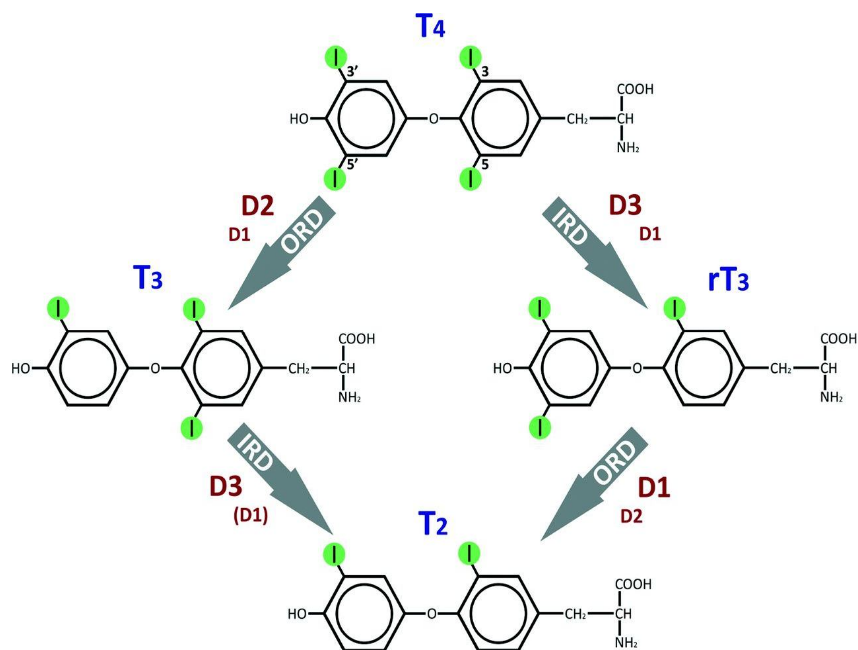

Figure 6: Deiodinase-mediated activation or inactivation of T_4 and T_3 (From Darras and Van Herck, 2012).

Integration of the activities of the iodothyronine deiodinases plays an important role in maintaining concentrations of serum T_3: Bianco et al. have pointed out to the role of the mutual changes in the activity of the main activating (D2) and inactivating (D3) deiodinases in maintaining thyroid gland homeostasis in response to variations in plasma T_4 and T_3 concentrations (Bianco *et al.*, 2002). Altered thyroid hormone metabolism related to abnormalities in iodothyronine deiodinases has been observed in a number of clinical conditions including critical illness, referred to as non-thyroidal illness or low T_3 syndrome. It is associated with lower levels of circulating T_3 and even T_4 in severe cases, as well as increased levels of rT_3 and thyrotropin (TSH) levels that are within the normal range (Mebis and van den Berghe, 2009).

In addition to deiodination, other alternative metabolic pathways of THs include conjugation of the phenolic hydroxyl group of the iododthyronines with sulfate (sulfation) or glucuronic acid (glucuronidation) (Wu *et al.*, 2005): Sulfation of T_4 completely blocks its ORD, while it robustly enhances the IRD of both T_3 and T_4. On the other hand, sulfated T_3 (T_3S) can act as a reservoir for the biologically active T_3 that is recovered via the action of tissue sulfatase (Wu *et al.*, 2005). The glucuronidated iodothyronines are excreted in bile and then eliminated through fecal excretion or recycled in the enterohepatic cycle (Wu *et al.*, 2005). Oxidative

deamination of the alanine side-chain of T_3 and T_4 leads to the formation of the acetic acid derivatives, 3,3′,5-triiodothyroacetic acid (triac) and tetraiodothyroacetic acid (tetrac), respectively (Wu *et al.*, 2005). The acetic acid derivatives of THs are mainly metabolized via deiodination as well as by conjugation (sulfation and glucuronidation) that might be followed by monodeiodination (Wu *et al.*, 2005). In addition, the endogenous biologically active thyronamines (TAMs), namely 3-iodothyronamine (3-T_1AM) and thyronamine (T_0AM), are derived from the iodothyronine precursors via decarboxylation of their alanine side-chain. Recently, it has been shown that, in addition to decarboxylation, the biosynthesis of TAMs involves both phenolic and tyrosyl rings deiodination mediated by D1 or D2 (phenolic) as well as D1 or D3 (tyrosyl) deiodinases (Gereben *et al.*, 2008b; Piehl *et al.*, 2011). While the physiological effects exerted by TAMs were found to be opposite to those of THs, it has been suggested that TAMs can either refine or act as antagonists of THs (Gereben *et al.*, 2008b; Piehl *et al.*, 2011). Finally, ether link cleavage (ELC) is considered to be a minor metabolic pathway of THs, the importance of which is illustrated during infections as it exhibits bacterial killing activity (Wu *et al.*, 2005).

1.3.4. Control of THs synthesis and secretion

The plasma levels of THs are under the regulation of the hypothalamic-pituitary-thyroid (HPT) axis (Stathatos, 2012). At low serum levels of T_4 and T_3, the heterodimeric glycoprotein thyrotropin or thyroid-stimulating hormone (TSH) is released from the anterior pituitary thyrotropes. TSH is considered to be the key regulatory factor controlling the synthesis and secretion of THs. The release of TSH itself is under the regulation of the thyrotropin-releasing hormone (TRH) secreted from the hypothalamus. The actions of TSH are mediated via the TSH-receptor (TSHR), a seven-transmembrane G protein-coupled receptor located at the basolateral membrane of the TFCs (Stathatos, 2012). Binding of TSH to its receptor leads to the activation of the enzyme adenylate cyclase (AC) via an activated Gs protein. The consequent increase in cAMP intracellular levels together with the activation of cAMP-dependent protein kinase A (PKA) mediate the TSH-dependent synthesis of THs as well as the development of TFCs (Yen, 2001; Rivas and Santisteban, 2003; Bursuk, 2012; Stathatos, 2012). Nearly every step along the process of THs' synthesis and secretion is stimulated by TSH. TSH stimulates the synthesis of Na^+/I^- symporter, thyroid peroxidase, and thyroglobulin, which are involved in iodide uptake,

organification, and formation of iodinated Tg, respectively. As well, TSH stimulates the generation of H_2O_2 via the activation of PLC-Ca^{2+} / DAG cascade, a key control stage in the synthesis process. Moreover, TSH stimulates the internalization of Tg by TFCs, its degradation, and the subsequent release of THs into the blood circulation. Hence, TSH assures the sufficient uptake of iodine by the TFCs and its efficient release in the circulation as THs (Dunn and Dunn, 2001). On the other hand, high plasma levels of THs exert a negative feedback action on the HPT axis leading to the inhibition of TSH secretion and the concomitant decrease in synthesis and secretion of THs (Stathatos, 2012). The mechanism leading to the inhibition of TSH secretion involves the type 2 deiodinase (D2)-mediated conversion of T_4 to T_3 in the pituitary (Yen, 2001). Moreover, TSH production is negatively regulated, either directly or indirectly, via THs. Direct negative regulation of TSH occurs by TH-mediated decrease in transcription of the glycoprotein hormone α- and TSH β-subunit genes (Yen, 2001). Similarly, THs negatively regulate the transcription of TRH which leads at last to the decrease in transcription of TSH mRNA (Hulbert, 2000; Yen, 2001).

The other major regulator of THs' synthesis and secretion, in addition to TSH, is iodine availability. An inverse relationship between the amounts of iodine available within the TFCs and the synthesis of THs is observed: inadequate amounts of iodine lead to increased TSH stimulation and uptake of iodine, faster iodine turnover, and increased production of T_3 relative to T_4. However, excess amounts of iodine hinder TPO activity by inhibiting the production of H_2O_2 (the Wolff-Chaikoff effect) (Wolff and Chaikoff, 1948), thus blocking Tg iodination and finally inhibiting the synthesis of THs (Dunn and Dunn, 2001; Stathatos, 2012). In addition, tissue-specific as well as hormone-dependent regulation of expression of the THs' metabolic enzymes, the three iodothyronine deiodinases, represent an additional layer of regulation exerted on the synthesis and secretion of THs (Santisteban and Bernal, 2005).

1.3.5. Actions of THs

Thyroid hormones play critical roles in regulating a large number of body functions including growth, differentiation, and metabolism. The physiological effects of THs affects nearly all tissues (Table II) (Kirsten, 2000).

Table II: Physiologic effects of thyroid hormones (modified from Kristen, 2000).

Tissue/Process	Effects
Bone	- Promote bone formation during growth - Regulate bone turnover in adults
CNS	- Play crucial role in fetal brain development and maturation: • Regulate migration and differentiation of neural cells, myelination, and synapse formation - Indispensable for normal mental development in infants - Regulate cognition, memory, and emotions in adults
Heart	- Increase cardiac output - Enhance cardiac contractility - Modulation of membrane ion channels - Increase heart rate
Hematopoietosis	- Direct regulation of cell population growth and apoptosis of human hematopoietic cells
Gastrointestinal tract (GIT)	- Regulate gastric and intestinal functions - Regulate gastric mobility
Lung	- Involved in regulation of fetal lung development • Control synthesis of lung surfactant
Metabolism	- Increase oxygen consumption and body heat production - Regulate synthesis and degradation of glucose, lipids, and proteins
Skin	- Stimulate epidermis, dermis, and hair - Play role in cutaneous wound repair

1.3.5.1. Genomic actions of THs

In target tissues, the intracellular genomic effects of the THs are elicited by their specific binding, with high affinity, to the nuclear thyroid hormone receptors (TRs), the two major isoforms of which are TRα and TRβ. TRα and TRβ are members of a large superfamily of nuclear hormone receptors (steriod/thyroid hormone receptor superfamily) that mediate ligand-dependent transcriptional regulation subsequent to recognition and binding to specific DNA sequences, the TH response elements or TREs, located at the regulatory regions of THs' responsive genes (Yen, 2001; Huang *et al.*, 2008). Through alternative splicing, the TRα gene encodes several proteins, among which TRα-1 is the only authentic TR while the others may act as inhibitors. On the other hand, the two isoforms of TRβ, TRβ-1 and TRβ-2, are generated as a

result of alternate promoter choice. Both TRβ-1 and TRβ-2 are authentic TRs that can mediate TH-mediated transcriptional regulation (Yen, 2001; Huang *et al.*, 2008).

TRs are capable of binding to TREs in the form of monomers or homodimers, the role of which in the transcriptional regulation is not clearly understood (Yen, 2001; Viguerie and Langin, 2003; Huang *et al.*, 2008). However, heterodimerization with TR auxiliary proteins (TRAPs), mainly retinoid X receptors (RXRs), leads to enhancement of TR binding to TREs (Yen, 2001). In addition, interactions with other nuclear proteins, such as coactivators or corepressors, are involved in either transcriptional activation or repression of basal transcription, respectively (Yen, 2001; Viguerie and Langin, 2003; Huang *et al.*, 2008). Upon binding of the natural ligand of TR, T_3, the TR/RXR heterodimer undergoes conformational changes that ultimately lead to the substitution of a corepressor complex by a coactivator one. The transcriptionally active coactivator complex bears a histone acetyl transferase (HAT) activity that eventually leads to an accessible chromatin structure and hence a marked increase of gene transcription above its basal level (Viguerie and Langin, 2003). In the absence of ligand, the TR/RXR heterodimer interacts with a corepressor complex having either a histone deacetylase activity (HDAC) or able to interact directly with the basal transcriptional machinery thus leading to repression of transcription (Viguerie and Langin, 2003). In addition to the ligand-dependent modulation of TRs' transcriptional activity, it has been shown that phosphorylation of TRs regulates their transcriptional activity by altering DNA binding ability as well as tissue-specific stability (Yen, 2001; Chen *et al.*, 2003; Huang *et al.*, 2008).

1.3.5.2. Nongenomic (extranuclear) actions of THs

Although the majority of the actions exerted by the THs involve the nuclear TRs (genomic actions), a number of non-genomic actions, not directly influencing nuclear gene expression, have been described (Bassett *et al.*, 2003; Cheng *et al.*, 2010; Davis *et al.*, 2011). Such nongenomic actions are initiated at the plasma membrane, in the cytoplasm, or in intracellular organelles, such as the mitochondria (Cheng *et al.*, 2010; Davis *et al.*, 2011). Moreover, the nongenomic actions of THs are mediated via either extranuclear TRs or cell surface receptors (certain integrins) and they are associated with the release of intracellular secondary messengers and the activation of a number of protein kinase signalling pathways (Bassett *et al.*, 2003; Davis *et al.*, 2011). In fact, certain alterations in gene transcription might

occur due to effects of THs initiated at the plasma membrane integrin receptor (Davis *et al.*, 2011). Moreover, other nongenomic actions of T3, involving extranuclear TR isoforms, can start in the cytoplasm and ends with gene transcription (Moeller *et al.*, 2006; Lei *et al.*, 2008). Hence, it is the site of initiation that differentiates between genomic and nongenomic actions of THs rather than whether or not altered gene expression occurs (Davis *et al.*, 2011). In contrast to the genomic actions of THs, the nongenomic ones are characterized by being rapid (occur within seconds to minutes), unaffected by inhibitors of both transcription and translation, and exhibit agonist/antagonist affinity different from those of classical TRs (Bassett *et al.*, 2003).

Among the nongenomic actions of THs occurring at the plasma membrane is the regulation of the basal activity of a number of plasma membrane ion pumps. Such an action is mediated via a TH cell surface receptor found on integrin αvβ3 and leads to the activation of the mitogen activated protein kinase (MAPK) signal transduction cascade (Davis *et al.*, 2005; Davis *et al.*, 2010). Furthermore, other non-genomic actions induced mainly by T_4 and to a lesser extent by T_3 include nuclear translocation of TRβ1 and those resulting in the activation of gene transcription leading to the modulation of angiogenesis and tumor cell proliferation (Davis *et al.*, 2009; Cheng *et al.*, 2010; Davis *et al.*, 2011). Moreover, trafficking of intracellular proteins (shuttling of TRα1 as well as several other proteins from cytoplasm to nucleus) and the transcription of genes involved in glucose metabolism are T_3-induced nongenomic actions mediated via the plasma membrane receptor on integrin αvβ3T3 and involves the activation of the signal transducing protein phosphatidyl Iinositol 3-Kinase (PI3K) (Cheng *et al.*, 2010). At the TH receptor on integrin αvβ3, both the binding and actions of THs is blocked by the deaminated derivative of T_4, tetraiodothyroacetic acid (tetrac), at both the T_4/T_3 site and the T_3-specific site (Cheng *et al.*, 2010; Lin *et al.*, 2011).

In the cytoplasm, interactions between T_3 and the extranuclear TRs isoforms, TRβ1 and TRα1, involve the activation of the signal transducing protein PI3K as is observed in the plasma membrane. Consequently, transcription of genes involved in glucose metabolism as well as plasma membrane insertion and activation of Na^+,K^+-ATPase. In addition, it was reported that a truncated form of TRα1 (TRΔα1) mediated the T_4 and rT_3-induced regulation of actin polymerization (Cheng *et al.*, 2010).

In the mitochondria, it has been shown that the extranuclear effect of T_3, leading to the stimulation of global mitochondrial gene expression, is mediated via the truncated forms of both TRα1 and TRβ1. Such a finding points to the coordination between nuclear and extranuclear actions of T_3 in regulating the mitochondrial transcriptional apparatus since at least two TH-dependent general mitochondrial transcription factors, the thermogenic PPAR γ coactivator-1 (PGC-1) and the mitochondrial transcription factor A, are encoded by the nuclear genome (Goglia *et al.*, 1999; Cheng *et al.*, 2010). Moreover, it has been shown that 3,5- diiodothyronine (T_2) is an active iodothyronine as it exhibits direct impacts on the energy transduction apparatus by binding with some components of the respiratory chain (Goglia *et al.*, 1999; Moreno *et al.*, 2008b).

2. Congenital Hypothyroidism (CH)

2.1. Definition and classification

Deficiency of thyroid hormones (THs) at birth is referred to as congenital hypothyroidism (CH) (Van Vliet and Deladoëy, 2012). CH diagnosed because of the observation of clinical manifestations (before the neonatal screening era) showed an incidence of 1 in 6,100 to 1 in 6,900 (Grosse and Van Vliet, 2011). However, owing to the introduction of the neonatal screening programs for CH, the incidence of CH has increased to up to 1:2,500-1:4,000 newborns in iodine-sufficient regions worldwide, thus becoming the most common neonatal endocrine disorder (Toublanc, 1992; Deladoëy *et al.*, 2011). Due to the neonatal screening programs and early treatment, CH is considered to be one of the most common preventable causes of mental retardation (Klein and Mitchell, 1996; Grosse and Van Vliet, 2011). However, subtle lasting intellectual deficits were still observed among adolescents and young adults in spite of early high-dose initial treatment. The median age at treatment was 9 days and the median starting dose of levothyroxine was 14.7 µg/kg/d. A low performance intelligence quotient (IQ) was observed especially among severe cases (CH due to athyreosis) showing an IQ loss of 10 points compared to controls (Dimitropoulos *et al.*, 2009; Hauri-Hohl *et al.*, 2011).

According to the persistence of THs deficiency observed at birth, CH is classified into either **permanent**, with lasting deficiency that requires lifelong replacement therapy, or **transient** where the deficiency reported at birth is recovered to normal within the first few weeks

to months after birth (Szinnai, 2013). In 60-85% of cases, permanent CH is due to developmental abnormalities of the thyroid gland collectively known as thyroid dysgenesis (TD: OMIM 218700) (Deladoëy *et al.*, 2011), while it is due to recessively inherited inborn errors of thyroid hormone synthesis or dyshormonogenesis in 10-15% of patients (TDHG; OMIM 274400–274900), both entities represent permanent primary CH characterized by high TSH levels corresponding to the extent of TH deficiency (Knobel and Medeiros-Neto, 2003; Moreno *et al.*, 2003; Van Vliet and Deladoëy, 2012). The molecular mechanisms underlying **permanent primary CH** will be discussed in detail in following sections.

Permanent central CH (CCH) is rare with an estimated incidence of around 1:20,000 (van Tijn *et al.*, 2005). It is due to defects within either the pituitary gland (secondary) or the hypothalamus (tertiary) resulting in deficiency of TSH or TRH, respectively in the presence of low levels of THs (Park and Chatterjee, 2005; Van Vliet and Deladoëy, 2012). CCH is usually associated with deficiencies in other pituitary hormones in addition to TSH deficiency causing combined pituitary hormone deficiency (van Tijn *et al.*, 2005). Mutations in genes encoding the pituitary transcription factors HESX1 (Dattani *et al.*, 1998; Thomas *et al.*, 2001), LHX3 (Netchine *et al.*, 2000), LHX4 (Machinis *et al.*, 2001), PIT1 (Tatsumi *et al.*, 1992) and PROP1 (Wu *et al.*, 1998) have been involved in the pathogenesis of familial combined pituitary hormone deficiency, with or without other syndromic features (Kelberman and Dattani, 2007). More rarely, isolated CCH occurs due to mutations in either the TSH β subunit gene (Hayashizaki *et al.*, 1989; Doeker *et al.*, 1998) or those in the TRH receptor gene (Collu *et al.*, 1997) resulting in isolated TSH deficiency and TRH resistance, respectively. Recently, loss-of-function mutations in immunoglobulin superfamily member 1 (*IGSF1*), a gene encoding a plasma membrane immunoglobulin superfamily glycoprotein, have been reported in an X-linked syndrome of central hypothyroidism and testicular enlargement. The resulting central hypothyroidism occurred secondary to impaired TRH receptor signalling (Sun *et al.*, 2012). In addition, novel *IGSF1* mutations have been also identified in male patients with CCH and variable prolactin (PRL) deficiency (Nakamura *et al.*, 2013).

Defects in either transport, metabolism, or action of THs are categorized as **peripheral CH**. Thyroid hormone cell membrane transport defects (THCMTD), thyroid hormone metabolism defects (THMD), and resistance to thyroid hormones (RTHs), all lead to reduced sensitivity to TH or RSTH (Dumitrescu and Refetoff, 2013). Mutations in *MCT8* (*SLC16A2*), the

gene encoding the transmembrane TH transporter monocarboxylate transporter 8 (MCT8), were reported in male patients with the X-linked mental retardation Allan-Herndon-Dudley syndrome. The mutations are associated with severe X-linked psychomotor retardation and high serum T_3 concentrations with low to normal T_4. The neurological abnormalities are due to the impaired entry of T_3 in the neurons via MCT8, resultin in defective T_3 action and metabolism, and eventually abnormal brain development (Dumitrescu *et al.*, 2004; Friesema *et al.*, 2004). In addition, patients with inherited TH metabolism defects (THMD) due to mutations in the selenocysteine insertion sequence (SECIS) binding protein 2 (*SECISBP2* or *SBP2*) gene have been reported. Homozygous or compound heterozygous *SBP2* gene mutations leading to partial SBP2 deficiency and a relatively mild phenotype have been described in patients with transient growth retardation together with abnormal thyroid function tests, low T_3, high T_4 and reverse T_3, as well as slightly elevated TSH. The partial SBP2 deficiency affects the expression of a subset of selenoproteins, among which is the iodothyronine deiodinases type 1 (D1) (Dumitrescu *et al.*, 2005; Di Cosmo *et al.*, 2009; Hamajima *et al.*, 2012). However, a more complex phenotype was reported due to *SBP2* gene mutations leading to severe SBP2 deficiency resulting in reduced synthesis of the majority of the known human selenoproteins (Azevedo *et al.*, 2010; Schoenmakers *et al.*, 2010). RTH, commonly undetected by neonatal screening, occurs mainly due to dominantly inherited mutations in the *TRβ* gene. Mostly, the affected individuals are euthyroid, although some hypothyroid individuals have been described. High serum levels of T_3, T_4, and rT_3 are reported without suppression of TSH (Weiss *et al.*, 1993; Adams *et al.*, 1994; Collingwood *et al.*, 1998). Non TR-RTH has been reported in 15% of families with features of RTH but no mutations in the *TRβ* gene (Sadow *et al.*, 2000). Non TR-RTH is neither clinically nor biochemically variable from RTH due to *TRβ* gene mutations (Weiss *et al.*, 1996). Recently, RTH due to mutations in the *TRα* gene has been reported. Unlike RTH with or without *TRβ* gene mutations, the thyroid function tests in the reported cases included low serum T_4, high T_3, and very low rT_3 (Bochukova *et al.*, 2012; van Mullem *et al.*, 2012).

Transient CH can be due to maternal or neonatal factors (Bhavani, 2011). Besides the endemic iodine deficiency seen in many countries (see map on iodine status worldwide from WHO; http://www.who.int/vmnis/iodine/status/summary/median_ui_2007_color.pdf?ua=1), other more common forms of transient primary CH particularly in North America and in Europe include: prenatal and postnatal iodine excess (Bartalena *et al.*, 2001; Markou *et al.*, 2001),

transplacental TSHR blocking antibodies (Brown *et al.*, 1996; Yang *et al.*, 2005), maternal antithyroid medications (Chan and Mandel, 2007), mono- and biallelic *DUOX2* gene mutations (Moreno *et al.*, 2002; Maruo *et al.*, 2008), and isolated hyperthyrotropinemia CH (Leonardi *et al.*, 2008; Zung *et al.*, 2010). Transient central CH, associated with low T_4 concentrations and a low or normal TSH, might occur due to untreated maternal hyperthyroidism leading to suppression of fetal TSH (Liebrand *et al.*, 2006), treatment of critically ill neonates using steroids and dopamine, withdrawal of transplacental T_4 transfer in premature infants (Bhavani, 2011), or isolated hypothyroxinemia prevalent in premature and very low birth weight (LBW) neonates (Williams and Hume, 2011).

2.2. Epidemiology

As previously mentioned, the worldwide incidence of CH is 1:2,500-1:4,000 newborns (Toublanc, 1992; Deladoëy *et al.*, 2011). However, different rates of CH incidence have been reported among various populations which reflects the use of different protocols with different sensitivities. The incidence of CH was reported to be 1:1,800 in Lebanon and Thailand (Daher *et al.*, 2003; Panamonta *et al.*, 2003), 1:800 among newborns from the Greek Cypriot population over an 11 year period (Skordis *et al.*, 2005), 1:1,300 newborns in the Netherlands (Loeber, 2007), 1:2,500-1:2,800 live births in Indian population (Jain *et al.*, 2008), 1:357-1:1,465 in different areas of Iran (Karamizadeh *et al.*, 2011; Dalili *et al.*, 2012), and an incidence of 1:2,135-1:2,300 newborns in the Italian population (Olivieri *et al.*, 2013).

Several studies have shown that the incidence of CH varies depending on various factors among which are race and ethnicity. Recently, Harris *et al.* have shown that the incidence of CH is highest in the Asian population (1:1,016) followed by the Hispanic one (1:1,559), while a lower incidence was observed among both the White (1:1,815) and the American Black (1:1,902) populations (Harris and Pass, 2007). In accordance with the impact of ethnicity on the incidence of CH, Stoppa-Vaucher *et al.* have shown that, among various ethnic groups, a higher incidence of CH due to TD (CHTD) was observed in Caucasians compared to Blacks (Stoppa-Vaucher *et al.*, 2011). Actually, many other risk factors are known to influence the prevalence of CH among which are the birth weight and gestational age where CH is related to low birth weight (LBW) and postdate delivery (Dalili *et al.*, 2012).

In the United States, an increase in the global incidence of CH (including all underlying etiologies) has been reported over the past two decades (Harris and Pass, 2007; Olney *et al.*, 2010). The reported increased incidence has been attributed to the identification of more milder (transient) cases of CH and to demographic factors, including the increase in number of births among Hispanics and of LBW babies in the United States (Hinton *et al.*, 2010; Parks *et al.*, 2010). Moreover, it has been shown that a high incidence rate of CH in the United States is associated with twin (1:876) and multiple births (1:575) compared to singletons (1:1,765) and with advanced maternal age (1:1,328) compared to younger age (1:1,703) (Harris and Pass, 2007).

In Europe, changes in screening procedures by lowering TSH cutoff during initial screening (from 20 to 10 mU/liter), led to the increase in global incidence of CH consequent to the increased case detection, mainly of premature and LBW neonates (Corbetta *et al.*, 2009; Mengreli *et al.*, 2010). The impact of lowering TSH threshold during the second screening test (from 15 to 5 mU/liter) on the incidence of CH, both globally and according to etiologic subgroups, has been assessed in Quebec over a period of 20 yr (Deladoëy *et al.*, 2011). The identification of additional mild cases of CH (with thyroid gland *in situ*) rather than severe ones (TD and TDHG cases) was behind the observed increased incidence of CH. When weaning off treatment is tested, half of these mild CH cases (which can be defined as isolated hyperthyrotropinemias) are transient (Deladoëy *et al.*, 2011; Oren *et al.*, 2013).

2.3. Clinical presentations

Due to the relative non-specificity of the clinical signs and symptoms of hypothyroidism and because of the residual thyroid function documented in many cases, the majority of newborns with CH appear normal with no noticeable physical signs (Büyükgebiz, 2013). Prior to the introduction of the neonatal screening program, diagnosis of CH was also delayed since the overt clinical signs in the minority of symptomatic CH cases are of later onset, and evolve gradually. Consequently, belated diagnosis of CH leads to severe mental retardation due to the critical dependence of the developing brain on THs for the first two to three postnatal years of life (Zoeller and Rovet, 2004; Bernal, 2007). On the first day of birth, unexplained postmaturity and macrosomia with an open posterior fontanel can be observed (Van Vliet and Deladoëy, 2012). Other common clinical symptoms are prolonged jaundice, poor feeding, lethargy, and

constipation (Counts and Varma, 2009; Van Vliet and Deladoëy, 2012). Additional common signs include flat nasal bridge, macroglossia, abdominal distention, umbilical hernia, hypothermia, hypotonia, hoarse cry, dry and mottled skin, and myxedematous facial features. Moreover, clinically detectable goiter is observed in a few of the newborns (Van Vliet and Deladoëy, 2012). Immediate measurement of thyroid functions (serum TSH and free T_4) is mandatory in any infant exhibiting signs/symptoms of hypothyroidism, regardless the collection of blood specimen for biochemical screening (Jain *et al.*, 2008; Van Vliet and Deladoëy, 2012).

2.4. Diagnosis

2.4.1. Newborn screening (NBS)

In an attempt to ensure prompt diagnosis of CH and rapid institution of thyroid hormone replacement therapy, the newborn screening programs for CH were initiated in the 1970s after the development of an assaying method for thyroxine (T_4) from dried blood spots (Dussault and Laberge, 1973). This has been shown to effectively avoid damage to the brain and the development of later neurocognitive and intellectual abnormalities. The mean IQ of children with CH was 85 prior to the introduction of newborn screening where less than 20% of affected infants were diagnosed within three months after birth, whereas deficits in fine motor control and learning disabilities were observed in infants with normal IQ. However, upon application of the screening programs and the rapid onset of treatment, the average development of infants with CH was within the normal range. Subtle cognitive and health impairments may persist in the most severely affected cases (Van Vliet and Deladoëy, 2012). Thus, newborn screening for CH is considered to be one of the most effective newborn medical prevention procedures (Grosse and Van Vliet, 2011).

For newborn screening, a heel-prick blood sample, spotted on special filter paper cards, is obtained 48 h after birth (3-5 d of life). The timing of sampling is crucial to avoid false positives resulting from the physiologic neonatal TSH surge after birth (Büyükgebiz, 2013). However, earlier timing of sample collection (before 24 h) is reported in cases of term neonates with acute illness who need to be transferred to another hospital, infants who die, and in case of blood transfusion (Van Vliet and Czernichow, 2004; Van Vliet and Deladoëy, 2012). Between the second and sixth week of age, a second specimen is routinely obtained by many screening programs to detect cases with delayed either TSH or T_4 rise (LaFranchi, 2010; Salim and Varma,

2013). Both total T_4 (bound and unbound) and TSH can be measured accurately in blood specimens spotted on filter paper (Van Vliet and Deladoëy, 2012). In addition, screening for CH on cord blood has been used by some programs (Van Vliet and Czernichow, 2004). However, false negatives may result in the case of monozygotic twins due to fetal blood mixing between a hypothyroid and an euthyroid twin, thus repeat screening needs to be done automatically at 2 weeks of age for all same-sex twins (Perry *et al.*, 2002). Moreover, use of cord blood for mass screening turned out to be impracticable due to associated risks as well as difficulties in handling and transportation (Rastogi and LaFranchi, 2010; Büyükgebiz, 2013).

By the beginning of the newborn screening era, two main approaches were used: Most of the screening programs applied a primary T_4 test with a follow-up TSH if T_4 levels are below a selected cutoff while the others used a primary TSH test approach (Van Vliet and Deladoëy, 2012). In addition to primary hypothyroidism detection, a primary T_4 test is able to detect the rare CCH cases and those with low total T_4 associated with prematurity, T_4-binding globulin (TBG) deficiency or severe non-thyroidal illness (Van Vliet and Czernichow, 2004). However, as most children (up to 80%) with CCH have normal thyroid function at birth, the initial T4 screening also failed to diagnose CCH and has been abandoned in many jurisdictions (Nebesio *et al.*, 2010). Moreover, some infants with thyroid ectopy will be missed when screened with the primary T_4 approach due to their T_4 levels that might be still above the selected cutoff, but their TSH is almost steadily high (Van Vliet and Czernichow, 2004). On the other hand, the primary TSH approach is more specific for detection of mild/sub-clinical primary hypothyroidism in which T_4 is initially normal with elevated TSH (LaFranchi, 2010; Salim and Varma, 2013). Nevertheless, cases with peripheral hypothyroidism are not detected by either of the two approaches (Rastogi and LaFranchi, 2010). For all the classes of CH to be detected, the most sensitive combined T_4 and TSH measurement was used by some programs and showed a higher incidence of CH (LaFranchi, 2010; Rastogi and LaFranchi, 2010). Noticeably, the two screening approaches show an equal recall rate (0.05%) for primary hypothyroidism. However, the false positive rate is high in case of initial T_4 screening programs (Büyükgebiz, 2013). Nowadays, most of the screening programs worldwide use the primary TSH test approach due to both the high rates of false positives in initial T_4 tests and to the improved sensitivity of TSH assays (Van Vliet and Deladoëy, 2012; Salim and Varma, 2013). Some of the newborn screening programs, among which is the Quebec screening program, measure T_4 as a second step when the blood-spot

TSH value is slightly elevated (Van Vliet and Deladoëy, 2012). For the shift between programs, age-related TSH cutoffs need to be considered due to the postnatal TSH surge and the subsequent drop by the age of one week. Likewise, age-related TSH cutoffs need also to be considered in case of specimens collected before 48 h of age that are subjected to initial T_4 test with a follow-up TSH (LaFranchi, 2010).

It should be noted that the cutoff values for recalling infants with abnormal screening results are set according to each screening program (e.g. Quebec newborn screening program, Figure 7) (Deladoëy *et al.*, 2011). Usually, infants are recalled when the screening results of T_4 is below the 10th percentile and/or that of whole blood TSH is higher than 15 mU/L (Rastogi and LaFranchi, 2010; LaFranchi, 2011; Salim and Varma, 2013). It should be noted that lower TSH cutoffs at initial screening have been reported in Europe (Korada *et al.*, 2008; Corbetta *et al.*, 2009; Mengreli *et al.*, 2010) and for the second blood specimen in Quebec (Deladoëy *et al.*, 2011) as previously mentioned. Neonates with abnormal thyroid screening tests are recalled immediately (within two weeks of age) for further investigation, which involves measurement of serum TSH and free T_4 (Van Vliet and Deladoëy, 2012).

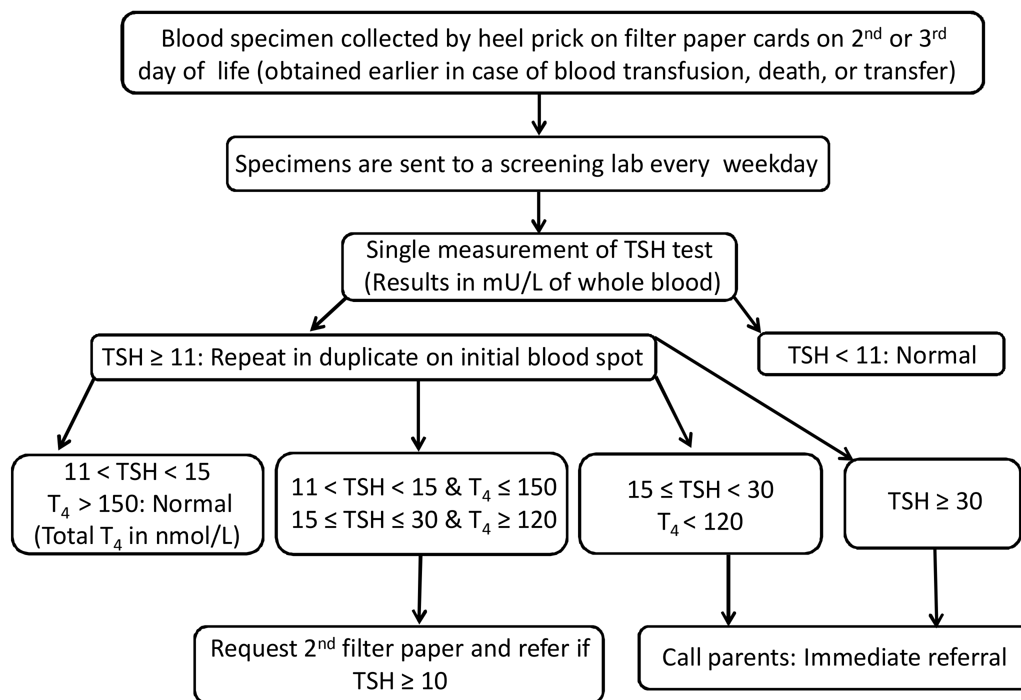

Figure 7: Screening algorithm for CH in Quebec (adapted from Deladoëy *et al.*, 2011).

2.4.2. Other diagnostic studies

Upon confirming the diagnosis of CH, further examinations should be performed to unravel the underlying etiology. In addition, these studies are also helpful in defining whether the established CH is transient or permanent. Moreover, they provide guidance in CH cases with borderline thyroid function test outcomes. It should be noted that the onset of treatment must not be delayed as it is independent of the findings of such additional investigations (Jain *et al.*, 2008; Van Vliet and Deladoëy, 2012).

Besides the confirmatory thyroid function tests (serum TSH and free T_4), the additional diagnostic studies undertaken are imaging techniques (thyroid scintigraphy and ultrasonography) that determine both the location and the size of the thyroid tissue. Newborn thyroid scintigraphy (scan), either with radioactive iodine (^{123}I) or ^{99m}Tc pertechnetate ($^{99m}TcO_4$), identifies the various forms of TD (athyreosis, hypoplasia, or ectopia) (Figure 8A) or a large gland with increased uptake consistent with dyshormonogenesis (Figure 8B) (Van Vliet, 2003; Jain *et al.*, 2008; Rastogi and LaFranchi, 2010; Van Vliet and Deladoëy, 2012). In the latter case, defects in iodide oxidation and organification can be identified using the perchlorate discharge test. Genetic studies can confirm such an inborn error in TH biosynthesis (LaFranchi, 2011). In infants with no thyroid tissue detected by scintigraphy, serum Tg should be measured: undetectable serum Tg concentration, reported in 50% of these infants, indicates true athyreosis. However, a hypoplastic and hypofunctional thyroid tissue is to be found in the rest of cases. If normally located, this hypoplastic thyroid gland occurs consequent to either TSHR-inactivating mutations associated with normal Tg levels (apparent athyreosis) or due to transplacental passage of maternal thyrotropin receptor blocking antibody (TRB-Ab) leading to a transient CH (Van Vliet and Deladoëy, 2012).

Thyroid scintigraphy is more sensitive than ultrasonography in detecting the position of the ectopic thyroid gland (Grüters and Krude, 2012; Van Vliet and Deladoëy, 2012). However, previous studies have pointed to the improved sensitivity of color Doppler ultrasonography (CDU) in diagnosing thyroid ectopy (Supakul *et al.*, 2012). By detecting an eutopic thyroid in the absence of thyroid gland uptake, the ultrasonography is able to differentate between true athyreosis and other situations associated with lack of thyroid uptake including TSHβ gene mutations, TSHR-inactivating mutations, iodide-trapping defects, and transplacental passage of

maternal thyrotropin receptor blocking antibody (TRB-Ab). In addition, ultrasonography can confirm the presence of an enlarged thyroid gland suggestive of dyshormonogenesis (LaFranchi, 2011).

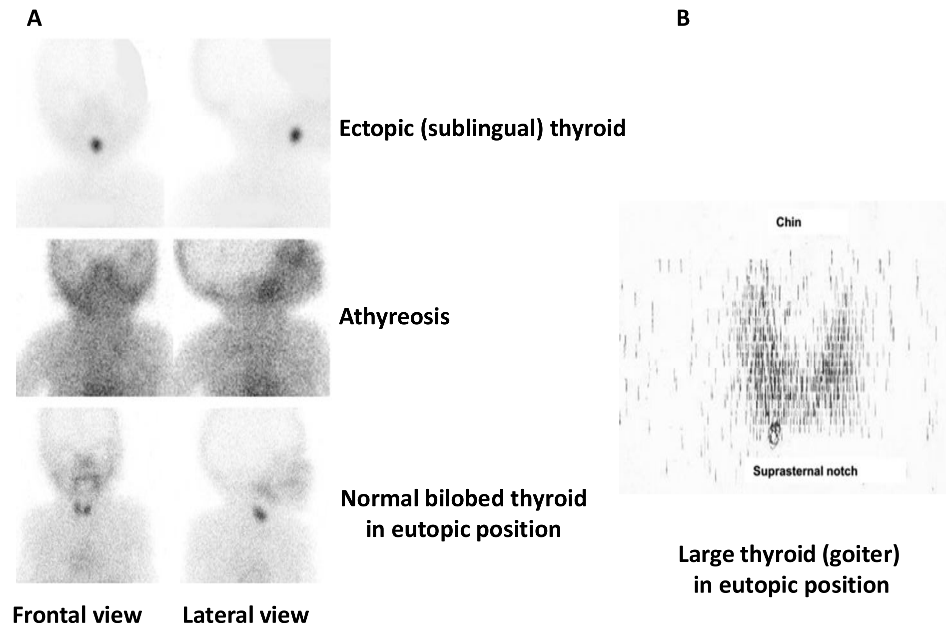

Figure 8: Thyroid scintigraphy. **8A:** Newborn thyroid scintigraphy, using ^{99m}Tc, showing examples of TD (modified from Van Vliet, 2003). **8B:** Tc-99m scan showing a large thyroid gland in eutopic position (modified from Rastogi and LaFranchi, 2010).

Born to a mother with autoimmune thyroid disease, infants exhibiting both absence of thyroid uptake and a hypoplastic or a normal-sized thyroid gland are highly likely to have CH due to transplacental passage of maternal TRB-Ab (Brown *et al.*, 1993; Pacaud *et al.*, 1995). Presence of TRB-Abs can be confirmed by the thyrotropin-binding inhibitor immunoglobulin (TBII) test (Rastogi and LaFranchi, 2010; LaFranchi, 2011).

To confirm either suspected iodine deficiency in a neonate with CH or excessive maternal iodine ingestion or neonatal iodine exposure, urinary iodine measurement will indicate either low or high levels of iodine, respectively, as it approximates iodine intake (LaFranchi, 2011). Of note, iodine deficiency is now extremely rare in industrialized countries but remains a public health issue in Africa and China (http://www.isns-neoscreening.org/).

Thyroid hormones (THs) are known to play a crucial role in normal bone growth. Hence, the degree of skeletal maturation (bone age) is among the parameters that evaluate the severity of CH and thus the eventual intellectual outcome (Wasniewska *et al.*, 2003). Deficiency of THs leads to retardation of bone maturation (BM) as evident by the absence of knee epiphyses on X-ray in newborns with CH (Van Vliet *et al.*, 2003). Previously, it has been shown that different degrees of BM retardation indicate different extents of thyroid hormone deficiency. In addition, retardation of BM is usually associated with athyreosis and a lower developmental quotient (DQ) during the first year of life, thus implying the importance of BM routine assessment among infants with CH before treatment initiation (Wasniewska *et al.*, 2003). Moreover, it has been shown that BM differentiates between neonatal transient hypothyroidism (NTH) and TDHG and evaluates the risk of the latter as well (Niu *et al.*, 2004).

Due to the low yield of the reported germline mutations, especially in case of TD (as will be discussed in later sections), testing for specific germline mutations associated with CH are probably confined to cases with a known family history or those presenting with suggestive presentations (Van Vliet and Deladoëy, 2012). Mutations of *NKX2.1*, the gene encoding thyroid transcription factor NKX2.1 are suggested in case of unexplained respiratory distress, hypotonia, and choreoathetosis (Krude *et al.*, 2002; Pohlenz *et al.*, 2002; Maquet *et al.*, 2009) whereas cleft palate and spiky hair encourage testing for *FOXE1* mutations (Bamforth *et al.*, 1989; Clifton-Bligh *et al.*, 1998; Castanet *et al.*, 2002; Baris *et al.*, 2006). In case of infants with isolated thyroid hypoplasia or apparent athyreosis, mutations in *PAX8* are suggested by a dominant pattern of inheritance (Macchia *et al.*, 1998; Al Taji *et al.*, 2007), while a recessive pattern points to TSH receptor (TSHR) mutations (Abramowicz *et al.*, 1997). On the other hand, it has been shown that sequence variants in *NKX2.5*, the gene encoding the thyroid-related transcription factor NKX2.5, are not disease-causing in a persuasive manner, unless probably combined with another genetic hit (Hermanns *et al.*, 2011). Analysis of *GLIS3* mutations are prompted in non-consanguineous pedigrees with neonatal diabetes, polycystic kidneys, glaucoma, hepatic fibrosis and exocrine pancreatic deficiency (Senée *et al.*, 2006; Dimitri *et al.*, 2011). Other genes (*SALL1*, *TBX1*, and *URB1*) are mutated in syndromes associated with generally mild thyroid dysfunction together with other dysmorphisms (Zenker *et al.*, 2005; Fagman *et al.*, 2007; Choi *et al.*, 2010). Finally, yet unknown genes are involved in some multiplex families (Castanet *et al.*, 2005).

2.5. Treatment

For the best cognitive outcome, treatment of infants with abnormal screening tests is started immediately as soon as the diagnosis is made and even before the results of confirmatory tests are obtained (Van Vliet and Deladoëy, 2012). In addition to the early onset of treatment, an adequate postnatal treatment is mandatory. During the first decade after the implementation of newborn screening programs, treatment was started at a T_4 doses of 5 to 6 µg/kg/day at a mean age of 20 to 35 days (Van Vliet and Deladoëy, 2012). However, it was noticed that treatment with these doses of T_4 was associated with a delayed bone maturation till the age of three years (Van Vliet G, 1989) and yet a lower IQ in severe cases (Derksen-Lubsen and Verkerk, 1996). Later, a higher initial dose of T_4, 10-15 g/kg/day, started within 2 weeks after birth was recommended (Van Vliet and Deladoëy, 2012). According to the severity of CH, it has been reported that infants with athyreosis usually need higher doses of T_4 compared with those with thyroid ectopy, whereas lower doses are required by infants with thyroid dyshormonogenesis (Hanukoglu *et al.*, 2001). The goal of the above mentioned treatment strategy is to achieve normal developmental and growth rates by maintaining the total or free T_4 serum levels in the upper half of the reference range during the first year of life (T4: 10-16 g/dl (130-206 nmol/L) and fT_4: 1.4-2.3 ng/dl (18-30 pmol/L). In addition, TSH has to be kept within the normal range (between 0.5–2.0 mU/L) (Jain *et al.*, 2008; Rastogi and LaFranchi, 2010). Compared to CH patients with total serum T_4 levels higher than 10 µg/dl during the first year of life, an association between persistent total T_4 serum levels below 10 g/dl and a lower IQ score has been reported (Heyerdahl, 1996). On the other hand, counteractive effects accompanying overtreatment, including acceleration of skeletal maturation, behavioural and temperamental problems, and inadequate attention in school attendance patients, have been reported (Rastogi and LaFranchi, 2010; LaFranchi, 2011). Recently, it has been shown that CH overtreatment during the first two years, as reflected by fT_4 concentrations above the individual steady state concentration (SSC) range, is associated with lower cognitive outcomes at 11 yr compared with undertreatment which leads to normal cognitive development if not complicated with overtreatment (Bongers-Schokking *et al.*, 2013). However, this latter study needs to be confirmed.

Although the frequency of follow-up visits varies considerably between different clinics, it is highly recommended that patients with CH under replacement therapy be followed-up very closely during their first three years of life. The following is an example of a monitoring schedule according to the recommendations of both the American Academy of Pediatrics (AAP) and the European Society for Pediatric Endocrinology (Table III) (LaFranchi, 2011).

Table III: Monitoring schedule for serum total or free T_4 and TSH (modified from LaFranchi, 2011).

Schedule	Condition/Age
2 and 4 wk	after initiation of L-T_4 treatment
Every 1 to 2 months	0-6 months of age
Every 2 to 3 months	6 months-3 yr of age
Every 6 to 12 months	until growth is completed
4 wk	after any change in L-T_4 dosage

3. Permanent primary CH due to thyroid dyshormonogenesis (TDHG)

The pathogenesis underlying congenital hypothyroidism due to thyroid dyshormonogenesis (TDHG) includes genetic defects in almost each of the multiple biochemical steps involved in the biosynthesis of THs (Grasberger and Refetoff, 2011; Grüters and Krude, 2012). In general, such genetic defects follow an autosomal recessive mode of inheritance and are highly recurrent in consanguineous families (Kopp, 2002; Park and Chatterjee, 2005).

Among the most common causes of inborn errors of thyroid hormone biosynthesis are defects in *TPO*, the gene responsible for the iodination and coupling of tyrosyl residues of Tg and their coupling to produce T_4 and T_3 (Grasberger and Refetoff, 2011; Cangul *et al.*, 2013). Inactivating mutations in *TPO* lead to goitrous or multinodular goitrous (MNG) CH consequent

to total iodide organification defects (TIOD) (Abramowicz *et al.*, 1992; Ris-Stalpers and Bikker, 2010; Belforte *et al.*, 2012; Lee *et al.*, 2013). The recessive mode of inheritance of TIOD is negated in some cases due to either monoallelic expression of *TPO* (Fugazzola *et al.*, 2003; Neves *et al.*, 2010) or to partial uniparental isodisomy (UPD) of chromosome 2 (Bakker *et al.*, 2001). Moreover, pseudodominant inheritance of goitrous CH due to *TPO* mutations has been also reported (Deladoëy *et al.*, 2008). In addition to TIOD, partial iodide organification defects (PIOD) has also been reported in patients bearing less severe inactivating mutations of *TPO* (Kotani *et al.*, 2003; Nascimento *et al.*, 2003).

Mutations in the gene encoding DUOX2 are also commonly involved in the etiology of dyshormonogenesis consequent to H_2O_2 deficiency (Grasberger, 2010; Fugazzola *et al.*, 2011). Both mono- and biallelic *DUOX2* gene mutations have been reported and found to be associated with either transient CH with PIOD or permanent CH with TIOD, respectively (Moreno *et al.*, 2002). Nonetheless, PIOD consequent to biallelic *DUOX2* gene mutations has also been reported (Vigone *et al.*, 2005; Varela *et al.*, 2006). Moreover, Maruo *et al.* have shown that biallelic *DUOX2* gene mutations are also associated with transient CH (Maruo *et al.*, 2008). The association between transient CH and inactivation of both *DUOX2* alleles has been also demonstrated by another group, thus suggesting the involvement of other pathophysiological components in the etiology of this sort of dyshormonogenesis (Hoste *et al.*, 2010). Recently, in an apparently normal patient during newborn screening, a delayed-onset of transient CH has been reported due to biallelic *DUOX2* mutations as well as maternal iodine excess. This is one example of misleading of screening results due to the impact of the environment on a genetically based CH (Kasahara *et al.*, 2013).

Deficiency in H_2O_2 generation, leading to PIOD, has been also detected in a patient with mild permanent CH due to dyshormonogenesis as a result of biallelic inactivating mutations in the gene encoding for the dual oxidase maturation factor 2 (*DUOXA2*). The milder CH phenotype observed, compared to that associated with biallelic *DUOX2* mutations, suggests partial compensation of *DUOXA2* deficiency by its paralog *DUOXA1* (Zamproni *et al.*, 2008). Recently, such a *DUOXA2* mutation together with another novel heterozygous insertion mutation, both leading to early termination of protein translation due to the generation of a premature stop codon, were considered to be the cause of mild CH in another patient (Yi *et al.*, 2013). Furthermore, it has been assumed that a homozygous *TSHR* mutation affecting the

TSHR/IP_3/Ca^{2+} signalling pathway, the positive control system for H_2O_2 generation in the thyroid, could be a plausible cause in cases of unexplained PIOD defects (Grasberger *et al.*, 2007).

In contrast to the DUOX2 system and beside the reported large deletion affecting both DUOX, no other mutations in either *DUOX1* or in *DUOXA1* have been detected as an underlying cause of dyshormonogenesis in patients with primary CH (Grasberger *et al.*, 2012). Likewise, it has been reported that mice spontaneously deficient in *Duox2* (*Duox2*^{thyd}) (Johnson *et al.*, 2007) rather than *Duox1* are congenitally hypothyroid (Donkó *et al.*, 2010). Recently, a mouse model in which the two *Duoxa* paralogs were knocked-out (*Duoxa*$^{-/-}$) was described by Grasberger *et al.* whom showed that animals deficient in the two *Duoxa* genes, *Duoxa1 and Duoxa2*, develop postnatal goitrous CH due to TIOD consequent to DUOX deficiency. Such a phenotype was not observed among *Duoxa*$^{-/-}$ mutants subjected to chronic postnatal T_4 replacement. These findings points to the important role of the DUOXA proteins in the maturation process of the DUOX isoenzymes who were proven to be the only source of H_2O_2 required in the process of THs biosynthesis (Grasberger *et al.*, 2012).

The uncommon iodide transport defects (ITD) identified among patients with CH due to dyshormonogenesis are attributed to biallelic mutations in the Na^+/I^- symporter gene (*SLC5A5* or *NIS*) (Fujiwara, 1997; Pohlenz and Refetoff, 1999; Szinnai *et al.*, 2006; Montanelli *et al.*, 2009). The intrinsic plasma membrane Na^+/I^- symporter (NIS) is responsible for the active iodide (I^-) uptake at the basolateral membrane of the thyroid follicular cells (TFCs) (Bagchi and Fawcett, 1973; De La Vieja *et al.*, 2000; Dohán *et al.*, 2003; Darrouzet *et al.*, 2014). In addition to limited or lack of radioiodide uptake in the thyroid and other *SLC5A5*-expressing organs (salivary glands and stomach) and the low iodide saliva-to-plasma (S/P) ratio, patients with ITD due to *SLC5A5* mutations present with variable degrees of CH and diffuse or nodular goiter (Stanbury and Dumont, 1983; Spitzweg and Morris, 2010). Likewise, biallelic mutations in *SLC26A4*, the gene encoding the multifunctional anion exchanger pendrin, lead to variable impaired ability of the thyroid to organify iodide (Everett *et al.*, 1997; Kopp, 2000; Campbell *et al.*, 2001; Huang *et al.*, 2013a). The resulting PIOD has been reported in 10% of the patients with Pendred syndrome (PDS), an autosomal recessive disorder defined by a triad of congenital hearing impairment, hypothyroidism due to PIOD, and goiter (Morgans and Trotter, 1958; Kopp and Bizhanova, 2011). In many patients, elevated levels of serum Tg are observed according to the size of the

goiter (Sheffield *et al.*, 1996). The role of environmental factors or other modifiers in compensating the inactivation of pendrin has been suggested by the lack of thyroid phenotype in the majority of patients with *SLC26A4* mutations and in *Slc26a4*$^{-/-}$ mice (Park *et al.*, 2003; Tsukamoto *et al.*, 2003; Park *et al.*, 2005; Calebiro *et al.*, 2011). Actually, sufficient iodide intake has been shown to modify the thyroid phenotype in patients with PDS where 90% of them are reverted to an euthyroid state while the remaining 10% present with goiter (Reardon *et al.*, 1997).

Synthesis defects in thyroglobulin (Tg) consequent to inactivating mutations in the gene encoding it (*Tg*) have been reported in patients with CH due to dyshormonogenesis (Ieiri *et al.*, 1991; Citterio *et al.*, 2013). The estimated incidence of defective Tg due to *Tg* gene mutations is approximately 1 in 100,000 newborns (Targovnik *et al.*, 2010). In fact, various phenotypes associated with *Tg* mutations have been reported that range from euthyroid to either mild or severe goitrous hypothyroidism, with goiter detection either at birth or shortly after it (Targovnik *et al.*, 2010). In addition, Tg defects in a goitrous individual are suggested by low serum thyroglobulin levels and normal organification of iodide indicated by a negative perchlorate discharge test (Medeiros-Neto *et al.*, 2002; Knobel and Medeiros-Neto, 2003). In spite of the usual autosomal recessive transmission of *Tg* defects, an autosomal dominant mode of inheritance has been proposed in one kindred (de Vijlder *et al.*, 1983).

In addition to the above-mentioned genes, biallelic homozygous mutations in the iodotyrosine deiodinase (known also as *DEHAL1*) gene (*IYD*) have been reported. In humans, *IYD* mutations lead to iodotyrosine deiodinase deficiency associated with defective intrathyroidal iodine recycling and excessive urinary secretion of MIT and DIT (Afink *et al.*, 2008; Moreno *et al.*, 2008a). Patients bearing *IYD* mutations are missed by newborn screening for CH, but they present with severe goitrous hypothyroidism at any time point between the neonatal period and adolescence (Moreno *et al.*, 2008a); mental retardation was reported in two cases, born to consanguineous parents, who presented with hypothyroidism during infancy. In addition to homozygous *IYD* mutations, patients carrying heterozygous mutations that might be associated with goiter or a hypothyroid phenotype have been also reported (Afink *et al.*, 2008). The variability in the phenotype and in age of onset of hypothyroidism might be attributed to either environmental factors (iodine intake) or genetic ones (severity of the involved mutations)

(Moreno and Visser, 2010). Alternative to L-T_4 treatment, it has been shown that iodine supplementation could effectively treat patients with *IYD* deficiency (Hirsch *et al.*, 1986).

4. Permanent primary CH due to thyroid dysgenesis (CHTD)

In contrast to the classical Mendelian recessive inheritance of thyroid dyshormonogenesis, thyroid dysgenesis (TD) is generally considered to be a sporadic disorder with an unknown underlying etiology in the vast majority of cases (Deladoëy, 2012). However, evidence indicating a possible role of genetic components have been reported, among which is the familial occurrence in a minority (2%) of CHTD cases with either ectopic thyroid or athyreosis, a rate that is 15 fold higher than would be expected by chance and that supports the contribution of a familial component in the pathogenesis of CHTD (Castanet *et al.*, 2000). The occurrence in some families of both types of TD, thyroid ectopy and athyreosis, among affected members of the same family points to a possible common underlying mechanism for both forms of the disorder (Castanet *et al.*, 2001). Moreover, further studies have shown that the incidence of familial cases of TD is even higher than previously described when the presence of asymptomatic thyroid developmental anomalies (TDA) in first degree relatives of infants with TD are prospectively screened for. Such findings represent an additional argument for a genetic component in the etiology of CHTD (Léger *et al.*, 2002; Adibi *et al.*, 2008; Kumorowicz-Czoch *et al.*, 2012).

Among the other lines of evidence supporting the possible involvement of genetic factors in the pathogenesis of TD is the female predominance in isolated cases of CHTD (especially for thyroid ectopy), where the female to male ratio is 3:1. Thus, the role of the individual's genetic background and/or the involvement of sex-modified factors in the etiology of TD is suggested (Devos *et al.*, 1999). On the other hand, the reported female/male predominance among familial cases of CHTD was similar in both thyroid ectopy and athyreosis, but lower when compared to isolated cases (Castanet *et al.*, 2001). Furthermore, the high frequency of additional extrathyroidal congenital malformations, notably cardiovascular ones, observed in CHTD cases compared to the general population suggests that common genetic factors may underlie TD and congenital heart diseases (Siebner *et al.*, 1992; Devos *et al.*, 1999; Castanet *et al.*, 2001; Olivieri

et al., 2002; Kreisner *et al.*, 2005; El Kholy *et al.*, 2007; Gu *et al.*, 2009; Kempers *et al.*, 2009; Kumar *et al.*, 2009; Reddy *et al.*, 2010; Razavi *et al.*, 2012).

In addition, variation by ethnicity in the incidence of TD is an additional argument which supports the role of genetic background in the pathogenesis of CHTD (Stoppa-Vaucher *et al.*, 2011). As discussed in a previous section it has been reported that the incidence of CH (all etiologic sub-groups included) varies in different racial and ethnic groups with a high incidence being reported among newborns that are Asians or Hispanics while the incidence among non-Hispanic black newborns was found to be low compared to non-Hispanic white ones (Lorey and Cunningham, 1992; Waller *et al.*, 2000; Harris and Pass, 2007; Hinton *et al.*, 2010; Chen *et al.*, 2013). Taking the etiology of CH (TD or TDHG) in consideration, Stoppa-Vaucher *et al.* have shown that the incidence of CHTD varies by ethnicity where Blacks, due to their genetic diversity, are less liable to develop TD compared to Caucasians who are more prone to TD because of the decreased genetic diversity and hence increased deleterious genetic variation among them (Lohmueller *et al.*, 2008; Stoppa-Vaucher *et al.*, 2011). Thus, the observed variation by ethnicity in patients with TD strongly supports the role of genetic susceptibility as an underlying molecular mechanism of its pathogenesis.

4.1. Molecular mechanisms of CHTD

4.1.1. Monogenic mechanisms

As previously mentioned in section **1.1.1.**, the embryonic development of the thyroid gland is regulated via an interplay between the TFCs enriched transcription factors NKX2.1, PAX8, FOXE1, and HHEX (De Felice and Di Lauro, 2004; Parlato *et al.*, 2004; De Felice and Di Lauro, 2011). Hence, they are considered to be the most pertinent candidate genes of CH due to TD. Actually, various animal models deficient of genes encoding these transcription factors develop TD, thus confirming their direct involvement in thyroid development (Table IV). In addition to the thyroid-specific transcription factors, other candidate genes **(section 1.1.1.)** have been also identified based on either animal models and/or on their function in human syndromes comprising CH associated with TD (Table IV). Extensive screening of patients with CHTD revealed the presence of germline mutations in the thyroid transcription factors NKX2.1, PAX8, FOXE1 and the thyroid related transcription factor NKX2.5 in a minor subset (3%) of the screened cases (Narumi *et al.*, 2010). In addition, inactivating mutations in the *TSHR* gene,

leading to TSH resistance, have also been identified in several patients (Sunthornthepvarakui *et al.*, 1995; Abramowicz *et al.*, 1997; Alberti *et al.*, 2002). However, no mutations among patients with TD have been detected either in the gene encoding the thyroid transcription HHEX nor in the new candidate gene *ISL1*(Al Taji *et al.*, 2007; Ferrara *et al.*, 2011). Likewise, no mutations were identified among TD patients screened for *TAZ* (*WWTR1*), a gene coding for one of the transcriptional co-factors of PAX8 and NKX2.1 (Ferrara *et al.*, 2009). Collectively, animal models deficient of transcription factors that regulate thyroid morphogenesis and gene expression as well as the corresponding reported germline mutations in patients with syndromic or nonsyndromic TD support the hypothesis that TD has a genetic component. This issue will be discussed in the following sections.

Table IV: Animal models and human genes of thyroid dysgenesis (TD) (modified from Deladoey, 2012).

Gene	Features	Thyroid phenotype	Additional phenotype
Zebrafish			
ace (Wendl *et al.*, 2007)	Growth factor; Fgf8	Hypoplasia	Lack of cerebellum and mid-hindbrain-boundrary
bon (Elsalini and Rohr, 2003)	Mixer transcription factor	Athyreosis	Overall reduction of endoderm
cas (Elsalini *et al.*, 2003)	Sox transcription factor	Athyreosis	Absence of endoderm
cyc (Elsalini *et al.*, 2003)	Nodal ligand	Hypoplasia	Overall reduction of endoderm, defects of neural tube, and cyclopia
fau (Reiter *et al.*, 2001)	GATA5 transcription factor	Athyreosis	Aplasia of liver, pancreas, and thymus
hand2 (Wendl *et al.*, 2007)	bHLH transcription factor	Athyreosis or hypoplasia	Heart, pharynx, pectoral fin defects
hhex (Elsalini *et al.*, 2003)	Homeobox transcription factor	Athyreosis or hypoplasia	Liver aplasia
nk2.1a (Elsalini *et al.*, 2003)	Homeodomain transcription factor	Athyreosis	Forebrain defect
noi (pax2.1) (Wendl *et al.*, 2002)	Paired-box transcription factor	Athyreosis	Lack of pronephric duct and mid-hindbrain-boundrary
oep (Elsalini *et al.*, 2003)	Nodal cofactor	Athyreosis	Absence of endoderm
Mouse			
Chordin (Bachiller *et al.*, 2003)	Extracellular BMP antagonist	Hypoplasia	Cardiac outflow tract defects, aplasia of thymus and parathyroid
Edn1 (Kurihara *et al.*, 1995)	Endothelin-1; signaling peptide	Hypoplasia, absent isthmus	Craniofacial, cardiac, and thymus defects

Table IV: continued.

Eya1 (Xu *et al.*, 2002)	Eya transcription factor	Hypoplasia	Aplasia of kidneys, thymus, and parathyroid
Fgf10 (Ohuchi *et al.*, 2000)	Growth factor	Athyreosis	Aplasia of limbs, lungs, pituitary, and salivary glands
Fibulin-1 (Cooley *et al.*, 2008)	ECM protein	Hypoplasia	Craniofacial, cardiac, and thymus defects
Foxe1 (De Felice *et al.*, 1998)	Forkhead transcription factor	Ectopia or athyreosis	Cleft palate
FRS2α (Kameda *et al.*, 2009)	Transducer of FGF signaling	Bilobation defect, hypoplasia, UBB defects	Thymus and parathyroid defects
Hes1 (Carré *et al.*, 2011)	Basic helix-loop-heix transcription factor	Hypoplasia	Hypoplastic UBB
Hhex (Martinez Barbera *et al.*, 2000)	Homeobox transcription factor	Athyreosis	Forebrain truncations, liver aplasia, and complex heart malformations
Hoxa3 (Manley and Capecchi, 1995; 1998)	Homeobox transcription factor	Hypoplasia, bilobation defects	Cardiovascular and skeletal defects
Hoxa5 (Meunier *et al.*, 2003)	Homeobox transcription factor	Empty thyroid follicle	
Hoxb3 (Manley and Capecchi, 1998)	Homeobox transcription factor	Ectopy in *Hoxa3*$^{-/-}$, *Hoxb3*$^{-/-}$ double mutants	Cardiovascular and skeletal defects
Hoxd3 (Manley and Capecchi, 1998)	Homeobox transcription factor	Ectopy in *Hoxa3*$^{-/-}$, *Hoxd3*$^{-/-}$ double mutants	Thymus and parathyroids agenesis
Isl1 (Westerlund *et al.*, 2008)	LIM homeodomain transcription factor	Hypoplasia of thyroid placode	Heart, pancreas, and neural defects
Nkx2.1 (Kimura *et al.*, 1996)	Homeodomain transcription factor	Athyreosis	Pulmonary aplasia, neural defects
Nkx2.5 (Dentice *et al.*, 2006)	Homeodomain transcription factor	Hypoplasia of thyroid placode	Cardiac defects

Table IV: continued.

Pax3 (Franz, 1989)	Paired-box transcription factor	Hypoplasia, bilobation defects	Thymus and parathyroid defects
Pax8 (Mansouri *et al.*, 1998)	Paired-box transcription factor	Athyreosis	Reproductive tract defects
Shh (Fagman *et al.*, 2004)	Secreted morphogen	Hemiagenesis	Holoprosencephaly, midline defect, aberrant carotid arteries, and short digits
Tbx1 (Fagman *et al.*, 2007; Lania *et al.*, 2009)	T-box transcription factor	Hypoplasia, bilobation defects	Cardiac outflow tract defects, aplasia of thymus and parathyroids
twisted (Petryk *et al.*, 2004)	Extracellular modulator of BMP signaling	Loss of Hhex expression at bud-stage	Vertebral defects, spectrum of midline defects, agnathia
Human			
FOXE1 (Bamforth *et al.*, 1989)	Forkhead transcription factor	Athyreosis	Cleft palate, choanal atresia, and spiky hair
GLIS3 (Senée *et al.*, 2006)	Zinc finger transcription factor	Hypoplasia	Neonatal diabetes, cystic kidneys, and cholestasis
NKX2.1 (Krude *et al.*, 2002)	Homeodomain transcription factor	Thyroid *in situ* with primary hypothyroidism	Respiratory failure, choreoathetosis
NKX2.5 (Dentice *et al.*, 2006)	Homeodomain transcription factor	Thyroid *in situ* with primary hypothyroidism	Congenital heart malformations
PAX8 (Macchia *et al.*, 1998)	Paired-box transcription factor	Hypoplasia	Unilateral renal agenesis
SALL1 (Choi *et al.*, 2010)	Zinc finger transcription factor	Thyroid *in situ* with primary hypothyroidism	Townes-Brocks Syndrome
TBX1 (Stagi *et al.*, 2010)	T-box transcription factor	Thyroid *in situ* with primary hypothyroidism	DiGeorge with congenital heart malformations
URB1 (Zenker *et al.*, 2005)	E3 ubiquitin ligases of the N-end rule pathway	Thyroid *in situ* with primary hypothyroidism	Johanson-Blizzard Syndrome

4.1.1.1. *TSHR*

The cell surface TSH receptor (TSHR) is a member of the glycoprotein hormone receptor (GpHR) subfamily of heterotrimeric G protein-coupled receptors (GPCRs) (Nagayama *et al.*, 1989; Parmentier *et al.*, 1989; Misrahi *et al.*, 1990). The human TSHR gene (*TSHR*) is located on chromosome 14q31 while in mice *Tshr* is located on chromosome 12. *TSHR/Tshr* each consists of ten exons, encoding a protein of 764 amino acids which shows a high degree of homology between species (Libert *et al.*, 1990; Rousseau-Merck *et al.*, 1990; Taylor *et al.*, 1996). At the amino acid levels, homology between the human and mouse TSHRs is greater than 87% (Patibandla *et al.*, 1997). The extracellular domain (ECD) of the receptor is responsible for high-affinity hormone (TSH) binding and is encoded by the first nine exons and part of exon ten. The seven-transmembrane domain (TMD) is involved in signal transduction where it activates both the adenylyl cyclase (AC) and phospholipase C (PLC) systems leading to the generation of the second messenger molecules cyclic adenosine monophosphate (cAMP) and Inositol triphosphae/Diacylglycerol (IP3/DAG). Both the TMD and the intracellular domain of the receptor are encoded by exon ten (Szkudlinski *et al.*, 2002).

During thyroid development in rodents, *Tshr* mRNA expression is scarcely detected by E14 and E15 in mice and rat, respectively and profoundly up-regulated by E17-E18 (Postiglione *et al.*, 2002; De Felice *et al.*, 2004). The expression pattern of *Tshr* mRNA is coincident with the completion of migration of the thyroid primordium, up-regulation of thyroid-specific genes, beginning of colloid formation and follicular development (Postiglione *et al.*, 2002). In humans, the probable onset of *TSHR* expression is by the end of the first trimester (10-12 wk) of gestation with increased levels detected by the middle period of a term pregnancy (18-20 wk) (Brown, 2004). Hence, these findings suggest that the early steps of thyroid organogenesis (0 to 8 wk) are independent of the TSH/TSHR signalling (De Felice *et al.*, 2004).

Fundamental knowledge concerning the role of Tsh/Tshr signalling in controlling the morphology as well as the differentiation of the developing thyroid were obtained from mutant mouse models with impaired Tsh/Tshr signalling due to deprivation of either TSH or a functional TSHR (Postiglione *et al.*, 2002). The mouse model pit^{dw}/pit^{dw} (formerly Snell dwarf or *dw/dw*) represents a model of TSH deprivation (Snell, 1929; Bartke, 1964; Cordier *et al.*, 1976). Among the mutant models deprived of a functional TSHR, $Tshr^{hyt}/Tshr^{hyt}$ mice (formerly *hyt/hyt* mice) bears a spontaneous point mutation in the coding sequence of the *Tshr* gene leading to defective

binding of TSH (Beamer *et al.*, 1981; Stein *et al.*, 1994). Another mutant model is the *Tshr*-knockout, a mouse model in which the *tshr* gene was inactivated by homologous recombination in embryonic stem cells (Marians *et al.*, 2002). Both the pit^{dw}/pit^{dw} and $Tshr^{hyt}/Tshr^{hyt}$ models exhibited severe hypothyroidism associated with thyroid hypoplasia (Cordier *et al.*, 1976; Stein *et al.*, 1994). During embryonic development, Postiglione *et al.* have shown that absence of a functional Tshr had no impact on the morphology of the developing thyroid in the mutant embryos in contrast to what is observed after birth (Postiglione *et al.*, 2002). Moreover, the expression of the thyroid-specific genes *Tpo* and *Nis* was undetectable in the mutant embryos while no effect was observed on that of either Tg or the thyroid-enriched transcription factors Nkx2.1, Pax8 and Foxe1 (Postiglione *et al.*, 2002). The described role of Tsh/Tshr signalling in controlling expression of some but not all of the thyroid specific genes during embryonic life in mice is consistent with its role during adult life (Marians *et al.*, 2002). Collectively, these data indicate that the Tsh/Tshr signalling is not involved in controlling the growth and morphology of the thyroid gland during embryonic development as it is during adulthood. On the other hand, the Tsh/Tshr signalling pathway seems to be a major regulator of genes involved in iodide metabolism (*Tpo* and *Nis*) in the developing mouse thyroid gland (Postiglione *et al.*, 2002).

In addition to mouse models of impaired TSH/TSHR signalling, the role of Tshr during embryonic thyroid development has been also investigated in a zebrafish model in which the tshr protein was knocked down using a *tshr*-specific morpholino antisense oligonucleotide (MO) (Opitz *et al.*, 2011). Initially, the authors cloned the zebrafish *tshr* gene and showed a high degree of conservation with *Tshr* from other teleosts and with the mammalian TSHR. Furthermore, they have shown that the expression of *tshr* mRNA in zebrafish embryos occurs mainly in the thyroid and is coincident with its budding, a process occurring, distinctly in zebrafish, concomitant to an early onset of the expression of the gland's functional differentiation markers (Opitz *et al.*, 2011). Furthermore, a TSH-dependent up-regulation of the functional differentiation markers *slc5a5*, *tshr*, *tpo*, and *iyd* was observed in zebrafish embryos treated with the hormonogenesis inhibitor phenylthiourea (PTU), a finding comparable to what has been observed in mammals (Levy *et al.*, 1997; Milenkovic *et al.*, 2007) and amphibians (Opitz *et al.*, 2009).

Contrary to what has been previously reported (Alt *et al.*, 2006a), Opiz and his colleagues have shown the presence of an obvious thyroid phenotype among the zebrafish *tshr* morphants

compared to their wild type counterparts. They have demonstrated an impaired functional differentiation of the thyroid encompassing, in addition to the reduced number and size of functional thyroid follicles, a reduced expression of thyroid transcription factors (*nkx2.1a* and *Pax8)* together with down-regulation of the gland's functional differentiation markers (*tg*, *tpo*, *slc5a5*, and *iyd*). The observed reduced expression of the thyroid transcription factors along with the down-regulation of thyroid differentiation markers recapitulate the thyroid phenotype reported in a transgenic mouse with defective cyclic adenosine monophosphate (cAMP) signalling (Nguyen *et al.*, 2000) and point to a plausible positive feedback signalling between expression of thyroid transcription factors and TSHR signalling in the zebrafish thyroid (Opitz *et al.*, 2011). In spite of the obvious role of TSHR signalling in regulating the developing gland's differentiation program, its impact on the growth of the developing gland was not easily revealed. Nevertheless, the observed thyroid phenotype resulting from *tshr* knockdown was partially rescued by means of inducible *tshr* overexpression. Collectively, these data provide evidence for the involvement of the TSH/TSHR signalling in regulating the differentiation of the developing thyroid in zebrafish and that its impairment leads to a thyroid phenotype that matches, in many features, that of mouse models of defective Tshr and cAMP signalling (Opitz *et al.*, 2011).

In humans, loss-of-function (LOF) mutations in the *TSHR* gene have been reported in association with various degrees of TSH resistance (OMIM #275200) (Sunthornthepvarakui *et al.*, 1995; Abramowicz *et al.*, 1997; Alberti *et al.*, 2002). The spectrum of phenotypes associated with *TSHR* LOF mutations ranges from euthyroid hyperthyrotropinemia (i.e. elevated TSH with fT4 in normal range) to severe congenital hypothyroidism with a hypoplastic thyroid gland, variable presentations that are dependent on the extent of TSH resistance (Refetoff, 2003; Persani *et al.*, 2010). Itself, the extent of TSH resistance is dependent on the number of mutated alleles and on the severity of the functional impairment of the receptor consequent to the mutation (Persani *et al.*, 2010). Recessively inherited homozygous or compound heterozygous biallelic mutations of the *TSHR* gene that are associated with complete lack of the receptor's function lead to severe CH and thyroid hypoplasia (complete or uncompensated TSH resistance). However, less severe biallelic LOF mutations are associated with milder forms of hypothyroidism characterized by elevated TSH levels, free thyroid hormones concentrations in the normal range, and a thyroid gland that is normal/reduced in size and can be compensated by

adequate increment of serum TSH levels (partial or compensated TSH resistance) (Sunthornthepvarakui *et al.*, 1995; Abramowicz *et al.*, 1997; Gagné *et al.*, 1998; Bretones *et al.*, 2001; Park *et al.*, 2004; Persani *et al.*, 2011; Cassio *et al.*, 2013). On the other hand, monoallelic LOF mutations, following an autosomal dominant mode of inheritance, have been reported in patients with nonautoimmune subclinical hypothyroidism (NASH) characterized by slight to moderate elevations in serum levels of TSH, normal serum concentrations of free thyroid hormones, and mainly normal sized (hypoplastic in a few cases) thyroid gland (Alberti *et al.*, 2002; Camilot *et al.*, 2005; Nicoletti *et al.*, 2009; Rapa *et al.*, 2009; Persani *et al.*, 2010; Calebiro *et al.*, 2012; Cassio *et al.*, 2013).

The main feature of *TSHR* gene mutations is the high level of phenotypic variability whereby the same mutation can be seen with different levels of thyroid function either among members of the same family or unrelated subjects. In addition, thyroid hypoplasia has been reported in association with heterozygous mutations (Cassio *et al.*, 2013). Hence, the involvement of other factors that might be responsible for the phenotypic variability has been suggested, among which are genetic rearrangements or mutations in yet unanalyzed regions or elements of the *TSHR* gene, other genes in addition to *TSHR* gene (digenic inheritance), or environmental factors (Lado-Abeal *et al.*, 2011; Sriphrapradang *et al.*, 2011; Cassio *et al.*, 2013).

Compiled data from several studies indicated that LOF *TSHR* mutations are more common among TD patients than expected with a reported prevalence of 4.3% for mono- and biallelic mutations (Szinnai, 2013). Furthermore, a high prevalence (11–29%) of *TSHR* mutations has been recently reported in children with a mild form of TSH resistance in the form of hyperthyrotropinemia without a history of CH or of autoimmune thyroid disease (Nicoletti *et al.*, 2009; Rapa *et al.*, 2009; Calebiro *et al.*, 2012; Cassio *et al.*, 2013). Collectively, these data indicate that LOF *TSHR* gene mutations are the most common genetic causes of TD (due to thyroid hypoplasia) and are frequent cause of NASH (Cassio *et al.*, 2013; Szinnai, 2013). **This said, it is important to bear in mind that TSHR mutations are not associated with thyroid ectopy or athyreosis, as TSHR is dispensable for thyroid migration and survival** (Gagné *et al.*, 1998).

4.1.1.2. *NKX2.1*

NKX2.1, also known as TTF-1 (thyroid transcription factor-1), T/EBP (thyroid-specific-enhancer-binding protein) or TITF-1, is a homeodomain-containing protein that belongs to the NKX.2 family of transcription factors (Civitareale *et al.*, 1989; Guazzi *et al.*, 1990). Both the mouse *Nkx2.1* gene (located on chromosome 12) as well as the human *NKX2.1* gene (located on chromosome 14q13) contain two and three exons, respectively, and encode highly homologous transcription factors. The amino acid sequences of human and mouse *NKX2.1* exhibit 98% sequence similarity, with a complete conservation of the 60-amino-acid homeodomain (Guazzi *et al.*, 1990; Oguchi *et al.*, 1995; Hamdan *et al.*, 1998). During the early stages of embryonic development in rodents, *Nkx2.1* transcripts and the corresponding encoded protein were identified in the primitive pharynx exclusively in the thyroid anlage at the time of its specification, at later developmental stages, as well as during adulthood (Lazzaro *et al.*, 1991; De Felice and Di Lauro, 2004). *Nkx2.1* is essential for the survival of pTFCs, for folliculogenesis, and in differentiated TFCs, for regulating the expression of thyroid specific genes *Tg, Tpo*, *Tshr,* and *Slc5a5* (*Nis*) (Civitareale *et al.*, 1989; Francis-Lang *et al.*, 1992; Civitareale *et al.*, 1993; Kimura *et al.*, 1996; Endo *et al.*, 1997). Moreover, transcripts of *Nkx2.1* have been identified in parafollicular C cells as well as in the epithelial cells of UBB (Mansouri *et al.*, 1998; Suzuki *et al.*, 1998). It has been shown that Nkx2.1 is crucial for maintaining the survival of the UBB cells during their migration as well as their dissemination into the thyroid diverticulum (Kusakabe *et al.*, 2006a). In the adult murine thyroid, it has been shown that Nkx2.1 is required for the maintenance of ordered follicular architecture and function of the differentiated thyroid (Kusakabe *et al.*, 2006b). Expression of *Nkx2.1* was also detected in the endodermal cells of the lung bud with the subsequent constant expression in the bronchial epithelium throughout all its early differential stages (Guazzi *et al.*, 1990; Lazzaro *et al.*, 1991; Stahlman *et al.*, 1996). In the lung, Nkx2.1 regulates the expression of SP (surfactant protein)-A, SP-B, SP-C, and other lung-specific genes (Bohinski *et al.*, 1994; Bruno *et al.*, 1995; Kelly *et al.*, 1996; Zhang *et al.*, 1997; Besnard *et al.*, 2007). In the central nervous system (CNS), the expression of *Nkx2.1* is observed in restricted regions of the forebrain structures including the hypothalamus (Lazzaro *et al.*, 1991; Nakamura *et al.*, 2001), where it is involved in interneuron specification and migration during forebrain development and contributes in the regulation of the circadian oscillations in gonadotrophin-releasing hormone (GnRH) gene transcription (Butt *et al.*, 2008; Nóbrega-Pereira

et al., 2008; Matagne *et al.*, 2012). In humans, starting from the developmental day (d) 32 and 33, transcripts of *NKX2.1* are weekly expressed in the median thyroid anlage (Trueba *et al.*, 2005; Szinnai *et al.*, 2007). The expression is continued to be detected when the gland reaches its final position and in the fetal gland by d 48 (Trueba *et al.*, 2005). Szinnai *et al.* have shown that the expression of *NKX2.1* remained stable before and after 11 gestational weeks (GW). In addition, expression of *NKX2.1* is detected in the lung bud and forebrain by developmental d 32 and 33, respectively. From 9 GW on, the expression of *NKX2.1* is detected in the lung only in the epithelium of the most recently formed branches (Trueba *et al.*, 2005).

The phenotype of *Nkx2.1* null mice is consistent with the expression pattern of the gene. They exhibit impaired morphogenesis of the lung, lack both the thyroid and pituitary glands, have extensive defects in the ventral forebrain, and are dead at birth (Kimura, 1996). Disruption of *Nkx2.1* at earlier developmental stages (E10) revealed the formation of thyroid anlage in *Nkx2.1* null mutants with its subsequent degeneration and final elimination via apoptosis around E12-13 (Kimura *et al.*, 1999). These latter findings emphasise the role of *Nkx2.1* in maintaining the survival of pTFCs by inhibiting their apoptosis rather than their specification and that athyreosis can be due to a single heritable genetic disorder (Parlato *et al.*, 2004; Montanelli and Tonacchera, 2010). On the other hand, mice heterozygous for *Nkx2.1* gene deletion ($Nkx2.1^{+/-}$) displayed mild non-goitrous hypothyroidism in addition to mild neurological defects in the form of poor coordination (Pohlenz *et al.*, 2002). Moeller *et al.* have attributed the thyroid phenotype observed in the $Nkx2.1^{+/-}$ mice to the reduced expression of the TSH receptor (*Tshr*) gene as a consequence of *Nkx2.1* haploinsufficiency. The mild hypothyroidism observed among the $Nkx2.1^{+/-}$ mice could be overcome when a higher level of Tshr saturation is attained consequent to the administration of an increased amount of exogenous TSH (Moeller *et al.*, 2003).

In humans, the majority of the reported *NKX2.1* mutations alter the DNA-binding ability of the protein consequent to its truncation, either before or within the DNA-binding homeobox domain localized in exon 3 (Peall *et al.*, 2013). Haploinsufficiency (due to mutation or deletion) of *NKX2.1* has been reported in patients with the triad of brain, lung, thyroid syndrome (BLTS, OMIM #610978) characterized by various combinations of CH, infant respiratory distress syndrome (IRDS), and benign hereditary chorea (BHC) (Devriendt *et al.*, 1998; Krude *et al.*, 2002; Pohlenz *et al.*, 2002; Willemsen *et al.*, 2005). Such an association of symptoms reflects the role of NKX2.1 in the development and function of the thyroid gland, basal ganglia, and lung in

accordance with its expression pattern in humans (Trueba *et al.*, 2005; Szinnai *et al.*, 2007). Nevertheless, the signs and symptoms of the three disorders are not always observed in combination and moreover their extent of severity varies greatly (Iwatani *et al.*, 2000; Breedveld *et al.*, 2002; Krude *et al.*, 2002; Moya *et al.*, 2006; Carré *et al.*, 2009; Maquet *et al.*, 2009; Hamvas *et al.*, 2013; Peall *et al.*, 2013). According to a systematic review of all the reported cases with variants of *NKX2.1*, it has been shown that *NKX2.1* mutations occur either *de novo* or via an autosomal dominant mode of inheritance. In addition, this review showed that only 50% of the cases had the involvement of the three organs (full triad of the syndrome), 30% showed CH and a neurologic disorder, 13% presented with isolated BHC, and 7% exhibited absence of BHC (Carré *et al.*, 2009). Concerning the functional and morphological thyroidal abnormalities observed among the reported cases, both a higher prevalence of hyperthyrotropinemia (66%), compared to overt hypothyroidism (39%), have been noted, with a preponderance of normal thyroid morphology (55%) over abnormal morphologies (hypoplasia or hemiagenesis, 35%; athyreosis, 10%) (Carré *et al.*, 2009). In contrast to syndromic cases of CH, no mutations of *NKX2.1* were detected in the three screened cohorts of non-syndromic CH (Al Taji *et al.*, 2007; Yuan *et al.*, 2008; Narumi *et al.*, 2010). However, both normal *NKX2.1* sequencing and copy number analysis have been recently reported in a case with BLTS bearing a 14q13.1–3 deletion with a breakpoint mapped to 192 kb proximal to the 3′ end of the *NKX2.1* gene and no physical interruption of it. The plausible involvement of an enhancer element, located about 400 kbp 3′ to the gene, in the developmental regulation of the *NKX2.1* gene has been postulated. In addition, partial explanation of the patient's phenotype might be attributed to heterozygosity for genes involved in neuronal differentiation and lung development, or other genes mapped within the deleted region (Barnett *et al.*, 2012).

4.1.1.3. *PAX8*

The paired box gene 8 (*PAX8/Pax8*) is one of the identified *PAX/Pax* genes encoding a family of nine transcription factors in humans (PAX1-PAX9) and mice (Pax1-Pax9), known for their crucial role in embryogenesis. The paired box family is characterized by the presence of a highly conserved 128-amino acid paired-box DNA binding domain (PD) at their N-terminal, initially identified in the *Drosophila* pair-rule segmentation gene *paired* (*prd*) (Mansouri *et al.*, 1996; Wang *et al.*, 2008). The members of the paired box family are divided into subfamilies based on the presence or absence of two additional DNA-binding domains, an octapeptide (OP)

and a prd-like homeodomain (HD) (Stuart *et al.*, 1994; Mansouri *et al.*, 1996). The subfamily II, including *PAX8* together with *PAX2* and *PAX5* genes, is characterized by the presence of an octapeptide (OP) and a truncated homeodomain (HD) (Mansouri *et al.*, 1996; Goode and Elgar, 2009). The mouse *Pax8* gene is localized on chromosome 2 and the human *PAX8* gene, having 12 exons, maps to chromosome 2q12–q14 (Plachov *et al.*, 1990; Poleev *et al.*, 1992; Stapleton *et al.*, 1993).

During embryonic development of the mouse, *Pax8* is expressed in the developing thyroid from the time of its specification through all the developmental stages of the TFCs as well as in adulthood (Plachov *et al.*, 1990; Mansouri *et al.*, 1998; De Felice and Di Lauro, 2004). Moreover, it is known to play a crucial role in initiating as well as maintaining the thyroid cell differentiation state (Mansouri *et al.*, 1998; Pasca di Magliano *et al.*, 2000). In synergy with *Nkx2.1*, it enhances the expression of the thyroid differentiation markers Tg, Tpo, and Nis (Pasca di Magliano *et al.*, 2000; Di Palma *et al.*, 2003). In the developing CNS, *Pax8* is transiently expressed in myelencephalon and along the full length of the neural tube. On the other hand, no expression was detected in later developmental stages or in the adult brain. Moreover, its expression has been reported in the developing and adult kidney (Plachov *et al.*, 1990; De Felice and Di Lauro, 2004).

During development of the human thyroid, Trueba *et al.* have shown that the expression of *PAX8* is strongly detected in the median anlage and the fourth pharyngeal arch ectoderm by 32 d. In addition to the median anlage, *PAX8* expression was detected in the thyroglossal duct and the UBB by 33 d. After the fusion of the median and lateral thyroid primordia (48 d), the developing thyroid kept on strongly expressing *PAX8* with a persistent expression observed in TFCs at fetal stages (Trueba *et al.*, 2005). In addition to the developing thyroid, expression of *PAX8* was detected in the otic vesicle, CNS, and the developing kidney by 32 d. In the developing CNS, expression of *PAX8* is limited to the midbrain-hindbrain boundary, thereafter to the spinal cord and myelencephalon. Moreover, human *PAX8* is expressed in the condensed mesenchyme of the developing kidney, the mesonephric duct, the ureteric bud, and the collecting ducts (with no expression detected at their tips) (Trueba *et al.*, 2005).

The role of Pax8 during embryonic development has been demonstrated by studying *Pax8* null mice: even though *Pax8*$^{+/-}$ mice showed no phenotype, *Pax8* null mice were born at

the expected Mendelian frequency but showed growth retardation and died within 2-3 wk of age (Mansouri *et al.*, 1998). The *Pax8* null animals exhibited no brain or kidney defects, plausibly due to the redundant function of other *Pax* genes. On the contrary, the thyroid gland of *Pax8*$^{-/-}$ mice are severely affected: they are smaller in size compared to WT embryos (due to absence of pTFCs), they lack any follicular organization, and are composed almost entirely of calcitonin-producing C cells (Mansouri *et al.*, 1998; De Felice and Di Lauro, 2004). In accordance with the thyroid developmental defects observed in the *Nkx2.1* knockout mouse (Kimura *et al.*, 1999), the thyroid anlage is initially formed early during development in *Pax8* null mice. However, by E11.5, the anlage appeared to be smaller in size compared to the wild type one and at E12.5 the thyroid precursors are no longer detectable, thus Pax8 is seemingly required for the survival of pTFCs and not for their specification (Mansouri *et al.*, 1998; De Felice and Di Lauro, 2004). Moreover, it has been shown that Pax8 is required for the initiation and maintenance of *Foxe1* and *Hhex* expression in the thyroid anlage, respectively, and that it plays a crucial role in regulating the thyroid-differentiated phenotype as previously mentioned (Pasca di Magliano *et al.*, 2000; Di Palma *et al.*, 2003; Parlato *et al.*, 2004). Consequently, the *Pax8* null animals suffer from CH due to TD, the principle cause of their death, as evident from their inability to produce thyroid hormones and from being rescued by thyroxine replacement (Mansouri *et al.*, 1998; Friedrichsen *et al.*, 2004). Based on the reported anti-apoptotic function as well as impact on cell proliferation of many *PAX* genes (Muratovska *et al.*, 2003; Robson *et al.*, 2006), specifically *PAX8/Pax8* (Chen *et al.*, 2008; Fagman *et al.*, 2011), it has been proposed that the loss of pTFCs through apoptosis might be the underlying mechanism of the observed TD in *Pax8* null embryos (Fagman and Nilsson, 2010; De Felice and Di Lauro, 2011), as previously suggested (Parlato *et al.*, 2004).

In humans, *PAX8* mutations have been initially reported in both sporadic as well as familial cases of CHTD (Macchia *et al.*, 1998). To this day, all the reported *PAX8* mutations are heterozygous and are transmitted among familial cases via an autosomal dominant pattern of inheritance with incomplete penetrance and variable expressivity (Congdon *et al.*, 2001). The thyroid phenotype of the affected cases varies widely, even among the members of the same family; at the biochemical level, euthyroidism to severe hypothyroidism have been described, and thyroid gland morphology ranges from a normal-sized gland to athyreosis, but with the majority being hypoplastic (Macchia *et al.*, 1998; Esperante *et al.*, 2008; Narumi *et al.*, 2010;

Hermanns *et al.*, 2011; Hermanns *et al.*, 2013). However, ectopic thyroids were detected in only two patients, a relative small number given the number of CHTD cohorts which were screened for *PAX8* (Macchia *et al.*, 1998; Tonacchera *et al.*, 2007). A new heterozygous *PAX8* mutation has been recently identified in a family with six affected cases of CH covering three successive generations. The mutation was found to be associated with urogenital malformations in five of the six CH affected patients. On the other hand, no mutations have been identified in *PAX2*, a gene that together with *PAX8*, is essential for kidney development (Carvalho *et al.*, 2013). In addition to the variable penetrance and expressivity of *PAX8* gene mutations, the role of genetic background is highlighted by the variable phenotype, even among members of the same family (Montanelli and Tonacchera, 2010). Both the dominant effect of *PAX8* gene mutations and the contradiction between the presence and absence of a thyroid phenotype in patients with *PAX8* gene mutations and in the $Pax8^{+/-}$ mice, respectively, might be attributed to a number of molecular mechanisms. Among the potential mechanisms is haploinsufficiency, whereby the normal development of the thyroid is not supported by the reduced amount of PAX8 in the affected patients. Actually, haploinsufficiency involving other *PAX/Pax* genes has been previously reported (Wilm *et al.*, 1998; van Raamsdonk and Tilghman, 2000). In addition, a dominant-negative effect of the mutated protein might be another underlying mechanism of TD in patients with PAX8 gene mutations (De Felice and Di Lauro, 2011). However, cotransfection of at least three mutated PAX8 alleles with a WT one showed no dominant-negative effect (Congdon *et al.*, 2001; Vilain *et al.*, 2001; Narumi *et al.*, 2010). Moreover, dominant-negative effects have never been characterized in phenotypes resulting from mutations of any other *PAX* genes (Strachan and Read, 1994). Finally, monoallelic expression of *PAX8* in the affected patients might be an alternative mechanism, whereby a hypoplastic gland is formed from a heterogenous group of pTFCs encompassing cells that express the mutant *PAX8* and others expressing the WT gene. However, such a probable mechanism does not explain the severe hypolasia observed in some patients that requires the preferential expression of the mutated gene (De Felice and Di Lauro, 2004).

Orthologues of the *Pax2/5/8* gene family have been detected in the thyroid of zebrafish and *Xenopus* (Heller and Brandli, 1999; Wendl *et al.*, 2002). In zebrafish, the expression pattern of *Pax8* is comparable to that observed in mammals: It is detected in the eyes, midbrain-hindbrain boundary region, as well as in the pronephros and nephric ducts (Pfeffer *et al.*, 1998).

Furthermore, it was detected in the developing thyroid from about 28 hours post-fertilization (hpf) and throughout gland development (Wendl *et al.*, 2002). In addition to *Pax8*, it has been shown that another member of the *Pax2/5/8* gene family, *Pax2.1*, is expressed in the thyroid anlage, even before *Pax8* is expressed (around 24 hpf) and continues to be expressed during the developmental process of the gland (Wendl *et al.*, 2002; Porazzi *et al.*, 2009). It has been suggested that *Pax2.1*, acting upstream of *Pax8*, is essential for the proper development and differentiation of thyroid follicles in zebrafish and that it has a role comparable to that of *Pax8* in mammalian thyroid development (Wendl *et al.*, 2002; Elsalini *et al.*, 2003). Mutation of the *Pax2.1* gene in zebrafish gives rise to the *no isthmus* (*noi*$^{-/-}$) phenotype (Brand *et al.*, 1996; Macdonald *et al.*, 1997; Lun and Brand, 1998; Majumdar *et al.*, 2000), which comprises defects comparable to those observed in humans and mice deficient in *PAX2*/*Pax2* (Favor *et al.*, 1996; Macdonald *et al.*, 1997; Favor and Neuhauser-Klaus, 2000; Porteous *et al.*, 2000). Moreover, the thyroid phenotype observed in *noi*$^{-/-}$ embryos was comparable to that in *Pax8*$^{-/-}$ mice (Mansouri *et al.*, 1998) i.e. *noi*$^{-/-}$ embryos completely lack T_4-positive follicles, in spite of the initial specification of the thyroid primordium (Wendl *et al.*, 2002). However, some T_4 production is independent of *pax2.1* as evident by the detection, in the anterior non-follicular domain of *noi*$^{-/-}$ zebrafish mutants, of a bound form of T4 which might not compensate for the absence of thyroid follicles (Wendl *et al.*, 2002). On the other hand, *Pax2* is not involved in the gland's development in mice, where no expression of this gene was detected in wild type embryos. Moreover, the thyroid appears normal in size and shape in *Pax2*$^{-/-}$ mouse embryos (Wendl *et al.*, 2002). In support of the notion that *PAX2*/*Pax2* plays no role in mammalian thyroid development is the lack of thyroid phenotype in humans with *PAX2* muations (renal-coloboma syndrome) (Favor *et al.*, 1996; Porteous *et al.*, 2000).

In contrast to the observed expression of *Pax8* during the development of the mammalian thyroid (Plachov *et al.*, 1990; Mansouri *et al.*, 1998; Trueba *et al.*, 2005), its expression was not detected in the thyroid gland of *Xenopus* embryos (Heller and Brandli, 1999). Instead, *Pax2* is detected in place of *Pax8* in the thyroid anlage, coincidently with its appearance during embryonic development. Its expression starts to decline upon thyroid lobulation until it is no longer detected by the time of follicular cell formation (Heller and Brandli, 1999).

4.1.1.4. *FOXE1*

The forkhead box E1 (FOXE1), also known as thyroid transcription factor 2 (TITF2) and formerly as forkhead drosophila homolog-like 15 (FKHL15), is a member of the forkhead/winged-helix family of transcription regulators (Civitareale *et al.*, 1994; Chadwick *et al.*, 1997). Members of the forkhead/winged-helix family have in common a highly conserved forkhead (FKH) DNA-binding domain (FHD) that is comprised of 110 amino acids (Weigel and Jackle, 1990; Kaufmann and Knochel, 1996). The FOX proteins play crucial roles during embryonic development and metabolism. In addition, their deregulation is associated with a number of human genetic diseases and cancer (Carlsson and Mahlapuu, 2002; Lehmann *et al.*, 2003; Benayoun *et al.*, 2011; Katoh *et al.*, 2013). Earlier, FOXE1 was identified as a thyroid-specific DNA-binding factor that recognizes, under hormonal stimulation, a specific DNA sequence on the promoters of the two thyroid-specific genes *Tg* and *TPO* (Civitareale *et al.*, 1989; Santisteban *et al.*, 1992; Aza-Blanc *et al.*, 1993). The human *FOXE1* genetic locus, named *FOXE1*, is located on chromosome 9q22, contains a single exon, and encodes a 42-kDa protein having 367 amino acids (Chadwick *et al.*, 1997), while in the mouse, *Foxe1* is located on chromosome 4 (Zannini *et al.*, 1997).

In the developing mice, transcripts of *Foxe1* are detected starting from E8.5 and beyond in the endodermal layer all over the foregut including the thyroid anlage. Moreover, its expression is observed in the epithelium lining the anterior pharynx and the pharyngeal arches but not in the pouches and their derivatives later during development (thymus, parathyroid, and UBB). Caudally, the entire foregut, including the future esophagus, also exhibits *Foxe1* expression (Zannini *et al.*, 1997; Dathan *et al.*, 2002; De Felice and Di Lauro, 2004). Consequently, at later developmental stages, *Foxe1* is found in the thyroid, tongue, epiglottis, palate, and esophagus, tissues derived from the pharyngeal arches and pharyngeal wall, with higher and lower expression levels observed during adulthood in thyroid and esophagus, respectively. In addition to the foregut and its derivatives, expression of *Foxe1* has been detected in the ectoderm and tissues derived from it, among which is the Rathke's pouch, the epithelium of the oral cavity, nasal choanae, whiskers, and hair follicles. However, the reported expression in the developing pituitary was proven to be transient (Zannini *et al.*, 1997; Dathan *et al.*, 2002; De Felice and Di Lauro, 2004).

During human development, the expression of *FOXE1* is detected in the thyroid anlage at d 34, later compared to what is observed in mouse, and continues to be expressed in the thyroid throughout the developmental process (Trueba *et al.*, 2005). In addition, its expression was detected outside of the thyroid, in the thymus and at lower levels in the oropharyngeal epithelium at d 47-48, and at later developmental stages (at 11 weeks) in the esophageal and tracheal epithelium (Trueba *et al.*, 2005). In adults, expression of *FOXE1* is maintained in the thyroid and is detected in the epidermis, hair follicles, and pre-pubertal testis (Chadwick *et al.*, 1997; Clifton-Bligh *et al.*, 1998; Dathan *et al.*, 2002; Sequeira *et al.*, 2003).

Although the heterozygous *Foxe1* KO mice (*Foxe1*$^{+/-}$) are euthyroid and exhibit no obvious phenotype, the homozygous ones (*Foxe1*$^{-/-}$), born at the expected ratio, die shortly after birth likely due to cleft palate. In addition, the mutant animals lack any orthotopic thyroid tissue and suffer from severe CH documented by elevated levels of TSH and undetectable levels of T_4 (De Felice *et al.*, 1998). During earlier developmental stages, a detailed analysis of thyroid morphogenesis in *Foxe1* null mice compared to wild-type (WT) counterparts revealed that at E9.5 the pTFCs in null embryos are still attached to the floor of the pharynx while those of WT embryos are detached and start to descend caudally. At later developmental stages (E15.5), 50% of the *Foxe1* null embryos exhibit a small thyroid remnant that is still attached to the floor of the pharynx while the other 50% have no thyroid tissue at all (De Felice *et al.*, 1998).

The variability of the phenotype observed among *Foxe1* null embryos can probably be attributed to either stochastic events occurring during the gland's development or due to the influence of differing genetic backgrounds or hormonal milieu (De Felice and Di Lauro, 2004). In spite of the incomplete migration of the thyroid precursors observed in half of the *Foxe1*$^{-/-}$ embryos, the differentiation process of the thyroid cells is completed, as evident by the production of Tg (De Felice *et al.*, 1998). These findings point to the incomplete dependence of the differentiation process on the proper localization of the TFCs, consistent with reported patients who possess fully functional ectopic lingual thyroids (Fisher and Klein, 1981; Gillam and Kopp, 2001; Van Vliet, 2003). Collectively, the data obtained from the homozygous *Foxe1* KO mice point to the crucial role of *Foxe1* in thyroid migration during morphogenesis and in follicular cells survival at a different time point than *Nkx2.1* and *Pax8* (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2010). The thyroid phenotype of *Foxe1*mutant mouse embryos favors the involvement of active cell migration in thyroid morphogenesis (De Felice *et al.*,

1998). Such a finding has been supported by the rescued thyroid phenotype by knock-in of *Foxe1* into the *Nkx2.1* locus of *Foxe1* deficient mice (Parlato *et al.*, 2004). Alternatively, it has been proposed that non-cell-autonomous morphogenetic mechanisms might be involved in the migration of pTFCs during development including the differential growth of the embryo as a whole or the remodeling of the embryonic vessels (Fagman *et al.*, 2006; Gasser, 2006).

Due to the perinatal death of *Foxe1* null mice, the role of Foxe1 in the adult mouse thyroid is not yet elucidated. However, it has been reported that FOXE1 can act as a transcriptional activator. By binding to the compacted chromatin of the inactive *TPO* promoter, FOXE1 alters the chromatin structure thus enabling other regulatory factors to access the chromatin leading to the subsequent expression of *TPO* (Cuesta *et al.*, 2007). In addition to the role of FOXE1 as a transcriptional activator, it has been suggested that FOXE1 also acts as a promoter-specific transcriptional repressor based on the interaction between its repression domain and coactivator proteins involved in the activation of thyroid-specific gene expression (Perrone *et al.*, 2000).

Genes homologous to *Foxe1* have been identified in *Xenopus* (Kenyon *et al.*, 1999; El-Hodiri *et al.*, 2005), amphioxus (Yu *et al.*, 2002), in the invertebrate chordate *C. intestinalis* (Ogasawara and Satou, 2003; Hiruta *et al.*, 2005), and zebrafish in which *Foxe1* shares a common pattern of expression with that of mouse during embryonic development but not during adulthood (Nakada *et al.*, 2009). However, it has been shown that in zebrafish, *foxe1* plays no role in thyroid development, since no thyroid phenotype was observed upon knocking-down the zebrafish *foxe1* ortholog unlike the findings in the *Foxe1* null mice embryos (Nakada *et al.*, 2009). On the contrary, the role of *Foxe1* in craniofacial development seems to be conserved among the two species, where knocking-down *foxe1* in zebrafish results in cleft palate and degeneration of cartilages in line with the craniofacial developmental defects (cleft palate) observed in *Foxe1* null mice and human patients presenting with Bamforth-Lazarus syndrome (Nakada *et al.*, 2009).

Indeed, homozygous mutations in *FOXE1* in humans have been reported in patients with a syndromic form of CH, the Bamforth-Lazarus syndrome (OMIM #241850), characterized by TD (mainly athyreosis or severe hypoplasia), cleft palate, and spiky hair with or without bilateral choanal atresia or bifid epiglottis (Bamforth *et al.*, 1989). In contrast to thyroid ectopy or

athyreosis, the two phenotypes observed in *Foxe1* null mice, patients with *FOXE1* mutations exhibit athyreosis as the etiology of TD (Bamforth *et al.*, 1989; Clifton-Bligh *et al.*, 1998; Castanet *et al.*, 2002; Carré *et al.*, 2014). The additional features, choanal atresia or bifid epiglottis, were not seen in the *Foxe1* KO mice and the spiky hair was not examined due to the perinatal death of the mutant animals (Gillam and Kopp, 2001). Indeed, the majority of *FOXE1* mutations were reported in familial forms of Bamforth-Lazarus syndrome as they were inherited from heterozygous carrier parents who were usually consanguineous (Bamforth *et al.*, 1989; Clifton-Bligh *et al.*, 1998; Castanet *et al.*, 2002; Carré *et al.*, 2014). A sporadic case, inherited via uniparental disomy (UPD), in a non-consanguineous family has also been described (Castanet *et al.*, 2010b). All the reported *FOXE1* mutations occurred within the forkhead domain (FHD) and led to either partial or complete loss of the DNA-binding ability and of the transcriptional activity of the mutated protein (Clifton-Bligh *et al.*, 1998; Castanet *et al.*, 2002; Baris *et al.*, 2006; Castanet *et al.*, 2010b). On the other hand, a homozygous missense mutation of *FOXE1*, recently identified in a patient with Bamforth-Lazarus syndrome born to consanguineous parents, was associated with increased transcriptional activity of the mutated protein. Hence, it has been noted that both gain- and loss-of-function mutations of *FOXE1* were found to be associated with Bamforth-Lazarus syndrome (Carré *et al.*, 2014).

Although systematic screening of *FOXE1* mutations in cohorts of either syndromic (with cleft palate) or isolated CH due to TD revealed their identification in a few patients with syndromic CH (Al Taji *et al.*, 2007; Narumi *et al.*, 2010), *FOXE1* has been suggested as being a susceptibility gene for TD, via its polyalanine tract, rather than being a disease-causing gene (Carré *et al.*, 2007). In a large cohort of patients with CH due to TD (athyreosis or ectopy), Carré *et al.* have shown that risk of TD was inversely correlated with the length of the *FOXE1* gene polyalanine tract where a high and low risk of TD were found to be associated with gene possessing 14 and 16 alanines, respectively (Carré *et al.*, 2007). Moreover, these findings were confirmed by both transmission-disequilibrium testing and *in vitro* functional studies, which showed differences in transcriptional activity between FOXE1 proteins with 14 versus 16 alanines (Carré *et al.*, 2007). Taken together, these findings indicate that the role of FOXE1 in abnormal thyroid development is to modulate the genetic susceptibility to TD through its polymorphic polyalanine tract (Carré *et al.*, 2007).

4.1.1.5. *NKX2.5/CSX*

In addition to the homeobox *Nkx2.1* gene, other members of the *NKx2* family, *Nkx2.3, Nkx2.5/Csx* and *Nkx2.6*, are key players in pharyngeal development (Harvey, 1996). They are expressed in the developing pharyngeal endoderm, including the thyroid primordium and other tissues (Lints *et al.*, 1993; Biben *et al.*, 1998; Biben *et al.*, 2002). In humans, the *NKX2.5/CSX* locus is located at 5q34 of chromosome 5 near the boundary of 5q34 and 5q35 and comprises two exons encoding for a 324 amino acid protein (Shiojima *et al.*, 1995), while the mouse homologue, *Nkx2.5/Csx*, is located at the *t*-locus of chromosome 17 (Himmelbauer *et al.*, 1994). During early embryonic development of mouse, *Nkx2.5* is expressed in the progenitors of the myocardial cells, continues to be expressed in the atrial and ventricular myocardium throughout development, and in the adult heart. In addition, transcripts of *Nkx2.5* were detected in the pharyngeal endoderm, the origin of the cardiac inducer (Tanaka *et al.*, 1998). Moreover, its expression is detected in the developing thyroid (from E8.5 up to E11.5) and tongue as well as in the embryonic spleen and stomach (Lints *et al.*, 1993; Tanaka *et al.*, 1998). In accordance with the *Nkx2.5* mRNA expression pattern, Kasahara *et al.* detected Nkx2.5 protein signals throughout all stages of heart development as well as in the spleen, distal stomach, and tongue. Contrary to the previously detected *Nkx2.5* mRNA in the murine thyroid (Lints *et al.*, 1993), they did not detect Nkx2.5 protein expression in thyroid gland of mouse embryos. However, it was detected in the anterior larynx, liver, and a subset of cranial skeletal muscles (Kasahara *et al.*, 1998).

Because of the early expression of *Nkx2.5* in the developing thyroid (Lints *et al.*, 1993), Dentice *et al.* have analyzed the phenotype of the thyroid primordium in *Nkx2.5* null embryos (Dentice *et al.*, 2006). In spite of normal budding of the gland evident by the expression of the thyroid-specific transcription factors *Nkx2.1, Foxe1, and Pax8*, at E9.5 a smaller sized thyroid bud was detected in *Nkx2.5* null embryos compared to a normally sized bud in wild type (WT) counterparts, thus suggesting that *Nkx2.5* is required during the organogenesis of the thyroid. The expression of Nkx2.5 was detected until E11.5 but no longer after that (Dentice *et al.*, 2006). A plausible functional redundancy between the *Nkx2.3, Nkx2.5* and *Nkx2.6* genes during early embryonic development is suggested based on their overlapping expression domains in the pharyngeal endoderm and the thyroid anlage (Biben *et al.*, 2002). In support of this is the observed redundant role of *Nkx2.3* and *Nkx2.5* in cardiogenesis reported in *Xenopous* embryos as well as the lack of abnormalities in the pharynges of *Nkx2.5* null embryos but the essential role

of *Nkx2.6* in pharyngeal development (Biben *et al.*, 2002). In addition, in double-mutant *Nkx2.5* and *Nkx2.6* embryos, lack of pharyngeal pouch formation is observed due to the elimination of the overlapping functions of both genes, thus supporting their redundant function (Tanaka *et al.*, 2001). Collectively, these data suggest that the absence of one of these genes in the developing thyroid could be compensated by another member of the sub-family (Tanaka *et al.*, 2001; Biben *et al.*, 2002).

Animal models have pointed to the crucial role of *Nkx2.5* in the morphogenesis and normal physiology of the developing heart (Lints *et al.*, 1993; Lyons *et al.*, 1995; Biben and Harvey, 1997; Kasahara *et al.*, 2000). Indeed, mutations in the coding region of the *NKX2.5* gene and sequence variants within its promoter region have been detected in patients with congenital heart disease (CHD) (Schott *et al.*, 1998; Reamon-Buettner and Borlak, 2010; Stallmeyer *et al.*, 2010) (Pang *et al.*, 2012). In addition, a new *NKX2.5* mutation has been described that is associated with congenital as well as adult-onset of heart disease (Costa *et al.*, 2013). On the basis of the detected *Nkx2.5* expression in the developing heart and thyroid as well as the high incidence of CHD in children with CHTD compared to the general population (Siebner *et al.*, 1992; Devos *et al.*, 1999; Kreisner *et al.*, 2005), it is assumed that *NKX2.5* is a candidate gene for the pathogenesis of CH due to TD (Dentice *et al.*, 2006). Actually, three heterozygous mutations of *NKX2.5* have been identified in CHTD patients with either thyroid ectopy or athyreosis. The reported mutations, inherited from unaffected parents, are functionally characterized by impaired binding of DNA, reduced transactivation as well as a dominant negative effect (Dentice *et al.*, 2006). Among the described variants, p.R25C, is a SNP (rs2893667) that is found in 1% of the general population and has been identified in several patients with CHD with no abnormalities in thyroid organogenesis (Tennessen *et al.*, 2012; Beffagna *et al.*, 2013). Hence, such a variant seems not to be involved in the pathogenesis of TD (van Engelen *et al.*, 2012). Together with a maternally inherited mutation in the *PAX8* promoter region, a paternally inherited *NKX2.5* mutation, exhibiting reduced function, has been reported in a girl with TD (Hermanns *et al.*, 2011). Being carried by a healthy parent, sibling, and grandmother as well as being associated with a mutation in *PAX8* that might account for the TD phenotype, the role of this *NKX2.5* mutation in the pathogenesis of TD has perhaps been overestimated (van Engelen *et al.*, 2012). Moreover, a previously reported mutation, p.A119S, in *NKX2.5* (Dentice *et al.*, 2006) has been recently described in a proband of a family with four affected individuals with CHD and a

sporadic CHD patient. The mutation was not associated with CHD in the familial case and none of the mutation carriers exhibited a thyroid phenotype (van Engelen *et al.*, 2012). In contrast to what has been previously reported (Dentice *et al.*, 2006), functional analysis of the mutant protein revealed no transactivation differences compared to its wild type counterpart (van Engelen *et al.*, 2012). Except for the reported *NKX2.5* mutations (Dentice *et al.*, 2006; Hermanns *et al.*, 2011; van Engelen *et al.*, 2012), screening of several TD cohorts, including patients with CHD, revealed no additional mutations (Al Taji *et al.*, 2007; Ramos *et al.*, 2009; Narumi *et al.*, 2010; Passeri *et al.*, 2011; Brust *et al.*, 2012). From all the above, it has been suggested that *NKX2.5* mutations are not a major contributor in the pathogenesis of TD, although its role as a genetic modifier cannot be ruled out (van Engelen *et al.*, 2012).

4.1.1.6. *HHEX/PRH*

Like the thyroid transcription factors NKX2.1, PAX8, and FOXE1, the transcription factor HHEX/PRH is expressed in the mature thyrocytes as well as their precursors (pTFCs) (Thomas *et al.*, 1998; Pellizzari *et al.*, 2000). It is their combined coexpression that provide the pTFCs with a specific molecular signature distinguishing them from other cells in the primitive pharynx (Parlato *et al.*, 2004). The haematopoietically expressed homeobox (HHEX) protein, also named PRH (proline-rich homeodomain), is encoded by a member of the Homeobox genes, a family of transcription factors that share a highly conserved 60 amino acid DNA-binding domain known as the homeodomain (Bedford *et al.*, 1993; Hromas *et al.*, 1993). HHEX was initially identified in various progenitors of hematopoietic cells (Crompton *et al.*, 1992; Bedford *et al.*, 1993). In mice, the genomic locus encoding Hhex, termed *Hhex*, is located on chromosome 19, while in humans it is called *HHEX* and is located on chromosome 10q23.32 (Hromas *et al.*, 1993; Ghosh *et al.*, 1999). *HHEX* encodes a protein that is 270 amino acids and has a molecular weight of 30 KDa. It shows a 94% identity to the 271 amino acids protein that is encoded by the *Hhex* locus in mice (Bedford *et al.*, 1993). In addition to the homeodomain, the HHEX protein contains an acidic C-terminal and an N-terminal proline-rich domains, where the latter is plausibly involved in regulating the transcription of target genes (Crompton *et al.*, 1992; Tanaka *et al.*, 1999; Swingler *et al.*, 2004). *Hhex*/*HHEX* encodes a tissue-specific transcription factor that either activates or represses the transcription of target genes using both direct and indirect mechanisms (Soufi and Jayaraman, 2008).

During early developmental stages of the mouse, the expression of *Hhex* is detected in the anterior visceral endoderm (AVE) and the definitive endoderm. Subsequently, expression of *Hhex* was found in the endothelial and hematopoietic precursors. Starting from E8.5 onward, the primordia of several organs derived from the foregut, including thyroid, liver, gall bladder, thymus, pancreas, and lungs are marked by the expression of *Hhex*, among which both developing and adult thyroid showed the highest level of *Hhex* expression (Bogue *et al.*, 2000; Martinez Barbera *et al.*, 2000).

In accordance with the expression of *Hhex* in the definitive endoderm during early development, it has been shown that it is crucial for the development of the forebrain, liver, and thyroid (Martinez Barbera *et al.*, 2000). In *Hhex* null mice embryos, severe (class I of embryos) to mild (class III) truncations of the forebrain were observed together with liver and thyroid hypoplasia. In *Hhex*$^{-/-}$, although the liver is specified and proliferation of its precursors is initiated, migration of these cells into the septum transversum did not occur with the subsequent degeneration of the liver diverticulum and disruption of the differentiation of precursor cells. In an initial attempt to determine the role of *Hhex* in the development of the thyroid gland, Martinez Barbera *et al.* have suggested its involvement in the specification of the thyroid gland. They have shown that at E9.5, the *Hhex*$^{-/-}$ mice embryos exhibit either lack of the thyroid primordium or, in the majority of embryos, hypoplasia, with no gland detected by E13.5. In addition, no expression of the thyroid transcription factors Nkx2.1 and Foxe1 was detected in the thyroid bud (Martinez Barbera *et al.*, 2000). However, the thyroid defects exhibited by the *Hhex*$^{-/-}$ mice embryos might be attributed to the general deficiency of the anterior endoderm (Martinez Barbera *et al.*, 2000) rather than being thyroid-specific (Fagman and Nilsson, 2010). On the other hand, the role of *Hhex* in specification of the thyroid cells has been ruled out by Parlato *et al.* as they have demonstrated the appropriate formation of the thyroid anlage at E9 together with the expression of the thyroid transcription factors *Nkx2.1*, *Pax8, and Foxe1* in the absence of *Hhex*. At subsequent developmental stages (E10), the absence of *Hhex* was associated with severe impairment of thyroid bud morphology, a profound alteration in the number of thyroid cell precursors as well as down-regulation of *Nkx2.1*, *Pax8, and Foxe1*. Hence, the described thyroid phenotype in absence of *Hhex* reflects its role in regulating the proliferation of thyroid cells and is correlated with its crucial role in maintaining the expression of *Nkx2.1*, *Pax8, and Foxe1* during thyroid development (Parlato *et al.*, 2004).

In common with the phenotype of *Hhex*$^{-/-}$ mouse embryos, Elsalini *et al.* have shown that thyroid specification in zebrafish deficient in *hhex* occurred with subsequent failure of development (Elsalini *et al.*, 2003). In addition, the authors have suggested that, in zebrafish, *hhex* can regulate the proliferation of the thyroid primordial cells after their specification with the subsequent regulation of thyroid size during later developmental stages. Such suggestions are consistent with what has been recently shown in mouse, where the number of thyroid primordial cells determines embryonic thyroid size and is controlled by a mesodermal Tbx1-dependent signal derived from cells located lateral and ventral to the pharyngeal endoderm, mediated via the fibroblast growth factor 8 (Fgf8) (Lania *et al.*, 2009).

In humans, systematic screening for genetic defects in thyroid-related transcription factors in a phenotype-selected cohort of patients with non-goitrous congenital and early-onset hypothyroidism CH revealed no *HHEX* mutations. In addition, only a very low incidence of genetic abnormalities in the other thyroid transcription factors *NKX2.1*, *FOXE1,* and *NKX2.5* has been observed (Al Taji *et al.*, 2007).

4.1.1.7. Inductive signals and other genes

In addition to the above mentioned thyroid transcription factors *NKX2.1*, *PAX8*, *FOXE1,* and *HHEX* as well as the thyroid-related genes *TSHR* and *NKX2.5,* their role in the developmental process of the thyroid gland and their contribution, although low, in the pathogenesis of CH due to TD, studies carried out in animal models for TD have pointed to the role of non-cell-autonomous inductive signals and other endodermal genes in the induction of a thyroid fate and organogenesis of the thyroid gland, respectively (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2010).

4.1.1.7.1. Inductive signals

Nodal is a member of the transforming growth factor beta (TGFβ) superfamily of signalling molecules, which includes TGF-βs, activins, growth/differentiation factors (GDFs), and bone morphogenic proteins (BMPs) (Schier, 2009). In vertebrates, Nodal-related signals are known for their involvement in the specification and patterning of the mesoderm and endoderm germ layers and for the morphological rearrangements that take place during gastrulation (Weng and Stemple, 2003; Tian and Meng, 2006). In zebrafish, it has been shown that the development of the thyroid gland relied on Nodal signalling: Zebrafish mutants for the *one-eyed pinhead*

(*oep*), a co-receptor for Nodal ligands, showed no specification of the endoderm and lack of formation of the gut tube including the thyroid (Schier *et al.*, 1997). In addition, the thyroid primordium was found to be completely absent in zebrafish mutants of *casanova (cas)* and *bonnie and clyde (bon)*, genes acting downstream of Nodal signalling (Elsalini and Rohr, 2003; Elsalini *et al.*, 2003). In these mutants, the activation of the endoderm determining gene *sox17*, is disrupted (Aoki *et al.*, 2002; Tam *et al.*, 2003; Sinner *et al.*, 2004). A more thyroid-specific impact of Nodal signalling was observed in zebrafish mutants of *faust (fau)/gata5*, another downstream Nodal regulator of sox17. In these mutants, expression of the *Nkx2.1* ortholog, *nk2.1*, is lost in the region of the potential thyroid primordium. However, only a mild impact on the early gut tube formation was observed (Reiter *et al.*, 2001). On the contrary, thyroid gland specification and initial morphogenesis occur in mouse embryos with inactivated *Sox17,* suggesting the involvement of other mechanisms downstream of Gata signalling in the development of the thyroid gland (Kanai-Azuma *et al.*, 2002). In zebrafish mutants of the Nodal ligand Cyclops (*cyc*), the thyroid anlage is specified but is small when compared to wild-type embryos, possibly reflecting an overall reduction of the pharyngeal endoderm. Moreover, the number of follicles in *cyc*$^{-/-}$ mutants is reduced later during development (Elsalini *et al.*, 2003). Taken together, data from the *cyc*$^{-/-}$ mutants points to the role of the number of cells specified at early stages of organogenesis in determining the final size of the organ (Fagman and Nilsson, 2010). Such a finding is supported by previous studies showing that the pancreas size is limited by the number of its embryonic progenitors (Stanger *et al.*, 2007).

Various studies have pointed to the importance of **mesodermal inductive signals** in the specification of the gut region and the derived organs (Jung *et al.*, 1999; Kumar *et al.*, 2003; Serls *et al.*, 2005; Manfroid *et al.*, 2007). Interactions between both the mesoderm and endoderm have been shown to play a central role in the specification and subsequent development of the thyroid anlage in zebrafish. It has been shown that in zebrafish, the specification of the thyroid is influenced, in a non-cell-autonomous manner, by the pharyngeal mesodermal signals mediated through **hand2**, a bHLH transcription factor involved in heart development. In zebrafish mutants for *hands off* (*han, hand2*), defects in cardiac morphogenesis are associated with complete absence of a functional thyroid gland. Likewise, the zebrafish ***ace* (*fgf8*)** mutant exhibited similar thyroid developmental flaws as did the *han* mutants, thus indicating that Fgf8 acts, in a non-cell-autonomous manner, in thyroid development. It has been shown that Fgf signals act either

downstream or in parallel to hand2 signalling in regulating thyroid development. Hence, the thyroid defects in zebrafish *han* mutants are due to the loss of Fgf-expressing tissue (Wendl *et al.*, 2007).

In mice, the role of Fgf signalling in thyroid gland development has been revealed by the absence of the gland in embryos deficient in the Fgf receptor 2 isoform IIIb **(Fgfr2b)** or its ligand, Fgf10, despite the initial development of the thyroid anlage (Celli *et al.*, 1998; Ohuchi *et al.*, 2000; Revest *et al.*, 2001). In addition, the docking protein fibroblast growth factor receptor substrate 2 **(Frs2alpha or Frs2α)**, that links the Fgf receptors (Fgfrs) to various signalling pathways, has been shown to regulate different morphogenetic steps of the thyroid. In the $Frs2\alpha^{2F/2F}$ mutant (-/-) mice, the thyroid gland was completely absent or hypoplastic and C cells were missing or markedly decreased in number (Kameda *et al.*, 2009).

Tbx1, the major gene whose human homolog is associated with the 22q11 deletion syndrome (22q11DS/Di George syndrome), encodes a T-box transcription factor that is expressed in both the pharyngeal epithelia (ectoderm and endoderm of the pharyngeal apparatus) and the pharyngeal mesoderm (Wurdak *et al.*, 2006), but not in the thyroid primordium at any embryonic stage (Fagman *et al.*, 2007). However, the primordium was found to be partially surrounded by a caudal mesodermal expression domain of *Tbx1* (Fagman *et al.*, 2007; Lania *et al.*, 2009). Fagman *et al.* have shown that *Tbx1* is involved, in a non-cell-autonomous mechanism, in late developmental stages of the thyroid gland. They have shown that *Tbx1* deficient embryos exhibit thyroid hypoplasia and hemiagenesis due to improper growth and translocation of the specified thyroid placode (Fagman *et al.*, 2007). In mouse, a close association between the developing thyroid and large embryonic blood vessels originating from the cardiac outflow tract has been previously shown (Fagman *et al.*, 2006). Hence, it has been suggested that the mechanism underlying the thyroid phenotype in $Tbx1^{-/-}$ mice is the inability of the embryonic thyroid to achieve contact with vessels derived from the cardiac outflow tract, an interaction crucial for proper bilateral growth and lobulation of the developing gland (Fagman *et al.*, 2007). Lately, an earlier role of *Tbx1* in thyroid development has been shown whereby it regulates the size of the early thyroid primordium by means of its expression in the adjacent mesoderm (Lania *et al.*, 2009). Knowing that Tbx1 is a transcriptional regulator of *Fgf8* in the mesodermal domain surrounding the thyroid primordium (Zhang *et al.*, 2006) and that Fgf8 is important for endodermal cell proliferation (Park *et al.*, 2006), it has been postulated that Fgf8

mediates the Tbx1-dependent interactions between mesodermal cells and the pTFCs (Lania *et al.*, 2009). Consistent with the role of *fgf8* in zebrafish thyroid development (Wendl *et al.*, 2007), Lania *et al.* have described a *Tbx1*-dependent role for *Fgf8* in mammalian thyroid development as well. They have shown that Tbx1, via Fgf8, is involved in determining the size of the thyroid early during mouse development probably via regulating the proliferation of its endodermal-derived progenitors (Lania *et al.*, 2009).

In vertebrates, members of the Hedgehog family of secreted proteins are key regulators of embryonic development. They are involved in various developmental processes among which is tissue patterning and organogenesis from all three germ layers (McMahon *et al.*, 2003). Among the Hedgehog members, sonic Hedgehog **(*shh*)** is known to be crucial for foregut development (Litingtung *et al.*, 1998). Fagman *et al.* have assessed the role of *Shh* in early and late stages of thyroid development (Fagman *et al.*, 2004). The thyroid phenotype (hypoplasia and hemiagenesis) described in *Tbx1* null mice (Fagman *et al.*, 2007) has been observed as well in mice deficient in *Shh*, the positive regulator of Tbx1 (Fagman *et al.*, 2004). In spite of the normal specification of the thyroid primordium, a trivial delay in both its budding and dislocation has been reported in *Shh*$^{-/-}$ embryos. Later during organogenesis, a defect in the bifurcation and localization of the developing gland was observed, resulting in an abnormal, unilateral (mostly left sidedness), single thyroid mass (hemiagenesis) in the mutant embryos. However, deficiency of *shh* exhibited no impact on the terminal differentiation of the gland. In addition, ectopic Nkx2.1 and Foxe1-positive cells developed in the presumptive trachea of *Shh*$^{-/-}$ embryos, thus indicating the role of shh in repressing the thyroid differentiation program in non-thyroidal tissues (Fagman *et al.*, 2004). Indeed, neither *Shh* nor its patched 1 (Ptc1) receptor mRNA expression was detected in the pTFCs nor in the surrounding mesenchyme throughout the developmental process of the gland (Fagman *et al.*, 2004). Hence, it has been suggested that the impact of shh on thyroid development is indirect and that the observed phenotype is probably consequent to morphological defects within the shh-regulated tissues in cervical regions, ultimately leading to vascular defects (Fagman *et al.*, 2004). Vascular defects in the head/neck region have been previously reported in both *Shh* or *Tbx1* deficient early mouse embryos (Yamagishi *et al.*, 2003). In addition, Alt *et al.* have shown that the thyroid gland is co-developing with the major arteries, the ventral aorta and carotid arteries in zebrafish and mouse,

respectively. They have suggested that these major vessels play role in determining thyroid tissue localisation (Alt *et al.*, 2006b).

Retinoic acid **(RA)**, a derivative of vitamin A, is involved in a variety of essential signalling pathways during vertebrate organogenesis (Duester, 2008). In addition to the established role of RA in the patterning of both the central nervous system (CNS) and the mesoderm (Chazaud *et al.*, 1999; Niederreither *et al.*, 2000; Begemann *et al.*, 2001; Grandel *et al.*, 2002; Niederreither *et al.*, 2002), it has been shown that, in a number of vertebrates, RA signalling influences the early regionalization of the developing endoderm. In zebrafish, inhibition of the RA signalling pathway had no impact on the specification of the thyroid gland in contrast to the evident defects in the specification of the hepatopancreatic progenitors. However, the position of the thyroid is altered as evident by the lack of *hhex* expressing cells in the thyroid expression domain consequent to interruption of the antero-posterior (AP) patterning instructions (Stafford and Prince, 2002). Similarly, specification of the thyroid occurs in chick embryos either in the absence of, or at low levels, of RA, whereas it is prevented upon application of exogenous RA. Moreover, the expression of the thyroid progenitors' markers *Hex* and *Nkx2.1* was repressed (Bayha *et al.*, 2009). Likewise, Wang *et al.* have shown recently that, in *Xenopus* embryos, addition of exogenous RA leads to the expression of markers of differentiated lung in the presumptive thyroid, with a lack of expression of the early transcription factors normally seen (*pax2, foxe4*, and *hhex*), thus indicating that development of the thyroid gland occurs in the absence of RA signalling (Wang *et al.*, 2011b). Studies carried out in mouse embryos also indicated that RA signalling is less likely involved in the early morphogenetic events of the thyroid gland (Desai *et al.*, 2004; Goss *et al.*, 2009; Chen *et al.*, 2010a). Collectively, these data indicate that RA plays a crucial role in pre-patterning of the foregut endoderm and positioning of the buds of organs along the digestive tract (Duester, 2008) but not in the determination/specification of the cells destined to become the thyroid.

From all of the above, we can conclude that the main initiator signal(s) of the thyroid anlage specification is still unknown. Although using pluripotent embryonic stem cells (ESCs) as an *in vitro* model could enhance our knowledge about the factor(s) and mechanism(s) required for acquiring a thyroid fate, the outcomes of the research work carried on ESC is not yet conclusive (Nilsson and Fagman, 2013). Previous studies have shown that differentiation of mouse ESC into thyroid cells occurs either in a TSH-dependent (Jiang *et al.*, 2010; Davies *et al.*,

2011) or TSH-independent activin A mediated manner (Ma *et al.*, 2009). In the case of activin A mediated differentiation, the resultant thyroid cells probably resemble a group of pre-programmed cells that are turned on after prolonged culture of ESC, following their nodal/activin-mediated differentiation into definitive endoderm (Sui *et al.*, 2013). In addition to TSH and its importance for the induction and specification for thyrocytes from ESCs, Arufe *et al*. have shown that both insulin and insulin-like growth factor-1 (IGF-1) are essential for their maturation (Arufe *et al.*, 2009). Recently, common specification inductive mechanisms for lung and thyroid have been suggested (Longmire *et al.*, 2012). Both murine Nkx2.1^{+} lung and thyroid progenitors have been derived and purified from definitive endodermal precursors, following transforming growth factor beta/bone morphogenetic protein (TGFβ/BMP) inhibition and subsequent combinatorial induction of BMP4 and FGF2 signalling. Such a cell fate restriction was accompanied by epigenetic silencing of Oct4 in Nkx2.1^{+} and altered histone methylation of the *Nkx2.1* locus in Nkx2.1-negative cells (Longmire *et al.*, 2012). Moreover, Antonica *et al.* have shown that mouse ESCs can be induced *in vitro* to differentiate into TFCs with the subsequent self-formation of functional thyroid follicles via transient overexpression of the two thyroid transcription factors Nkx2.1 and Pax8 followed by recombinant human TSH (rhTSH) treatment. These findings pave the way for the implementation of ECS technology in the treatment of hypothyroidism, keeping in mind that CH is the most common congenital endocrine disorder in humans (Antonica *et al.*, 2012).

4.1.1.7.2. Other genes

The ***Hox*** genes encode a class of transcription factors characterized by the presence of a highly conserved DNA binding domain, the *Antennapedia* homeodomain. They are organized in four clusters (*Hox A*, *B*, *C*, and *D* clusters) that are located on four different chromosomes (Krumlauf, 1994). In humans, the clusters *HOX A*, *B*, *C*, and *D* map to chromosomes 7p14, 17q21, 12q13 and 2q31, respectively (Mark *et al.*, 1997; Quinonez and Innis, 2014). During embryogenesis, the *Hox* genes play a crucial role in patterning the anterior-posterior axis of the developing embryo (Manley and Capecchi, 1995). Among the *Hox* genes, *Hoxa3* is expressed in the hindbrain, migrating neural crest, neural-crest-derived mesenchymal cells, and the pharyngeal pouch endoderm of the 3rd and 4th pharyngeal arches but not in the thyroid primordium (Manley and Capecchi, 1998). Hence, *Hoxa3* mutants exhibited multiple developmental defects that included deletions and malformations of the throat cartilage and

cranial nerves, absence of both thymus and parathyroids as well as thyroid hypoplasia (Manley and Capecchi, 1995; 1998). In *Hoxa3* mutants, Manley and Capecchi have shown that the observed thyroid phenotype is due to defects involving the number and organization of both the endoderm-derived follicular cells and the parafollicular C cells, derivatives of the mesenchyme neural crest cells. They described alterations in the UBB (precursors of C cells) with a reduction in parafollicular C cells that was mainly unilateral. In addition, severe structural defects in the follicular lobes of the thyroid have been shown that included a variable overall shape and size of the thyroid lobes and the displacement or complete loss of the isthmus (Manley and Capecchi, 1995). Furthermore, analysis of mice double mutants of *Hoxa3* and either of its paralogs, *Hoxb3* or *Hoxd3,* has revealed an exacerbation of the thyroid phenotype that corresponds to severe aberrations in the UBB, thus reflecting the crucial role of *Hox3* paralogs in UBB development and migration (Manley and Capecchi, 1998).

Hoxa5 is among the *Hox* genes that have been shown to affect thyroid development. Targeted disruption of the *Hoxa5* gene revealed its involvement in axial and appendicular specification of the cervicothoracic region (Jeannotte *et al.*, 1993; Aubin *et al.*, 1998) as well as in the functional maturation of foregut (Aubin *et al.*, 1997; Aubin *et al.*, 2002) and midgut (Aubin *et al.*, 1999) derivatives. Collectively, most of the defects resulting from the loss of *Hoxa5* function are limited to the cervicothoracic region which also includes the pharyngeal glands including the thyroid. Hence, the role of *Hoxa5* during the development of the thyroid gland was assessed in *Hoxa5* mutants (Meunier *et al.*, 2003). Foremost, expression of *Hoxa5* was detected at E12.5 in regions flanking the UBB and the expanding thyroid tissue as well as in the surrounding tracheal mesenchyme; expression disappeared by E18.5. Lack of *Hoxa5* function led to a transient altered expression of the thyroid transcription factors *Nkx2.1*, *Foxe1* and *Pax8* at various developmental stages of the thyroid gland. In spite of the normal onset of thyroid morphogenesis in *Hoxa5*$^{-/-}$ mutants, deficiency of *Hoxa5* led to late gestational disturbances at the level of both development as well as the structural organization of the thyroid gland including the detection of a large proportion of thyroglobulin (Tg)-depleted follicles by E17.5 and E18.5. The observed abnormal accumulation of Tg in the thyroid follicles is plausibly consequent to the defective transport of Tg that hinders its proper distribution in the lumen of the thyroid follicles. Apart from Tg-depleted follicles, no other significant differences in either the production or the distribution of Tg, calcitonin, and T_4 was detected at later fetal as well as

postnatal stages. Finally, *Hoxa5*$^{-/-}$ mice exhibited transient postnatal growth retardation as well as retarded eye opening and ear extension, a postnatal phenotype resembling that reported in *hyt*/*hyt* mouse and the TG66-19 transgenic mouse line, both of which are animal models of congenital hypothyroidism (Beamer *et al.*, 1981; Adams *et al.*, 1989; Wallace *et al.*, 1995). Together, these data point to the transient impact exerted by *Hoxa5* depletion on thyroid development (Meunier *et al.*, 2003).

Eya1 is one of the members of the *Eya* gene family, which includes several homologues (four genes in mammals) of the *Drosophila eyes absent (eya)* gene (Duncan *et al.*, 1997; Hanson, 2001). During mouse development, a robust expression of *Eya* genes in the pharyngeal region and its derivatives has been detected starting from E9.5 (Xu *et al.*, 2002). Xu *et al.* have assessed the role of *Eya1* in the development of the pharyngeal organs among which the thymus, parathyroid and the thyroid glands. In *Eya1*$^{-/-}$ embryos, the organ primordia for both thymus and parathyroids fail to form. Comparable to what is observed in *Hoxa3* null embryos (Manley and Capecchi, 1995), *Eya1*$^{-/-}$ mice presented hypoplastic thyroid lobes together with severe reduction in the number of parafollicular C cells, lack of fusion between the UBB and the thyroid lobes as well as absence of the isthmus (Xu *et al.*, 2002). Although these data indicate that *Eya1* is required for the formation of a mature thyroid gland, it has been postulated that the defects in thyroid lobes associated with absence of *Eya1* are due to the lack of fusion with the UBB rather than a direct involvement of *Eya1* in the morphogenesis of the gland (Xu *et al.*, 2002). Supporting this hypothesis is the lack of expression of *Eya1* in the thyroid diverticulum and the observation of thyroid defects resembling those observed in *Hoxa3* and *Eya1* mutants in mice deprived of *Pax3* or *Endothelin-1*, two genes known to be implicated in the development of neural crest-derived structures (Franz, 1989; Kurihara *et al.*, 1995). In humans, mutations in the *EYA1* gene have been identified in the Bronchio-Oto-Renal (BOR) syndrome, an extrathyroidal syndrome without TD (Abdelhak *et al.*, 1997).

Hes1 (hairy/enhancer of split 1), a Notch target gene product, is a widely distributed proline basic-helix-loop-helix (bHLH) protein characterized by the presence of another two domains, the Orange domain and a C-terminal Trp-Arg-Pro-Trp (WRPW) domain involved in the selection of bHLH heterodimer partners and transcription repression, respectively (Iso *et al.*, 2003; Kageyama *et al.*, 2007). As a transcriptional repressor of bHLH target genes, including the C cell expressed *Mash1*, Hes1 regulates the morphogenesis of various tissues by maintaining the

undifferentiated state of the progenitor cells (Kageyama *et al.*, 2007). Moreover, it plays a crucial role in maintaining the proliferation of progenitor cells through the transcriptional repression of the cyclin-dependent kinase inhibitors $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$(Carré *et al.*, 2011). In addition, Hes1 was found to be involved in controlling the development of endodermal endocrine tissues, the pancreas and the pituitary (Jensen *et al.*, 2000; Raetzman *et al.*, 2007). Indeed, $Hes1^{-/-}$ mice embryos exhibited severe neuronal defects and profound both thymic and pancreatic hypoplasia (Murata *et al.*, 2005). Furthermore, Carré *et al.* have shown that *Hes1* is involved in determining the normal size of the thyroid gland as it regulates the number of progenitors of thyrocytes and C cells encompassed in the two thyroid anlagen. Furthermore, they have shown that *Hes1* is crucial for normal organogenesis of the developing gland as it assures the proper fusion of the thyroid anlagen. In addition to the documented hypoplasia and abnormal fusion of the thyroid anlagen in $Hes1^{-/-}$ mutants, a reduced endocrine function of both the thyrocytes and the C cells was demonstrated, who have a profound decrease in both T_4 and calcitonin (CT)/NKx2.1 positive surface areas (Carré *et al.*, 2011). Recently, Kameda *et al.* have confirmed the previously reported defects of the pharyngeal endoderm-derived organs, including the thymus, parathyroid, and thyroid glands, in $Hes1^{-/-}$ null mice, with the most striking malformations occurring in the form of aplasia of both the UBB (precursors of thyroid C cells) and parathyroid glands. In addition, consequent to the defective survival of neural-crest-derived mesenchymal cells in the absence of Hes1, they have attributed the observed hypoplasia or aplasia of the pharyngeal endoderm-derived organs to the scarcity of these cells in the pharyngeal region, the signals from which are essential for the differentiation, migration and survival of the pharyngeal endoderm-derived developing organs (Kameda *et al.*, 2013).

Isl1 is among the novel genes found to be involved in the development of the thyroid gland. It encodes Islet1 (Isl1), one of the LIM homeodomain transcription factors, named for the LIM domain-containing transcription factors Lin11, Isl1, and Mec3. The LIM protein super family possesses, in addition to the DNA binding homeodomain, a LIM homeodomain (LIM-HD) which is a cysteine-rich zinc-coordinating domain consisting of two tandemly repeated zinc fingers. In vertebrates, the 12 LIM homeodomain genes identified to date encode transcription factors known to play crucial roles in cellular development and differentiation of various tissues including the pituitary and pancreas (Hunter and Rhodes, 2005).

The expression pattern of *Isl1* in the developing thyroid has been assessed by Westerlund *et al*. (Westerlund *et al.*, 2008). They have shown that *Isl1* is expressed in both the median and lateral primordia of the developing thyroid gland and the mesenchyme adjacent to the thyroid bud. Continuous expression of *Isl1* was observed in the two anlagen until their fusion, where a prominent expression of *Isl1* was detected only in the lateral primordia. Later during development, *Isl1* expression was detected only in immature C cells before their terminal differentiation program is started. Moreover, in the adult gland, the number of *Isl1* positive cells was lower compared to those expressing calcitonin and might represent a subpopulation of C cells that preserve properties of progenitor cells in adult life. Collectively, these data suggest the involvement of Isl1 in regulating thyroid development before the terminal differentiation of its precursors (Westerlund *et al.*, 2008).

Although the thyroid median anlage is specified in *Isl1* mice null embryos, Westerlund *et al.* have shown that the size of the placode is reduced. Hence, *Isl1* might be involved in regulating the growth and expansion of the thyroid precursors during the budding of the thyroid anlagen and afterwards. Though, it could be attributed to the generalized growth retardation reported in *Isl1* null embryos that particularly affects the mesoderm, the endoderm, and their derivatives. Moreover, the authors have indicated that comparable to the endodermal *Isl1*, the abundantly expressed *Isl1* in the mesenchyme located adjacent to the pharyngeal endoderm and the thyroid bud exhibited no impact on the specification of the thyroid placode (Westerlund *et al.*, 2008).

EphA4 is one of the Eph receptors (named for the expression in an *E*rythropoetin-*P*roducing *H*epatocellular carcinoma cell line) representing the largest mammalian tyrosine kinase family of receptors. Eph receptors and their membrane-bound ligands, the ephrins, affect the morphogenesis of various tissues as well as maintaining homeostasis in many adult organs (Pasquale, 2008; Miao and Wang, 2009). The Eph-ephrin receptor-ligand systems transmit their signals in a bidirectional (forward and reverse) manner exerted by Eph kinases and ephrins on the opposing cells, respectively (Miao and Wang, 2009). Based on their binding preferences, both Ephs and their ligands are classified as A- and B-types. Exceptionally, EphA4 is the only Eph receptor that can bind to and be activated by both A-and B-type ephrins (Durbin *et al.*, 1998).

Lately, it has been reported that the EphA4 receptor is steadily expressed in the pTFCs in early and late developmental stages and in the mouse thyroid beyond birth till adulthood. Hence, it has been suggested that Eph/ephrin signalling confers a regulatory role on the development of the thyroid gland (Andersson *et al.*, 2011). During the developmental process of the thyroid, no signs of thyroid dysgenesis or defective folliculogenesis were observed in *EphA4* deficient late embryos. In addition, both markers of specification (*Nkx2.1*) and functional differentiation (*Tg*) were normally expressed. Although the *Eph4*-null adult mice are euthyroid and have a normal-sized thyroid, they exhibited fine but marked phenotypic modifications involving the size, number, and shape of follicles. On the other hand, a more severe phenotype characterized by flattened follicular epithelium and a reduced number of C cells was detected in mutant animals with a truncated receptor that is unable to convey a forward signal (Andersson *et al.*, 2011). The reduction in the number of C cells might be due to either a decreased proliferation rate or to a disturbed migration of C cells; both processes are affected by Eph receptor tyrosine kinases (Pasquale, 2005). Together, these data indicate that EphA4 is a novel regulator of postnatal thyroid morphogenesis and is probably involved in regulating the morphogenesis of the differentiated gland via modulating the development of both the thyroid follicular and the C cells (Andersson *et al.*, 2011).

Dicer, an RNaseIII endonuclease, is involved in the functional maturation of the small non-coding RNAS, the microRNAs (miRNAs) (Starega-Roslan *et al.*, 2011). Several animal studies have revealed the significant role of small RNAs in the organogenesis of various tissues (Rodriguez *et al.*, 2012). The role of small RNAs in thyroid organogenesis has been assessed using two mouse models, *Pax8(Cre/+); Dicer$^{flox/flox}$* and *Tg(Cre/+); Dicer$^{flox/flox}$*, in which *Dicer* was conditionally knocked-out (cKO) in TFCs either early during development (E8.5) or at later stages (E14.5), respectively (Rodriguez *et al.*, 2012). In accordance with previous results (Frezzetti *et al.*, 2011), inactivation of *Dicer* in either model showed no impact on early stages of thyroid development and an *in situ* bi-lobed gland was observed. However, at the age of one month, the two *Dicer* cKO mutants exhibited severe hypothyroidism associated with low total T_4 and increased TSH plasma levels resulting from lack of appropriate follicular organization. Follicular disorganization, in the form of a low number of follicles with appreciable lumen compartment, could lead to the thyroid hypoplasia exhibited by the mutant animals. Consequently, the mutant animals die around weaning time with an observed delay of two weeks

upon thyroid hormone replacement therapy. In the minority of viable animals on treatment, a progressive de-differentiation of the thyroid is seen, as revealed by the significant reduction of thyroid related genes (specifically *Nis* together with *Pax8*, *Foxe1*,*Tpo*, *Tg*, and *Tshr*), with the exception of *Nkx2.1*, at both mRNA and protein levels. Moreover, remarkably increased proliferation rates were observed in *Dicer* cKO mutants compared to their wild-type counterparts, although no significant differences in the number of follicular cells was observed. Indeed, the increased rate of proliferation is less likely to be due to a Tshr-mediated cAMP signalling hyperstimulation (Rodriguez *et al.*, 2012). In addition to thyroid differentiation markers, it has been previously reported that in absence of Dicer, the expression of the thyroid polarity markers, Cdh1 (E-cadherin) and Cdh16 (ksp-cadherin), is either reduced or completely lost, respectively, thus influencing cell polarity and consequently thyroid function (Frezzetti *et al.*, 2011).

The thyroid de-differentiation observed in the above described mutant animals resembles that observed during human thyroid tumorigenesis with the notable lack of *Nis* expression followed by that of *Tshr* and *Tpo* (Gérard *et al.*, 2003). The lack of *Nis* and *Tpo* expression could be attributed to the down-regulation of *Pax8* and *Foxe1*, the two crucial regulators of their expression (Rodriguez *et al.*, 2012). The increased rate of follicular cell proliferation observed in the two *Dicer* cKO models, in spite of the down-regulation of *Tshr*, reflects the role of miRNAs in regulating the expression of genes implicated in controlling cellular proliferation (Chen *et al.*, 2010c). Collectively, the previous data point to the importance of an intact miRNA biogenesis machinery, mediated via the RNase III Dicer, that ensures the inhibition of genes hindering normal differentiation of TFCs or driving cancer initiation and/or progression, knowing that a crucial role of miRNAs down-regulation in thyroid carcinogenesis has been previously suggested (Visone *et al.*, 2007; Braun *et al.*, 2010). Interestingly, humans carrying *DICER1* heterozygous mutations develop a number of rare embryonal tumors as well as thyroid pathology consisting of multinodular goiters, although this autosomal dominant familial cancer syndrome displays a wide phenotypic variability (Rio Frio *et al.*, 2011; Choong *et al.*, 2012).

4.1.2. Alternative mechanisms

As previously mentioned, germline mutations in the thyroid-specific genes *NKX2.1*, *PAX8*, and *FOXE1* and the thyroid-related *TSHR* and *NKX2.5* genes have been reported in only a

few patients with TD (Clifton-Bligh *et al.*, 1998; Macchia *et al.*, 1998; Breedveld *et al.*, 2002; Castanet *et al.*, 2002; Krude *et al.*, 2002; Dentice *et al.*, 2006; Narumi *et al.*, 2010). In addition, their role in the pathogenesis of TD has been excluded in some multiplex families of TD (Castanet *et al.*, 2005). Hence, the pathogenesis of CHTD in the majority of patients is not explained by a Mendelian mechanism. Furthermore, the implication of environmental factors in the pathogenesis of TD is highly unlikely, since neither seasonal nor temporal trends for CHTD have been reported and maternal folate supplementation showed no impact on its incidence (Deladoëy *et al.*, 2007b; Deladoëy *et al.*, 2011). Taken together, these data led to the consideration of other mechanisms for the vast majority of human CHTD cases (Deladoëy *et al.*, 2007a).

4.1.2.1. Multigenic model of TD

The lack of a clear Mendelian transmission of TD in families with at least one affected member and the high incidence of asymptomatic thyroid anomalies among first degree relatives suggest the involvement of modifier genes in the pathogenesis of TD and hence a possibly multigenic origin. In support of this suggestion is the incomplete penetrance and variable expressivity of the inherited *PAX8* and *NKX2.5* mutations reported in familial cases of CHTD (Macchia *et al.*, 1998; Congdon *et al.*, 2001; Dentice *et al.*, 2006). Amendola *et al.* have provided the evidence for the multigenic origin of TD in mice (Amendola *et al.*, 2005). Contrary to the singly deficient heterozygous mice, they have shown that mice which are double heterozygotes for *Nkx2.1* (*Titf1*) and *Pax8*-null alleles (DHTP) exhibit severe hypothyroidism characterized by thyroid hypoplasia and increased incidence of hemiagenesis in adult mice. Moreover, it has been shown that the TD phenotype is strain-specific with two potential modifier genes responsible for such specificity, among which one locus exhibits the major effect (Amendola *et al.*, 2005): this modifier gene for hypothyroidism was mapped to chromosome 2 and named hypothyroidism-related chromosome 2 (HTRC2); it encodes Dnajc17, a member of the type III heat-shock protein-40 (Hsp40) family. Dnajc17 is highly expressed in the thyroid and known for its crucial role in mouse embryonic development and its ability to modify the expression of the *Tg* promoter *in vitro* (Amendola *et al.*, 2010).

In humans, a similar mechanism encompassing multiple loci in the pathogenesis of TD has been suggested. In line with an oligogenic origin of TD is the recently reported low

incidence of CHTD in Blacks when compared to Caucasians who exhibit decreased genetic diversity and tend to accumulate pathogenic variants (Lohmueller *et al.*, 2008; Stoppa-Vaucher *et al.*, 2011). Recently, CH with no goiter has been reported in individuals of a consanguineous kindred bearing both homozygous and compound heterozygous *TSHR* and *TPO* mutations, respectively (Sriphrapradang *et al.*, 2011). Likewise, TSH resistance in association with CH, caused by inactivating mutations in both *TSHR* and adenylate cyclase-stimulating G alpha protein subunit (*GNAS*), has been reported in several members of a family with CH (Lado-Abeal *et al.*, 2011). Interestingly, heterozygous mutations in the *PAX8* promoter and *NKX2.5* gene have been identified in a girl with TD (Hermanns *et al.*, 2011). However, no TD cases with digenic defects were identified in either a cohort of 102 Japanese patients with CH (Narumi *et al.*, 2009; Narumi *et al.*, 2010) or in a cohort of 170 Czech patients with CHTD or early-onset hypothyroidism (Al Taji *et al.*, 2007) screened for genetic aberrations in genes encoding the transcription factors NKX2.1, PAX8, FOXE1, HHEX, and NKX2.5 and the TSH-receptor. This implies that putative genetic modifiers are outside the coding region of the current candidate genes or that other (yet unknown) genes account for CHTD in humans. Moreover, there is also an urgent need for considering other sources of genetic and/or epigenetic variation such as (i) *de novo* germ line copy number variants (CNVs), (ii) early somatic mutations and (iii) epigenetic modifications. Indeed, CNVs can act as genetic modifiers of the phenotype by either directly or indirectly affecting its penetrance (Cooper *et al.*, 2013). In addition, non-Mendelian mechanisms, consistent with the sporadic occurrence of CHTD in the majority (98%) of cases and the high discordance rate (92%) between monozygotic (MZ) twins, are strongly suggested as plausible underlying pathogenic mechanisms for CHTD. Among these mechanisms are postzygotic (somatic) either genetic or epigenetic modifications in thyroid and extrathyroid genes involved in the embryonic development of the gland (Deladoëy *et al.*, 2007a; Deladoëy, 2012). The three sources of variability will be discussed below.

4.1.2.2. Copy number variations (CNVs)

Copy number variations (CNVs) are submicroscopic chromosomal abnormalities, ranging in size between one kilobase and one megabase, that alter the copy-number of a specific chromosomal region consequent to either deletion or duplication of DNA segments (Tang and Amon, 2013). They are either inherited or sporadic (*de novo*) (Stankiewicz and Lupski, 2010). Copy number variants occurring with an altered copy number frequency (ACNF) >1% in the

human population are classified as copy number polymorphisms (CNPs), that are heritable and enriched in regions of segmental duplications (Locke *et al.*, 2006). According to the underlying mechanism involved in their formation, CNVs are classified into either recurrent or nonrecurrent (van Binsbergen, 2011). Recurrent CNVs, representing 20–40% of the normal polymorphic CNVs and numerous *de novo* pathogenic CNVs, are developed during meiosis via non-allelic homologous recombination (NAHR), a mechanism mediated via pre-existing low-copy repeats (LCRs) or segmental duplications (SDs) (Kim *et al.*, 2008; Arlt *et al.*, 2012). On the other hand, nonrecurrent CNVs encompass the majority of normal polymorphic CNVs and a large portion of disease-related CNVs. Both the recombination-based mechanisms, including nonhomologous end joining (NHEJ) and microhomology-mediated end-joining (MMEJ), as well as those involving template switching events are responsible for the formation of nonrecurrent CNVs. Models based on template switching include the replication-based mechanisms named fork stalling and template switching/microhomology-mediated break-induced replication (FoSTeS/MMBIR) (Stankiewicz and Lupski, 2010; Arlt *et al.*, 2012).

Nowadays, CNVs are believed to encompass a larger number of nucleotides and to be more frequent than single nucleotide polymorphisms (SNPs) (Stankiewicz and Lupski, 2010; Brunham and Hayden, 2013). They cover approximately 13% of the human genome thus accounting for a notable source of human genomic evolution and inter-individual genetic diversity (Iafrate *et al.*, 2004; Stankiewicz and Lupski, 2010; van Binsbergen, 2011). In addition, it has been shown that CNVs are implicated in the etiology of various genomic disorders due to the higher *de novo* locus-specific mutation rate (μ) of genomic rearrangements compared to *de novo* single base mutations (Lupski, 2007; Stankiewicz and Lupski, 2010). They are associated with various sporadic and Mendelian diseases (Zhang *et al.*, 2009), birth defects among which congenital heart disease (Erdogan *et al.*, 2008; Lu *et al.*, 2008; Hitz *et al.*, 2012; Soemedi *et al.*, 2012; Southard *et al.*, 2012; Lalani *et al.*, 2013), as well as common complex traits (including Alzheimer disease, autism, epilepsy, pancreatitis, Parkinson disease, and schizophrenia) (Lee and Lupski, 2006; Sebat *et al.*, 2007; Henrichsen *et al.*, 2009; Stankiewicz and Lupski, 2010; Girirajan *et al.*, 2011; Coe *et al.*, 2012). In addition, CNVs have been also associated with susceptibility to other human diseases among which are HIV infection (Gonzalez *et al.*, 2005), Crohn's disease (Fellermann *et al.*, 2006; McCarroll *et al.*, 2008), systemic lupus erythematosus (Fanciulli *et al.*, 2007; Willcocks *et al.*, 2008), as well as numerous others (Stankiewicz and

Lupski, 2010). Moreover, both common (present at frequencies > 5%) and rare CNVs (with frequencies < 1%) influence genetic susceptibility to cancer, where a high cancer risk was observed in association with rare CNVs (Kuiper *et al.*, 2010; Krepischi *et al.*, 2012).

Based on the reported involvement of *de novo* CNVs in the pathogenesis of many sporadic diseases (Zhang *et al.*, 2009) and the fact that the majority of CHTD cases are sporadic, *de novo* CNVs were considered as potential underlying pathogenic mechanisms (Deladoëy, 2012). Using genome-wide array-based comparative genomic hybridization (array CGH), Thorwarth *et al.* have screened 80 CHTD patients and two pairs of discordant MZ twins to determine the role of CNVs in the etiology of TD (Thorwarth *et al.*, 2010). In contrast to previous studies (Bruder *et al.*, 2008), they found no differences in DNA copy number between discordant MZ twins. On the other hand, potentially pathogenic nonrecurrent CNVs, four deletions and six duplications, have been identified in patients with athyreosis and hypoplasia representing 8.75% of all patients screened. Interestingly, four out of the ten described CNVs (40%) occurred *de novo* while the other six CNVs were equally either maternally or paternally inherited. None of the identified CNVs encompassed genes known to be involved in thyroid morphogenesis except for a duplication of the 22q11.21 chromosomal region including the *TBX1* gene (Thorwarth *et al.*, 2010). Unfortunately, Thorwarth *et al.* provided no functional studies on the candidate genes located in the reportd CNVs.

Furthermore, somatic variations in DNA copy number (CNVs) in combination with gene expression analyses were assessed in three human ectopic (lingual) thyroid tissues. Three thyroid-specific CNVs (common variants) have been identified in the ectopic tissues and not in matched leukocytes. Moreover, the combined analysis revealed that the expression profile of the ectopic thyroids is different compared to normal thyroids. However, such a differential gene expression was independent of the identified thyroid-specific CNVs (Abu-Khudir *et al.*, 2010).

Collectively, based on the high rate of CNVs identified among the screened TD patients, a plausible role of CNVs in the pathogenesis of CHTD has been proposed. However, lack of their recurrence together with absence of differences in DNA copy number among discordant twins and lack of gene expression dependence on CNVs in ectopic thyroids, plead against a major role of CNVs in the pathogenesis of TD. In addition, they highlight the need for

considering, as disease-relevant, pathways involving the candidate genes located within the identified CNVs rather than regarding single disease-related genes (Thorwarth *et al.*, 2010).

4.1.2.3. Early somatic mutations

In addition to cancer, somatic mutations can be responsible for a wide range of other non-cancerous diseases. In the case of CH, both the sporadic occurrence of the majority of CHTD cases and the high discordance between MZ twins suggest the involvement of postzygotic (post-fertilization) events in the pathogenesis of CHTD. Among these events would be an early somatic mutation, with a dominant effect, that leads to a loss-of-function mutation in one of the genes involved in thyroid development. In order for a somatic loss-of-function mutation to lead to a phenotype, it has to occur very early during the developmental process in the common ancestral cell of all the cells committed to the thyroid fate (Deladoëy *et al.*, 2007a). Postzygotic loss-of-function events leading to a phenotype have been previously reported among which are mosaic karyotypes associated with variable features of Turner of syndrome (Costa *et al.*, 1998; Lebl *et al.*, 2001), somatic mosaicism of the androgen receptor (AR) observed in patients with androgen insensitivity syndrome (AIS) (Holterhus *et al.*, 1997; Köhler *et al.*, 2005), and *IRF6* somatic mutation reported in the affected twin of a MZ pair discordant for the Van der Woude syndrome (VWS; OMIM 119300) (Kondo *et al.*, 2002). The role of somatic mutations, including brain-only ones, in the development of human neurodevelopmental and neuropsychiatric disorders has been increasingly identified as reviewed by Poduri *et al.* (Poduri *et al.*, 2013). A high prevalence of somatic mutations in genes encoding a number of transcription factors including *TBX5*, *NKX2.5*, *GATA4*, *HEY2* and *HAND1*, among individuals with congenital heart defects (CHDs) has been previously reported (Reamon-Buettner and Borlak, 2004; Reamon-Buettner *et al.*, 2004; Reamon-Buettner and Borlak, 2005; 2006; Reamon-Buettner *et al.*, 2008; Reamon-Buettner *et al.*, 2009). The somatic mutations have been identified in DNA extracted from an archive of formalin-fixed cardiac tissues from individuals with atrial (ASD), ventricular (VSD), and atrioventricular septal defects (AVSD). However, these findings were not replicated by a number of later studies that used fresh-frozen cardiac tissues rather than formalin-fixed tissues (Draus *et al.*, 2009; Salazar *et al.*, 2011; Wang *et al.*, 2011a; Sabina *et al.*, 2013). The lack of detection of somatic mutations by these recent studies might be attributed to the mosaicism that may reduce the probability of detection or due the plausible involvement of these

mutations in other forms of CHD, knowing that CHD is clinically classified into at least 21 different types (Huang *et al.*, 2013b). Nevertheless, an association between somatic mutations in the *GATA6* gene and sporadic tetralogy of Fallot (TOF), the most common cyanotic CHD, has been lately reported (Huang *et al.*, 2013b).

Recently, a somatic mosaic heterozygous inactivating *PAX8* mutation has been reported in a female patient who is the mother of two half-siblings with CH and the same *PAX8* mutation inherited by germline (Narumi *et al.*, 2011). The thyroid function profile of the patient was normal but she was subjected to hemithyroidectomy due to a thyroid adenoma. In addition to normal thyroid tissue, histological analysis of the patient's thyroid revealed both adenoma tissue as well as fetal-like thyroid tissue in the follicular growth stage (TFCs with very small or absent follicles). Among the identified tissues, a *PAX8* mutation was detected in the fetal-like tissue, thus indicating the sensitivity of the follicular growth stage to *PAX8* gene dosage. In addition to the mutated thyroid tissue, a *PAX8* mutation was detected in lymphocytes and nails, tissues derived from the mesodermal and ectodermal embryonic layers, respectively as well as in germ cells, hence reflecting its early occurrence during embryogenesis (Narumi *et al.*, 2011).

This case report provides evidence for a postzygotic somatic mutation affecting *PAX8*, one of the genes involved in thyroid development. In his review, Szinnai has attributed the lack of a TD phenotype in the mother bearing the *PAX8* mutation to its mosaic state which prohibited the penetrance of a mild phenotype arising consequent to the mutation (Szinnai, 2013). On the contrary, her germline mutation carrier children developed a TD phenotype (thyroid hypoplasia) as all their TFCs, among all other body cells, are affected by the mutation. In addition, he correlated the severity of the TD phenotype with the time at which the somatic mutation occurs during the embryonic life: the earlier it occurs (after the commitment of endodermal cells), the greater the proportion of pTFCs that will be affected (Szinnai, 2013).

4.1.2.4. Epigenetic modifications

In addition to the possible role of early somatic mutations in the pathogenesis of CHTD, the contribution of epigenetic alterations, among which DNA methylation, is a hypothesis worth considering (Vassart and Dumont, 2005). The involvement of stochastic epigenetic mechanisms, leading to variable degree of gene or allele silencing, in the pathogenesis of TD is more likely than that of somatic mutations. This could be attributed to the fact that the low number of

founder cells in the embryo is more consistent with the frequency of epigenetic errors than of spontaneous somatic mutations (Mathis and Nicolas, 2002). Like somatic mutations, the hypothesis of epigenetic mechanisms is compatible with the sporadic nature of TD and with the high discordance rate between MZ twins (Deladoëy *et al.*, 2007a). According to this hypothesis, quantitative modulation of gene expression via stochastic epigenetic events will lead the cells of an organ bud to express single or two alleles of a gene or absolutely none. If ultimately fixed in the anlagen, developmental defects might arise consequent to the low expression of the randomly selected key gene(s) (Vassart and Dumont, 2005). Of note, discordance between MZ for various developmental defects could be attributed to monoallelic expression resulting from random silencing (Fraga *et al.*, 2005; Mansilla *et al.*, 2005; Wong *et al.*, 2005).

The term epigenetics was introduced by the developmental biologist Conrad Waddington in the early 1940s. Initially, Waddington defined epigenetics as the interactions of genes with their environment which bring the phenotype into being (Waddington, 1942). Currently, epigenetics is broadly used to describe mitotically and/or meiotically heritable alterations in gene function in the absence of changes in the primary DNA sequence (Dupont *et al.*, 2009) The epigenetic factors that modulate gene expression include DNA methylation, various post-translational modification of histones, regulatory non-coding RNAs (ncRNAs), and chromatin remodeling proteins (Mohn and Schubeler, 2009; Handel *et al.*, 2010; Bannister and Kouzarides, 2011; Inbar-Feigenberg *et al.*, 2013; Li, 2013). The cross-talk between the various epigenetic modifications mediates chromatin remodeling thus rendering it either permissive or repressive to the transcriptional machinery (Vaissière *et al.*, 2008; Murr, 2010). Much of our understanding about the role of DNA methylation and histone modifications in transcriptional regulation has come from studying genes that undergo imprinting (gene expression limited to only one allele of an autosomal pair in a parent-of-origin specific fashion) as well as genes silenced during sex-chromosome dosage compensation (X-chromosome inactivation) (Monk, 1986; 1988). However, it is now recognized that epigenetic mechanisms are involved in various cellular processes among which are genome reprogramming during early embryogenesis and gametogenesis. In addition, they are involved in initiation and maintenance of cell differentiation. The patterns of epigenetic marks (epigenome) are established early during development and differentiation. However, deregulation of these epigenetic patterns via endogenous and/or exogenous

(environmental) signals has been associated subsequently with number of human diseases, including cancer (Delcuve *et al.*, 2009).

The two major epigenetic modifications, DNA methylation and histone modifications, and their role in modulating gene expression will be reviewed in the following sections.

4.1.2.4.1. DNA methylation

DNA methylation is the most extensively studied epigenetic modification and the only one that involves a direct chemical modification of the DNA (Dolinoy and Faulk, 2012; Moore *et al.*, 2013). DNA methylation involves the covalent addition of a methyl group to the 5′ carbon position of cytosine residues to form 5-methylcytosine (5mC) (Auclair and Weber, 2012; Moore *et al.*, 2013). In mammalian genomes, methylation of cytosine bases occurs mainly in the context of 5′-CpG-3′ dinucleotides, though methylation of cytosine in the sequence context of CHG and CHH (H = A, C, T) or CpA has been detected in mouse and human ESCs, oocytes, sensory neurons and plants, but rarely in somatic mammalian cells (Ramsahoye *et al.*, 2000; Lister *et al.*, 2009; Piperi and Papavassiliou, 2011; Tomizawa *et al.*, 2011; Ziller *et al.*, 2011; Xie *et al.*, 2012).

In mammals, CpG dinucleotides are scarce in the majority of the genome due to the potential mutagenicity resulting from the spontaneous or enzymatic deamination of methyalated cytosines to thymidine (Coulondre *et al.*, 1978; Bird, 1980). In general, only 60-80% of the CpG dinucleotides in the human genome are methylated and less than 10% are found in CpG islands (CGIs), regions where a high density of CpG dinucleotides is observed (Deaton and Bird, 2011). CGIs are located within or near the promoters and/or the first exons of housekeeping and developmental regulating genes (Gardiner-Garden and Frommer, 1987; Larsen *et al.*, 1992; Saxonov *et al.*, 2006; Dolinoy and Faulk, 2012). Most promoter CGIs are not methylated, though some of them become methylated in a tissue-specific manner during development or upon differentiation (Weber *et al.*, 2007; Illingworth *et al.*, 2008; Portela and Esteller, 2010; Moore *et al.*, 2013). On the contrary, CGIs located in intergenic and gene body regions as well as in CpG island shores (CGI shores), regions of lower CpG density located ~2 kb from CGIs, exhibit tissue-specific methylation patterns (Doi *et al.*, 2009; Irizarry *et al.*, 2009; Moore *et al.*, 2013).

DNA methylation is initiated (*de novo* methylation) and maintained (maintenance methylation) by the enzymatic activity of the DNA methyltransferases (DNMTs) (Goll and

Bestor, 2005). Both DNMT3A and DNMT3B enzymes catalyze the establishment of a *de novo* methylation pattern of unmodified DNA molecules during development, thus they are known as *de novo* DNMTs (Okano *et al.*, 1999). On the other hand, DMNT1, the maintenance DNMT, is responsible for maintaining the methylation pattern of the parent DNA molecule during the process of replication (Leonhardt *et al.*, 1992; Bestor, 2000). DNMT1 has a preference for hemimethylated DNA that limits its *de novo* methyltransferase activity (Pradhan *et al.*, 1999). DNMT3L, has no a catalytic domain, but it interacts with DNMT3A to establish allele-specific methylation in imprinted regions and methylation of several repetitive elements of the genome (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Kato *et al.*, 2007). In vertebrates, members of the ten-eleven translocation (TET) family of enzymes (TET1, TET2, and TET3) can oxidize 5mC into 5-hydroxymethylcytosine (5hmC), an intermediate of the cytosine demethylation reaction that is abundantly found in human and mouse brain and ESCs (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009; Ito *et al.*, 2010). The TET can then catalyze the oxidation of 5hmC into the additional intermediates of demethylation 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He *et al.*, 2011; Ito *et al.*, 2011). In addition to DNA demethylation, a role for 5hmC in gene regulation has been proposed (Valinluck *et al.*, 2004; Wu *et al.*, 2011). Recently, several somatic mutations have been reported in DNMT3A and DNMT1 as well as in the TET protein TET2 in cancer (Kanai *et al.*, 2003; Langemeijer *et al.*, 2009; Walter *et al.*, 2011; Yan *et al.*, 2011), whereas germline mutations in DNMT1 and DNMT3B have been reported in neurological and immunological disorders, respectively (Berdasco and Esteller, 2013).

The canonical function of DNA methylation is to mediate transcriptional repression at promoter elements (Reddington *et al.*, 2013). The impact of DNA methylation on transcriptional activity at promoter regions is dependent upon the local CpG-density (Hackett and Surani, 2013; Reddington *et al.*, 2013). At low (poor) CpG-density promoters (LCP) that are mostly methylated, no correlation between DNA methylation and gene silencing is observed (Weber *et al.*, 2007). On the other hand, methylated high CpG-density promoters (HCP) are strongly associated with gene silencing whereas, when unmethylated, the transcriptional activity of the gene depends on whether or not it is silenced by other forms of repression (Meissner *et al.*, 2008). Excluding the imprinted genes and the inactive X-chromosome, the HCPs are rarely methylated during development (Hackett and Surani, 2013). Finally, methylation status of intermediate CpG-density promoters (ICP) is strongly correlated with the transcriptional activity

of genes and is responsible for tissue-specific gene regulation (Meissner *et al.*, 2008; Borgel *et al.*, 2010). It has been shown that DNA methylation is involved in stably silencing ICPs associated with germline-specific genes in somatic cells due to their probable involvement in carcinogenesis (Simpson *et al.*, 2005; Maatouk *et al.*, 2006; Meissner *et al.*, 2008; Janic *et al.*, 2010; Velasco *et al.*, 2010).

DNA methylation-mediated gene silencing at promoter regions occurs via either preventing the direct binding of transcription factors (TFs) to their DNA binding motifs, essential for the recruitment of the transcriptional machinery (Watt and Molloy, 1988; Iguchi-Ariga and Schaffner, 1989; Santoro and Grummt, 2001; Wiench *et al.*, 2011), or by recruitment of methyl-CpG-binding proteins (MeCPs) that subsequently attract repressive complexes such as histone deacetylases (HDACs) or recruiting histone methyltransferases that add the repressive histone H3 lysine 9 (H3K9) methylation, and eventually leads to chromatin compaction and transcriptional repression of the gene (Jones *et al.*, 1998; Nan *et al.*, 1998; Bird and Wolffe, 1999; Bird, 2002; Fuks *et al.*, 2003). The canonical function of DNA methyaltion is considered an important mechanism for regulating tissue-specific gene expression (Figure 9) (Reddington *et al.*, 2013). In addition to single-copy gene promoters, DNA methylation of repetitive transposable elements (TEs) and viral ones leads to their inactivation thus maintaining genomic stability and integrity (Yoder *et al.*, 1997; Walsh *et al.*, 1998; Jackson-Grusby *et al.*, 2001).

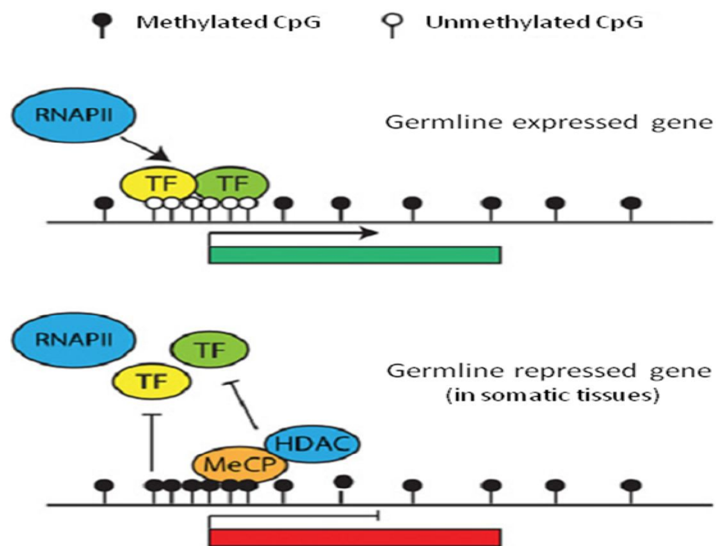

Figure 9: Canonical function of DNA methylation and tissue-specific gene regulation (modified from Reddington *et al.*, 2013).

In addition to the established canonical functions of DNA methylation at promoter regions, yet unclear possible roles in regulating other genomic regions have been revealed (Reddington *et al.*, 2013). In mammals, DNA methylation within the gene body (beyond the first exon) has been reported (Figure 10A) (Portela and Esteller, 2010; Jones, 2012; Moore *et al.*, 2013; Reddington *et al.*, 2013). Previously, the association between active transcription and gene body DNA methylation has been confirmed on the active X chromosome (Hellman and Chess, 2007) as well as by shotgun bisulphite sequencing of animal and plant genomes (Cokus *et al.*, 2008; Lister *et al.*, 2009; Feng *et al.*, 2010). Among the potential roles of gene body methylation is the regulation of the co-transcriptional mRNA processing via splicing modulation (Figure 10Ai) (Laurent *et al.*, 2010; Reddington *et al.*, 2013). In humans, it has been shown that increased DNA methylation of an exon favors its incorporation in a mature transcript (Shukla *et al.*, 2011). In addition, gene body DNA methylation, although speculative, might prevent the initiation of spurious transcription within gene bodies and the consequent formation of cryptic products (Figure 10Aii) (Suzuki and Bird, 2008; Reddington *et al.*, 2013). It has been shown that gene body methylation can prevent initiation of transcription, where the activity of the *Shank3* gene was negatively correlated with a tissue-specific methylated CGI that functioned as an alternative promoter for it (Maunakea *et al.*, 2010).

In addition to gene body, a role of methylated inter- and intragenic (orphan) CGIs has been suggested (Reddington *et al.*, 2013). A proportion of the orphan CGIs might be promoters for either unannotated transcripts, developmentally regulated alternative promoters of known transcripts, or promoters of non-coding transcripts such as long ncRNAs (Illingworth *et al.*, 2010; Maunakea *et al.*, 2010; Reddington *et al.*, 2013). Upon methylation of the orphan CGI, its promoter activity will be inhibited (in a tissue-specific manner) (Figure 10Bi) (Illingworth *et al.*, 2010). In addition, the methylation status of the orphan CGIs can modulate the recruitment of chromatin remodeling proteins (Figure 10Bii) (Blackledge *et al.*, 2010; Thomson *et al.*, 2010). In addition to orphan CGIs, DNA methylation of CGI shores might be involved in reducing transcription of nearby genes, by acting as alternative promoter, in a tissue-specific manner as they exhibit tissue-specific DNA methylation pattern (Irizarry *et al.*, 2009; Ji *et al.*, 2010). However, it has been suggested that methylation of CGI shores is not correlated with gene expression and that they exhibit no significant levels of differential DNA methylation (Deaton *et al.*, 2011).

Finally, a role of DNA methylation in regulating the function of distal regulatory elements, among which are enhancers, has been suggested (Jones, 2012; Reddington *et al.*, 2013). Enhancers, mostly CpG poor, are situated at varying distances from gene promoters and are known to control gene expression (Jones, 2012). It has been suggested that the presence or absence of TFs at enhancer elements can modulate their methylation status by either preventing or allowing the binding of DNMTs (Figure 10Ci) (Stadler *et al.*, 2011; Thurman *et al.*, 2012). In addition, methylation of the enhancer element can modulate the protein-DNA interaction either directly by blocking the binding of TF or via a chromatin condensed state mediated by MeCPs and HDACs (Figure 10Cii) (Reddington *et al.*, 2013).

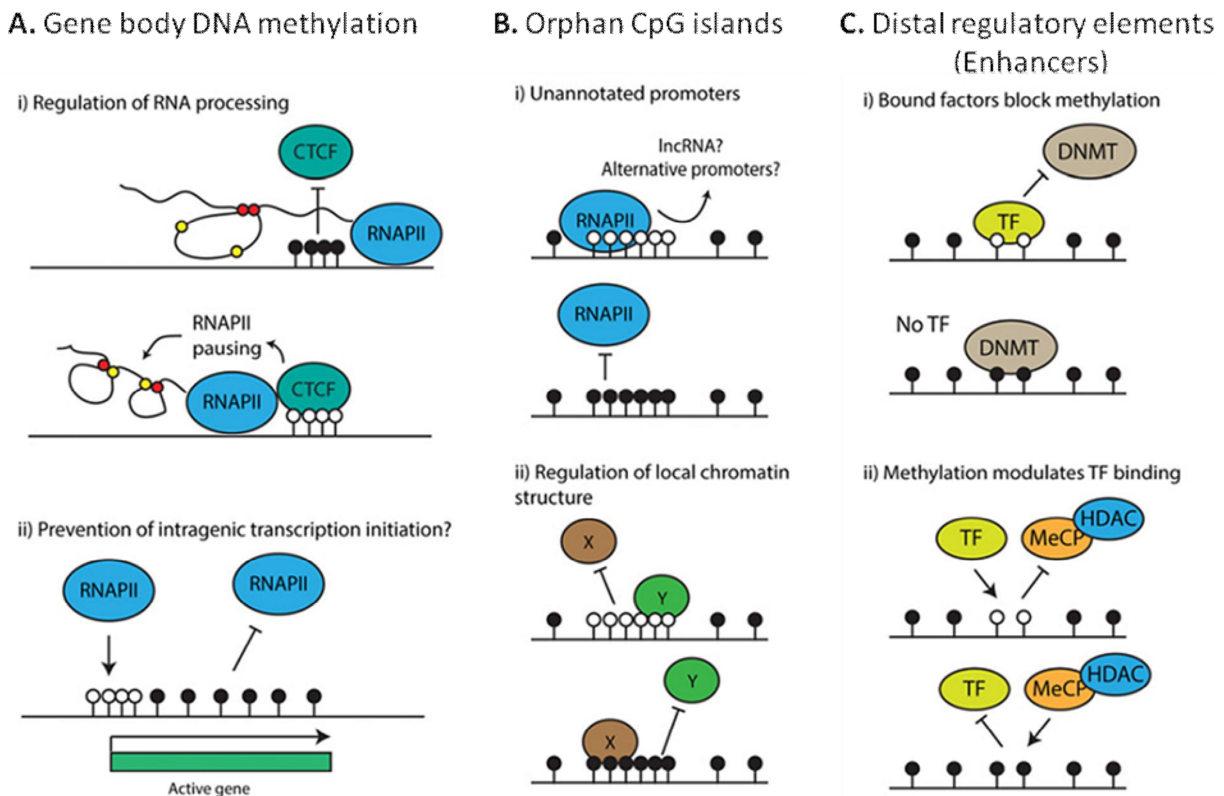

Figure 10: DNA methylation-mediated transcriptional regulation at genomic regions other than promoter elements (modified from Reddington *et al.*, 2013).

4.1.2.4.2. Histone modifications

The basic repeating structural unit of chromatin is the nucleosome, a DNA-protein complex including 147 base pairs of genomic DNA wrapped around an octamer of histone

proteins, two copies each of H2A, H2B, H3, and H4 (Luger and Richmond, 1998; Kornberg and Lorch, 1999). Mainly, the flexible histone tails are subjected to several covalent post-translational modifications (PTMs) including, among others, acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation (Strahl and Allis, 2000; Kouzarides, 2007; Mehler, 2008; Bannister and Kouzarides, 2011; Gardner *et al.*, 2011; Tan *et al.*, 2011; Tammen *et al.*, 2013). In addition, modifications within the globular core region of the H3 and H4 proteins have been also identified (Hyland *et al.*, 2005; Ye *et al.*, 2005; Das *et al.*, 2009; Shukla *et al.*, 2009; Füllgrabe *et al.*, 2011). Histone modifications regulate chromatin dynamics and downstream functions (transcription, replication, and DNA repair) via two different mechanisms: (i) altering the charges on histone tails leading to the disruption of contacts between histones and DNA or between neighboring nucleosomes (acetylation and phosphorylation) and (ii) recruitment of non-histone proteins (e.g. chromatin remodelers and transcriptional co-activators/repressors) that recognize the 'histone code' representing the distinct patterns of histone PTMs (Figure 11) (Izzo and Schneider, 2010; Bannister and Kouzarides, 2011; Lothrop *et al.*, 2013; Tammen *et al.*, 2013).

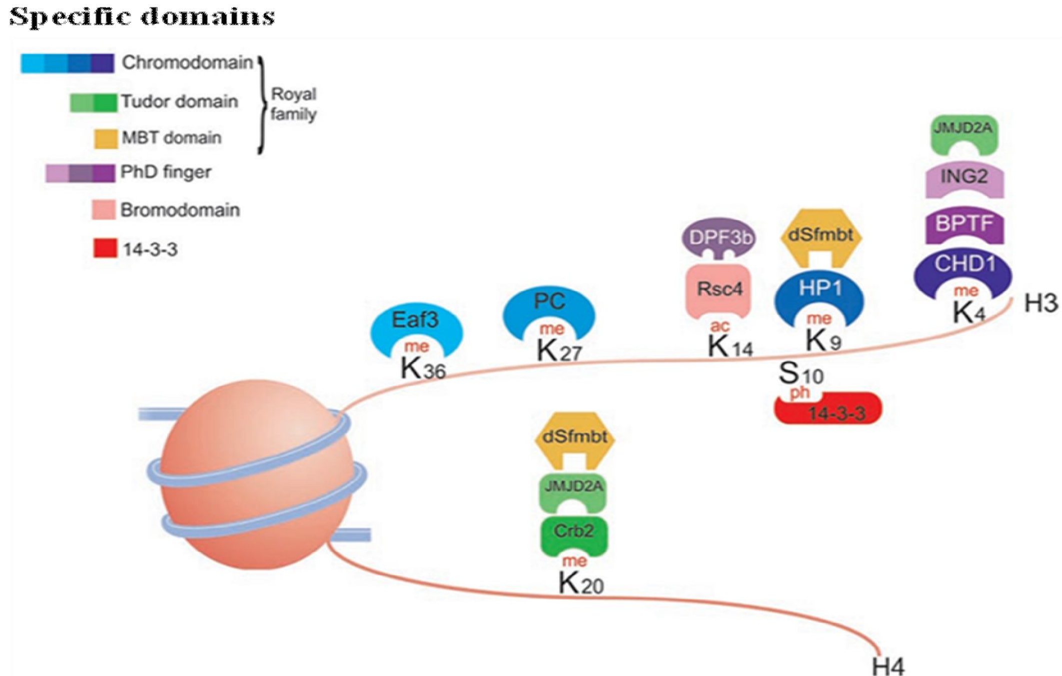

Figure 11: Examples of the recruitment of proteins to modified histones (modified from Bannister and Kouzarides, 2011).

The dynamic histone PTMs are catalyzed by histone-modifying enzymes that either add or remove the histone modifications in response to various intracellular and extracellular stimuli (Mehler, 2008). In addition, the histone-modifying enzymes can regulate cellular processes associated with gene expression by exerting actions on non-histone proteins. Together with other multiple chromatin-modifying enzymes, the histone-modifying enzymes are components of larger multi-protein complexes involved in regulating gene expression (Rosenfeld *et al.*, 2006; Ruthenburg *et al.*, 2007; Mehler, 2008). In humans, aberrations in histone-modifying enzymes have been associated with the development of cancer (Sharma *et al.*, 2010; Füllgrabe *et al.*, 2011; Butler *et al.*, 2012; Campbell and Turner, 2013). However, it has been reported that only a few of the known residues to be modified are incorporated (Kouzarides, 2007). Moreover, alterations in histone variants have been reported in cancer as well (Kapoor *et al.*, 2010; Khare *et al.*, 2011).

The overall impact of histone modifications on chromatin structure and subsequent downstream functions is refined by the cross-talk between the various modifications (Figure 12) (Izzo and Schneider, 2010; Bannister and Kouzarides, 2011). The mechanisms mediating such an interplay include: (i) antagonism between multiple modifications targeting the same amino acid, (ii) the dependence of one modification on the other (*trans*-regulation), (iii) alteration of the catalytic activity of an enzyme consequent to the modification of its recognition site, (iv) disruption of protein binding to a specific modification because of an adjacent one, and (v) the possible collaboration between different modifications to attain efficient recruitment of binding proteins (Fischle *et al.*, 2005; Nelson *et al.*, 2006; Kouzarides, 2007; Vermeulen *et al.*, 2010; Xhemalce and Kouzarides, 2010; Bannister and Kouzarides, 2011).

Arrowhead= Positive effect
Flat-head= Negative effect

Figure 12: Cross-talk between histone modifications (modified from Bannister and Kouzarides, 2011).

4.1.2.4.3. Interplay between DNA methylation and histone modifications

It has been reported that changes in chromatin structure induced by DNA methylation are mediated via alterations in histone modifications (Kondo, 2009). Several reviews have pointed to the interplay between DNA methylation and histone modifications (Bernstein *et al.*, 2007; Vaissière *et al.*, 2008; Cedar and Bergman, 2009; Kondo, 2009; Muthusamy *et al.*, 2010; Auclair and Weber, 2012), though the order of events is not clearly empathized and that it might be dependent on cell type, genomic locus, as well as physiological conditions (Vaissière *et al.*, 2008; Muthusamy *et al.*, 2010). In addition to the direct inhibition of transcription factors binding (Watt and Molloy, 1988; Iguchi-Ariga and Schaffner, 1989; Santoro and Grummt, 2001; Wiench *et al.*, 2011), DNA methylation mediates transcriptional gene repression through the methyl-CpG-binding domain (MBD)-containing proteins (MBDs), such as MeCP2 or MBD2. The MBDs are known to recruit repressor complexes encompassing HDACs that remove histone acetylation and lead to the formation of a more compact DNA-histone complex and hence trasncriptional repression (Jones *et al.*, 1998; Nan *et al.*, 1998; Bird and Wolffe, 1999; Bird, 2002). Also, an enhancement of chromatin repression, mediated by MeCP2, is attained via the

recruitment of HMTs that methylates histone H3 at lysine 9 (H3K9) (Fuks *et al.*, 2003). Furthermore, it has been shown that DNA methylation inhibits histone H3 lysine 4 methylation (H3K4me) (Okitsu and Hsieh, 2007; Weber *et al.*, 2007). Trimethylation of histone H3 lysine 4 methylation (H3K4me3) is an activating modification and a hallmark of unmethylated CGIs (Mikkelsen *et al.*, 2007). It has been suggested that, in somatic cells, promoters are protected from *de novo* DNA methylation by the methylation of H3K4 (Appanah *et al.*, 2007; Okitsu and Hsieh, 2007; Weber *et al.*, 2007). Moreover, the binding of the DNMTs to H3 histone tails is impaired with the subsequent prevention of DNA methylation due to the presence of H3K4me3 (Ooi *et al.*, 2007; Zhang *et al.*, 2010). In contrast to the evident association between DNA methylation and the activation mark H3K4me3, a limited link between DNA methylation and repressive histone modifications has been reported in mammalian genomes (Auclair and Weber, 2012). It has been shown that in ESCs *de novo* DNA methylation is enhanced by the histone H3K9 methyltransferase G9a, in a way that is independent of its histone methyltransferase (HMT) activity (Dong *et al.*, 2008; Epsztejn-Litman *et al.*, 2008; Tachibana *et al.*, 2008). Likewise, DNA methylation leads to the deposition of H3K9 dimethylation (H3K9me2), a mark of repressive chromatin, via the interaction of G9a and DNMT1 during replication (Hashimshony *et al.*, 2003; Estève *et al.*, 2006). A number of studies have proposed the association of the repressive mark H3K36me3 and DNA methylation, an association that occurs through the recognition of H3K36me3 by the PWWP domain of the DNMT3A (Dhayalan *et al.*, 2010). Trimethylation at histone H3 lysine 27 (H3K27me3) is a histone modification involved in the regulation of genes encoding developmental regulators and in early steps of X-chromosome inactivation (Cao and Zhang, 2004). The histone H3 Lysine 27 (H3K27) methylation of nucleosomes is posited by the histone methyltransferase enhancer of zeste 2 (EZH2), the catalytic component of the transcriptional polycomb repressive complex 2 (PRC2) (Kuzmichev *et al.*, 2002). H3K27me3 mediates repression of transcription through the formation of compact chromatin (Pietersen and van Lohuizen, 2008; Schwartz and Pirrotta, 2008). In addition to the suggested role of histone H3 Lysine 9 in directing DNA methylation, it has been proposed that the Polycomb group (PcG)-mediated histone H3 Lysine 27 methylation (H3K27me) might be also involved in triggering *de novo* DNA methylation on a number of target genes under certain conditions (Hershko *et al.*, 2003; Mikkelsen *et al.*, 2007; Fouse *et al.*, 2008; Meissner *et al.*, 2008; Mohn *et al.*, 2008). The induction of *de novo* DNA methylation associated with

H3K27me3 is mediated via the interaction between EZH2 and DNMTs via another domain that is independent of that responsible for H3K27 methylation (Viré *et al.*, 2006). Notably, other factors are implicated in stimulating the *de novo* DNA methylation of those genes enriched with either H3K9 or H3K27 histone methylations. This is suggested by the notion that the presence of such histone methylation does not always lead to *de novo* DNA methylation (Cedar and Bergman, 2009; Kondo, 2009).

Collectively, the above mentioned data confirm the concept of a bidirectional interplay or crosstalk between the two major epigenetic modifications, DNA methylation and histone modifications. Mainly, it occurs at the level of the modifying enzymes, the DNA methyltransferases and the SET domain histone methyltransferases, thus leading to the coordination of these two major epigenetic modifications (Cedar and Bergman, 2009).

5. Hypothesis and project objectives

Congenital hypothyroidism from thyroid dysgenesis (CHTD) is mainly a sporadic disorder (only 2% of cases are familial) (Castanet *et al.*, 2000) and exhibits a high discordance rate (92%) between MZ twins (Perry *et al.*, 2002). CHTD is characterized by both female and Caucasian predominances (Stoppa-Vaucher *et al.*, 2011; Deladoëy, 2012). Moreover, germline mutations in the thyroid-related transcription factors NKX2.1, PAX8, FOXE1, and NKX2.5 have been identified by systematic genetic screening in at most 3% of patients with sporadic CHTD (Narumi *et al.*, 2010). Linkage analysis has excluded these genes in rare multiplex families with CHTD (Castanet *et al.*, 2005). Of note, evidence of non-penetrance of mutations in genes such as *NKX2.5* in close relatives of patients (Castanet *et al.*, 2005) suggests that modifiers, possibly additional germline mutations such as CNVs and/or somatic genetic or epigenetic mutations, are involved in the pathogenesis of CHTD. In this regard, we hypothesized that a two (or more)-hit model combining germline mutations together with somatic mutations or epimutations in threshold-sensitive genes involved in thyroid development could account for the pathogenesis of TD (Deladoëy *et al.*, 2007a). Such a unifying two (or more)-hit model could account for the lack of a clear familial transmission and the sporadic nature of CHTD. In familial cases, the first hit could be a germline rare inherited or a *de novo* mutation, while the additional hits could also be germline or otherwise somatic mutations or epimutations in other loci. In sporadic cases, the mutational hits occur *de novo* either in germline, in thyroid tissue or both. By analogy, focal congenital hyperinsulinism (CHI), a sporadic endocrine disorder that is less common compared to TD, results from such a two-hit mechanism: a paternally inherited mutation in the sulfonylurea receptor gene (*SUR1*) or inward-rectifying potassium channel gene (KIR6.2) gene occurs together with loss of the maternal 11p15 allele (loss of heterozygosity (LOH)), a locus containing many imprinted genes. The LOH is a somatic event found only in the pancreatic lesion (Giurgea *et al.*, 2006).

Therefore, <u>the three main objectives of my doctoral studies were</u>:

I. To assess the role of somatic genetic or epigenetic variations in the pathogenesis of CHTD by:

a) Analysis of a genome-wide gene expression profile of eutopic (normally located) versus ectopic (abnormally located) thyroid tissues using microarray analysis.

b) Assessment of whether or not somatic changes in DNA methylation play a role in dysregulation of gene expression in ectopic thyroids. Methylation profiling was assessed by methylated DNA immunoprecipitation (MeDIP) and MeDIP-chip assays.

c) Assessment of the role of thyroid-specific CNVs (absent in matched leukocytes) in shaping the gene expression profile in ectopic thyroids using array comparative genomic hybridization (aCGH) of the ectopic thyroid DNA with matched leukocytes.

II. Based on the finding that no differences in calcitonin expression levels between ectopic and normal (orthotopic) thyroids was identified in our genome-wide gene expression study of ectopic thyroid tissues (part I), we aimed to determine whether calcitonin-producing C cells are present in ectopic lingual thyroids by performing calcitonin immunolabeling and transcript detection on ectopic lingual thyroids compared with normal thyroid tissues.

III. Based on the finding that no differentially methylated regions (DMRs) in ectopic versus eutopic were detected (part I) and on the fact that CpG dinucleotides and DMRs are genetic and epigenetic mutational hotspots, we aimed to determine whether the promoter methylation profile was different between thyroid and leukocytes using genome-wide and candidate gene approaches (for the thyroid-related transcription factors FOXE1, PAX8 and NKX2.1). The assessment involved:

a) The determination of the genome-wide methylation profile of thyroids and leukocytes using the methylated DNA immunoprecipitation (MeDIP) and MeDIP-chip assays.

b) The characterization of the methylation profile of the human *FOXE1, PAX8* and *NKX2.1* promoters by using bisulfite sequencing.

c) The assessment of the functional impact of CpG methylation of the *FOXE1* promoter on its expression using global and regional (patch) *in vitro* methylation coupled to transient transfections and reporter gene assays.

CHAPTER 2: RESULTS

1. Transcriptome, Methylome and Genomic Variations Analysis of Ectopic Thyroid Glands.

Rasha Abu-Khudir*[1,4], Jean Paquette*[1], Anne Lefort[2], Frederick Libert[2], Jean-Pierre Chanoine[3], Gilbert Vassart[2], Johnny Deladoëy[1,4].

[1] Endocrinology Service and Research Center, CHU Sainte-Justine, Department of Pediatrics, University of Montreal, Montreal H3T 1C5, Canada.

[2] IRIBHM, Free University of Brussels ULB, Campus Erasme, B-1070 Brussels, Belgium.

[3] Endocrinology and Diabetes Unit, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

[4] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

*These authors contributed equally to this work.

Key terms: ectopic thyroid, congenital anomalies, hypothyroidism, epigenetics, methylation, copy number variants, transcriptome.

Address all correspondence and requests for reprints to:

Johnny Deladoëy, M.D. Ph.D.
CHU Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

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Contributions of authors:

Rasha Ab-Khudir: Performed the experiments, analyzed the data, and wrote the first draft of the manuscript.

Jean Paquette: Performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Anne Lefort: Performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Frederick Libert: Performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Jean-Pierre Chanoine: Analyzed the data, contributed reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Gilbert Vassart: Conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Johnny Deladoëy: Conceived and designed the experiments, performed the experiments, analyzed the data, contributed by reagents/materials/analysis tools, and wrote the first draft of the manuscript.

Transcriptome, Methylome and Genomic Variations Analysis of Ectopic Thyroid Glands.

Rasha Abu-Khudir*[1,4], Jean Paquette*[1], Anne Lefort[2], Frederick Libert[2], Jean-Pierre Chanoine[3], Gilbert Vassart[2], Johnny Deladoëy[1,4].

[1] Endocrinology Service and Research Center, CHU Sainte-Justine, Department of Pediatrics, University of Montreal, Montreal H3T 1C5, Canada.

[2] IRIBHM, Free University of Brussels ULB, Campus Erasme, B-1070 Brussels, Belgium.

[3] Endocrinology and Diabetes Unit, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

[4] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

*These authors contributed equally to this work

Address all correspondence and requests for reprints to:

Johnny Deladoëy, M.D. Ph.D.
CHU Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

Key terms: ectopic thyroid, congenital anomalies, hypothyroidism, epigenetics, methylation, copy number variants, transcriptome.

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Abstract

Background: Congenital hypothyroidism from thyroid dysgenesis (CHTD) is predominantly a sporadic disease characterized by defects in the differentiation, migration or growth of thyroid tissue. Of these defects, incomplete migration resulting in ectopic thyroid tissue is the most common (up to 80%). Germinal mutations in the thyroid-related transcription factors NKX2.1, FOXE1, PAX-8, and NKX2.5 have been identified in only 3% of patients with sporadic CHTD. Moreover, a survey of monozygotic twins yielded a discordance rate of 92%, suggesting that somatic events, genetic or epigenetic, probably play an important role in the etiology of CHTD.

Methodology / Principal Findings: To assess the role of somatic genetic or epigenetic processes in CHTD, we analyzed gene expression, genome-wide methylation, and structural genome variations in normal *versus* ectopic thyroid tissue. In total, 1011 genes were more than two-fold induced or repressed. Expression array was validated by quantitative real-time RT-PCR for 100 genes. After correction for differences in thyroid activation state, 19 genes were exclusively associated with thyroid ectopy, among which genes involved in embryonic development (e.g. *TXNIP*) and in the Wnt pathway (e.g. *SFRP2* and *FRZB*) were observed. None of the thyroid related transcription factors (FOXE1, HHEX, NKX2.1, NKX2.5) showed decreased expression, whereas PAX8 expression was associated with thyroid activation state. Finally, the expression profile was independent of promoter and CpG island methylation and of structural genome variations.

Conclusions / Significance: This is the first integrative molecular analysis of ectopic thyroid tissue. Ectopic thyroids show a differential gene expression compared to that of normal thyroids, although molecular basis could not be defined. Replication of this pilot study on a larger cohort could lead to unraveling the elusive cause of defective thyroid migration during embryogenesis.

285 words

Introduction

Permanent primary hypothyroidism is the most common congenital endocrine disorder. In up to 85% of cases, it results from thyroid dysgenesis, a condition comprised of defects in the differentiation, migration or growth of thyroid tissue. Of these defects, incomplete migration resulting in ectopic thyroid tissue (sub-lingual thyroid) is the most common (up to 80%). The etiological diagnosis is established through thyroid scintigraphy [1]. Ectopic thyroids are smaller (i.e. they lack the lateral lobes that are characteristic of orthotopic thryroids) but are otherwise normal (i.e. they have a normal follicular architecture and their capacity to trap and organify iodine and to produce thyroid hormones and thyroglobulin is intrinsically normal [2, 3, 4, 5]. This suggests that the hypothyroidism of subjects with thyroid ectopy is due to a smaller amount of tissue (hypoplasia), which is a consequence of the migration defect, and not to defects in differentiation or in histological organization of the thyroid follicular cells.

Congenital hypothyroidism from thyroid dysgenesis (CHTD) is a heterogeneous disease, which exists in familial (2%) and non-familial (sporadic, 98%) forms [6]. Moreover, the results

of a survey of monozygotic twins yielded a discordance rate of 92% [7], which together with the female predominance in CHTD [8] suggest that complex non-Mendelian mechanisms underlie this condition. On the other hand, environmental causes operating *in utero* are unlikely because: (i) no temporal or seasonal trends for CHTD have been observed [9] and (ii) MZ twins who are discordant for CHTD have similar birth weight (G. Van Vliet, personal communication). Germinal mutations in thyroid related transcription factors NKX2.1, FOXE1, PAX-8, and NKX2.5 have been identified in only 3% of patients with sporadic CHTD and linkage analysis excluded these genes in some multiplex families with CHTD, which is consistent with a complex genetic contribution [10]. Together these findings indicate the involvement of novel genes and pathways and underlines the importance of somatic epigenetic or genetic events [11].

Combining data of gene expression, DNA methylation and DNA copy number has led to the identification of novel genetic regulators of cancer [12, 13]. Consistent with this approach, we aim to assess whether the transcriptome of ectopic thyroids is shaped by somatic genomic or epigenomic variations (Figure 1).

Results

Expression array identified 1011 genes that are more than two-fold induced or repressed.

We used microarray analysis to compare the genome-wide RNA expression profile of normal (orthotopic; n=1) versus abnormal (ectopic; n=3) thyroid tissue. We identified 1833 differentially expressed genes, and a total of 1011 genes were induced (n=522) or repressed (n=489) more than two-fold. The forty genes with the highest differential expression are listed in Table 1. To validate the differential expression identified by microarray analysis, we performed quantitative real-time PCR (qRTPCR) of 100 genes in ectopic thyroids compared with the same commercial control (Ambion) used for the arrays; these 100 genes included highly differentially expressed genes and genes known to play a role in the thyroid function. Overall, there was a highly significant correlation between microarray and qRTPCR (Pearson correlation coefficient of 0,86, $p<2.2$ e-16)(Figure S1).

Functional annotation clustering of the 1011 differentially expressed genes showed enrichment for developmental processes.

To assess whether the differentially expressed genes are related to development and organogenesis, we classified the 1011 differentially expressed genes into gene ontology (GO) groups using DAVID (Database for Annotation, Visualization and Integrated Discovery) with medium classification stringency. Table S2 shows the top three clusters for the 1011 differentially expressed genes (more than two-fold induced or repressed) with enrichment scores greater than 6 (i.e. $p < 1$ E -06). Two of them are clusters of genes enriched for developmental processes. We next clusterized separately the induced (n=522) and the repressed (n=489) genes using DAVID according to GO terms with high classification stringency. The top five clusters of induced genes with enrichment scores greater than 5.5 (i.e. $p < 0.5$ E -06) show genes important for development, vasculogenesis, the extracellular matrix, immune system development and collagen whereas the top five clusters for repressed genes with enrichment scores greater than 4 (i.e. $p < 1$ E -04) show genes important for histone function, apoptosis, chromatin function, organelle and contractile functions (Table S3). Finally, the analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with DAVID shows enrichment ($p<0.05$) for eight pathways, among which three are associated with cell-to-cell interaction (i.e. MAPK signaling pathway, focal adhesion and cell communication) (Figure 2).

Additional validation against age-matched hyperfunctioning thyroid tissue selects 19 genes whose expression is related to thyroid ectopy and independent of thyroid activation state.

Level of activity (i.e. thyroid hormone production) and consequently expression of genes involved in thyroid hormone production is sensitive to thyroid stimulating hormone (TSH) [14,15]. CHTD patients have high TSH at diagnosis, after which the level of their TSH may vary depending on the compliance to the treatment; or the TSH is high for years in cases of delayed treatment, as in our case 1 (Table S4). To correct for differential TSH-dependent activation of thyroid tissue, we extended the qRTPCR analysis for the 100 validated genes to three hyperfunctioning thyroid nodules (i.e. somatic activation of the TSH receptor) that had arisen in otherwise normal (orthotopic) thyroids. Of the 100 genes, 19 showed consistently induction or repression when compared to all types of controls (i.e. hyperfunctioning thyroid nodules and the commercial control) (Table 1). The 81 remaining genes showed divergent expression: i.e. they

were either induced in ectopic thyroid when compared to the commercial control but repressed when compared to the three hyperfunctioning thyroid nodules or *vice versa* (Table S5), suggesting that the expression of those 81 genes was dependent of thyroid activation state.

Pathway analysis of the 19 selected genes showed association with Wnt signalling pathway whereas the 81 excluded genes were enriched for thyroid hormonogenesis and function.

We then asked whether the use of two different types of control was able to exclude genes that are dependent on thyroid activation state and to identify genes associated exclusively with defective migration of the thyroid. To consider the level of expression (which is not possible with DAVID), we used the Ingenuity Pathways Analysis software for GO annotation and pathway analysis (Ingenuity® Systems, www.ingenuity.com). The 19 selected genes (Table 2) were enriched for the Wnt pathway, dendritic cell maturation pathway, and embryonic developmental functions (for each enrichment scores greater than 1.5; $p < 0.02$ and Benjamini-Hochberg multiple correction $p < 0.05$). As expected, the 81 genes (Table S5) excluded because of divergent expression are enriched for thyroid hormonogenesis and function: thyroglobulin *(TG),* thyroid peroxidase *(TPO),* deiodinase type II (*DIO2),* deiodinase type I *(DIO1)*, dual oxidase 2 (*DUOX2)*, paired box gene 8 *(PAX8),* thyroid stimulating hormone receptor *(TSHR)* and thyroid hormone responsive SPOT14 homolog *(THRSP)* (i.e for GO term endocrine system disorders, the enrichment score is 2; $p<0.01$ and Benjamini-Hochberg multiple correction $p < 0.01$). To control for tissue quality, we also analyzed 10 unregulated genes which are either well described thyroid-related transcription factors (FOXE1, HHEX, NKX2.1, NKX2.5) [16] or genes involved in the Wnt pathway (CTNNB1, GSK3B, CDH1, APC, AXIN1, AXIN2) [17]. CTNNB1 and CDH1 showed divergent expression; APC, AXIN1, AXIN2, FOXE1 and NKX2.1 showed increases of convergent expression; and NKX2.5 expression was massively increased when compared to orthotopic thyroids but only slightly increased when compared to the commercial control, which therefore might be considered as divergent. None of the thyroid related transcription factors (FOXE1, HHEX, NKX2.1, NKX2.5) showed decreased expression regardless of the control used (Table 3).

Differential gene expression in ectopic thyroid is independent of methylation.

The next step was to assess whether somatic changes in DNA methylation play a role in dysregulation of gene expression in ectopic thyroids. Methylation profiling by methylated DNA

immunoprecipitation (MeDIP) and MeDIP-chip was performed by hybridizing pairs of enriched methylated fraction (IP) and normal fraction (IN) of genomic DNA from our three ectopic thyroids and three controls orthotopic hyperfunctioning thyroid nodules. The methylation profile was similar between the ectopic and orthotopic thyroids: after multiple test correction, there was no statistically significant difference (i.e. no region with a less stringent False Discovery Rate threshold of 0.1) (data not shown). Consequently, no correlation was found between the differential expression in ectopic thyroids and the global methylation profile.

Differential gene expression in ectopic thyroid is independent of thyroid-specific CNVs.

To assess whether thyroid-specific (i.e. absent in matched leucocytes) CNVs shape gene expression in ectopic thyroids, we used array comparative genomic hybridization (aCGH) of the ectopic thyroid DNA with matched leucocytes. By analyzing data as described in *Materials and Methods*, we found four thyroid-specific CNVs (three validated by qPCR), which are reported variants as reported in the Database for Genomic Variants (http://projects.tcga.ca/variation) (Table S6). No correlation was found between thyroid-specific CNVs and differentially expressed genes in ectopic thyroids.

Discussion

Generally, CHTD is sporadic and shows discordance between MZ twins [7]. Somatic genetic or epigenetic events might therefore have a role in the etiology of this condition. The objective of this study was to assess whether somatic molecular events account for the failure of migration of ectopic thyroids. Therefore, we conducted the first integrative analysis of transcriptome, DNA methylation and structural variants (CNV) in ectopic thyroids.

We found altered expression in genes and pathways that might play a significant role in abnormal thyroid development (e.g. Wnt signaling pathway). Interestingly, none of the thyroid related transcription factors (FOXE1, HHEX, NKX2.1, NKX2.5) showed decreased expression, whereas PAX8 expression was associated with thyroid activation state. This is a direct indication that the expression of these known candidate genes is at least neutral in ectopic thyroid and is consistent with the observation that the coding sequences for FOXE1, NKX2.1 and PAX8 were normal in case #1 [5].

Four pathways identified by analysing the results of expression arrays (i.e. focal adhesion, antigen processing and presentation, cell communication, cell adhesion molecules and Type I diabetes) have been identified independently in hyperfunctioning thyroid nodules [18]. However, our results identify mostly induced genes in these pathways (Figure 1) whereas repression of these genes was observed in the aforementioned study [18]. To obtain, in ectopic thyroid, an opposite expression pattern when compared to that of orthotopic hyperfunctional thyroid (i.e. thyroid with somatic TSH receptor activating mutation) is plausible, but it underlines also the need to consider the differential activation of the TSH-receptor signaling pathways in our samples. Consequently, we have excluded 81 validated genes for which expression was associated with TSH-driven thyroidal activity.

The 19 selected genes whose expression was dependent on thyroid location (i.e. ectopy) were enriched for pathways involved in cellular movement (i.e. Wnt pathway and dendritic cell maturation pathway). This association has biological plausibility especially for the Wnt pathway. First, non-canonical Wnt pathway is crucial for cell migration [19] and development of organs of endodermal origin (e.g. intestine, lung, pancreas) [20]. There is indirect evidence for the involvement of the non-canonical Wnt pathway in the developing thyroid in mice [21], even though the canonical Wnt/beta-catenin pathway seems to be inactive during thyroid development in mice and humans [21, 22]. Second, as Wnt signaling is implicated in development and cancer [23], to find an association between Wnt pathway and thyroid ectopy (i.e. failure of proper thyroid migration during development) makes biologically sense. Indeed, SFRPs have been associated with embryonic patterning [24], inhibition of meduloblastoma cell proliferation [25] and inhibition of glioma cell motility [26]. Inhibition of the Wnt pathway by Wnt5-a has also been shown to suppress tumor activity in thyroid carcinoma [27].

This study has several limitations. First, the expression profiles in tissue collected and analyzed postnatally may not reflect embryonic expression. Consequently, whether the differences we observed are causes or consequences of the ectopic location of the thyroid remains to be tested. Second, even though clusters of genes involved in histone and chromatin function have repressed expression in ectopic thyroids, we have not formally excluded a role of differential histone methylation or acetylation on differential gene expression in ectopic thyroids. Third, the arrays used for the CNVs and methylome analyses have their own limits in definition

and genome coverage. Lastly, the sample number is small but our preliminary findings justify testing a larger number of samples.

This study identifies interesting candidate pathways that may play important roles in the migration of the embryonic thyroid and provides a prototype approach for the study of congenital disorders difficult to explain by classical genetics.

Methods

Ethic Statement

This study was approved by the Ethics Committee of the CHU Sainte-Justine (ERB number 94). All the parents gave written informed consent.

Patients and Tissue Collection

We obtained flash-frozen samples of ectopic thyroid tissue removed from 3 girls aged 8, 10 and 15 yr, because it caused local symptoms (i.e. dysphagia). For controls, we used (i) thyroid tissue from 2 girls (aged 15 and 16 yr) and 1 boy (aged 4 yr) who were operated for a single hyperfunctioning thyroid nodule that had arisen in an orthotopic thyroid and (ii) commercially available RNA from normal thyroid when appropriate (Table S6).

Functional clusters and pathways analysis

We submitted the 1011 differentially expressed genes into gene ontology (GO) groups using the DAVID database (http://david.abcc.ncifcrf.gov) for cluster analysis according to Gene Ontology (GO) terms with medium or high classification stringency. To provide a refined analysis, the 100 validated gene were analyzed through Ingenuity Pathways Analysis (IPA; http:www.ingenuity.com), a software that also considers the level of gene expression. With either DAVID or IPA, the proportion of each gene in the submitted list is compared with the one in the whole genome to compute the P value of the Fisher's test, the enrichment scores (i.e. geometric mean of the inverse log of each P value) and the Benjamini-Hochberg multiple correction P value.

Expression Arrays

After surgical resection, the samples were immediately frozen in liquid nitrogen and stored at -70 Celsius until use. Total RNA was extracted as per manufacturer recommendations

using the QIAzol kit (QIAGEN Inc., Ontario, Canada). RNA was DNase-treated to minimize DNA contamination. RNA quantity was measured by ND-1000 (Nanodrop, Wilmington, DE, USA). RNA quality was assessed by electropherograms on the Agilent 2100 Bioanalyzer. Microarray hybridization was performed on three different ectopic thyroids (two in duplicate, one in quadruplicate) and compared to RNA of thyroid tissue from a Caucasian female, age 68 y with gall bladder cancer (Ambion, #AM6872). Double-stranded cDNA was synthesized from 1 µg of total RNA, followed by production of antisense RNA containing the modified nucleotide 5-(3-aminoallyl)-UTP using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, Texas, USA). After labeling with Cy3 or Cy5 (GE Healthcare Bio-Sciences, New Jersey, USA), sample pairs were hybridized onto Human Exonic Evidence Based Oligonucleotide HEEBO slides (Stanford Functional Genomics Facility, CA, USA). The oligonucleotide set consists of 44544 70-mer probes that were designed using a transcriptome-based annotation of exonic structure for genomic loci. Hybridizations were replicated with dye swap. Slides were scanned using a Molecular Devices 4000B Laser scanner and expression levels were quantified using GenePix Pro 6.1 image analysis software (Axon Instruments, CA, USA). Image acquisitions were performed with automatic photomultiplier gains (PMT) adjustment. Artefact-associated spots were eliminated by both visual and software-guided flags, as were spots with a signal/background fluorescence ratio less than 2. The fluorescence values were imported into Acuity 4.0 software package (Molecular Devices, Union City, CA, USA). A non-linear locally weighted scatter plot (Lowess) normalization method applied to each individual block (print-tip option) was carried out using Acuity 4.0 software package (Molecular Devices, Union City, CA, USA) [28]. The identification of genes with significant differences in expression levels was performed using the significance analysis of microarray method (SAM one class) [29]. SAM estimates the percentage of genes identified by chance, the false discovery rate (FDR). We assessed the statistical significance of the differential expression of genes by computing a q-value (i.e. minimum FDR) for each gene (Table 1). Genes were considered to be differentially expressed when the absolute normalized fold change between ectopic thyroids and control was determined to be greater than 2.0 or less than 0.5 in at least one pair of the hybridized arrays. Full access to the primary array data is available on the GEO web site (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE16804.

Quantitative Real-Time RT-PCR

Validation of the expression levels of 100 genes of interest was carried out using TaqMan low density array (TLDA) technology (Applied Biosystems, Ontario, Canada). Probes and primers have been selected with the publically available software http://www5.appliedbiosystems.com/tools/ and can be retrieved by using the assay ID reported in Tables 2, 3 and S5. The expression levels were normalized to the expression level of the 18S rRNA. Induced (n=49) and repressed (n=51) genes were selected from the 1011 differentially regulated genes found in the HEEBO expression microarray analysis. Total RNAs from thyroids were first treated with the DNA-free kit to remove residual contamination of genomic DNA (Ambion Inc.). DNA-free total RNA (175 ng) was subjected to reverse transcription using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). An aliquot of the cDNA was mixed to TaqMan® Gene Expression Master Mix, loaded on the TLDA plates then centrifuged for distribution of the material in the 384 wells. Gene target amplifications were performed in triplicate using the 7900HT Real-Time PCR System (Applied Biosystems). Sequence Detection System software version 2.2.2 (Applied Biosystems) was used for comparative gene expression analysis using the ΔCt method. In a first analysis, expression levels found in the normal orthotopic thyroid from Ambion were compared to the mRNA levels present in the ectopic thyroids. Expression levels in the hot nodules were then compared to the levels found in the ectopic thyroids. For analysis, the cut-off $\log_2$ value was 0.5. Then, to compare the results of the quantitative real-time RT-PCR with those of expression arrays, Pearson correlation was calculated with the free statistical software R [30].

Methylation Profiling by Methylated DNA Immunoprecipitation (MeDIP) and MeDIP-chip.

The MeDIP-chip was performed using pairs of enriched methylated fraction (IP) and normal fraction (IN) of genomic DNA from our three ectopic thyroids and three controls (i.e. hyperfunctioning thyroid nodules). The methylated fraction of genomic DNA was enriched using the methylated DNA immunoprecipitation (MeDIP) assay [31] and interrogated on human Promoter plus CpG Island Tiling Arrays with a ChIP design for CpG islands and promoter regions (n=28,226) from HG18 using 385,020 Probes selected from CGH probe bank with a median spacing of 101bp (Roche NimbleGen, Madison, WI). Briefly, 4 µg *Mse*I digested genomic DNA was immunoprecipitated with monoclonal mouse anti 5-methylcytidine antibody

(New England Biolab, Pickering, Ontario and Abcam Inc.Cambridge, MA 02139). After washes and purification steps, immunoprecipitated material and a sample of input DNA were amplified using GenomePlex Complete Whole Genome Amplification (WGA) kit (Sigma-Aldrich, Saint Louis, Missouri 63103 USA). The resulting products (4 µg) were labeled, cohybridized and scanned by the NimbleGen Customer Service (Roche NimbleGen, Madison, WI). For each sample, NimbleScan detects peaks by searching for at least two probes above a P-value cutoff (-$\log_{10}$) of 2 and peaks within 500bp are merged (gff files on GEO web site). Then, peak data were analyzed to compare the methylation profile between ectopic thyroids and orthotopic thyroids using the Loess normalized $\log_2$ (ChIP/input) ratios with the one-way ANOVA tool of the Partek Genomics Suite (PGS) software. A FDR less than 0.1 was considered as significant. Full access to the primary array data is available on the GEO web site (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE17581.

Array Comparative Genomic Hybridization and validation with quantitative real-time PCR

Array comparative genomic hybridization (aCGH) was performed using pairs of thyroid tissues and leucocytes from the three ectopic thyroids. We used the Nimblegen X1 HG18 whole genome CGH design (version 2). The 385,815 probes are distributed across the genome with a median spacing of 7073 bp. Probes are 60-mers, with a Tm target of 80 degrees. Labeling, hybridization, washing and scanning was performed by the NimbleGen Customer Service (Roche NimbleGen, Madison, WI). After normalization, the $\log_2$ (test/reference) signals were analyzed using a circular binary segmentation algorithm (segMNT) with the PGS Software to identify somatically acquired segmental copy number changes. To call a copy number change, segMNT required a segment to span a minimum of 5 consecutive probes with a p-value threshold of 0.001 and a signal to noise ratio of 0.3. Then, reported regions were set at segMNT $\log_2$ ratio below 0.3 or above 0.2 in all three samples with a p-value threshold of 0.01. Full access to the primary array data is available on the GEO web site (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE17463. Validation of the aCGH with quantitative real-time PCR was performed with the TaqMan technology. Identified CNVs were validated using TaqMan Gene Copy Number Assays from ABI. Probes and primers have been selected with the public available software (http://www5.appliedbiosystems.com/tools/cnv/) and can be retrieved by using the assay ID reported in Table S6.

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Figure Legends:

Figure 1. Breakdown of the experimental design and overview of the results.

Figure 2. Expression array: gene pathway distribution in ectopic thyroid according to KEGG. The pathways are classified according to the number of regulated genes (HEEBO array results). P-values of Fisher's exact test for each KEGG pathways are listed to the right of the boxes. Dark gray, induced genes; gray, repressed genes.

Figures:

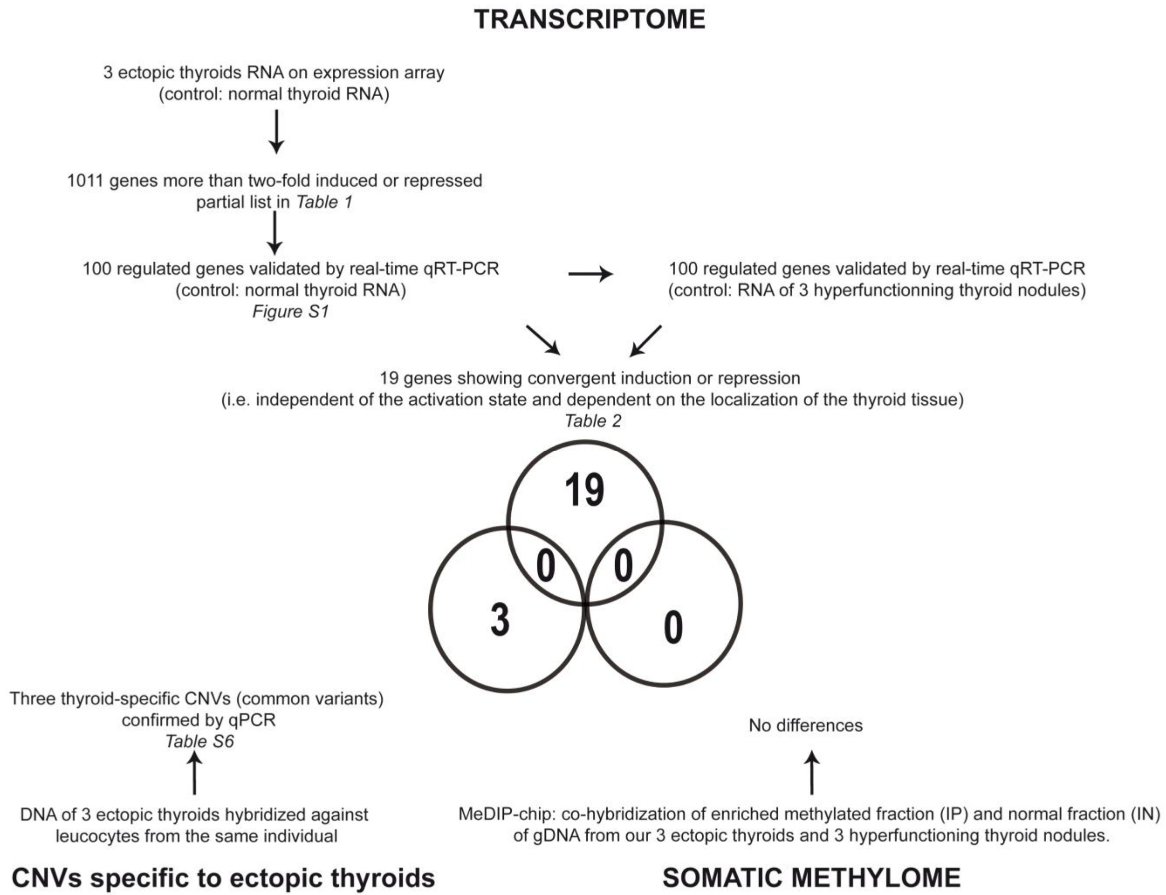

Figure 1

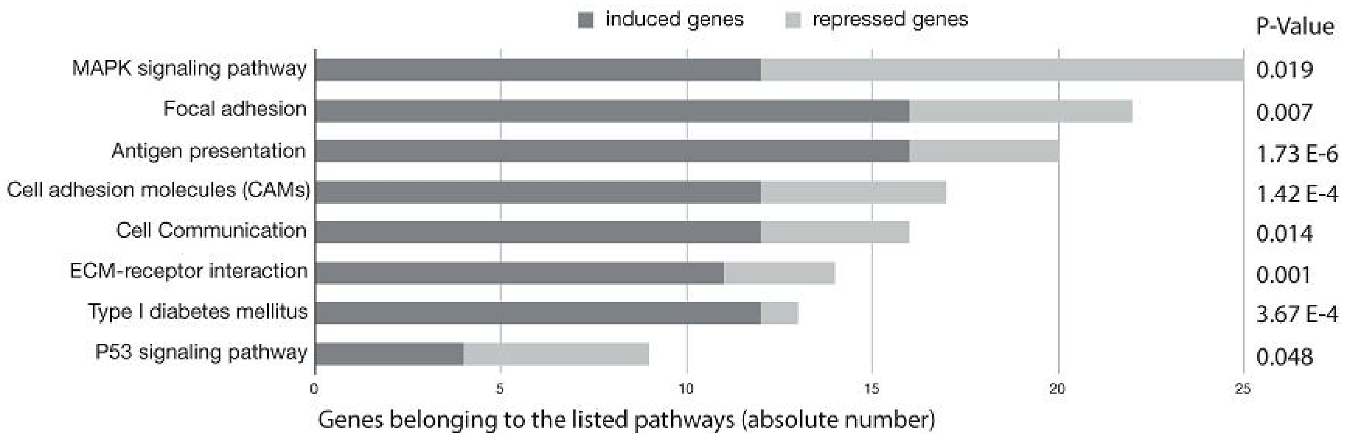

Figure 2

Tables:

Table 1. Result of the expression array: the top twenty induced (upper panel) and top twenty repressed (lower panel) genes.

TOP 20 induced genes

Entrez Gene Name	Entrez Gene ID	Array[a]	q-value (%)[b]	qRTPCR RQ value [c]
LYZ	4069	5,99	0,0000	5,41
FOSB	2354	5,48	0,0000	5,62
IGJ	3512	5,44	0,0000	
TRA@	6955	5,23	0,7897	
PAX8	7849	4,68	0,0000	2,55
CYBB	1536	4,62	0,0000	
TRA@	6955	4,60	0,0000	
HLA-DQA1	3117	4,56	0,0000	
CECR1	51816	4,49	0,0000	
HLA-DQB1	3119	4,41	0,0000	
EGR1	1958	4,29	0,0000	
RNASE6	6039	3,98	0,0000	
SFRP2	6423	3,91	0,0000	4,74
GPNMB	10457	3,87	0,0000	4,97
PABPC1	26986	3,85	0,0000	
MS4A6A	64231	3,84	0,0000	
KLF4	9314	3,74	0,7897	
HADHA	3030	3,61	0,7897	
FOS	2353	3,61	0,0000	4,46
IGHG4	3503	3,52	0,7897	

TOP 20 repressed genes

Entrez Gene Name	Entrez Gene ID	Array[a]	q-value (%)[b]	qRTPCR RQ value[c]
ABCA13	154664	-6,06	0,0000	-4,12
CKM	1158	-5,86	0,0000	-10,31
ACTA1	58	-5,31	0,0000	-5,06
C9orf70	84850	-4,85	0,0000	
CHGA	1113	-4,83	0,0000	-8,38
MYBPC1	4604	-4,60	0,0000	
ATP2A1	487	-4,19	0,0000	
TNNC2	7125	-4,09	0,0000	-4,64
IGSF1	3547	-4,00	0,0000	
EEF1A2	1917	-3,87	1,9070	
FLJ32115	121506	-3,79	0,7897	
PCSK2	5126	-3,66	1,9070	
RASD1	51655	-3,53	2,7615	-0,51
RARRES1	5918	-3,52	1,9070	
EDN3	1908	-3,40	1,9070	-1,83
PRKCE	5581	-3,32	2,7615	1,98
GSTT1	2952	-3,24	4,2100	
FLJ11127	54491	-3,20	3,2882	
TCAP	8557	-3,17	3,9587	
LOC440696	3322	-3,17	3,2882	

[a]Array mean ratio are expressed in $\log_2$.

[b]Q-values (i.e. minimal false discovery rate) are expressed in percent, all P-values are less than 0.001.

[c]Validation with qRTPCR: RQ values are expressed in log_2. Bold characters indicate genes that are validated (13 of 14 tested) among these 40 genes.

Table 2. Validated genes (n=19) with convergent induced (n=16) and repressed (n=3) expression in ectopic thyroid tissue (i.e. independent of the activation state and dependent on the localization of the thyroid tissue).

Entrez Gene Name	Enterz Gene ID	Array[a]	qRTPCR vs normal thyroid[a]	qRTPCR vs hot nodules[a]	Taq Man Assay ID for qRTPCR
SFRP2	**6423**	**3,91**	**4,74**	**5,48**	Hs00293258_m1
TBX1	6899	1,52	3,21	1,57	Hs00271949_m1
GPNMB	10457	3,87	4,97	1,45	Hs01095669_m1
DUSP6	1848	1,98	3,09	1,32	Hs01044001_m1
FRZB	**2487**	**0,83**	**1,83**	**1,24**	Hs00173503_m1
COL1A1	1277	2,71	5,93	1,10	Hs00164004_m1
FGL2	10875	2,46	4,22	1,00	Hs00173847_m1
LYZ	4069	5,99	5,41	0,94	Hs00426231_m1
COL3A1	1281	3,39	5,12	0,89	Hs00164103_m1
TYROBP	7305	2,25	3,62	0,76	Hs00182426_m1
TNFAIP2	7127	3,45	4,63	0,68	Hs00196800_m1
BGN	633	1,09	3,14	0,63	Hs00959143_m1
PBX4	80714	1,83	3,67	0,58	Hs00257935_m1
PLCXD1	55344	1,71	2,83	0,58	Hs00383111_m1
CPEB4	**80315**	1,28	2,6	0,54	Hs00270923_s1
MKRN1	*23608*	*2,63*	*1,18*	*0,53*	Hs00831972_s1
TXNIP	10628	−2,81	−1,78	−0,51	Hs01006900_g1
ABCA13	154664	−6,06	−4,06	−0,71	Hs00541549_m1
ENO3	2027	−1,99	−0,86	−1,15	Hs00266551_m1

[a] Array mean ratio and qRTPCR RQ are expressed in log_2.
Bold characters indicate genes that are directly associated with the Wnt pathways (canonical and non-canonical).
Italic characters indicate genes that are regulators of the Wnt/beta-Catenin pathway (Major *et al.*, 2008).

Table 3. Quantitative RTPCR for 10 controls.

Entrez Gene Name	Entrez Gene ID	RQ of qRTPCR vs normal thyroid [a]	RQ of qRTPCR vs hot nodules[a]	Taq Man Assay ID for qRTPCR
APC	324	2,31	0,13	Hs01568269_m1
AXIN1	8312	1,39	0,2	Hs00394718_m1
AXIN2	8313	2,05	0,62	Hs01063168_m1
CTNNB1	1499	1,35	-0,53	Hs00355045_m1
CDH1	999	2,07	-0,35	Hs00170423_m1
FOXE1	2304	2,58	0,47	Hs00538731_s1
GSK3B	2932	2,1	0	Hs00275656_m1
HHEX	3087	1,62	0,05	Hs00242160_m1
NKX2.1	16002	1,43	0,36	Hs00163037_m1
NKX2.5	1482	0,53	6,93	Hs00231763_m1

[a] Array mean ratio and qRTPCR RQ are expressed in $\log_2$.

Supplementary figure S1:

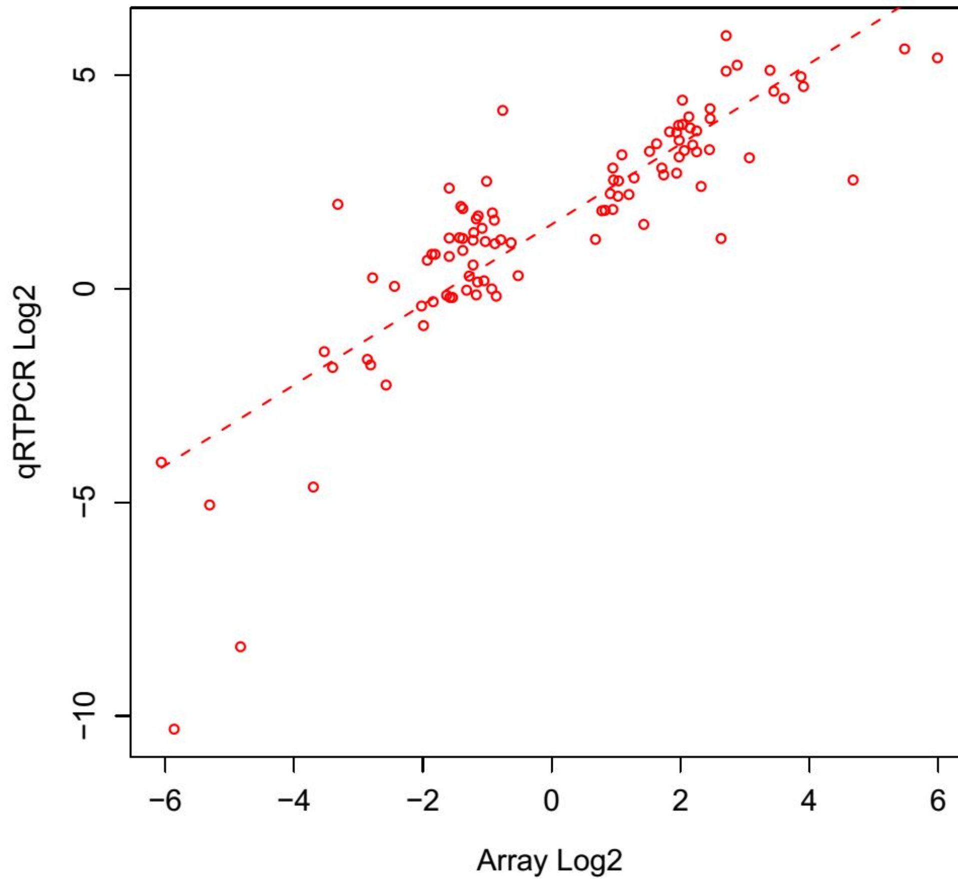

Figure S1: Reliability of the HEEBO expression array was confirmed by calculating the Pearson correlation coefficient (r = 0,86; P<2.2 e-16, n = 100 genes; ectopic thyroid (n = 3) vs normal thyroid - Ambion, #AM6872) between microarray and qRT-PCR results. Results are expresed in log2 ratio.

Supplementary tables:

Table S1: The top three clusters for the 1011 differentially expressed genes (more than two-fold induced or repressed).

Functional Group 1	enrichment score: 9.62[a]			
Category	Term	Hits[b]	P-Value[c]	Benjamini[d]
GOTERM_BP_ALL	GO:0006950~response to stress	110	6.85 E-15	3.61 E-11
GOTERM_BP_ALL	GO:0009605~response to external stimulus	71	2.67 E-11	4.68 E-8
GOTERM_BP_ALL	GO:0009611~response to wounding	51	2.41 E-9	1.58 E-6
GOTERM_BP_ALL	GO:0006952~defense response	59	2.62 E-8	1.37 E-5
GOTERM_BP_ALL	GO:0006954~inflammatory response	38	6.81 E-8	2.55 E-5
Functional Group 2	**enrichment score: 7.84**			
Category	Term	Hits	P-Value	Benjamini
GOTERM_BP_ALL	GO:0032502~developmental process	231	2.40 E-12	6.31 E-9
GOTERM_BP_ALL	GO:0048856~anatomical structure development	163	1.30 E-10	1.36 E-7
GOTERM_BP_ALL	GO:0048869~cellular developmental process	141	1.37 E-9	1.20 E-6
GOTERM_BP_ALL	GO:0030154~cell differentiation	141	1.37 E-9	1.20 E-6
GOTERM_BP_ALL	GO:0048513~organ development	106	6.17 E-9	3.60 E-6
GOTERM_BP_ALL	GO:0048731~system development	130	7.98 E-8	2.62 E-5
GOTERM_BP_ALL	GO:0007275~multicellular organismal development	160	3.42 E-7	9.00 E-5
GOTERM_BP_ALL	GO:0009653~anatomical structure morphogenesis	91	3.50 E-7	8.76 E-5
GOTERM_BP_ALL	GO:0032501~multicellular organismal process	213	7.58 E-4	0.0561
Functional Group 3	**enrichment score: 6.38**			
Category	Term	Hits	P-Value	Benjamini
GOTERM_BP_ALL	GO:0032502~developmental process	231	2.40 E-12	6.31 E-9
GOTERM_BP_ALL	GO:0048869~cellular developmental process	141	1.37 E-9	1.20 E-6
GOTERM_BP_ALL	GO:0030154~cell differentiation	141	1.37 E-9	1.20 E-6
GOTERM_BP_ALL	GO:0016265~death	76	2.81 E-8	1.23 E-5
GOTERM_BP_ALL	GO:0008219~cell death	76	2.81 E-8	1.23 E-5
GOTERM_BP_ALL	GO:0006915~apoptosis	72	5.11 E-8	2.06 E-5
GOTERM_BP_ALL	GO:0012501~programmed cell death	72	7.39 E-8	2.59 E-5
GOTERM_BP_ALL	GO:0048468~cell development	95	1.74 E-6	3.65 E-4
GOTERM_BP_ALL	GO:0042981~regulation of apoptosis	49	1.19 E-5	0.0018
GOTERM_BP_ALL	GO:0043067~regulation of programmed cell death	49	1.60 E-5	0.0024
GOTERM_BP_ALL	GO:0043066~negative regulation of apoptosis	25	1.90 E-4	0.0206
GOTERM_BP_ALL	GO:0043069~negative regulation of programmed cell death	25	2.32 E-4	0.0236
GOTERM_BP_ALL	GO:0006916~anti-apoptosis	14	0.0526	0.7418

a) Enrichment score is the negative log of geometric mean of each member's P-value in the cluster.
b) Number of dysregulated genes found in this list of GO-term.
c) P-value is calculated by Fisher exact tests.
d) Benjamini-Hochberg is the P-value corrected for multiple comparisons.

Table S2: Clusters for the induced (n = 522) and repressed (n = 489) genes.

induced genes				
Functional Group 1	Enrichment score (a): 10.44			
Category	Term	Hits	PValue (b)	Benjamini (c)
GOTERM_BP_ALL	GO:0048856~anatomical structure development	107	7.91 E-13	4.15 E-9
GOTERM_BP_ALL	GO:0048731~system development	88	1.48 E-10	1.94 E-7
GOTERM_BP_ALL	GO:0007275~multicellular organismal development	106	3.83 E-10	3.36 E-7
Functional Group 2	Enrichment score: 7.06			
Category	Term	Hits	PValue	Benjamini
GOTERM_BP_ALL	GO:0001568~blood vessel development	22	8.90 E-9	5.84 E-6
GOTERM_BP_ALL	GO:0001944~vasculature development	22	1.18 E-8	6.21 E-6
GOTERM_BP_ALL	GO:0048514~blood vessel morphogenesis	19	1.63 E-7	5.04 E-5
GOTERM_BP_ALL	GO:0048646~anatomical structure formation	19	5.08 E-7	1.48 E-4
GOTERM_BP_ALL	GO:0001525~angiogenesis	17	5.21 E-7	1.44 E-4
Functional Group 3	Enrichment score: 6.79			
Category	Term	Hits	PValue	Benjamini
SP_PIR_KEYWORDS	extracellular matrix	23	1.39 E-8	2.96 E-6
GOTERM_CC_ALL	GO:0005578~proteinaceous extracellular matrix	27	4.61 E-7	1.33 E-4
GOTERM_CC_ALL	GO:0031012~extracellular matrix	27	6.44 E-7	1.39 E-4
Functional Group 4	Enrichment score: 5.84			
Category	Term	Hits	PValue	Benjamini
GOTERM_BP_ALL	GO:0048534~hemopoietic or lymphoid organ	20	9.35 E-7	2.45 E-4
GOTERM_BP_ALL	GO:0030097~hemopoiesis	19	1.42 E-6	3.41 E-4
GOTERM_BP_ALL	GO:0002520~immune system development	20	2.19 E-6	5.01 E-4
Functional Group 5	Enrichment score: 5.76			
Category	Term	Hits	PValue	Benjamini
SP_PIR_KEYWORDS	triple helix	11	2.84 E-9	1.01 E-6
SP_PIR_KEYWORDS	hydroxylysine	10	6.70 E-8	8.92 E-6
SP_PIR_KEYWORDS	hydroxyproline	10	1.15 E-7	1.36 E-5
INTERPRO	IPR008160:Collagen triple helix repeat	13	4.68 E-7	0.0027
INTERPRO	IPR008161:Collagen helix repeat	11	6.05 E-7	0.0017
SP_PIR_KEYWORDS	hydroxylation	11	2.08 E-6	1.71 E-4
GOTERM_BP_ALL	GO:0006817~phosphate transport	13	3.49 E-6	6.54 E-4
SP_PIR_KEYWORDS	collagen	12	5.54 E-5	0.0036
GOTERM_BP_ALL	GO:0015698~inorganic anion transport	14	2.02 E-4	0.0276
GOTERM_BP_ALL	GO:0006820~anion transport	15	4.35 E-4	0.0570
repressed genes				
Functional Group 1	Enrichment score: 6.24			
Category	Term	Hits	PValue	Benjamini
SMART	SM00427:H2B	7	1.65 E-7	1.00 E-4
INTERPRO	IPR000558:Histone H2B	7	1.95 E-7	5.79 E-4
PIR_SUPERFAMILY	PIRSF002050:histone H2B	7	4.72 E-7	0.001
INTERPRO	IPR009072:Histone-fold	9	6.66 E-6	0.013
Functional Group 2	Enrichment score: 5.24			
Category	Term	Hits	PValue	Benjamini
GOTERM_BP_ALL	GO:0006915~apoptosis	40	1.30 E-6	7.60 E-4
GOTERM_BP_ALL	GO:0012501~programmed cell death	40	1.62 E-6	8.55 E-4
GOTERM_BP_ALL	GO:0016265~death	41	2.34 E-6	9.47 E-4
GOTERM_BP_ALL	GO:0008219~cell death	41	2.34 E-6	9.47 E-4
GOTERM_BP_ALL	GO:0048468~cell development	46	5.35 E-4	0.086
Functional Group 3	Enrichment score: 5.14			
Category	Term	Hits	PValue	Benjamini
INTERPRO	IPR007125:Histone core	12	3.44 E-10	2.03 E-6
SP_PIR_KEYWORDS	Nucleosome core	12	1.38 E-9	7.38 E-7
GOTERM_BP_ALL	GO:0006334~nucleosome assembly	13	5.01 E-7	4.39 E-4
GOTERM_CC_ALL	GO:0000786~nucleosome	12	5.87 E-7	1.02 E-4
GOTERM_BP_ALL	GO:0031497~chromatin assembly	13	2.21 E-6	0.0010
INTERPRO	IPR009072:Histone-fold	9	6.66 E-6	0.0130
GOTERM_BP_ALL	GO:0006333~chromatin assembly or disassembly	14	1.74 E-5	0.0057
GOTERM_BP_ALL	GO:0065004~protein-DNA complex assembly	14	3.30 E-5	0.0086
SP_PIR_KEYWORDS	Chromosomal protein	12	7.27 E-5	0.0077
GOTERM_CC_ALL	GO:0000785~chromatin	14	4.76 E-4	0.0194
GOTERM_BP_ALL	GO:0006323~DNA packaging	18	7.10 E-4	0.0936
GOTERM_BP_ALL	GO:0006325~establishment and/or maintenance of	17	0.0015	0.1809
GOTERM_BP_ALL	GO:0051276~chromosome organization and	19	0.0026	0.2339
Functional Group 4	Enrichment score: 4.51			
Category	Term	Hits	PValue	Benjamini
GOTERM_CC_ALL	GO:0043229~intracellular organelle	228	1.09 E-5	0.0010
GOTERM_CC_ALL	GO:0043226~organelle	228	1.14 E-5	9.91 E-4
GOTERM_CC_ALL	GO:0043231~intracellular membrane-bound	199	8.33 E-5	0.0055
GOTERM_CC_ALL	GO:0043227~membrane-bound organelle	199	8.58 E-5	0.0053
Functional Group 5	Enrichment score: 4.33			
Category	Term	Hits	PValue	Benjamini
GOTERM_CC_ALL	GO:0043292~contractile fiber	10	2.36 E-5	0.0018
GOTERM_CC_ALL	GO:0030016~myofibril	9	4.46 E-5	0.0032
GOTERM_CC_ALL	GO:0044449~contractile fiber part	9	9.17 6E-5	0.0052

[a]Enrichment score is the negative log of geometric mean of each member's P-value in the cluster.
[b]P-value is calculated by Fisher's exact tests.
[c]Benjamini-Hochberg is the P-value corrected for multiple comparisons.

Table S3: Source of patients derived thyroid tissues.

	case 1	case 2	case 3	control 1	control 2	control 3	control AMBION
thyroid gland location	ectopic - lingual	ectopic - sublingual	ectopic - sublingual	orthotopic	orthotopic	orthotopic	orthtotopic
age at diagnosis / sexe	8.5 yr/F	2 d/F	2 d/F	4/M	15/F	16/F	68 / F
TSH (mU/L) prior to L-T4 treatment	6.6	N/A	N/A	N/A	N/A	N/A	N/A
fT4 (pmol/L) prior to L-T4 treatment	8.5	N/A	N/A	N/A	N/A	N/A	N/A
LT4 daily dose (µg)	75	68.5	50	N/A	N/A	N/A	N/A
age at surgery (yr)	9.5	9	14	4	15	16	68
TSH (mU/L) prior to surgery	0.08	3.9	3.4	0.08	<0.03	0.01	N/A
fT4 (pmol/L) prior to surgery	15.7	14.0	12.8	24.8	22.07	17.3	N/A

Table S4: Validated genes (n = 81) with divergent expression in ectopic thyroid tissue (i.e. dependent on the activation state and independent of the localization of the thyroid tissue).

Symbol	Gene product	Enterz Gene	mean log2 ratio on HEEBO array (3 ectopic thyroids VS control AMBION)	RQ of qRTPCR vs normal thyroid (log2 converted)	RQ of qRTPCR vs hot nodules (log2 converted)	Taq Man Assay ID
CHGA	chromogranin A (parathyroid secretory protein 1)	1113	-4,83	-8,38	5,15	Hs00900373_m1
TNNC2	troponin C2, fast	7125	-3,7	-4,64	4,67	Hs00268519_m1
ACTA1	actin, alpha 1, skeletal muscle	58	-5,31	-5,06	2,88	Hs00559403_m1
THRSP	thyroid hormone responsive (SPOT14 homolog, rat)	7069	-1,01	2,52	1,97	Hs00930058_m1
GNG5	guanine nucleotide binding protein (G protein), gamma 5	2787	-1,22	0,56	1,75	Hs00359134_g1
SMOC2	SPARC related modular calcium binding 2	64094	-1,32	-0,03	1,49	Hs00405777_m1
TEF	thyrotrophic embryonic factor	7008	-1,17	-0,13	1,21	Hs00162657_m1
CNTN6	contactin 6	27255	-2,86	-1,64	1,15	Hs00274291_m1
LRP8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	7804	-1,38	1,88	1,09	Hs00182998_m1
EDN3	endothelin 3	1908	-3,4	-1,84	0,99	Hs00171177_m1
FMR1	fragile X mental retardation 1	2332	-1,08	1,42	0,92	Hs00924547_m1
GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	2697	-1,59	2,36	0,92	Hs00748445_s1
CKM	creatine kinase, muscle	1158	-5,86	-10,31	0,74	Hs00176490_m1
SPRED1	sprouty-related, EVH1 domain containing 1	161742	-1,05	0,19	0,71	Hs00544790_m1
CHORDC1	cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein 1	26973	-1,93	0,67	0,62	Hs00854389_g1
PLEKHA3	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 3	65977	-1,21	1,32	0,57	Hs00604551_m1
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	10797	-1,86	0,81	0,48	Hs00759197_s1
SMAD9	SMAD, mothers against DPP homolog 9 (Drosophila)	4093	-0,52	0,31	0,42	Hs00195441_m1
SMAD5	SMAD, mothers against DPP homolog 5 (Drosophila)	4090	-0,79	1,15	0,42	Hs00195437_m1
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	27242	1,98	3,48	0,4	Hs00205419_m1
MUC1	mucin 1, transmembrane	4582	-1,63	-0,1	0,39	Hs00159357_m1
PRKCE	protein kinase C, epsilon	5581	-3,32	1,98	0,38	Hs00178455_m1
EMP3	epithelial membrane protein 3	2014	2,06	3,24	0,38	Hs00171319_m1
INHBB	inhibin, beta B (activin AB beta polypeptide)	3625	-2,02	-0,4	0,35	Hs00173582_m1
EVC2	Ellis van Creveld syndrome 2 (limbin)	132884	-1,43	1,19	0,34	Hs00377633_m1
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	9459	2,25	3,21	0,31	Hs00374462_m1
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	2353	3,61	4,46	0,26	Hs99999140_m1
TSHR	thyroid stimulating hormone receptor	7253	1,43	1,49	0,2	Hs01053846_m1
POLD4	polymerase (DNA-directed), delta 4	57804	0,95	1,86	0,2	Hs00221775_m1
RASD1	RAS, dexamethasone-induced 1	51655	-3,53	-1,47	0,12	Hs00607394_g1
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1956	-0,88	1,06	0,08	Hs00193306_m1
PKNOX1	PBX/knotted 1 homeobox 1	5316	-1,59	1,19	0,07	Hs00231814_m1
TCF4	transcription factor 4	6925	0,78	1,83	0,04	Hs00162613_m1
NAV1	neuron navigator 1	89796	1,74	2,67	0,01	Hs00368110_m1
FZD4	frizzled homolog 4 (Drosophila)	8322	0,91	2,23	0,01	Hs00201853_m1
PLXND1	plexin D1	23129	1,97	3,83	0	Hs00391129_m1
ACP2	acid phosphatase 2, lysosomal	53	1,04	2,53	0	Hs00155636_m1
SFRS2	splicing factor, arginine/serine-rich 2	6427	2,03	4,42	-0,01	Hs00293258_m1
DTX4	deltex 4 homolog (Drosophila)	23220	-1,84	-0,3	-0,02	Hs00392288_m1
CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	8727	-1,59	0,76	-0,03	Hs00169384_m1
PAX8	paired box gene 8	7849	4,68	2,55	-0,04	Hs00243335_m1
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	6387	1,94	2,71	-0,04	Hs00930455_m1
SFRP1	secreted frizzled-related protein 1	6422	2,88	5,24	-0,07	Hs00610060_m1
TG	thyroglobulin	7038	1,2	2,21	-0,07	Hs00174974_m1
EFNB2	ephrin-B2	1948	0,95	2,83	-0,07	Hs00187950_m1
ROBO4	roundabout homolog 4, magic roundabout (Drosophila)	54538	1,63	3,4	-0,1	Hs00219408_m1
ASPM	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	259266	-0,76	4,18	-0,12	Hs00396967_m1
FN1	fibronectin 1	2335	1,94	3,66	-0,13	Hs00415006_m1
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	10000	-1,14	1,71	-0,13	Hs00289302_s1
CYBRD1	cytochrome b reductase 1	79901	-1,17	1,63	-0,13	Hs00227411_m1
CXCR4	chemokine (C-X-C motif) receptor 4	7852	0,68	1,16	-0,15	Hs00237052_m1
FZD3	frizzled homolog 3 (Drosophila)	7976	-0,63	1,08	-0,15	Hs00184043_m1
AKT1	v-akt murine thymoma viral oncogene homolog 1	207	-0,89	1,8	-0,18	Hs00920503_m1
LMO3	LIM domain only 3 (rhombotin-like 2)	55885	-1,15	0,16	-0,18	Hs00375237_m1
NLK	nemo like kinase	51701	1,03	2,17	-0,18	Hs00212076_m1
DKK3	dickkopf homolog 3 (Xenopus laevis)	27122	2,15	3,77	-0,2	Hs00247429_m1
LRP8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	7804	-1,38	0,9	-0,2	Hs00182998_m1
CTGF	connective tissue growth factor	1490	2,45	3,26	-0,22	Hs00170014_m1
HSPA1B	heat shock 70kDa protein 1B	3304	-2,57	-2,25	-0,22	Hs00359147_s1
FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	2260	2,32	2,4	-0,23	Hs00241111_m1
KPNA4	karyopherin alpha 4 (importin alpha 3)	3840	-1,22	1,14	-0,25	Hs00927639_g1
SNX1	sorting nexin 1	6642	-1,38	1,17	-0,25	Hs00541723_m1
VEGFA	vascular endothelial growth factor	7422	3,07	3,07	-0,27	Hs00900055_m1
FZD1	frizzled homolog 1 (Drosophila)	8321	-1,28	0,3	-0,27	Hs00268943_s1
LAMA4	laminin, alpha 4	3910	2,13	4,03	-0,29	Hs00158588_m1
CDC42EP4	CDC42 effector protein (Rho GTPase binding) 4	23580	-1,54	-0,2	-0,29	Hs00201664_m1
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1051	-1,58	-0,2	-0,37	Hs00270923_s1
TPO	thyroid peroxidase	7173	-0,93	0	-0,43	Hs00174927_m1
DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	1756	-1,41	1,93	-0,49	Hs01049436_m1
FXR1	fragile X mental retardation, autosomal homolog 1	8087	-1,03	1,11	-0,64	Hs01096865_m1
DUOX2	dual oxidase 2	50506	2,46	3,99	-0,66	Hs00204187_m1
CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	595	0,96	2,55	-0,86	Hs00277039_m1
DIO1	deiodinase, iodothyronine, type I	1733	2,71	5,1	-0,89	Hs00174944_m1
DIO2	deiodinase, iodothyronine, type II	1734	2,03	3,85	-0,89	Hs00255341_m1
CLDN5	claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	7122	-2,44	0,06	-0,92	Hs01561351_m1
FGF12	fibroblast growth factor 12	2257	2,19	3,37	-1,03	Hs00374427_m1
CDH16	cadherin 16, KSP-cadherin	1014	-1,81	0,81	-1,43	Hs00187880_m1
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	1000	-0,86	-0,16	-2,0	Hs00169953_m1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	2354	5,48	5,61	-2,73	Hs00171851_m1
PAX2	paired box gene 2	5076	-0,92	1,78	-3,47	Hs01057416_m1
MYL2	myosin, light polypeptide 2, regulatory, cardiac, slow	4633	-2,78	0,26	-4,32	Hs00166405_m1

Table S5: Thyroid specific CNVs found in ectopic tissues.

chromosome	start	end	length (bps)	classification	genes (Entrez Gene ID)	Validation with qPCR: case (number of copies)	Assay ID		
1p36.32	3419310	3504625	85316	common CNV	MEGF6 (1953)	case 1 (3)	Hs02299905_cn	Hs03346798_cn	Hs03344891_cn
4p16.3	1696020	1781125	85106	common CNV	FGFR3 (2261); TACC3 (10460)	case 1 (4); case 3 (3)	Hs00091802_cn	Hs03518017_cn	
7q11.23	74616550	74711750	95201	common CNV	LOC750805 similar to PMS2L14 (750805); WBSCR16 (81554); GATSL2(729438)	ø	*	*	*
12q24.33	131582000	131678000	96001	common CNV	AUTS2L (304419); FBRSL1(57666)	case 1 (3); case 3 (3)	Hs03817872_cn	Hs03816047_cn	Hs03835002_cn

*Custom made qPCR for 7q11.23 are described in the table below.

Assay ID	Forward Primer Seq.	Reverse Primer Seq.	Probe
chr7_CCWRDIS	ATGTCCCCCTACTCAGTTCTCA	CTCCGAGGTCCCCAAAGATTG	CTCCGAGGTCCCCAAAGATTG
ch7-2_CCY89U8	GGCCCGGACCCTTCCTA	GTACCAGCTCATGGCAGTCT	GTACCAGCTCATGGCAGTCT
ch7-3_CC7ZW2S	GCACCAGCCTGGAAGGA	CAAACTCCCTCATTCCAGACAGAA	CAAACTCCCTCATTCCAGACAGAA

2. Evidence for Calcitonin-Producing Cells in Human Lingual Thyroids.

Isabelle Vandernoot*[1,3], Hervé Sartelet*[1], **Rasha Abu-Khudir[1,4]**, Jean-Pierre Chanoine[2], Johnny Deladoëy[1,4]

* These authors contributed equally to this work.

[1] Research Center of Sainte-Justine Hospital, Department of Pediatrics, University of Montreal, Montreal, Quebec H3T 1C5, Canada.

[2] Endocrinology and Diabetes Unit, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

[3] Institute of Interdisciplinary Research in Molecular Human Biology, Université Libre de Bruxelles, B-1070 Brussels, Belgium.

[4] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

All correspondence and requests for reprints should be addressed to:

Johnny Deladoëy, M.D. Ph.D.
Centre Hospitalier Universitaire Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

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Contributions of authors:

Isabelle Vandernoot: Designed and performed the experiments as well as wrote the first draft of the manuscript.

Hervé Sartelet: Designed and performed the experiments as well as wrote the first draft of the manuscript.

Rasha Abu-Khudir: Contributed by technical expertise and assistance to Isabelle Vandernoot and participated in the active discussion and correction of the manuscript.

Jean-Pierre Chanoine: Contributed by samples and participated in the correction of the manuscript

Johnny Deladoëy: Supervised, participated in the design of the experiments and in active discussion, and corrected the manuscript.

Evidence for Calcitonin-Producing Cells in Human Lingual Thyroids

Isabelle Vandernoot*[1,3], Hervé Sartelet*[1], **Rasha Abu-Khudir[1,4]**, Jean-Pierre Chanoine[2], Johnny Deladoëy[1,4]

* These authors contributed equally to this work.

[1] Research Center of Sainte-Justine Hospital, Department of Pediatrics, University of Montreal, Montreal, Quebec H3T 1C5, Canada.

[2] Endocrinology and Diabetes Unit, Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

[3] Institute of Interdisciplinary Research in Molecular Human Biology, Université Libre de Bruxelles, B-1070 Brussels, Belgium.

[4] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

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All correspondence and requests for reprints should be addressed to:

Johnny Deladoëy, M.D. Ph.D.
Centre Hospitalier Universitaire Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

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Précis: Evidence for calcitonin-producing cells in ectopic lingual thyroids challenges the concept of ultimobranchial bodies as unique source of calcitonin-producing cells in human thyroids. (25 words)

Abstract

Context: The thyroid contains two types of cells, the thyroid follicular cells and the calcitonin-producing cells. The site of origin of the thyroid follicular cells is the median thyroid anlage, an endothelial diverticulum in the midline of the ventral pharynx between the first and the second pharyngeal pouches. The ultimobranchial bodies (UBB), a pair of transient embryonic structures evaginated from the fourth pharyngeal pouch and located symmetrically on the sides of the developing neck, are the source of calcitonin-producing cells. In human embryos, the thyroid bud starts its migration at embryonic day 24 (E24) and reaches its final location in front of the trachea at E45-50. The UBB fuse with the primitive thyroid when thyroid migration is completed. Lingual thyroids result from the failure of the thyroid precursor cells to migrate from the primordial pharynx to the anterior part of the neck. Therefore, calcitonin-producing cells are not expected to be present in lingual thyroids.

Objectives: Our objective was to determine whether calcitonin-producing C cells are present in ectopic lingual thyroids.

Design, Setting, Patients, and Main Outcome Measure: We performed calcitonin immunolabeling and transcript detection on four flash-frozen ectopic lingual thyroids. Additional calcitonin immunolabeling was performed on two other paraffin-embedded ectopic lingual thyroids.

Results: We report evidence of calcitonin-producing cells in six independent cases of ectopic lingual thyroids.

Conclusion: The UBB are not the only source of calcitonin-producing cells in humans. Interactions between calcitonin-producing and thyroid follicular cells occur earlier than previously accepted.

245 words

Introduction

Congenital hypothyroidism is the most frequent endocrine disorder in newborns, with a birth prevalence of 1 case per 2,500 live births (1). It is caused by defects in thyroid migration that lead to lingual or sublingual ectopy, in about 50% of the cases (1, 2). Thyroid lingual ectopy results from a failure of the thyroid precursor cells to migrate from their origin in the primordial pharynx to their correct final anatomical location at the anterior part of the neck.

Thyroid hormones and calcitonin are produced in the thyroid gland by two distinct cell types, the thyroid follicular cells (TFCs) and the parafollicular or C cells, respectively. These two cell types originate from two different embryonic structures. The site of origin of the TFCs is the median thyroid anlage, an endothelial diverticulum in the midline of the ventral pharynx between the first and the second pharyngeal pouches. The ultimobranchial bodies (UBB), a pair of transient embryonic structures evaginated from the fourth pharyngeal pouch and located symmetrically on the sides of the developing neck, are considered to be the only source of the C cells (3). The migration of the UBB, from their primitive origin in the neural crest to their final location in the thyroid gland, was carefully studied in the chicken embryos in the 1970s (4). In humans, the thyroid bud starts its migration at embryonic day 24 (E24), to reach its final location in front of the trachea at E45-50 (2). While the human thyroid continues to grow and expand laterally, the UBB fuse with the primitive thyroid at around E60 (2). Considering the initial localization and migration path of the UBB, C cells are not expected to be present in lingual ectopic thyroids.

In a genome-wide gene expression study of ectopic thyroid tissue (Gene Expression Omnibus, accession number GSE16804) (5), we observed no differences in calcitonin expression levels between ectopic and normal (orthotopic) thyroids. This suggests that calcitonin is also expressed in ectopic lingual thyroids, which contradicts current knowledge of the embryologic development of the thyroid. Moreover, no calcitonin was detected by immunohistochemical staining in the few histological studies previously performed on non-tumoral ectopic thyroids in cats and dogs(6, 7). In contrast, a case of a sporadic form of cancer arising from C cells (i.e. medullary carcinoma of the thyroid) was observed in the lingual thyroid of a 45-year-old woman (8). All the tumor cells were positive for calcitonin, coexisting with a few TFCs. No systematic search for C cells has been performed in non-tumoral ectopic thyroids. The purpose of this study

was therefore to determine whether calcitonin-producing C cells are, as our genome-wide gene expression study suggested, present in ectopic lingual thyroids.

Material and Methods

Ethics Statement

This study was approved by the Ethics Committee of the CHU Sainte-Justine. The patients or the parents of the patients from whom samples were taken gave written informed consent.

Characteristics of the participants and tissue collection

We obtained flash-frozen samples of ectopic lingual thyroid removed from four girls aged 9.5, 14, 9 and 14 yr (lingual thyroids number 1 to 4), and two additional paraffin-embedded ectopic lingual thyroids removed from one boy aged 8 yr and one woman aged 26 yr (lingual thyroids number 5 and 6); in all these cases, surgery was performed because the ectopic lingual thyroid caused local symptoms (e.g. dysphagia) (5, 9). As positive control for the RT-PCR, we used tissue (obtained from near-total thyroidectomy) adjacent to papillary thyroid carcinomas from 3 girls aged 12, 14 and 16 yr. The non-tumoral nature of the healthy tissues used as controls was confirmed by histology. The fact that 5 of 6 lingual thyroid were obtained from females is consistent with the known female predominance of ectopic thyroid (1). As negative controls, we used RNA from the WRO thyroid cell line (follicular tumoral cells) and RNA from the leucocytes of a healthy adult male.

Immunohistochemical analysis

Immunohistochemistry was performed on paraffin-embedded sections using Ultraview Universal DAB detection kit (Ventana, Ventana medical system, Tuscon, AR). Antibodies against calcitonin (1/1000, rabbit polyclonal, Dako, Glostrup, Denmark) were applied for 32 min. This was followed by application of Ultraview Universal DAB detection kit reagents in accordance with manufacturer’s instructions. Alkaline phosphatase was used as a chromogen and hematoxylin was used as a counterstain. Normal rabbit IgG at the same concentration as the primary antibody served as negative control. A thyroid sample with C cell hyperplasia, obtained at prophylactic thyroidectomy from a 6 yr old boy carrier of the C634Y RET mutation, was used as positive control.

RNA extraction and reverse-transcriptase PCR

Calcitonin gene (*CALCA,* NCBI reference sequence NC_000011.9) mRNA expression was examined by reverse transcriptase polymerase chain reaction (RT-PCR) in four ectopic lingual thyroids, three orthotopic thyroids (flash-frozen healthy tissues adjacent to papillary thyroid carcinomas) and in negative controls (leucocytes and WRO cell line). Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN Inc., Ontario, Canada) according to the manufacturer's recommendations. RNA was DNase-treated to minimize DNA contamination and quantified using ND-1000 (Nanodrop, Wilmington, DE, USA). Performing electropherograms on the Agilent 2100 Bioanalyzer assessed RNA quality. Reverse-transcriptase reactions were performed with 500 ng of RNA, using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystem). The *CALCA* gene is transcribed into two different RNAs, one coding for calcitonin and katacalcin (the precursor protein of which is differentially cleaved) and another for alpha calcitonin gene-related peptide (CGRP). Therefore, RT-PCR was performed with specific primers for calcitonin/katacalcin cDNA, which do not amplify alpha CGRP cDNA. Thirty-five cycles of PCR were necessary to detect calcitonin transcripts even in normal thyroids, consistent with the fact that C cells are only a minute portion of the thyroid cellular mass. The ubiquitous gamma actin gene (*ACTG1*, NCBI reference sequence NM_001614.2) was used as a positive control to ensure the efficiencies of the RNA extraction, retro-transcriptase reaction and cDNA amplification. Primers sequences and RT-PCR protocols are available upon request.

Results

Immunolabeling of calcitonin-producing cells in human lingual thyroids.

To assess whether calcitonin-producing cells are present in human lingual thyroids, we performed immunostaining for calcitonin on samples from six human ectopic lingual thyroid specimens (5). All six samples showed a positive staining for calcitonin (Figure 1). The putative C cells identified were isolated and were located in both inter- and intrafollicular areas.

Detection of calcitonin transcript in human lingual thyroids.

Calcitonin gene (*CALCA*) transcripts were detectable through RT-PCR on samples from the four flash-frozen ectopic thyroid tissues (samples 1 to 4) (Figure 2). Indeed, low RNA quality from paraffin-embedded ectopic thyroids (samples 5 and 6) precluded any molecular analysis.

CALCA cDNA was detected in all analyzed thyroid samples, although in lower amounts (as shown by signal intensities) than in the three orthotopic thyroid tissues. However, considering the random nature of tissue sampling, which often results in variations in the number of counted C cells, an absolute quantitative comparison was not possible.

Discussion

Finding calcitonin-producing cells in ectopic lingual thyroid samples challenges the current view of thyroid development. This observation is indirectly supported by previous findings such as (i) the detection of a medullary carcinoma in a lingual thyroid (8), (ii) the equivalent calcitonin expression level in the comparative gene expression study between the ectopic and orthotopic thyroid tissue (5) and (iii) the presence of low (but detectable) circulating calcitonin levels in patients suffering from congenital hypothyroidism due to thyroid dysgenesis (10, 11).

Origins of C cells in ectopic lingual thyroid

Three possible explanations may account for this finding. First, UBB may aberrantly migrate towards the base of the tongue and join the primitive thyroid. However, there is no literature supporting aberrant UBB migration in developing thyroids and the systematic recurrence of aberrant migration in six independent ectopic thyroids seems unlikely. Second, some TFCs could differentiate towards heterotopic C cells, i.e. undergo transdifferentiation, in which a non-stem cell is transformed into a different cell type. One of the best examples of transdifferentiation is Barrett metaplasia, an abnormal change of the cells of the lower esophagus into intestinal mucin-secreting cells, caused by damage from chronic stomach acid exposure (12). Transdifferentiation of various cell types towards C cells is observed either in tumoral or in non-tumoral tissue. Some tumor cells can switch their differentiation program and acquire some of the properties of C cells (i.e. the secretion of calcitonin), as in cancers of the lung, breast or adrenal medulla(13). The recognition that a small group of primary tumors may undergo both C cell and TFC differentiation has given rise to speculation as to whether C cells are solely derived from neural crest or whether some are of follicular origin (14). Struma ovarii (i.e. the presence of thyroid tissue as major component of an ovarian tumor) can also be explained by a transdifferentiation phenomenon. There are also reported cases of non-tumoral ectopic thyroid masses in heart, lung or even in infra-diaphragmatic sites, such as the intestine. In one such

duodenal ectopic thyroid mass, staining for calcitonin was negative (15). These ectopic thyroids are usually observed in patients who have an orthotopic thyroid gland and, considering their anatomic position, arise more likely also from cell transdifferentiation: an aberrant migration of the thyroid precursors so far from their origin is not plausible. Finally, the third possible explanation is that the *in situ* differentiation of pluripotent stem cells gives rise to both C cells or TFCs in ectopic thyroids. Conversely, it has been suggested that UBB cells could differentiate into TFCs (16). Some authors maintain that most of the follicular content of the thyroid originates from the median anlage but the "lateral thyroid", corresponding to 1-30% of the gland total weight, is derived from UBB (17). Moreover, Williams *et al.* showed, in cases of thyroid ectopy, cystic structures in the region of the upper parathyroids which contained both TFC and C cells, suggesting that these intracystic follicular cells are derived from the UBB (18).

Developmental and marker gene expression in TFCs and C cells

There is evidence for an interconnection between the embryologic development of the primitive TFCs and of the UBB-derived cells. The two endocrine cell types have common markers, such as *FOXE1/TITF2, NKX2-1//TITF1* and *HES1,* which are expressed in both the median thyroid anlage and lateral UBB from the beginning of their specification, throughout development and in adult tissues (19, 20). *FOXE1* is expressed in the UBB but its absence in *Foxe1* -/- mice does not affect UBB development and function (21). *NKX2-1* is mandatory for UBB survival (20) and, in *Nkx2-1* haploinsufficiency, the UBB does not fuse with the thyroid diverticulum and C cells persist in clusters around the vesicular structures, as observed previously in *Nkx2-1 -/-* mice (20). In mature C cells , *NKX2-1* can be modulated and it coordinately regulates genes involved in calcium-homeostasis (22). *Hes1*, a gene notably involved in the development of endoderm-derived endocrine organs, such as the pancreas, has recently been shown to play a role in thyroid development. *Hes1* knockout mouse embryos had a significantly lower number of *Nkx2-1* positive progenitor cells, showed a delayed fusion of the UBB with the primitive thyroid and had decreased production of both thyroxin and calcitonin (19). Moreover, interactions between UBB and median precursor cells seem to be necessary for the last steps of thyroid organogenesis. For example, the invalidation of *Eya1* in mice resulted in two concomitant phenotypes: thyroid hypoplasia and a severe alteration in the migration of the UBB, which was accompanied by lack of fusion between these structures and the thyroid lobe. In addition, the primordial organ for both the thymus and parathyroid glands failed to form. Since

Eya1 is not expressed in the thyroid diverticulum, it is possible that the TFC defect arises because the UBB fail to fuse with the thyroid lobes (23). *Hoxa3* null mice (24) and mice carrying mutations in the *Pax3* (25) or *Endothelin-1* (26) genes show defects similar to those observed in mice deprived of *Eya1*. Another study demonstrated the variable penetrance of the phenotype in mice carrying various combinations of mutant in *Hoxa3* and its paralogs *Hoxb3* and *Hoxd3*, with a more marked thyroid defect that was associated with increased UBB migration failure (27). Other situations are however described where the absence of UBB formation seems to be not associated with a structural thyroid anomaly, as in *Pax9* defective mice (28). These interactions between the UBB and the thyroid diverticulum, which seem to be critical for correct morphogenesis, are a unique feature of the mammalian thyroid: in chicken and fish, the UBB remain a bilateral structure that does not merge with the thyroid diverticulum (29).

Functional links between TFCs and C cells

In addition to their embryologic links, TFCs and C cells also seem to show functional interconnections. The anatomic location of the C cells within the basement membrane of the thyroid follicles renders a paracrine interrelationship with the follicular epithelial cells plausible and also potentially exposes the C cells to high concentrations of thyroid hormones, their precursors and metabolites (30). C cell activity seems to be closely related to the state of TFCs, as shown by the alteration of C cells activity in rats with experimental models of hypo and hyperthyroidism (31). In addition to their calciotrophic function, which is related to calcitonin, C cells produce and secrete a number of regulatory peptides. Some of these act as local stimulators of thyroid function (serotonin, gastrin related peptide [GRP], helodermin), whereas others are inhibitors (somatostatin, thyrotropin-releasing hormone [TRH], calcitonin-gene related peptide [CGRP]) (32).

In summary, we report evidence of calcitonin-producing cells in six independent cases of ectopic lingual thyroids. This suggests that the ultimobranchial bodies are not the only source of calcitonin-producing cells in humans. Interactions between calcitonin-producing and thyroid follicular cells occur in an earlier stage of embryonic development than previously accepted.

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Figure legends:

Figure 1: Immunolabeling of calcitonin-producing cells in six independent human ectopic lingual thyroids (arrowheads in C-H); negative immunolabeling (normal IgG instead of primary antibody) in a normal thyroid (A); positive immunolabeling of calcitonin-producing cells in a thyroid with C cell hyperplasia (arrowheads in B). Scale bars, 20 µm.

Figure 2: Detection of calcitonin transcript in orthotopic and in ectopic lingual thyroids: lower signal intensities are observed in ectopic thyroids when compared to the orthotopic thyroids.

Figures:

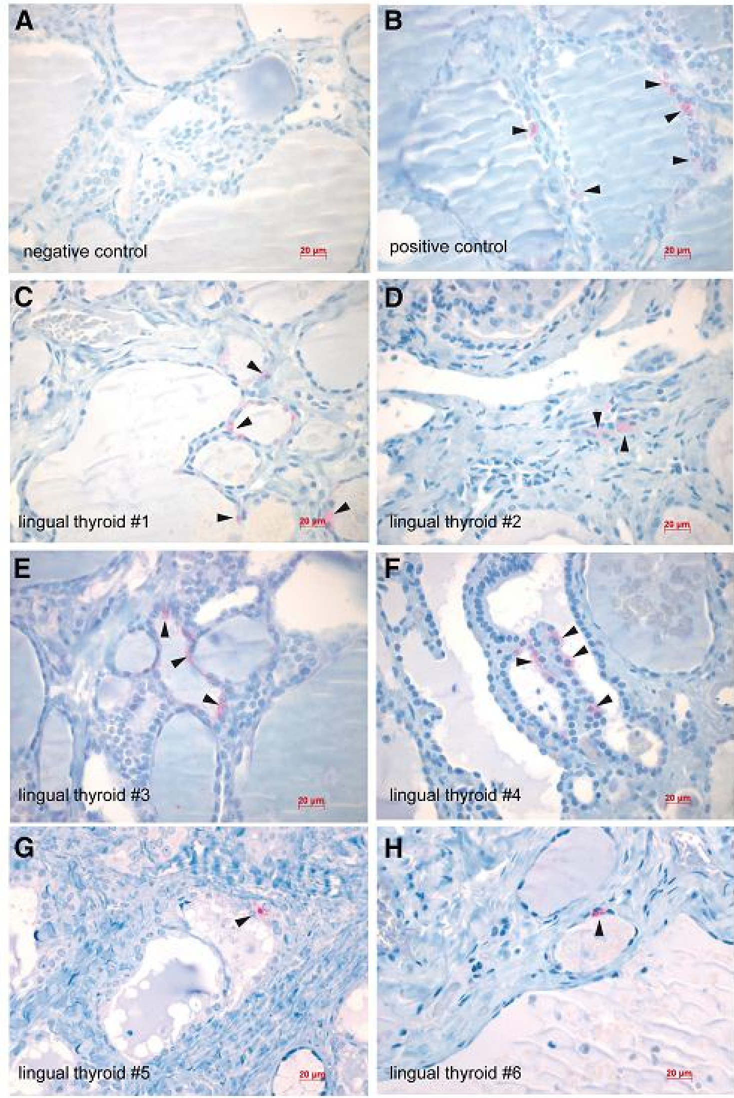

Figure 1

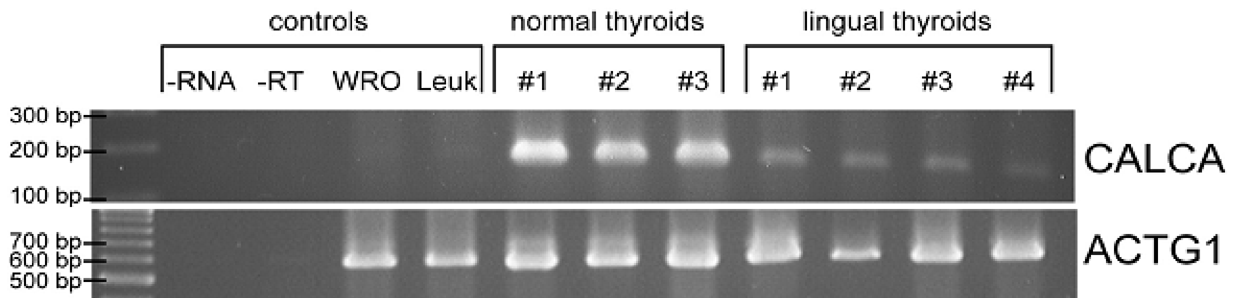

Figure 2

3. Role for tissue-dependent methylation differences in the expression of FOXE1 in non-tumoral thyroid glands

Rasha Abu-Khudir[1,2], Fabien Magne[1], Jean-Pierre Chanoine[3], Cheri Deal[1], Guy Van Vliet[1], Johnny Deladoëy[1,2].

[1] Endocrinology Service and Research Center, Centre Hospitalier Universitaire Sainte-Justine, Department of Pediatrics, University of Montreal, Montreal H3T 1C5, Canada.

[2] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

[3] Endocrinology and Diabetes Unit, Department of Pediatrics, British Columbia Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

Address all correspondence and requests for reprints to:

Johnny Deladoëy, M.D. Ph.D.
Centre Hospitalier Universitaire Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

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Contributions of authors:

Rasha Abu-Khudir: Designed and performed the experiments, analyzed the results, wrote the first draft of the manuscript and took care of the corrections that ensued.

Fabien Magne: Participated in active discussions and participated in the correction of the manuscript.

Jean-Pierre Chanoine: Contributed by samples and participated in the correction of the manuscript.

Cheri Deal: Supervised, participated in active discussion and in the correction of manuscript.

Guy Van Vlie: Participated in active discussion and in the correction of manuscript.

Johnny Deladoëy: Supervised, participated in the design of the experiments and in active discussion, and corrected the manuscript.

Role for tissue-dependent methylation differences in the expression of FOXE1 in non-tumoral thyroid glands

Rasha Abu-Khudir[1,2], Fabien Magne[1], Jean-Pierre Chanoine[3], Cheri Deal[1], Guy Van Vliet[1], Johnny Deladoëy[1,2].

[1] Endocrinology Service and Research Center, Centre Hospitalier Universitaire Sainte-Justine, Department of Pediatrics, University of Montreal, Montreal H3T 1C5, Canada.

[2] Department of Biochemistry, University of Montreal, Montreal H3C 3J7, Canada.

[3] Endocrinology and Diabetes Unit, Department of Pediatrics, British Columbia Children's Hospital, University of British Columbia, Vancouver V6H 3V4, Canada.

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Address all correspondence and requests for reprints to:

Johnny Deladoëy, M.D. Ph.D.
Centre Hospitalier Universitaire Sainte-Justine
3175 Côte Sainte-Catherine, Montréal QC, H3T 1C5, Canada
phone: 514-345-4735; fax: 514-345-4988

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Abstract

Background: Discordance of monozygotic twins for thyroid dysgenesis suggests that epigenetic mechanisms may underlie defects in thyroid gland development. This prompted us to evaluate whether differentially methylated regions (DMR) can be found between human thyroids (either eutopic or ectopic) and matched leukocytes.

Methods: To compare the genome-wide methylation profile of thyroids and leukocytes, immunoprecipated methylated DNA was interrogated on human promoter plus CpG island tiling arrays. In addition, the methylation profile of the human *FOXE1, PAX8* and *NKX2.1* promoter was examined using bisulfite sequencing. Finally, the functional impact of CpG methylation of the promoter on *FOXE1* expression was assessed with luciferase assays.

Results: Genome-wide methylation profiling and bisulfite sequencing of CpG islands of *PAX8* and *NKX2.1* promoters revealed no DMR between thyroid and leukocytes. However, bisulfite sequencing revealed that the methylation level of two consecutive CpG dinucleotides (CpG_{14} and CpG_{15}, which were not covered by the genome-wide array) in one CpG island of the *FOXE1* promoter (-1600 to -1140 from the transcription start site) is significantly higher in leukocytes than in eutopic or ectopic thyroid tissues, suggesting that methylation of this region may decrease *FOXE1* gene expression. Indeed, luciferase activities were decreased when *FOXE1* promoter constructs were methylated *in vitro*. Moreover, derepression of luciferase activity was observed when methylation of CpG_{14} and CpG_{15} was prevented by mutations.

Conclusion: We report a tissue-dependent DMR in the *FOXE1* promoter. This DMR contains two consecutive CpG dinucleotides which are epigenetic modifiers of *FOXE1* expression in non-tumoral tissues.

244 words

Introduction

The transcription factor forkhead box E1 (FOXE1) is a member of the forkhead/winged-helix family and plays an essential role in thyroid morphogenesis (1, 2). In humans, FOXE1 mutations have been identified in a few syndromic cases of athyreosis associated with spiky hair, cleft palate, sometimes with choanal atresia and bifid epiglottis (3-5). Animal studies have pointed to the critical role of Foxe1 in the embryonic migration of the thyroid. Homozygous

Foxe1 knockout mice have either ectopic or absent thyroid gland at embryonic day (E)11.5 while the thyroid is completely absent at birth in all (2). In addition, evidence suggests that the migration of the thyroid bud is a cell-autonomous event requiring the Pax8-dependent expression of Foxe1 in the migrating thyroid cells of mouse embryos (6). In humans, failure of the thyroid precursor cells to migrate from their origin in the primordial pharynx to their final anatomical location (the anterior part of the neck) results in thyroid ectopy (lingual or sub-lingual) while complete absence of the thyroid (athyreosis) may result either from lack of differentiation or from disappearance of the thyroid before birth. Ectopy and athyreosis are generally grouped under the term thyroid dysgenesis (TD), which is the commonest cause of congenital hypothyroidism (CH). The incidence of CH due to TD (CHTD) is 1 in 4,000 live births (7). Germline mutations in thyroid-related transcription factors NKX2.1 (8, 9), FOXE1 (4), PAX8 (10) and NKX2.5 (11) have been identified by candidate gene screening in a small subset (3%) of patients with sporadic CHTD (12). Linkage analysis has excluded these genes in rare multiplex families with CHTD (13). Indeed, CHTD is predominantly not inherited (98% of cases are non-familial (14)), has a high discordance rate of 92% in monozygotic (MZ) twins and a female and ethnic (i.e., Caucasian) predominance (15, 16). This, together with evidence of non-penetrance of mutations in close relatives of patients (e.g. NKX2.5 (11)), suggests that modifiers, possibly additional somatic epigenetic or genetic events, are associated with CHTD. CpG island hypermethylation is reported in many cancers and some DMRs in cancers are found in regions that are also differentially methylated among different non-tumoral tissues (17). Consequently, as FOXE1 CpG islands are known to be hypermethylated in cancers of the skin, pancreas and breast (18-20), it is plausible that FOXE1 DMR would account for differential FOXE1 expression in non-tumoral tissues. However, our recent integrative molecular analysis of ectopic thyroids did not find any alteration of genomic structure and methylation profile when compared to eutopic thyroids even though the expression profile differed (21). To find no DMR in a single tissue (thyroid) differing only in its location (ectopic versus eutopic) may be expected but DMR are likely to exist between thyroid and leukocytes, two tissues with different expression profiles. Moreover, CpGs and DMR are genetic and epigenetic mutational hotspots (22, 23). Thus, finding a DMR within the upstream regulatory region of thyroid-related transcription factors might pave the way for further studies in which cases with CHTD are screened for genetic variants within this DMR. Therefore, in the present study, we set out to determine whether

promoter methylation profile was different between thyroid and leukocytes using genome-wide and candidate gene approaches (i.e., FOXE1, PAX8 and NKX2.1).

Methods

Detailed descriptions of all experimental protocols are available in Supplemental Data.

Participant characteristics and tissue collection

We obtained three ectopic and four eutopic thyroids as described previously (21). Because the methylomes of ectopic and eutopic thyroids are similar (21), they were treated as one group for the present analysis. For controls, we used matched leukocytes (when available) from the above-mentioned cases and additional leukocytes from normal subjects (supplemental Table 1). This study was approved by the Ethics Committee of the CHU Sainte-Justine. All the parents and participants gave written informed consent.

Nucleic acid isolation

Genomic DNA and/or total cellular RNA were isolated from cell lines, thyroid tissues and matched leukocytes using pureLink genomic DNA Mini kit (Life Technologies, Burlington, Canada) and RNeasy Mini kit (Qiagen, Mississauga, Canada), respectively.

Methylation Profiling by Methylated DNA Immunoprecipitation (MeDIP) and MeDIP-chip

The MeDIP-chip was performed using pairs of enriched methylated fraction (IP) and normal fraction (IN) of genomic DNA from four thyroids and five leukocytes (four matched; see supplemental Table 1). The methylated fraction of genomic DNA was enriched using the methylated DNA immunoprecipitation (MeDIP) assay (24) and interrogated on human Promoter plus CpG Island Tiling Arrays (Roche NimbleGen, Madison, Wisconsin) as described previously (21).

Cell culture

The human thyroid cancer cell line WRO was a gift from Dr. H. Mircescu (University of Montreal), the human leukemia cell lines Jurkat (T-cell acute lymphoblastic leukemia), K562 (chronic myelogenous leukemia), and REH (acute lymphoblastic leukemia of the non-T, non-B type) were a gift from Dr. A. Ahmad (University of Montreal), the rat follicular thyroid PCCL3 cell line was a gift from Dr. F. Miot (Université Libre de Bruxelles, Institut de Recherche

Interdisciplinaire en Biologie Humaine et Moléculaire, Brussels, Belgium) and the immortalized human thyroid Nthy-ori 3-1 cells were obtained from Sigma (St. Louis, Missouri). Culture media and conditions are explained in the Supplemental Data.

Bisulfite genomic DNA sequencing and methylation analysis

Bisulfite treatment of genomic DNA was performed using the Zymo EZ DNA Methylation-Gold kit (Zymo Research, Irvine, California). The targeted promoter region of *FOXE1* (−1600 to −125 from transcription start site (TSS)), *PAX8* (−635 to −471 from ATG) and *NKX2.1* (−343 to −107 from ATG) were subsequently amplified with a nested PCR protocol in which two sets of forward and reverse primers were used. Primers designed using the MethPrimer software (www.urogene.org/methprimer/), reagents and PCR conditions are listed in the Supplemental Table 2.

Construction of luciferase reporter vectors

A pGL3-Basic plasmid incorporating a 2.38-Kb fragment that includes the 5′-upstream regulatory region of the human *FOXE1* (hFOXE1) gene from −1934 to +446 relative to the transcriptional start site (+1) was a generous gift from Dr. T. Eichberger (University of Salzburg, Salzburg, Austria). A deletion construct missing CpG island 1 was generated by restriction enzyme digestion using *Afl*II/*Nsi*I followed by blunt-end ligation. The two CpGs 14 and 15 within CpG island 1 located at −1417 and −1412 relative to TSS were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California). Primers are reported in the Supplemental Data.

In vitro global methylation assay

For *in vitro* methylation of pGL3-Basic and the different human *FOXE1* promoter constructs, whole plasmids were methylated using the site-specific CpG Methyltransferase M.*Sss*I (New England Biolabs, Whitby, Canada). Mock-methylated plasmids were subjected to the same treatment in the absence of M.*Sss*I. Following methylation, the plasmids were purified using the EZ-10 spin column DNA Gel Extraction Kit (Bio Basic, Markham, Canada). The extent of methylation was subsequently verified by digestion with the CpG methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs) and its isoschizomer the CpG methylation-insensitive *Msp*I (Thermo Scientific, Burlington, Canada). Only completely methylated

(M.*Sss*I+) and mock-methylated (M.*Sss*I–) plasmids were then used in the transfection experiments.

In vitro regional (patch) methylation assay

To allow the integration of methylated fragment of CpG island 1 in unmethylated plasmids, *Nde*I and *Nsi*I restriction sites were engineered into both the wild type and point mutated *FOXE1* promoter fragments using the QuikChange site-directed mutagenesis kit (Stratagene). The efficiency of ligation and equivalence of incorporated DNA into the methylated and mock-methylated constructs were confirmed by agarose gel electrophoresis as published elsewhere (25). In addition, the ligation reactions were transformed into chemically competent E. Coli to assess the ligation efficiency. To obtain optimal readouts, luciferase assays were performed in the immortalized human thyroid Nthy-ori 3-1 cells. See supplemental Figure 1 and detailed protocol in the Supplemental Data.

Transient transfection and luciferase reporter gene assay

Rat follicular thyroid PCCL3 cells were transiently transfected with polyethylenimine (PEI; Polysciences, Warrington, Pennsylvania) in triplicate in 24-well tissue culture plates (Corning, Corning, New York). The volume of PEI used is based on a 4:1 ratio of PEI (µg): total plasmid DNA (µg). Cells were seeded at a density of 0.3×10^5 cells/well 48 h prior to transfection. Cells were transfected with 0.32 g/well of the methylated or mock-methylated reporter vector pGL3-Basic (Promega, Madison, Wisconsin) and as well as the reporter plasmids including the different *FOXE1* promoter inserts. To correct for transfection efficiency and variation in cell viability between wells, cells were co-transfected with 0.005 g/well *Renilla* luciferase reporter vector (pRL-TK; Promega) as an internal control. The activities of the firefly luciferase and the *Renilla* luciferase were measured in cell lysates 24 h after transfection using the dual-luciferase reporter assay system (Promega). Luminescence was detected by the 2104 EnVision Multilabel Plate Reader (PerkinElmer, Waltham, Massashussets). For regional methylation, each of the religated methylated and mock methylated wild type and point mutated plasmids (0.75 µg DNA) were transfected in Nthy-ori 3-1 cells ($0.1x\ 10^5$ cells/well), plated 48 hrs before transfection, using X-tremeGENE 9 (Roche Diagnostics, Laval, Canada). In each experiment performed in triplicate, the pRL-TK plasmid (0.25 µg) was cotransfected for

normalization purposes. Luminescence was measured 48 h after transfection using the dual-luciferase reporter assay system (Promega).

Semiquantitative RT-PCR

The forkhead box E1 gene (*FOXE1,* NCBI reference sequence NT_008470.19) mRNA expression was examined by semiquantitative reverse transcriptase PCR (RT-PCR) in two ectopic lingual thyroids, three orthotopic thyroids (flash-frozen healthy tissue adjacent to papillary thyroid carcinomas) and leukocytes from normal subjects. In addition, *FOXE1* mRNA expression was determined in the thyroid cancer WRO cell line and the human leukemia cell lines Jurkat, K562, and REH. Primers' sequences are reported in the Supplemental Data.

Statistical analysis

All data are reported as means ± SEM. The data were subjected to Fisher's exact test or unpaired two-tailed Student's *t*-test, with correction for multiple comparisons with the Holm-Sidak method, when appropriate. A P-value of 0.05 or lower was considered statistically significant.

Results

Lack of tissue-specific methylation differences in CpG islands of PAX8 and NKX2.1 promoter

No methylation profile differences were observed in the CpG-rich region of the *PAX8* (−635 to −471 from ATG) and *NKX2.1* (−343 to −107 from ATG) promoters using either MeDIP arrays or bisulfite sequencing (Supplemental Figure 2).

A CpG island of the FOXE1 promoter is differentially methylated in human thyroid tissue when compared to matched leukocyte DNA

To determine whether the CpG rich 5′-flanking region of the *FOXE1* gene (−1600 to −125 from transcription start site) show differences in methylation between tissues with different levels of *FOXE1* expression, we used bisulfite sequencing of five thyroids, four of which were paired with leukocytes from the same donors. Findings were validated with bisulfite pyrosequencing on four thyroids and leukocyte tissues (three pairs) (Supplemental Table 1). Bisulfite sequencing revealed the presence of a DMR at CpG island 1 (−1600 to −1140 from transcription start site, +1) that was globally more methylated in leukocytes (10%) compared to

thyroids (3%, $P<0.001$, two-sided exact Fischer's test). Indeed, we found two consecutive CpG dinucleotides (i.e. CpG_{14} and CpG_{15}, located −1417 and −1412 relative to transcription start site (+1)) with a significantly higher methylation rate in leukocytes (51% for CpG_{14} and 43% for CpG_{15}) when compared to that in thyroids (i.e., 5%; $P<0.001$, two-sided exact Fischer's test) (Figure 2). These results were validated with bisulfite pyrosequencing, which showed an average methylation rate of 24% for GpC_{14} and 22% for GpC_{15} in leukocytes whereas the thyroid methylation rate was 12% for GpC_{14} and 11% for GpC_{15} ($P<0.05$, two-sided paired *t*-test, corrected for multiple comparison with Holm-Sidak method) (Figure 3). The other CpG island (CpG island 2, see Figure 1) encompassing the transcription start site of *FOXE1* promoter showed no differential methylation (see supplemental Figure 3) and no differences were observed at methylation-sensitive restriction enzyme sites close to the ATG (not shown). Of note, the dinucleotide stretches CpG_7-GpC_9 and CpG_{14}-GpC_{17} were not covered by the probes of the CpG Island Tiling Array, which explains why these subtle differences were not detected by this technology.

The methylation status of this DMR was also analyzed in bisulfite-treated DNA from the human leukemia cell lines (Jurkat, K562, and REH) as well as from the thyroid cancer cell line WRO. The human leukemia cell lines Jurkat and REH exhibited a high percentage of DMR methylation (51% and 86%, respectively), while a moderate percentage of methylation (15%) was detected in K562 cells. On the other hand, the DMR in the thyroid cancer WRO cells was almost unmethylated (4%, $p<0.001$), which represented a typical and significant decrease of methylation when compared to each of the leukemia cell lines (Figure 2).

No expression of FOXE1 was detected in normal leukocytes and human leukemia cell lines

Since altering DNA methylation in CpG islands is an essential mechanism involved in the regulation of gene expression, we examined whether the methylation pattern of the detected DMR in thyroids and leukocytes is implicated in the transcriptional status of *FOXE1*. Interestingly, lack of *FOXE1* expression was observed in leukocytes that exhibited a global hypermethylation of the DMR in comparison to thyroids (Figure 4A). As expected from the findings in normal leukocytes, no expression of *FOXE1* was detected in the three leukemia cell lines (Jurkat, K562, and REH) used in the present study (Figure 4B). On the other hand, *FOXE1* expression was detected in all thyroid tissues (mean FOXE1/γ-actin ratio of 1.06, range 0.95-

1.32, normalized to that of ectopic thyroid tissue # 3) (Figure 4A). In contrast, in the de-differentiated thyroid cancer WRO cells which show a globally unmethylated DMR (Figure 2C), *FOXE1* expression was still detected, but was about 2.5-fold lower than in thyroid tissues (Figure 4A), which is consistent with previous work (21, 26) and implies that the low expression levels of *FOXE1* in WRO can be attributed to other controlling mechanisms, such as partial repression through the polycomb repressive complex 2 (PRC2), as suggested by our chromatin immunoprecipitation (ChIP) assays (supplemental Figure 4). Altogether, methylation of CpG sites within the DMR correlates with the expression of *FOXE1*.

DNA methylation decreases transcription from the FOXE1 promoter

To investigate whether methylation of the 5′-upstream region of *FOXE1* gene has an impact on transcription from this promoter, a 5′ deletion reporter gene construct (the −1115/+446 bp deletion construct, from which CpG island 1 was deleted) and constructs with wild type and mutated CpG_{14}-CpG_{15} were *in vitro* methylated using the CpG methyltransferase M.*Sss*I (Figures 1 and 5). First, transfection of the mock-methylated construct with deletion of the CpG island 1 showed a marked increase of the luciferase expression (Figure 5), suggesting that transcriptional repressor may target the CpG island 1, which was confirmed by our ChIP assays targeting SUZ12 (a core protein of the PRC2) in immunoprecipitated chromatin of WRO cells (supplemental Figure 3). Then, transfection with the methylated and mock-methylated promoter constructs showed a profound decrease of luciferase expression levels upon methylation (Figure 5). Notably, the methylated constructs in which CpG_{14} and CpG_{15} were either point mutated or deleted (−1115/+446 bp deletion construct) exhibited a significant increase in luciferase activity (0.6 and 1.6 fold of the basic construct activity, respectively) compared to that of the methylated wild type construct (0.27 fold of the basic construct activity, $p<0.05$).

Although these results indicate that the promoter activity of *FOXE1* can be suppressed by DNA methylation, the observed decrease in luciferase activities could be due in part to methylation within the pGL3-Basic vector itself (27, 28) (Figure 5). Therefore, experiments where only CpG island 1 (either wild type or mutated) was methylated (i.e. regional “patch” methylation) and ligated into unmethylated pGL3-Basic vector were carried out to confirm the impact of methylation of CpG island 1 on *FOXE1* transcription. Indeed, regional methylation of CpG island 1 induced a significant decrease in luciferase activity (a decrease of 33%, from 3.27

to 2.19 fold of the basic construct, $p<0.05$; 48% if corrected for the background obtained for transfections with empty basic vector) whereas regional methylation of the CpG island 1 construct with mutated CpG_{14} and CpG_{15} did not affect *FOXE1* expression (Figure 6).

Discussion

Herein, we examined the impact of the tissue-dependent differential methylation on the expression of *FOXE1*. We found a DMR in the *FOXE1* promoter when comparing thyroid and leukocytes tissues. We then showed that: (i) *FOXE1* expression is repressed upon methylation of this DMR and (ii) the methylation of two specific consecutive CpG dinucleotides suffices to decrease *FOXE1* expression.

The role of DNA methylation in the regulation of tissue-specific gene expression has been previously reported (29, 30). Several genome-wide studies have shown that distinct regions of the mammalian genome exhibit a tissue-dependent pattern of DNA methylation and are increasingly reported to be associated with tissue-specific gene activity (30-36). These tissue-specific DMRs are found in either CpG-rich or -poor DNA sequences (37). In a recent attempt to identify DMRs in humans, it was shown that DMRs are enriched in promoter regions of genes exhibiting tissue-specific functions (promoter-like DMRs) (38). Hence, DNA methylation of promoters can be implicated in major cell lineage determination. On the other hand, DMRs enriched in enhancer elements (enhancer-like DMRs) are identified as cell-type specific DMRs. Thus, DNA methylation is a possible mechanism allowing cells to attain final lineage commitment or maintain a distinct cell type. Collectively, a tight association between differences in DNA methylation involving gene regulatory elements (promoters and enhancers) and gene activity has been established (38). Of note, transcriptional repression is not linearly related to methylation (39). Indeed, a tissue-dependent and gene-specific methylation threshold is required to attenuate gene expression (40), which explains why low-levels of methylation of *FOXE1* DMR did not hamper *FOXE1* expression in thyroid tissue (Figures 3 and 4).

Abnormal methylation patterns of *FOXE1* gene has been previously reported in primary pancreatic carcinomas as well as in pancreatic cancer cell lines. Expression of *FOXE1* was induced upon treatment with the demethylating agent 5-aza-2'-deoxycytidine in pancreatic cell lines and not in unmethylated non-neoplastic cells (19). Moreover, *FOXE1* hypermethylation in tumor-derived DNA released into the bloodstream of patients with breast cancer has also been

described (20). Kuang *et al.* determined the methylation status of *FOXE1* in Jurkat, K562, and REH cells, among other leukemia cell lines, using methylated CpG island amplification coupled to representational differential analysis (MCA/RDA) or a DNA promoter microarray (MCA/microarray) and validated their findings using bisulfite pyrosequecing (41). *FOXE1* was found to be aberrantly hypermethylated in leukemia cell lines when compared to normal peripheral lymphocytes (controls). The genomic region within the *FOXE1* promoter that was found to be hypermethylated in the abovementioned study is located within the region from −673 to −124 from TSS. According to our data, this region was not differentially methylated between leukocytes and thyroid. For that reason, we have not determined the methylation status of the region from −673 to −124 from TSS in leukemia cell lines. On the other hand, the DMR that we identified (−1600 to −1140 from TSS or CpG island 1) was not covered on the proximal promoter microarray, covering -1.0 kb upstream and +0.3 kb downstream from TSS, used in the study of Kuang *et al.* (41). Recently, Venza *et al.* (18) have shown that hypermethylation of CpG islands located within the promoter of *FOXE1* gene is frequent among patients with cutaneous simple squamous carcinoma (SCC). Although these authors did not specify the site of promoter methylation related to transcriptional repression, both the concordance between the methylation status of the *FOXE1* promoter and its mRNA expression together with the reactivation of expression upon treatment with 5-Aza-dc point to the involvement of DNA methylation in the transcriptional regulation of *FOXE1* in SCC (18). Moreover, a marked hypermethylation was found at the transcriptional start site (TSS) of the *FOXE1* gene in adenoid cystic carcinoma (ACC) of the salivary glands, suggesting the association of *FOXE1* aberrant methylation with the development and progression of ACC (42).

Herein, upon transfection with different globally methylated *FOXE1* promoter constructs, the luciferase activities were significantly reduced compared to the corresponding mock-methylated constructs. Given that: (i) the luciferase gene in the plasmids can also be methylated by M.*Sss*I methylase (27, 28) and (ii) regions outside CpG island 1 (i.e. the T-DMR of *FOXE1* promoter) were mainly unmethylated in leukocytes (supplemental Figure 3), further regional (patch) methylation was carried out to assess the specific impact of CpG island 1 methylation on the *FOXE1* expression. In this regard, regional methylation of CpG island 1 encompassing wild type CpG_{14} and CpG_{15} (at −1417 and −1412) leads to a significant decrease in reporter gene activity when compared to mock-methylated control. In contrast, no significant difference in the

activity was observed upon point mutating the two CpG dinucleotides of interest, which indicates their role in regulating the expression of the *FOXE1* gene. This is consistent with the previously reported involvement of site-specific methylation in tissue or cell-specific gene expression. Grant *et al.* have shown that the methylation status of three promoter CpG dinucleotides (−22, −54, and −455) is altered in a tissue-specific manner and that lactoferrin expression was detected in tissues exhibiting at least two unmethylated CpG dinucleotides out of three (43). Similarly, Boatright *et al.* have shown that tissue and site-specific methylation of the two CpG dinucleotides (−725 and −115) of the murine interphotoreceptor retinoid binding protein (*IRBP*) promoter decreased the promoter activity *in vitro* and correlated with *IRBP* expression *in vivo* (44). Moreover, repression of transcription mediated via single-site methylation in promoter regions has been previously reported within the promoter region of the Herpes simplex virus thymidine kinase (*tk*) gene (45), the calcium-binding protein gene *S100A2* (46), the *p16* gene in human bladder cell lines (47), the alternative reading frame (*ARF*) gene promoter (48), and the *p53* gene promoter (49). Collectively, these observations underline the ability of methylation at specific CpG sites to efficiently repress transcriptional activity. Generally, CpG methylation contributes to transcriptional suppression by directly preventing ubiquitous transcriptional regulators from binding to their target gene promoters (50) or by binding of methyl-CpG-binding proteins that subsequently recruit repressive complexes such as histone deacetylases (HDACs) that lead to chromatin compaction and in turn transcriptional repression of the gene (51). Among the methyl-CpG-binding protein, MeCP2 has been shown to bind to as few as one to three methylated cytosines (52), thus supporting the notion that site-specific methylation of CpG dinucleotides is involved in transcriptional repression. Whether the mechanism via which CpG_{14} and CpG_{15} mediate the differential expression of *FOXE1* involves recruitment of methyl-CpG-binding proteins or direct blocking of transcription factors binding needs further investigation.

Our luciferase assay also suggests that the unmethylated FOXE1 CpG island 1 binds to a transcriptional repressor (Figure 5). ChIP assays revealed that this repressor is the polycomb repressive complex 2, in which SUZ12 (target of ChIP) is a core component (supplemental Figure 4A). This result is consistent with SUZ12-ChIP sequencing data from ENCODE (http: genome.ucsc.edu/) showing SUZ12 binding in this region of the *FOXE1* promoter (supplemental Figure 4B). Unmethylayed CpG islands have a key role in polycomb complex recruitment and most PRC2 target genes actually remain constitutively unmethylated throughout development

(53, 54). Some genes are *de novo* repressed by PRC2-mediated methylation on H3K27 (such as WRO) but subsequently only a subset of these genes become definitively methylated and lose their epigenetic plasticity (54). This suggests that there are additional (as yet unknown) factors required for definitive DNA methylation (54).

In conclusion, the main outcome of the present study supports the concept that DNA methylation plays a role in the differential expression of the thyroid transcription factor FOXE1 in normal leukocytes (cells that do not express *FOXE1*) and in the thyroid, a tissue that abundantly expresses *FOXE1*. Evidence for the role of DNA methylation mainly depends on the CpG methylation profiling of human *FOXE1* that exhibited the presence of a DMR in its 5′-flanking regulatory region. One limitation of the present study is the low number of thyroid samples included that were obtained from female in the age range of 8-18 yrs. Tissue-specific age-related DMR have been reported in humans (55). Of note, over the 490 age-related DMR found through epigenome-wide scans by Bell *et al.*(55), none were found in *FOXE1*, *PAX8* and *NKX2.1*. Moreover, within this limited age range (8-18 yr), we saw no difference in methylation pattern among the different thyroid tissues investigated. In addition to age, sex influences genome-wide methylation in humans (56). Indeed, Liu *et al.* assessed the genome-wide methylation profile of 20,493 CpG sites and found 690 sex-related DMR in 432 genes (421 on X chromosome, 11 genes on autosomes); none were found in *FOXE1*, *PAX8* and *NKX2.1* (56). Consequently, there is currently no evidence that age or sex might have an impact on our results. However, further studies will determine whether these results are observed in a wider range of tissues (in term of age and sex) and will assess whether rare genetic variants in the *FOXE1* DMR are associated with congenital hypothyroidism due to thyroid ectopy.

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Figure legends:

Figure 1: CpG methylation profile of the human *FOXE1* promoter. *A,* CpG methylation profile was determined using European Molecular Biology Open Software Suite (EMBOSS) CpGblot (http: http://www.ebi.ac.uk/Tools/emboss/). *B,* schematic representation of the *FOXE1* promoter, numbered from the transcription start site (TSS, +1), with the CpG island 1 (−1600 to −1140 from TSS). *C,* nucleotide sequence of CpG island 1, with CpG dinucleotides numbered; CpG_{14} and CpG_{15} are highlighted in a box.

Figure 2: DNA methylation status of CpG island 1 (−1600 to −1140 from TSS) in the 5′-UTR of the *FOXE1* gene using bisulfite sequencing. *A-B*, CpG island of *FOXE1* (−1600 to −1140 from transcription start site, +1) is globally more methylated in leukocytes (10%) compared to thyroids (3%, P<0.001). In addition, two CpG dinucleotides (i.e. CpG_{14} and CpG_{15}, located −1417 and −1412 relative to transcription start site) show higher methylation in leukocytes (51% for CpG_{14} and 43% for CpG_{15}) when compared to thyroids (5%; P<0.001). *C,* Genomic DNA was extracted from 4 different human cell lines and subjected to sodium bisulfite sequencing to analyze the methylation profile of CpG island 1 of the *FOXE1* promoter. Seven (REH cells) to fourteen (other cell lines) different clones were sequenced. All leukemia cell lines showed significantly higher CpG island 1 methylation (Jurkat, 51%; K562, 15%; and REH, 86%) when compared to the thyroid cell line WRO (4.4% methylation; P<0.001 when compared with each leukemia cell line); this methylation difference is even more pronounced for CpG_{14} and CpG_{15} (Jurkat, 100%; K562 39%; REH 100% compared to 0% in WRO). Each line represent sequencing results of distinct tissues or cell lines. Circles represent the 33 CpG dinucleotides of CpG island 1 which are labeled as follows: black, methylation over 75%; dark grey, methylation of 50 to 75%; grey, methylation of 10 to 49%; white, methylation under 10%. Numbers of clones analysed for each tissue or cell line are listed on the right of the figure.

Figure 3: Confirmation of DNA methylation status by bisulfite pyrosequencing. The methylation profile of the first 17 CpG dinucleotides in CpG island 1 was analyzed using sodium bisulfite pyrosequencing. The figure represents the means ± SEM percentage of DNA methylation of the 17 CpG dinucleotides in leukocytes (in black) *vs* thyroid tissues (in grey) (*; P<0.05, two-tailed paired t-test, corrected for multiple comparisons with the Holm-Sidak method).

Figure 4: Semiquantitative RT-PCR analysis of FOXE1 expression. FOXE1 RT-PCR in normal leukocytes and thyroid tissues (A), in human leukemia cell lines (Jurkat, K562, and REH), and in thyroid cancer WRO cells (B). The numbers denote the ratio of FOXE1:γ-actin values, normalized to that of ectopic thyroid tissue # 3 (set as 1.0).

Figure 5: Effect of global CpG methylation on FOXE1 promoter activity. *FOXE1* promoter activity was tested in PCCL3 cells using either M.SssI methylated constructs (black bars) or mock methylated constructs (grey bars). Values are expressed as fold of the basic empty vector. Data represent means ± SEM of three independent experiments, each in triplicate (*, $P<0.05$; **, $P<0.01$; Student's t-test). All methylated constructs showed significant decreased luciferase activity. Methylated constructs with CpG_{14}-CpG_{15} mutations and with CpG island 1 deletion showed a significant derepression when compared with the methylated WT construct.

Figure 6: Effect of regional methylation of the DMR on FOXE1 promoter activity. *FOXE1* promoter activity was tested in Nthy-ori 3-1 cells using either M.SssI regionally methylated constructs (black bars) or mock methylated constructs (grey bars). Values are expressed as fold of the basic empty vector. Data represents means ± SEM of two independent experiments, each in triplicate (*, $P<0.05$; **, $P<0.01$; n.s., non significant; Student's t-test). Regional methylation of CpG island 1 encompassing the wild type CpG_{14} and CpG_{15} (at −1417 and −1412) leads to a significant decrease by reducing reporter gene activity by 33% when compared to mock methylated control (48% if corrected for the background obtained for transfections with empty basic vector). Regional methylation of the CpG island 1 construct with mutated CpG_{14} and CpG_{15} induced no difference between methylated and mock methylated constructs.

Figures:

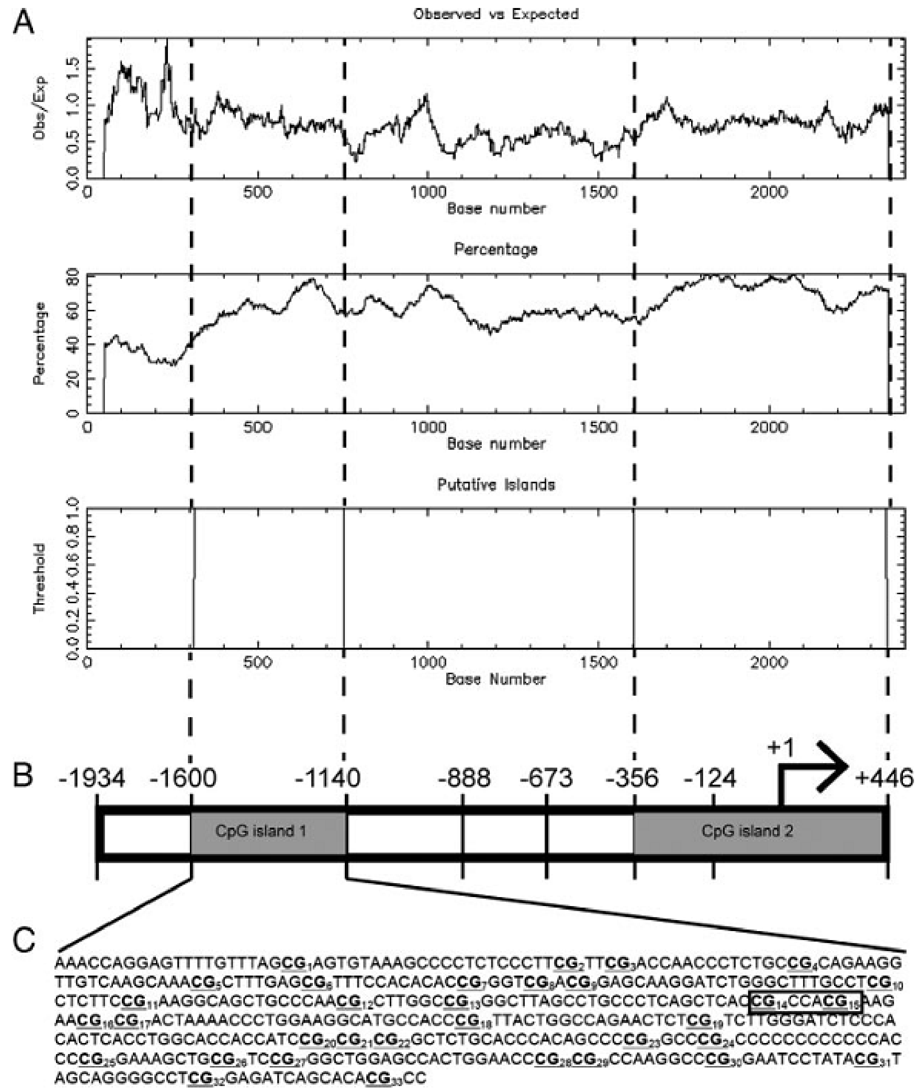


Figure 1

Figure 2

Figure 3

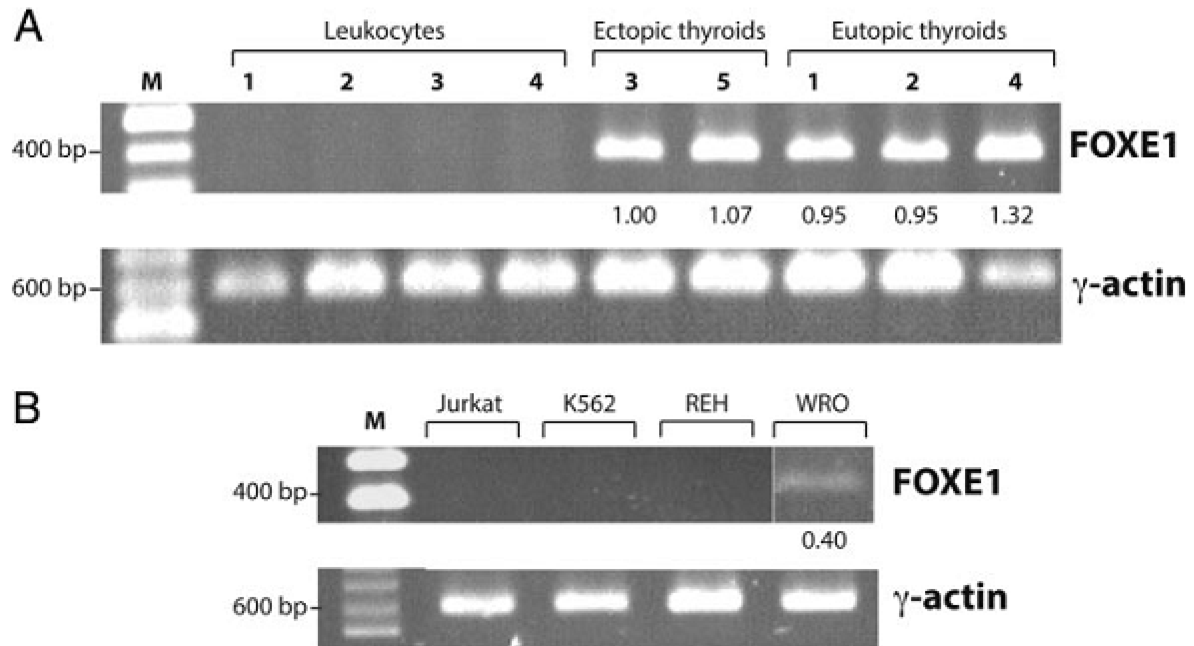


Figure 4

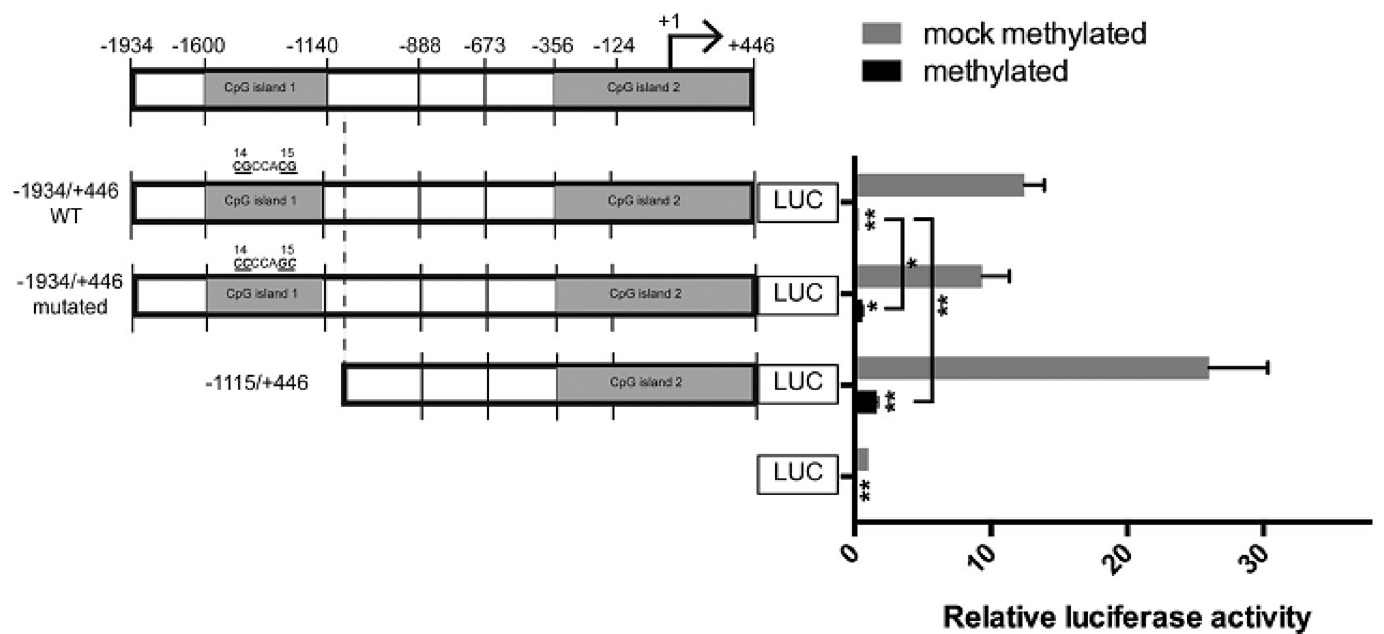


Figure 5

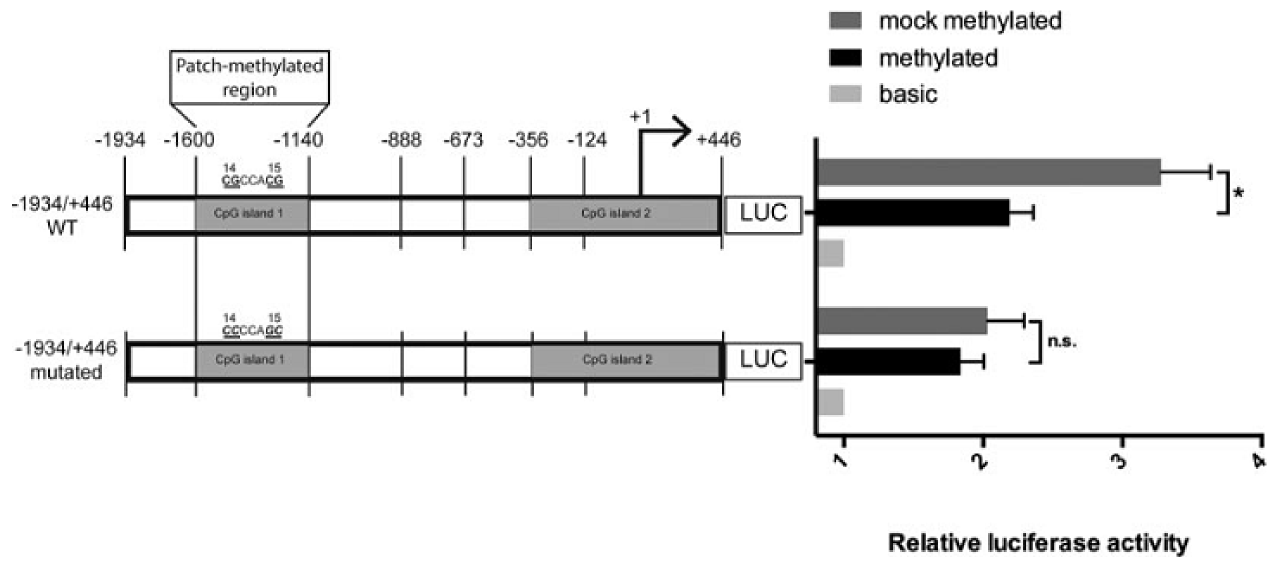


Figure 6

Supplemental Data

A) METHODS

Participant characteristics and tissue collection

We obtained flash-frozen samples of ectopic (lingual) thyroid tissue removed from 3 girls aged 8, 10 and 15 yr, because of obstructive symptoms and we obtained also normal eutopic thyroid tissue from 4 girls (aged 12, 15, 16, and 18 yr) and 1 boy (aged 4 yr) adjacent to a single hyperfunctioning thyroid nodule that had arisen in an orthotopic thyroid for which they had been operated. As the methylome of ectopic and eutopic thyroids are similar (1), they were considered as a single group for the present analysis. For controls, we used, matched leukocytes (when available) of the above-mentioned cases and additional leukocytes from normal subjects (supplemental Table 1). This study was approved by the Ethics Committee of the CHU Sainte-Justine (ERB number 94). All the parents and participants gave written informed consent.

Nucleic acid isolation

Genomic DNA and total cellular RNA were isolated from thyroid tissues and cell lines (the human thyroid cancer cell line WRO and the human leukemia cell lines Jurkat, K562, and REH) using the pureLink genomic DNA Mini kit (Life Technologies Inc., Burlington, ON, Canada) and the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) respectively. Isolation of total genomic DNA from peripheral blood leukocytes was carried out using the standard phenol/chloroform extraction method followed by ethanol precipitation while total cellular RNA was isolated using the PAXgene Blood RNA kit (Qiagen). Purity and concentration of nucleic acids were measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Samples were stored at -20°C (DNA) or -80°C (RNA) until time of analysis.

Methylation Profiling by Methylated DNA Immunoprecipitation (MeDIP) and MeDIP-chip

The MeDIP-chip was performed using pairs of enriched methylated fraction (IP) and normal fraction (IN) of genomic DNA from four thyroids and five leukocytes (four matched; see supplemental Table 1). The methylated fraction of genomic DNA was enriched using the methylated DNA immunoprecipitation (MeDIP) assay (2) and interrogated on human Promoter plus CpG Island Tiling Arrays with a ChIP design for CpG islands and promoter regions (n=28,226) from HG18 using 385,020 probes selected from CGH probe bank with a median

spacing of 101bp (Roche NimbleGen, Madison, WI). Briefly, 4 µg MseI digested genomic DNA was immunoprecipitated with monoclonal mouse anti 5-methylcytidine antibody (New England Biolabs, Pickering, Ontario and Abcam Inc., Cambridge, MA 02139). After washes and purification steps, immunoprecipitated material and a sample of input DNA were amplified using GenomePlex Complete Whole Genome Amplification (WGA) kit (Sigma-Aldrich, Saint Louis, MI). The resulting products (4 µg) were labeled, cohybridized and scanned by the NimbleGen Customer Service (Roche NimbleGen, Madison, WI). For each sample, NimbleScan detects peaks by searching for at least two probes above a P-value cutoff (-log10) of 2 and peaks within 500bp are merged. Then, peak data were analyzed to compare the methylation profile between ectopic thyroids and orthotopic thyroids using the Loess normalized log2 (ChIP/input) ratios with the one-way ANOVA tool of the Partek Genomic Suite (PGS) software. After Bonferroni correction for multiple testing, differences in methylation should achieve a p-value of less than 0.05 to be considered as significant.

Cell culture

The human thyroid cancer cell line WRO was a gift from Dr. H. Mircecsu (University of Montreal), the human leukemia cell lines Jurkat (T-cell acute lymphoblastic leukemia), K562 (chronic myelogenous leukemia), and REH (acute lymphoblastic leukemia of the non-T, non-B type) were a gift from Dr. A. Ahmad (University of Montreal) and the rat follicular thyroid PCCL3 cell line was a gift from Dr. F. Miot (Université Libre de Bruxelles, Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire, Brussels, Belgium), and the immortalized human thyroid Nthy-ori 3-1 (NT) cells were obtained from Sigma (St Louis, MO). WRO cells, Nthy-ori 3-1 cells, and the human leukemia cell lines were maintained in the RPMI 1640, HEPES culture medium (Gibco, Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamate, and antibiotics/anti-mycotics to a final concentration of 100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml amphotericin B (Gibco, Life Technologies). PCCL3 cells were maintained in Coon's modified Nutrient Mixture F-12 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 5% heat-inactivated FBS and a mixture of six hormones to a final concentration of 1 mU/ml TSH, 10 g/ml insulin, 5 g/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml glycyl-l-histidyl-l-lysineacetate, and 3.2 ng/ml hydrocortisone (Sigma-Aldrich, Oakville,

ON, Canada) together with prophylactic plasmocin (InvivoGen, San Diego, CA). All cells used in the present study were cultured at 37°C in 5% CO2 humidified atmosphere.

Semiquantitative RT-PCR

The forkhead box E1 gene (*FOXE1*, NCBI reference sequence NT_008470.19) mRNA expression was examined by semiquantitative reverse transcriptase PCR (RT-PCR) in two ectopic lingual thyroids, three orthotopic thyroids (flash-frozen healthy tissue adjacent to papillary thyroid carcinomas) and leukocytes from normal subjects. In addition, FOXE1 mRNA expression was determined in the thyroid cancer WRO cell line and the human leukemia cell lines Jurkat, K562, and REH. Total RNA was extracted as mentioned in nucleic acids isolation. First-strand complementary DNA was synthesized using 1 µg of RNA per reaction with the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). The ubiquitous γ-actin gene (ACTG1, NCBI reference sequence NM_001614.2) was used as a positive control to ensure the efficiencies of the RNA extraction, retro-transcriptase reaction, and cDNA amplification. Amplification of cDNA was performed using primers specific for *FOXE1* and γ-actin. Primers specific for *FOXE1* cDNA amplification were sense 5′-TGCTGGTAATTTCATGGCTGTTA-3′ and antisense 5′-AAGGGAAAGGGGGAGAGTTATTG-3′ and for γ-actin amplification were sense 5`-GACACCAGGGCGTCATGGTG-3` and antisense 5′-GCAGCTCGTAGCTCTTCTCC-3′. Protocols of RT-PCR are available upon request. The PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, and quantitated using the ImageJ 1.46r software.

Bisulfite genomic DNA sequencing and methylation analysis

Genomic DNA was isolated from cell lines, thyroid tissues and matched leukocytes as mentioned above in nucleic acid isolation. Bisulfite treatment of genomic DNA was performed using the Zymo EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The targeted region of *FOXE1* (-1600 to -125 from transcription start site (TSS)), *PAX8* (-635 to -471 from ATG) and *NKX2.1* (-343 to -107 from ATG) promoters were subsequently amplified with a nested Platinum Taq polymerase (Invitrogen, Life Technologies Inc., Burlington, ON, Canada) PCR protocol in which two sets of forward and reverse primers, designed using the MethPrimer software (www.urogene.org/methprimer/), were used. Primers, reagents and PCR conditions are listed in the Supplemental Table 2. PCR

amplification products were purified from a 1.5% agarose gel with the QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada). The gel-purified PCR fragments were TA cloned into the pCR4-TOPO vector (Invitrogen, Life technologies, Burlington, ON) and then introduced into the One Shot TOP10 chemically competent *E. coli* (Life technologies). To quantify methylation, individual clones were sequenced with T3 primer. Sequencing was performed using the DNA analyser ABI 3730 at the genomics platform of the Institute for Research in Immunology and Cancer (IRIC, University of Montreal). Data analysis and quality control have been performed using the BiQ Analyzer software (http:biq-analyzer.bioinf.mpi-sb.mpg.de/) using stringent criteria to avoid clonal effect and PCR bias (3). This stringent quality control explains why some samples have fewer data available for analysis. To assess whether the difference in methylation between thyroid and leukocytes were significant, two-sided exact Fischer's tests were performed for each CpG dinucleotide. To correct for comparison of multiple clones per individual samples and to focus on CpGs having the best possible likelihood of biological relevance, only P-values less than 0.001 were considered significant. Statistical analysis was performed using the statistical software R. To validate our results, DNA from four pairs of thyroids and leukocytes were pyrosequenced using Qiagen-Pyrosequencing PSQ MD system (EpigenDx, MA, USA). As data are expressed in percent of an arbitrary scale for each CpG, the results were analyzed with two-sided paired t-tests, and a P-value less than 0.05 were considered as significant.

Construction of luciferase reporter vectors

A pGL3-Basic plasmid incorporating a 2.38-Kb fragment that includes the 5′ upstream regulatory region of the human *FOXE1* (hFOXE1) gene from -1934 to +446, phFOXE1(-1934/+446)-Luc, relative to the transcriptional start site (+1) was a generous gift from Dr. T. Eichberger (University of Salzburg, Salzburg, Austria). A deletion construct missing CpG island 1(phFOXE1(-1115/+446)-Luc)was generated by restriction enzyme digestion using AflII/NsiI followed by blunt-end ligation. The two CpGs 14 and 15 within CpG island 1 located at -1417 and -1412 relative to TSS (+1), respectively were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Subsequently, a BseYI restriction site (5′-CCCAGC-3′) was created to facilitate further screening. The following primers were used in site-directed mutagenesis: sense 5′-GCCCTCAGCTCACCCCCAGCAAGAACGCGACTAAAAC-3′ and anti-sense 5′-GTTTTAGTCGCGTTCTTGCTGGGGGTGAGCTGAGGGC-3′. Restriction enzyme analysis

and DNA sequencing (IRIC, University of Montreal, Canada) confirmed the integrity of all the above constructs.

In vitro global methylation assay

For in vitro methylation of pGL3 Basic and the different human *FOXE1* promoter reporter plasmids, whole plasmids were methylated using the site-specific CpG Methyltransferase M.SssI (New England Biolabs Inc., Whitby, ON, Canada). Mock methylated plasmids were subjected to the same treatment in the absence of M.SssI. Following methylation, the plasmids were purified using the EZ-10 spin column DNA Gel Extraction Kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's recommendations. The extent of methylation was subsequently verified by digestion with the CpG methylation-sensitive restriction enzyme HpaII (New England Biolabs) and its isoschizomer the CpG methylation-insensitive MspI (Thermo Scientific, ON, Canada). Only completely methylated (M.SssI+) and mock-methylated (M.SssI-) plasmids were then used in the transfection experiments.

In vitro regional (patch) methylation assay

For regional (patch) methylation of CpG island 1 to allow integration of methylated fragment, NdeI and NsiI restriction sites were engineered into both the wild type and point mutated *FOXE1* promoter fragment using the QuikChange site-directed mutagenesis kit as well (Stratagene) according to the manufacturer's instructions. The following primers were used in site-directed mutagenesis:

NdeI-sense 5′-CATTACTTGGGAAGAAGGAAACCATATGTTTTGTTTAGCGAGTGTAAAG-3′, NdeI-antisense 5′-CTTTACACTCGCTAAACAAAACATATGGTTTCCTTCTTCCCAAGTAATG-3′, NsiI-sense 5′-GATCAGCACACGCCCTCCAGATGCATTTTAACAGAGTAGAACACTG-3′, and NsiI-antisense 5′-CAGTGTTCTACTCTGTTAAAATGCATCTGGAGGGCGTGTGCTGATC-3′.

Restriction enzyme analysis and DNA sequencing (IRIC; University of Montreal, Canada) confirmed the integrity of the constructs. Fourty micrograms of the phFOXE1(-1934/+446)-Luc plasmid containing either the wild type or the point mutated *FOXE1* promoter insert were either globally in vitro methylated (using M.SssI methylase) or mock-methylated.

The efficiency of in vitro methylation was carried out as mentioned above. The methylated and mock-methylated vectors were then double digested with NdeI/NsiI to excise the CpG island 1 fragments. After fractionation on a 1% agarose gel, the DNA bands corresponding to CpG island 1 were cut from the gel and isolated using the QIAquick Gel Extraction kit (Qiagen). To determine the impact of regional methylation on the expression of the reporter gene, the methylated and mock-methylated wild type and point mutated CpG island 1 DNA fragments were then ligated into phFOXE1(-1934/+446)-Luc vector between the NdeI and NsiI restriction sites. The ligation reaction was performed using T4 DNA Ligase (Thermo scientific) according to the manufacturer's instructions. According to the final yield of the purified methylated and mock-methylated CpG islands and due to the maximum amount of 500 ng total DNA/ligation reaction, 11 ligation reactions were carried out in which 50 ng of DNA/CpG island were ligated to 450 ng of back bone vector. Only one master mix was prepared that was then aliquoted in the different tubes. The ligation reactions were carried out overnight and then pooled together. The efficiency of ligation and equivalence of incorporated DNA into the methylated and mock-methylated constructs were confirmed by agarose gel electrophoresis (as published by McGowan *et al.* (4); see supplemental Figure 1A-B). In addition, a small aliquot of the ligation reaction were transformed into *E.coli* XL-10-gold chemically competent cells to check the ligation efficiency and not for amplification (see supplemental Figure 1C). Hence, the ligation products were transfected directly into the normal human thyroid Nthy-ori cells to attain a significant read-out due to the small proportion of either methylated or mock-methylated constructs of interest. Of note, direct transfection of the ligation products is the standard procedure used with path-methylayed plasmids (4-9), given that subcloning of the ligation products in *E. coli* erases the methylation mark.

Then, the pGL3 Basic vector with no promoter insert was used as a negative control. The effect of targeted site-specific methylation on the transcriptional activity of the inserted *FOXE1* promoter fragment was expressed as fold change in the luciferase reporter gene activity relative to the pGL3 Basic vector.

Transient transfection and Luciferase reporter gene assay

Rat follicular thyroid PCCL3 cells were transiently transfected with polyethylenimine (PEI; Polysciences, Inc., Warrington, PA) in triplicate in 24-well tissue culture plates (Corning

Inc., NY, USA). The volume of PEI used is based on a 4:1 ratio of PEI (µg): total plasmid DNA (µg). Cells were seeded at a density of 0.3 × 105 cells/well 48 h prior to transfection. Cells were transfected with 0.32 g/well of the reporter vector pGL3-Basic (Promega, Madison, WI) and reporter plasmids including the different *FOXE1* promoter inserts. To correct for transfection efficiency and variation in cell viability between wells, cells were co-transfected with 0.005 g/well Renilla luciferase repoter vector (pRL-TK; Promega) as an internal control. For methylated and mock-methylated plasmids, the activities of the firefly luciferase reporter gene and the Renilla luciferase were measured in cell lysates 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Luminescence was detected by the 2104 EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA). Following normalization with Renilla luciferase activity, luciferase activity corresponding to each plasmid was obtained relative to pGL3-Basic vector and the mean and standard error of the mean (SEM) from triplicate wells were calculated. Transfections were repeated for three independent experiments.

For regional methylation, due to the low read-out of the luciferase activities in PCCL3, the immortalized human thyroid Nthy-ori 3-1were transfected instead. Each of the religated methylated and mock methylated wild type and point mutated plasmids (0.75 µg DNA) were transfected in Nthy-ori 3-1 cells (0.1x 105 cells/well), plated 48 hrs before transfection, using X-tremeGENE 9 (Roche Diagnostics, Laval, QC, Canada). In each experiment (n=2 in triplicate), the pRL-TK plasmid (0.25 µg) was cotransfected for normalization purposes. Luminescence was measured 48 h after transfection using the dual-luciferase reporter assay system (Promega) as previously mentioned. Following normalization with Renilla luciferase activity, luciferase activity corresponding to each methylated or mock-methylated plasmid was obtained relative to pGL3-Basic vector and the mean and standard error of the mean (SEM) from triplicate wells were calculated. Transfections were repeated for two independent experiments.

In vitro methylation assay

For in vitro methylation of pGL3 Basic and the different human *FOXE1* promoter reporter plasmids, whole plasmids were methylated using the site-specific CpG Methyltransferase, M.SssI (New England Biolabs Inc., Whitby, ON, Canada) following the manufacturer's protocol. Mock methylated plasmids were subjected to the same treatment in the

absence of M.SssI. Following methylation, the plasmids were purified using the EZ-10 spin column DNA Gel Extraction Kit (Bio Basic Inc., Markham, ON, Canada) according to the manufacturer's recommendations. The extent of methylation was subsequently verified by digestion with the CpG methylation-sensitive restriction enzyme HpaII (New England Biolabs) and its isoschizomer the CpG methylation-insensitive MspI (Fischer Scientific, ON, Canada). Only completely methylated (M.SssI +) and mock-methylated (M.SssI-) plasmids were then used in the transfection experiments.

Chromatin Immunoprecipitation Assay (ChIP)

WRO cells, grown to ~90% confluence, were treated with thyroid-stimulating hormone (TSH) to a final concentration of 1mU/ml and insulin to a final concentration of 10µg/ml for 45 min at 37 ºC. Treated cells were cross-linked with 1% formaldehyde for 10 min by at room temperature and the cross-linking was stopped using glycine to a final concentration of 125 mM. Cells were lysed in SDS lysis buffer for 10 min on ice, sonicated to an average size of 300–500 bp using Fisher Scientific F 60 sonic dismembrator (Thermo Fisher scientifc) and lysates were clarified by centrifugation at 13 K for 10 min. Supernatants of sonicated lysates were diluted fivefold with ChIP dilution buffer and 10% of the chromatin solution was set aside to evaluate input DNA before immunoprecipitation. Samples were pre-cleared by incubating with 60µl of protein A agarose/salmon sperm DNA (Millipore, Billerica, MA) for 1h at 4°C with gentle agitation. Pre-cleared chromatin from 2 × 106 cells was used for each immunoprecipitation that was carried out for overnight at 4°C. The antibodies (3µg/each) used in the ChIP assays included anti-SUZ12 (D39F6) XP Rabbit mAb (Cell Signaling Technology, Whitby, Ontario), anti-Histone H3 (acetyl K27) Rabbit pAb (ab4729, Abcam Inc, Toronto, Ontario), anti-trimethyl Histone H3-Lys27 Rabbit pAb (07-449, Millipore), and control Rabbit anti-IgG. Immune complexes were collected by incubating with 60µl of protein A agarose/salmon sperm DNA (Millipore), washed, and protein or modified histone/DNA complexes were eluted with 500 µl of 1% SDS and 0.1 M NaHCO3. Cross-linking was reversed for 4h at 65°C and was followed by proteinase K digestion. DNA was purified by standard phenol/chloroform and ethanol precipitation and was amplified by a primer set related to the region -1529 to -1383 (from TSS) of the FOXE1 genomic locus. The primer set was as follows: -1529(sense), 5′-GCTTTGAGCGTTTCCACACACC-3′ and -1383 (antisense), 5′-TGCCTTCCAGGGTTTTAGTCG-3′. Initially, PCR was performed with different numbers of

cycles to determine the linear range of the amplification. After 28 cycles of amplification, PCR products were run on 1.5% agarose gel and analyzed by ethidium bromide staining. Composition of lysis, dilution, and washing buffers as well as PCR conditions are available upon request.

B) RESULTS & DISCUSSION

No large methylation profile differences observed in whole genome from thyroid and leukocytes.

To assess whether large differences in methylation (i.e., more than 200 bp in length, see Methods), we compared the genome-wide methylation profile of promoters and CpG islands using MeDIP arrays of four pairs of matched thyroids and leukocytes (with an additional unmatched leukocyte in the control group). After multiple test correction (Bonferroni), there was no statistically significant difference between thyroids and leukocytes (data not shown).

Lack of differential methylation of CpG islands in PAX8 and NKX2.1 promoter confirmed by bisulfite sequencing

To determine whether the CpG rich region of the *PAX8* (-635 to -471 from ATG) and *NKX2.1* (-343 to -107 from ATG) promoters show differences in methylation between tissues, we used bisulfite sequencing of matched thyroids and leukocytes. Due to technical constraints and stringent quality controls, the final data analysis was possible on three pairs of matched thyroids and leukocytes for *PAX8* and on one pair of unmatched thyroids and leukocytes for *NKX2.1*. No significant differences were observed. *NKX2.1* promoter was completely demethylated in leukocytes and thyroids, whereas *PAX8* promoter methylation showed large inter-individual variations both in leukocytes and thyroids (see supplemental Figure 1).

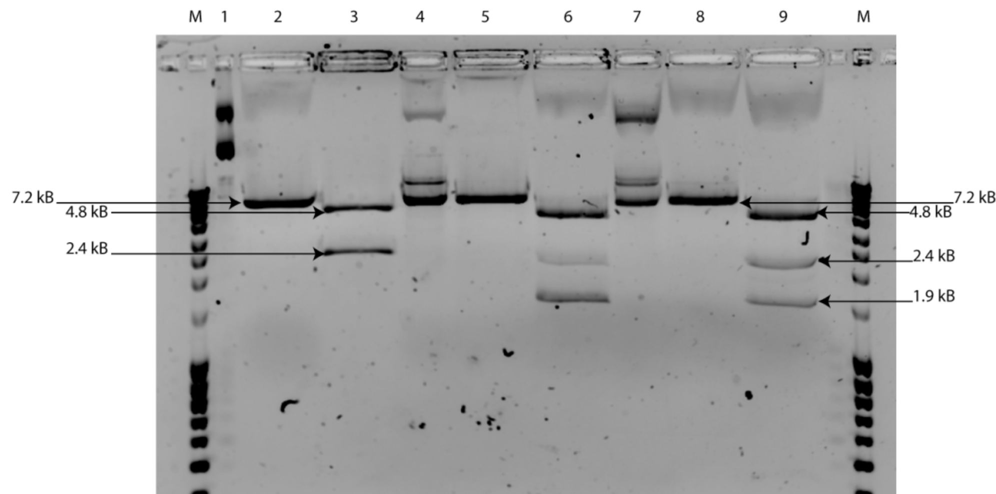
The epigenetic modifier SUZ12 binds to CpG island 1 of the human FOXE1 promoter

Our data revealed that Suppressor of zeste 12 (SUZ12), a component of the polycomb repressive complex 2 (PRC2), can bind to CpG island 1 and more specifically to the -1529/-1383 region of human FOXE1 gene locus, a result that is consistent with SUZ12-ChIP sequencing data available on the UCSC genome browser (supplemental Figure 3). Furthermore, we have assessed the methylation of histone H3K27 status within the same region knowing that the polycomb group (PcG) proteins are responsible for the deposition of the trimethylation of histone H3K27 (H3K27me3), the hallmark of PcG protein-mediated gene silencing. Such a

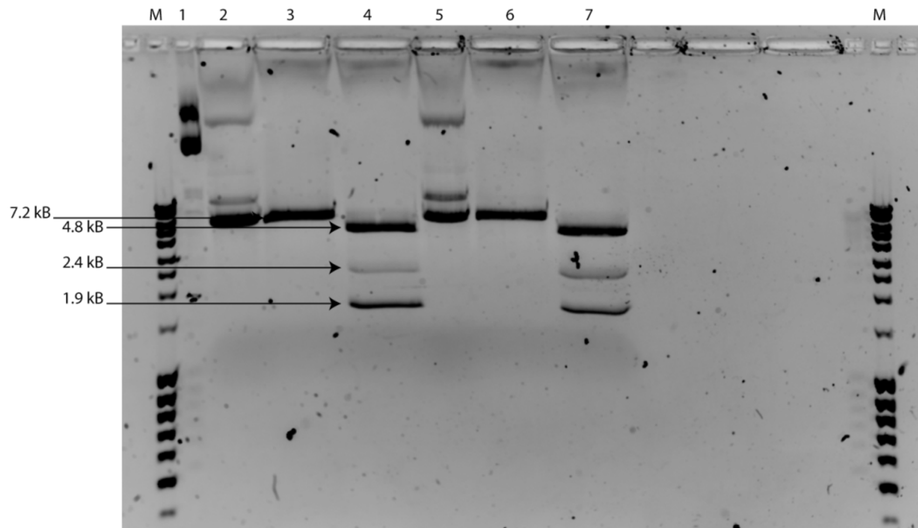
methyltransferase activity is mediated by the catalytic component of the PRC2, enhancer of zeste 2 (EZH2)(10). Being inactive on its own, EZH2 is assisted by two other core components of the PRC2, SUZ12 and embryonic ectoderm development (EED) to attain proper methyltransferase activity. On the other hand, faint levels of the activation mark histone H3K27 acetylation (H3K27Ac) were observed, a finding that is consistent with the PRC2-mediated prevention of H3K27 methylation as a mechanism of transcription repression. Of note, a bivalent histone modification is present in the promoter of many lineage-control genes (11) and plays a role in stem-cell differentiation and possibly in cancerous de-differentiation (10, 12). Collectively, these results indicate that SUZ12 binds to the CpG island 1 of the *FOXE1* promoter, within the region encompassing the two CpGs 14 and 15, and this binding activity is coincident with H3K27 trimethylation.

Supplementary figures:

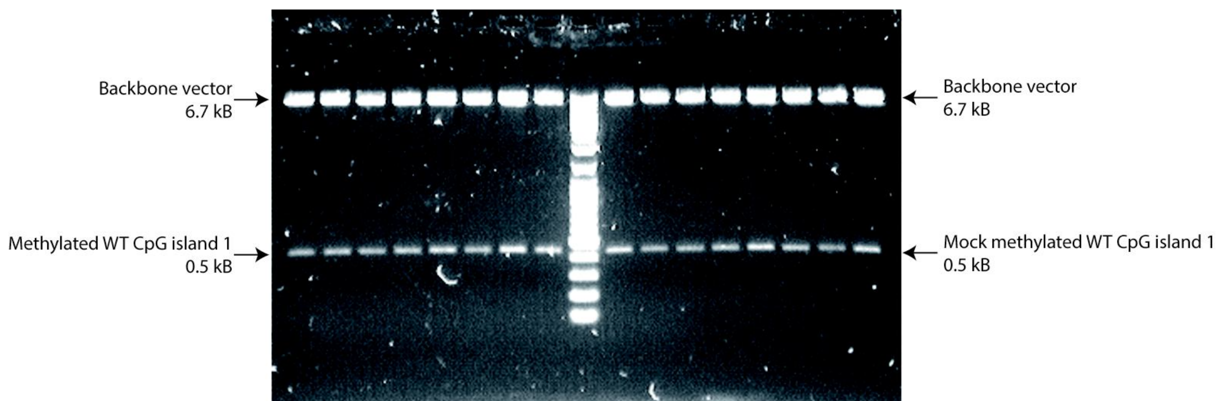
Supplemental figure 1:



A) Checking ligation of WT methylated and mock-methylated CpG island 1 to the vector back bone vector (pGL3-FOXE1 CpG island 1-less): The undigested methylated (lane 4) and mock-methylated (lane 7) WT ligation reactions were loaded on 1.5% agarose gel in parallel to original plasmid (lane 1). Digestion products, using *EcoR*V, of the original plasmid, methylated and mock-methylated WT ligations are loaded on lanes 2, 5, and 8, respectively. In addition, digestion products, using *Hind*III, of the original plasmid, methylated and mock-methylated WT ligations were loaded on lanes 3, 6, and 9, respectively. Band(s) are expected at 7.2 Kb in case of *Eco*RV, while in case of *Hind*III bands are expected at 4.8 and 2.4 Kb. In case of ligation reactions digested with *Hind*III, a third band of 1.9 Kb is observed, representing promoter region without CpG1 island insert.



B) Checking ligation of CpG14-15 mutated methylated and mock-methylated CpG island 1 to the vector back bone vector (pGL3-FOXE1 CpG island 1-less): The undigested methylated (lane 2) and mock-methylated (lane 5) PM ligation reactions were loaded on 1.5% agarose gel in parallel to original plasmid (lane 1). Digestion products, using *EcoR*V, of the methylated and mock-methylated PM ligations were loaded on lanes 3 and 6, respectively. In addition, digestion products, using *Hind*III, of the methylated and mock-methylated PM ligations were loaded on lanes 4 and 7, respectively. Band(s) are expected at 7.2 Kb in case of *EcoR*V, while in case of *Hind*III bands are expected at 4.8 and 2.4 Kb. In case of ligation reactions digested with *Hind*III, a third band of 1.9 Kb is observed, representing promoter region without CpG1 island insert.



C) Restriction digest, using *Nde*I/*Nsi*I, of minipreps from bacterial clones resulting from transformation of *E. coli* with ligation reactions of WT methylated and mock-methylated CpG island 1 (0.5 kB/each) to backbone vector (6.7 kB).

Supplemental figure 2:

	Patient ID	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
LEUCOCYTES	#1	clone 1																9
		clone 2																11
		clone 3																7
		clone 4																8
		clone 5																14
		clone 6																10
		clone 7																10
		clone 8																12
		clone 9																12
		clone 10																10
	#3	clone 1																7
		clone 2																1
		clone 3																1
	#4	clone 1																8
		clone 2																13
		clone 3																12
		clone 4																11
		clone 5																11
THYROIDS	#1	clone 1																11
		clone 2																12
		clone 3																7
		clone 4																11
		clone 5																11
		clone 6																1
		clone 7																5
		clone 8																0
		clone 9																0
	#3	clone 1																11
		clone 2																12
		clone 3																8
		clone 4																13
		clone 5																12
		clone 6																11
		clone 7																12
	#4	clone 1																1
		clone 2																1
		clone 3																0
		clone 4																4
LEUCOCYTES	18 clones	% methylated	8	13	9	13	15	12	15	12	14	8	8	13	14	--	12	167
THYROIDS	20 clones	% methylated	6	12	9	13	10	11	9	9	9	11	3	13	12	--	18	143
FISHER EXACT TEST	P-VALUE	NS	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
		<0.05																✓
		<0.001																

A) Bisulfite sequencing results for *PAX8* promoter CpG island (−635 to −471 from ATG); globally significant, but not consistent when analyzed by matched samples or by single CpG.

	Patient ID	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	TOTAL
LEUCOCYTES	#1	clone 1															0
		clone 2															0
		clone 3															0
THYROIDS	#3	clone 1															0
		clone 2															0
		clone 3															0
LEUCOCYTES	18 clones	% methylated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
THYROIDS	20 clones	% methylated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FISHER EXACT TEST	P-VALUE	NS	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑
		<0.05	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
		<0.001	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

B) Bisulfite sequencing results for *NKX2.1* promoter CpG island (−342 to −107 from ATG).

Supplementary figure 3:

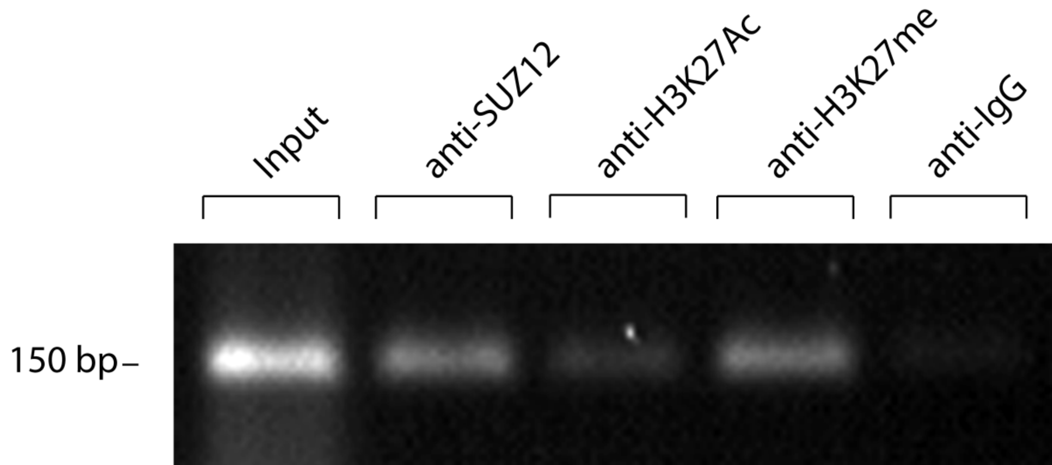
	Patient ID	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	TOTAL
LEUCOCYTES	#1	clone 1	■			■				■			■								■	5
		clone 2				■				■			■						■			4
		clone 3																				0
		clone 4																	■			1
		clone 5																				0
		clone 6																				0
		clone 7				■		■	■													3
		clone 8				■	■			■											■	4
		clone 9																			■	1
		clone 10											■									1
	#3	clone 1																				0
		clone 2																				0
		clone 3																				0
THYROIDS	#1	clone 1																				0
		clone 2																				0
		clone 3																				0
		clone 4																				0
		clone 5																				0
		clone 6																				0
		clone 7																				0
	#3	clone 1																				0
		clone 2																				0
		clone 3																				0
		clone 4																				0
		clone 5																■				1
		clone 6																				0
		clone 7																				0
LEUCOCYTES	13 clones	n methylated	1	0	0	4	1	1	1	3	0	0	3	0	0	0	0	0	2	0	3	19
THYROIDS	14 clones	n methylated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
FISHER EXACT TEST	P-VALUE	NS	☑	☑	☑	☐	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☐
		< 0.05	☐	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
		< 0.001	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☑

A) Bisulfite sequencing results for *FOXE1* promoter region −1140 to −880 from TSS (region between the CpG islands 1 and 2); globally significant, but not consistent when analyzed by matched samples or by single CpG.

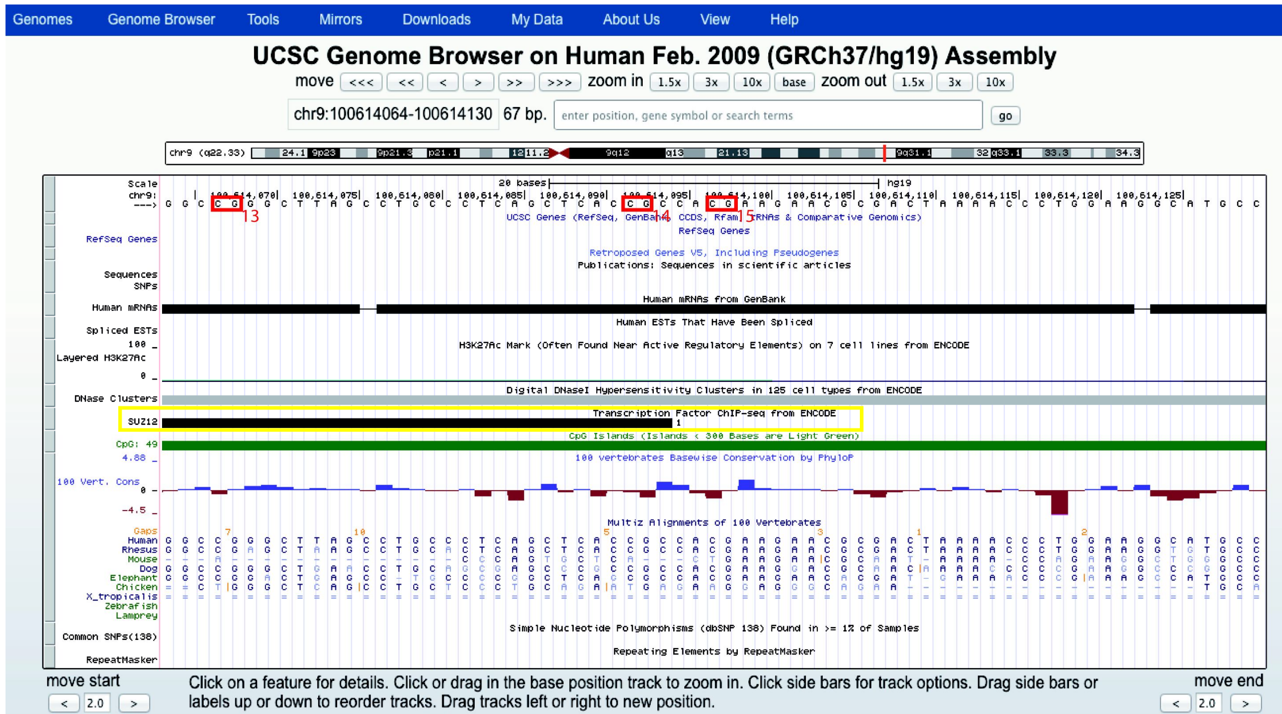
	Patient ID	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	TOTAL
LEUCOCYTES	#1	clone 1										■																							■			2
		clone 2																																				0
		clone 3																																				0
		clone 4																																	■			1
		clone 5																		■						■			■									3
	#2	clone 1																																				0
	#3	clone 1				■	■			■							■																		■			4
		clone 2				■											■																					2
		clone 3																																				0
		clone 4																																				0
		clone 5																																				0
		clone 6			■																														■			2
		clone 7																		■															■			2
		clone 8												■		■																	■					3
		clone 9																														■						1
		clone 10			■	■	■																	■											■			5
THYROIDS	#1	clone 1																														■						1
		clone 2														■		■																				2
		clone 3																							■	■												2
	#2	clone 1																																				0
		clone 2																																	■			1
		clone 3																																				0
		clone 4															■																					1
	#3	clone 1																																				0
		clone 2																																				0
		clone 3											■	■																					■			3
		clone 4																						■											■			2
		clone 5																						■											■			2
LEUCOCYTES	16 clones	% methylated	0	0	0	3	2	0	0	1	0	1	1	1	0	1	2	0	0	2	0	0	0	1	0	1	0	0	1	0	0	1	1	0	6	0	0	25
THYROIDS	12 clones	% methylated	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	2	1	1	0	0	0	0	0	1	0	0	4	0	0	14
FISHER EXACT TEST		P-VALUE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

B) Bisulfite sequencing results for *FOXE1* 5′ region (−660 to −125 from TSS) of the promoter upstream of and within CpG island 2.

Supplementary figure 4: SUZ12 binds to *FOXE1* promoter *in vivo*.



A) Chromatin of WRO cells was immunoprecipitated with the specific antibodies for SUZ12, H3K27Ac, H3K27me3, or control IgG then amplified by PCR with a primers' set specific for the −1529 to −1383 region.



B) Our results are consistent with SUZ12-ChIP sequencing data from ENCODE showing SUZ12 binding (in the *yellow box*) in this region of the *FOXE1* promoter (http: genome.ucsc.edu/). *Red boxes* indicate the CpG dinucleotides 13 to 15 of the CpG island 1.

Supplemental tables:

Supplemental table 1: Patient and sample characteristics with listing of data having pass QC check for each assay.

TISSUE	sample ID	patient ID	age (yr) / sex		DATA PASSED QC CHECK FOR THE FOLLOWING EXPERIMENTS				
				Chip ID	MeDIP-chip	FOXE1 bisulfite	FOXE1 pyrosequencing	PAX8 bisulfite	NKX2.1 bisulfite
leucocytes	1-EC	1	12 / F	--	☐	☑	☐	☑	☑
orthotopic thyroid	1-EC	1	12 / F	--	☐	☑	☐	☑	☐
leucocytes	2-LS	2	16 / F	--	☐	☑	☐	☐	☐
orthotopic thyroid	2-LS	2	16 / F	--	☐	☑	☐	☐	☐
ectopic thyroid	3-6F	3	10 / F	9735302	☑	☑	☑	☑	☑
leucocytes	3-6F	3	10 / F	24230702	☑	☑	☑	☑	☐
leucocytes	4-SP	4	16 / F	24278802	☑	☑	☑	☑	☐
orthotopic thyroid	4-SP	4	16 / F	24281002	☑	☑	☑	☑	☐
ectopic thyroid	5-22R	5	8 / F	9736402	☑	☑	☑	☐	☐
leucocytes	5-22R	5	8 / F	24264702	☑	☐	☑	☐	☐
ectopic thyroid	6-23V	6	15 / F	10059802	☑	☐	☐	☐	☐
leucocytes	6-23V	6	15 / F	24306002	☑	☐	☐	☐	☐
orthotopic thyroid	15-DM	15	4 / M	9735502	☑	☐	☐	☐	☐
orthotopic thyroid	16-TMJ	16	18 / F	--	☐	☐	☑	☐	☐
leucocytes	62-MF	17	38 / F	--	☐	☐	☑	☐	☐

Supplemental table 2: Primers, reagents and PCR conditions for amplification of bisulfite treated DNA.

FOXE1 (NCBI Reference Sequence: NT_008470.19)

region	step	internal ID	forward primer	reverse primer	product size	CpGs in product
FOXE1 -1731 to -745 from TSS	1	F7/R7	AAAGGTGTTTATAATATAATTTAGTTAAAA	TAAAAAACCCAAAATCTATCTCTATTTAAT	986	58
FOXE1 -1600 to -1140 from TSS	2	F8/R8	TTTTGTTTTTTTATTATTTGGGAA	AAAAAATCCAAAACCTATCCTTATC	533	34
FOXE1 -1140 to -880 from TSS	2	F9/R9	AATAGAGTAGAATATTGAGGTTTTG	AACAACCAAACAACCCCTTC	300	19
FOXE1 -1023 to + 14 from TSS	1	F10/R10	AGGAAAGGAATTTAGAAATTGGATATT	ACTTCCCCTCCTTAAAAAAACC	1010	69
FOXE1 -660 to -125 from TSS	2	F5b/R5b	AAGGATTTTTGAGAGATTTAGATTT	CTCCCCAAATAAAAACAACTATAAC	589	35

	initial denaturation-0	denaturation-1	annealing-2	amplification-3	cycles (1 to 3)
FOXE1 F7/R7	95 C x 5 min	94 C x 1 min	54 C x 2 min	72 C x 3 min	35
FOXE1 F8/R8	95 C x 5 min	94 C x 1 min	54 C x 2 min	72 C x 3 min	35
FOXE1 F9/R9	95 C x 5 min	94 C x 1 min	58 C x 2 min	72 C x 3 min	35
FOXE1 F10/R10	95 C x 5 min	94 C x 1 min	56 C x 2 min	72 C x 3 min	35
FOXE1 F5b/R5b	95 C x 5 min	94 C x 1 min	58 C x 2 min	72 C x 3 min	35

PAX8 (GenBank accession number AB062505.1)

region	step	internal ID	forward primer	reverse primer	product size	CpGs in product
PAX8 -816 to	1	F1/R1	TTTTTGGTGGTTAGTGATTTAGGAG	TAAAACCAACCTCAACCTAACCTAA	639	23
PAX8 -635 to -471 from ATG	2	F2/R2	ATGTTTTTTTTGTAAAATTTTTTTT	CTACAAACCCTCACTACTTAAATCC	213	13

	initial denaturation-0	denaturation-1	annealing-2	amplification-3	cycles (1 to 3)
PAX8 F1/R1	95 C x 5 min	94 C x 1 min	54 C x 2 min	72 C x 3 min	35
PAX8 F2/R2	95 C x 5 min	94 C x 1 min	58 C x 2 min	72 C x 3 min	35

NKX2.1 (GenBank accession number AB062425.1)

region	step	internal ID	forward primer	reverse primer	product size	CpGs in product
NKX2.1 F1/R1	1	F1/R1	TTTTTATTTTATTTTTTGTTTTTTT	ACCAAATATTCTAATATTACCTTAAC	441	21
NKX2.1 F2/R1	2	F2/R1	TTGAGATTTAAAAATTTTGATAAGT	AACAAATATTCTAATACCTTAAC	276	14

	initial denaturation-0	denaturation-1	annealing-2	amplification-3	cycles (1 to 3)
NKX2.1 F1/R1	95 C x 5 min	94 C x 1 min	54 C x 2 min	72 C x 3 min	35
NKX2.1 F2/R1	95 C x 5 min	94 C x 1 min	58 C x 2 min	72 C x 3 min	35

PCR MIX FOR ALL REACTIONS

	volume	final concentration
gDNA or 1st PCR product	5 µl	
Forward primer 30 µM	1 µl	600 nM
Reverse primer 30 µM	1 µl	600 nM
Bisulfite PCR buffer 10x**	5 µl	
dNTPs 2.5 mM each	4 µl	0.2 mM
ddH2O	33.6 µl	
Platinium Taq 5u/µl	0.4 µl	2 units
TOTAL	50 µl	

**Bisulfite PCR buffer 10x: $(NH_4)_2SO_4$ 166 mM; Tris (pH 8.8) 670 mM; $MgCl_2$ 67 mM and ß-mercaptoethanol 100mM

C) REFERENCES

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CHAPTER 3: GENERAL DISCUSSION

The main goal of this thesis was to assess the association of postzygotic (somatic) *de novo* events (genetic and/or epigenetic) with CH secondary to thyroid ectopy, a disorder that is mainly sporadic and is highly discordant between MZ twins. Recently, the importance of somatic mutations, including CNVs, in a various number of human diseases has been demonstrated (Erickson, 2010). Indeed, early postzygotic mutational events resulting in somatic mosaicism have been identified in MZ twins discordant for otopalatodigital syndrome (OPD) spectrum disorders (Robertson *et al.*, 2006), Proteus syndrome (Saul *et al.*, 1990; Brockmann *et al.*, 2008), and for Dravet's syndrome (Vadlamudi *et al.*, 2010). In this regard, two main approaches were applied: 1) **a genome-wide approach,** which involved the integrative molecular analysis of ectopic thyroid tissues compared to eutopic (normally located) thyroids and 2) **a candidate-gene approach,** which mainly focused on *FOXE1*, the only transcription factor that has been shown to result in thyroid ectopy in KO mice.

With regards to the **genome-wide approach**, two major outcomes were obtained: (i) the first article presented in this thesis reveals the enrichment of genes involved in the regulation of the Wnt signalling pathway in the transcriptome of ectopic thyroids, a finding that was independent of genetic (CNVs) and epigenetic (promoter and CpG island methylation) events at the level of definition used in the analysis (Abu-Khudir *et al.*, 2010) and (ii) surprisingly, the expression level of calcitonin was similar between ectopic and eutopic thyroid glands , a fact that was confirmed by immunohistochemistry and reverse transcriptase PCR (RT-PCR) in our second article (Vandernoot *et al.*, 2012). Indeed, the Wnt signalling pathway is involved in the development of various organs and tissues (Grigoryan *et al.*, 2008; Petrie *et al.*, 2009; Minami *et al.*, 2010; Sugimura and Li, 2010; Lade and Monga, 2011; Rodriguez-Seguel *et al.*, 2013). Moreover, **a vital role of Wnt signalling for thyroid organogenesis** is further supported by recent zebrafish studies showing thyroid abnormalities in transgenic *tg(hsp70l:dkk1)* and *tg(hsp70l:wnt8)* embryos in which the activity of the Wnt pathway was globally modulated via heat-shock treatment during different steps of thyroid organogenesis (Opitz & Costagliola, unpublished data).

As for the **candidate gene approach**, our article on the epigenetic control of *FOXE1* revealed a tissue-dependent DMR in *FOXE1* promoter upon comparing leukocytes with thyroid

tissues and cell lines. Given the described functional impact of the reported DMR on *FOXE1* expression, we believed that this differentially methylated region is a hotspot for functional variants associated with CHTD, a hypothesis that seemed to be confirmed by our recent preliminary results as will be discussed later in the perspectives.

1. Genome-wide approach

The two main outcomes of our genome-wide approach were: (i) the association of the Wnt signalling pathway with thyroid ectopy and (ii) the conserved (and unexpected) expression of calcitonin in dysgenetic ectopic thyroid.

1.1. Wnt signalling and thyroid ectopy

In an attempt to assess whether the transcriptome of ectopic thyroids is shaped by somatic genomic or epigenomic variations, we conducted the first integrative analysis of transcriptome, DNA methylation and structural genomic variations (CNVs) in ectopic thyroids (Chapter 2, section 1, Figure 1). It is noteworthy that, novel genetic regulators of cancer have been identified by applying a similar integrative profiling approach (Adler *et al.*, 2006; Sadikovic *et al.*, 2009; Kresse *et al.*, 2012).

As our results indicated, microarray analysis of the genome-wide RNA expression profile revealed a differential gene expression in ectopic compared to normal orthotopic thyroids (Chapter 2, section 1, Table 1). Indeed, the highly differentially expressed genes and genes known to play a role in thyroid function (n=100) were validated using quantitative real-time PCR (qRT-PCR), which showed a highly significant correlation with microarray analysis (Chapter 2, section 1, Figure S1). Functional annotation of the differentially expressed genes showed that two of the top three clusters are enriched for developmental processes (Chapter 2, section 1, Table S1). Moreover, our functional annotation revealed that the top five clusters of genes induced in ectopic thyroids encompassed genes important for development, vasculogenesis, the extracellular matrix, immune system development and collagen, while the top five clusters for repressed genes included genes important for histone function, apoptosis, chromatin function, organelle and contractile functions (Chapter 2, section 1, Table S2).

According to our results, pathway distribution of the differentially expressed, induced genes in ectopic thyroids showed their enrichment for eight pathways, three of which are

associated with cell-to-cell interaction (MAPK signaling pathway, focal adhesion and cell communication) (Chapter 2, section 1, Figure 2). Interestingly, four of the identified pathways, including focal adhesion, antigen processing and presentation, cell communication, cell adhesion molecules, and Type I diabetes pathways were independently identified in "hyperfunctioning" thyroid nodules from familial non-autoimmune hyperthyroidism (FNAH) patients (Hébrant *et al.*, 2009). Contrary to our findings, Hébrant *et al.* found genes that were down-regulated in thyroid tissues of FNAH patients, including those involved in focal adhesion and cell adhesion that mediate cell/cell as well as cell/extracellular matrix (ECM) interactions (Hébrant *et al.*, 2009). In addition, they and others also described down-regulated genes in FNAH tissues and in thyroid autonomous adenomas which are involved in the immune response and inflammation, including antigen processing and presentation (Wattel *et al.*, 2005; Hébrant *et al.*, 2009).

Due to the difference in the TSH-dependent activation state of ectopic and orthotopic thyroid tissues, we have extended our gene expression analysis of the 100 validated genes to normally located "hyperfunctioning" thyroid nodules, occurring mainly due to constitutively activating TSH receptor (TSHR) mutations (Parma *et al.*, 1993; Paschke and Ludgate, 1997). Indeed, genes whose expression is dependent on TSH-driven thyroidal activity, enriched for thyroid hormonogenesis and function, were excluded (Chapter 2, section 1, Table S4). We believe that the list of TSH-dependent genes described in this study represents the most exhaustive list of TSH-driven genes published to date, and many hits were reported by either previous or subsequent publications from other groups (Sato *et al.*, 1995; Matowe *et al.*, 1996; de Cristofaro *et al.*, 2012). In addition, the list recapitulates all the known TSH-dependent genes (*TSHR*, *TPO*, *Tg*, *PAX8*, *DUOX2*), substantiating the validity of our data. Moreover, it also proposes a lot of new hits related to cell-cell interactions (e.g. cadherin 2 and 16, claudin, and fibronectin 1).

On the other hand, the expression of 19 genes is dependent only on thyroid localization. This group of 19 genes is enriched for the Wnt signalling pathway, the dendritic cell maturation pathway, and embryonic developmental functions (Chapter 2, section 1, Table 2). Of note, none of the genes encoding the thyroid related transcription factors *FOXE1*, *HHEX*, *NKX2.1*, *NKX2.5* showed decreased expression in ectopic thyroids when compared to either normal or "hyperfunctioning" thyroids (Chapter 2, section 1, Table 3).

Although we have shown that the differential expression determined in our ectopic thyroids is independent of the DNA methylation profile of the glands (Chapter 2, section 1, Figure 1), the role of histone modifications cannot be ruled out. Various histone modifications enriched in promoter regions are associated with either active or repressed genes (Bernstein *et al.*, 2005; Kim *et al.*, 2005; Roh *et al.*, 2005; Vakoc *et al.*, 2005; Boyer *et al.*, 2006; Lee *et al.*, 2006; Squazzo *et al.*, 2006). Moreover, crosstalk between DNA methylation and histone modifications has been previously reported (Fuks, 2005; Cedar and Bergman, 2009; Ikegami *et al.*, 2009). Our results have shown that the expression of genes involved in histone modifications and chromatin function are repressed in ectopic thyroids (Chapter 2, section 1, Table S2), thus their probable contribution to the differential expression observed in ectopic thyroids deserves further investigation.

However, none of our validated common CNVs were correlated with the differentially expressed genes detected in the ectopic thyroid tissues (Chapter 2, section 1, Figure 1 and Table S5). Recently, potentially pathogenic germline CNVs were identified in TD patients, the majority of which have not been previously associated with TD (Thorwarth *et al.*, 2010). The high rate of CNVs identified in the screened TD patients is in favor of the involvement of these genetic variants in the etiology of TD. However, their lack of recurrence and their lack of enrichment among the screened patients points to the importance of considering not only these candidate genes but the pathways in which they play a role (Thorwarth *et al.*, 2010). Such a consideration is in support of our findings revealing the enrichment of pathways involved in cellular movement, among which is the Wnt signalling pathway, in our analyzed ectopic tissues.

An association between the Wnt signalling pathway and abnormal thyroid migration during development is biologically acceptable. In multicellualr organisms, the Wnt signalling pathway is involved in various aspects of embryonic development and adult tissue homeostasis. Consequently, its aberrant regulation leads to serious developmental defects or pathological conditions, especially cancer, later in adult life (Barker, 2008; Giunta, 2009; Herr *et al.*, 2012). The Wnt ligands induce several signalling pathways: the beta (β)-catenin-dependent signalling pathway (canonical pathway) and the β-catenin-independent pathways (non-canonical pathway), including the Planar cell polarity (PCP) signalling and the Wnt–Ca^{2+} pathway (Figure 13) (Sastre-Perona and Santisteban, 2012).

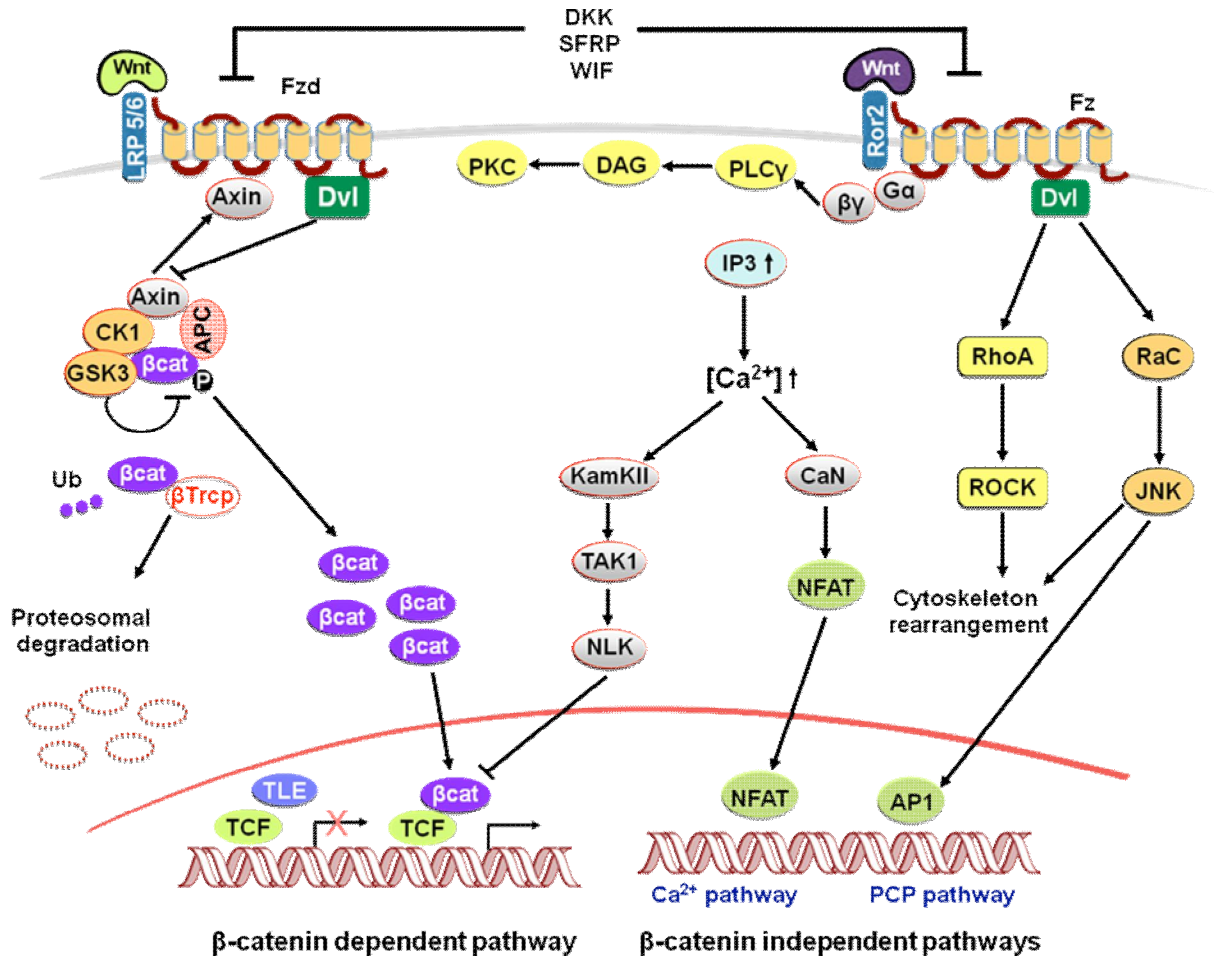

Figure 13: The Wnt signalling pathways (adapted from Sastre-Perona and Santisteban, 2012).

The non-canonical Wnt signalling pathway mediates cell migration and polarity during embryogenesis (Veeman *et al.*, 2003; Kohn and Moon, 2005) and several animal studies have supported that role (Rauch *et al.*, 1997; Heisenberg *et al.*, 2000; Hamblet *et al.*, 2002; Wallingford and Harland, 2002; Curtin *et al.*, 2003; Wang *et al.*, 2006; Etheridge *et al.*, 2008; Yen *et al.*, 2009). Moreover, mouse mutants for the Wnt antagonists *Sfrp1*, *Sfrp2*, and *Sfrp5* provide additional evidence for the importance of the Wnt/PCP signalling pathway in regulating the morphogenetic movements during mammalian gastrulation (Satoh *et al.*, 2008).

In *Xenopus* and zebrafish, Wnt signalling is known to play an essential role during gastrulation and endoderm fate determination (Zorn *et al.*, 1999; Schier and Talbot, 2005; Zorn and Wells, 2007), in endoderm patterning (McLin *et al.*, 2007; Goessling *et al.*, 2008), and subsequently, Wnt signals enhance the specification and differentiation of the endoderm-derived

organs including lungs, liver, pancreas, stomach, and intestine (Murtaugh, 2008; Verzi and Shivdasani, 2008; Goss *et al.*, 2009; Lade and Monga, 2011; Poulain and Ober, 2011). In *Xenopus*, it has been suggested that endoderm patterning is mediated via several Wnt ligands that signal via both **the canonical and non-canonical (Wnt/JNK) pathways** which promote hindgut fate and morphogenesis in the posterior endoderm, respectively (McLin *et al.*, 2007; Li *et al.*, 2008; Reed *et al.*, 2009).

Moreover, analysis of conditional loss- and gain-of-function mutations of β-catenin in mice revealed the crucial role of the canonical Wnt/β-catenin signalling pathway in mammals where it controls the development of numerous tissues and organs including endoderm-derived ones (e.g. intestine, pancreas, liver, and lung) (Grigoryan *et al.*, 2008). **The canonical Wnt/β-catenin signalling** is implicated in liver bud expansion and differentiation, postnatal growth and development as well as liver regeneration and carcinogenesis (Lade and Monga, 2011; Tremblay, 2011). Similar to what is observed in *Xenopus* and zebrafish (McLin *et al.*, 2007; Goessling *et al.*, 2008; Li *et al.*, 2008), the Wnt/β-catenin signalling is required for the specification of pancreas during early endoderm development in murine and is implicated as well in fetal pancreas growth and/or differentiation, especially for exocrine acinar cells. The role of Wnt/β-catenin signalling in development or function of endocrine islets is a matter of debate. However, independent studies in mouse and humans have pointed to the role of the signalling pathway in maintaining adult islet function (Murtaugh, 2008). Recently, **the non-canonical Wnt pathway** has been identified as a potential developmental regulator of the pancreatic versus liver cell fate specification, in a β-catenin-independent manner, in *Xenopus* embryos and mammalian cells (Rodriguez-Seguel *et al.*, 2013).

Earlier, Helmbrecht *et al.* have described a functional Wnt/β-catenin signalling pathway in normal human thyroid cells that expressed the major effectors of the pathway including the Wnt proteins (Wnt-2, Wnt-3, Wnt-4, Wnt-5a, and Wnt-10b), members of the Frizzled (Fzd) receptor family (Fzd-1, -2, and -6), and the intracellular effectors the Disheveled (DVL) isoforms DVL-1, -2, and -3. In addition, the β-catenin degradation complex consisting of β-catenin, the tumor suppressor Adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β), was detected in human thyroid cells (Helmbrecht *et al.*, 2001). Moreover, an increase in rat and human thyroid cell proliferation has been identified upon TSH-dependent over-expression of Wnt1 and inhibition of GSK3β by adenoviral-interference (Kim *et al.*, 2007; Chen

et al., 2010b). Taken together, these data point to the existence of a functional Wnt pathway in thyroid cells that is related to their proliferation. On the other hand, in the mouse embryo, no nuclear β-catenin could be detected in thyroid progenitor cells, thus indicating that the canonical Wnt/β-catenin pathway seems to be inactive during specification and migration of the thyroid anlage in mice (Fagman *et al.*, 2003).

Several studies have pointed to the role of **the canonical Wnt/β-catenin pathway** in thyroid cancer (Sastre-Perona and Santisteban, 2012). The contribution of Wnt/β-catenin signalling pathway to thyroid carcinoma is supported by the modulation of the pathway via the thyroid hormone T_3 and its receptor TRβ in a mouse model of the well-differentiated follicular thyroid carcinoma harboring a dominant negative mutation of TRβ (TRβPV/PV mice) (Guigon *et al.*, 2008; Lu *et al.*, 2012). On the other hand, activation of **the non-canonical** Wnt/Ca^{2+} signalling pathway via the increased expression of its activator Wnt5a leads to inhibition of growth, migration, invasiveness, and clonogenicity in a thyroid carcinoma cell line. These effects were related to the antagonistic action of Wnt-5a to the canonical Wnt signalling (Kremenevskaja *et al.*, 2005).

Among the identified genes that exhibited convergent expression in our analyzed ectopic thyroid tissues are *SFRP2* and *FRZB*, encoding the negative modulators (antagonists) SFRP2 and SFRP3, respectively, of the Wnt signalling pathway (Chapter 2, section 1, Table 2). The secreted frizzled-related proteins (SFRPs), are among the antagonists which directly interact with Wnt proteins or Frizzled (Fzd) receptors and block all Wnt signalling pathways (Kawano and Kypta, 2003). However, it has been recently demonstrated that SFRPs can either promote or suppress Wnt/β-catenin signalling, depending on their concentration, cellular context, and the expression pattern of Fzd receptors (Xavier *et al.*, 2014). As recently reviewed by Surana *et al.*, down-regulation of SFRPs, mainly via DNA hypermethylation, has been reported in a variety of malignancies, not including the thyroid, and this is often correlated with poor prognosis (Surana *et al.*, 2014). In addition, the inhibitory capacity of SFRPs (SFRP1, SFRP2 or SFRP3) on migration, invasion, and growth of a number of cancer cells has been previously demonstrated. These inhibitory actions occur via attenuation of either the canonical or non-canonical Wnt signalling pathway (Roth *et al.*, 2000; Chung *et al.*, 2009; Kongkham *et al.*, 2010; Ekström *et al.*, 2011).

1.2. Expression of calcitonin in ectopic lingual thyroids

In our genome-wide gene expression study of ectopic thyroids (Gene Expression Omnibus, accession no. GSE16804), we unexpectedly observed no differences in the expression level of calcitonin between ectopic and normal (orthotopic) thyroids, thus suggesting the presence of calcitonin-producing C cells in ectopic lingual thyroids (Abu-Khudir *et al.*, 2010). In our second article (Chapter 2, section 2), we confirmed the presence of calcitonin-producing C cells in six independent ectopic lingual thyroids (Chapter 2, section 2, Figures 1 and 2), an observation that was further confirmed on two additional paraffin-embedded ectopic thyroids (J. Deladoëy, unpublished observation). This unprecedented finding in non-tumoral ectopic thyroids contradicts current knowledge of the embryological development of the thyroid.

During the embryonic development of the human thyroid, the ultimobranchial bodies (UBB), considered to be the only source of the C cells (Pearse and Carvalheira, 1967), merge with the primitive thyroid at around embryonic days (E) 60 i.e. ten days after the thyroid bud reaches its final location in front of the trachea at E45–50 (De Felice and Di Lauro, 2004). Accordingly, C cells are not expected to be present in lingual ectopic thyroids, tissues that failed to complete their migratory path. Consequently, our finding indicates that the UBB are not the sole source of calcitonin-producing C cells in humans and that the interactions between calcitonin-producing and TFCs occur earlier than previously accepted. Our results showing that human ectopic lingual thyroids contain calcitonin producing C cells are indirectly supported by the previously reported observations showing the presence of low but detectable circulating calcitonin levels in patients with CHTD (Chanoine *et al.*, 1990; Body *et al.*, 1993) and the detection of medullary carcinoma (cancer arising from C cells) in a lingual thyroid (Yaday *et al.*, 2008).

Several hypotheses can be generated to explain the cellular origin of calcitonin-producing C cells in ectopic lingual thyroids. First, C cells can occur in ectopic thyroids subsequent to the differentiation of some TFCs towards C cells (TFCs transdifferentiation). Transdifferentiation of various cell types towards C cells is observed either in non-tumoral or in tumoral tissues. Non-tumoral ectopic thyroid masses have been previously reported in the thoracic or abdominal cavities (Bando *et al.*, 1993; Muysoms *et al.*, 1997; Casanova *et al.*, 2000; Gungor *et al.*, 2002). Also, non-tumoral ectopic thyroid follicles have been identified in the submucosa of the

duodenum, though stained negative for calcitonin (Takahashi *et al.*, 1991). For tumoral tissues, it has been demonstrated that properties of C cells, represented by secretion of calcitonin, are acquired by some tumor cells (e.g. carcinomas of breast, lung, and adrenal medulla) after switching their differentiation program (Coombes *et al.*, 1976; Hillyard *et al.*, 1976; Calmettes, 1984). Another explanation for the presence of calcitonin-producing C cells in ectopic lingual thyroids is the *in situ* differentiation of pluripotent stem cells to both TFCs and C cells within the ectopic thyroid. Similarly, the occurrence of medullary and either papillary or anaplastic components in the thyroid may suggest that the tumor had arisen from a common stem cell (Shiroko *et al.*, 2001; Tohidi *et al.*, 2013). On the contrary, it has been previously suggested that UBB cells could differentiate into TFCs and might be a source of both follicular as well as C cells (Weller, 1933; Williams *et al.*, 1989). However, some authors maintain that most of the follicular content of the thyroid originates from the median anlage, while the lateral thyroid is derived from UBB (Bersaneti *et al.*, 2011). Moreover, the presence of cystic structures in cases of ectopic thyroids containing both TFCs and C cells suggests that these intracystic follicular cells are derived from the UBB (Williams *et al.*, 1989). Finally, a less likely considered explanation for the presence of C cells in ectopic thyroids is the aberrant migration of the UBB during thyroid development, where none has been previously reported. In addition, the recurrent occurrence of such a developmental defect in the analyzed cases (n=6) is unlikely.

2. Candidate gene approach

In order to determine the role of somatic epimutations in the pathogenesis of CHTD, in this section of the thesis, we sought to determine whether DNA methylation differences exist between ectopic and normally located (orthotopic) thyroids within the promoter regions of the human thyroid-specific transcription factors *FOXE1, PAX8* and *NKX2.1*. As previously mentioned, we focused our attention on *FOXE1*, the only thyroid-specific transcription factor that has been shown to result in ectopic thyroid in knockout mice (Parlato *et al.*, 2004).

As our integrative molecular analysis of ectopic and orthotopic thyroids has shown (Abu-Khudir *et al.*, 2010), no differences in promoter methylation profiles of the candidate genes *FOXE1, PAX8* and *NKX2.1* were observed between the two types of thyroid tissues. Finding no differentially methylated region(s) DMR(s) in thyroid tissues differing only in their location (ectopic versus eutopic) might be anticipated. However, we believed that a DMR(s) is(are) likely

to exist between thyroid and leukocytes, two tissues exhibiting different expression profiles. In this regard, we sought to determine whether the promoter methylation profile was different between thyroid tissue and leukocytes using MeDIP arrays or bisulfite sequencing. Of note, association between DMRs and genetic as well as epigenetic variations has been previously proposed, thus they are considered as hotspots for disease-associated mutations (Beaudet and Jiang, 2002; Cooper *et al.*, 2010).

As previously mentioned in the introductory chapter, DNA methylation is the most common epigenetic modification (Dolinoy and Faulk, 2012; Moore *et al.*, 2013). It involves the covalent addition of a methyl group to the 5′-position of cytosine in the context of 5′-CpG-3′ dinucleotides, a process catalyzed and maintained by enzymes of the DNA methyltransferase (DNMT) family (Auclair and Weber, 2012; Kohli and Zhang, 2013; Moore *et al.*, 2013). During development, DNA methylation plays a crucial role in the establishment of tissue-specific patterns of gene expression, where various patterns of DNA methylation exist between different tissues (Futscher *et al.*, 2002; Ching *et al.*, 2005; Klose and Bird, 2006; Bogdanović and Veenstra, 2009). Several genome-wide studies have shown that distinct regions of the mammalian genome exhibit a tissue-dependent pattern of DNA methylation, known as tissue-specific differentially methylated regions (T-DMRs) (Song *et al.*, 2005; Eckhardt *et al.*, 2006; Khulan *et al.*, 2006; Kitamura *et al.*, 2007; Illingworth *et al.*, 2008; Rakyan *et al.*, 2008; Byun *et al.*, 2009; Maunakea *et al.*, 2010). Tissue-specific DMRs are found in either CpG-rich or -poor DNA sequences (Ohgane *et al.*, 2008), with a high prevalence in CpG-poor regions in both the human and mouse genome (Rakyan *et al.*, 2008; Yagi *et al.*, 2008; Byun *et al.*, 2009; Liang *et al.*, 2011; Nagae *et al.*, 2011). Such a finding is supported by the evident existence of tissue-specific DNA methylation in regions outside CGIs or CGI flanking regions (Slieker *et al.*, 2013). Moreover, it has been shown that T-DMRs, identified in multiple peripheral and internal human tissues, are mapped to genes with tissue-specific expression. T-DMRs are hypomethylated specifically in the tissue expressing those genes, which is in accordance with the inverse relationship between DNA methylation and gene expression (Slieker *et al.*, 2013). Recently, Zhang *et al.* have shown that in humans, DMRs are robustly associated with gene regulatory elements, promoters and/or enhancers, together with the active chromatin marks H3K4me3 and H3K4me1, respectively. In addition, they have shown that DNA methylation of promoters can be implicated in major cell lineage determination, whereas methylation of enhancer elements might

be involved in modulating gene expression and allowing the cells to possess a final lineage commitment or maintain a distinct cell fate (Zhang *et al.*, 2013).

As mentioned above, we sought to locate T-DMRs in the promoters of the *FOXE1, PAX8* and *NKX2.1* genes that might be involved in their differential expression. Our results have shown that genome-wide methylation profiling and bisulfite sequencing of CGIs located within the promoters of *PAX8* and *NKX2.1* revealed no T-DMR between thyroid and leukocytes. However, **we described a T-DMR in the *FOXE1* promoter**. Bisulfite sequencing revealed that the reported DMR in CpG island 1 of *FOXE1* promoter (−1600 to −1140 from the transcription start site (TSS; +1)) was globally more methylated in leukocytes compared to thyroid tissues (Chapter 2, section 2, Figure 2A and B). Moreover, within the DMR, the major methylation targets were the two consecutive CpG dinucleotides, CpG_{14} and CpG_{15}, which were not covered by the genome-wide array. The two CpG dinucleotides showed a significantly higher methylation rate in leukocytes when compared to thyroid tissues (Chapter 2, section 2, Figure 2A and B). These results were validated using bisulfite pyrosequencing (Chapter 2, section 2, Figure 3), thus suggesting that methylation of this region might be involved in the modulation of *FOXE1* gene expression.

According to our results, the methylation status of the reported DMR was correlated with *FOXE1* mRNA expression, with complete lack of expression in leukocytes versus a clearly detected one in thyroid tissues (Chapter 2, section 2, Figure 4A). DNA methylation can inhibit gene expression at different levels. In addition, the efficiency of repression is affected by various parameters, including position, length, and density of the methylated CpG dinucleotides (Kass *et al.*, 1993; Hsieh, 1994; 1997). Moreover, the relationship between gene repression and number of modified CpGs is nonlinear with an obvious overall level or a threshold of promoter methylation effect. Hence, transcriptional repression can spread only when a sufficient amount of CpGs are methylated (Curradi *et al.*, 2002; Choi *et al.*, 2009). In accordance with this threshold-dependent methylation-mediated model of repression, expression of *FOXE1* in our thyroid tissues is not hindered, in spite of low-levels of overall methylation, (Chapter 2, section 2, Figures 2B and 4A). This also explains the lack of significant difference in *FOXE1* expression among thyroid tissues, even though the eutopic tissue #2 showed an intermediate-low level of methylation in CpG_{14} and CpG_{15}, the two CpGs shown to modulate the differential expression of *FOXE1* as will be discussed later (Chapter 2, section 2, Figures 2B and 4A).

Several studies have pointed to the association between aberrant methylation patterns of *FOXE1* and a number of different cancers (Sato *et al.*, 2003; Rush *et al.*, 2004; Kuang *et al.*, 2008; Weisenberger *et al.*, 2008; Venza *et al.*, 2010; Bell *et al.*, 2011; Vincent *et al.*, 2011; Papadia *et al.*, 2014). Moreover, reactivation of *FOXE1* expression has been reported in a number of pancreatic and cutaneous SCC cancer cell lines. The reactivation of expression occurred following treatment of the cells with the demethylating agent 5-Aza-2′-deoxycytidine (5-AZA-dc), thus supporting the role of DNA methylation in regulating *FOXE1* expression (Sato *et al.*, 2003; Venza *et al.*, 2010).

As our results indicated, the methylation status of the reported T-DMR in the human leukemia cell lines (Jurkat, K562, and REH) ranged from moderate (K562) to high (Jurkat and REH) methylation (Chapter 2, section 2, Figure 2C). Such a methylation profile was correlated with the lack of *FOXE1* expression in the mentioned cell lines (Chapter 2, section 2, Figure 4B). Previously, Kuang *et al.* have analyzed *FOXE1* promoter-associated CGI methylation in various leukemia cell lines, including Jurkat, K562, and REH (Kuang *et al.*, 2008). They have shown that the region −673 to −124 from TSS (upstream of and within CpG island 2) is hypermethylated in the investigated leukemia cell lines. According to our results, this region showed no tissue-specific DNA methylation differences between leukocytes and thyroid tissues (Chapter 2, section 2, supplementary figure 3B), thus, we did not determine its methylation status in the leukemia cell lines. It should be noted that our reported T-DMR (encompassing CpG island 1) was not covered on the proximal promoter microarray used by Kuang *et al.* (Kuang *et al.*, 2008).

Contrary to what we have observed in the human leukemia cell lines, the DMR was almost unmethylated in the dedifferentiated thyroid cancer cells WRO (Chapter 2, section 2, Figure 2C). In accordance with previous studies (van Staveren *et al.*, 2007; Abu-Khudir *et al.*, 2010), we have reported that *FOXE1* expression was faintly detected in WRO cells (Chapter 2, section 2, Figure 4B). Indeed, it is quite interesting to show that *FOXE1* expression is slightly conserved even in this dedifferentiated cell line which shows no methylation at the two CpGs 14 and 15. As our results indicate, the low expression levels of *FOXE1* in WRO cells implies the involvement of other mechanisms, such as partial repression through the polycomb repressive complex 2 (PRC2), as suggested by our chromatin immunoprecipitation (ChIP) assay results (Chapter 2, section 2, supplemental figure 4A). The main function of PRC2 complex is to repress gene expression through imposing trimethylation on histone H3 lysine 27 residue (H3K27me3)

(Cao *et al.*, 2002). It has been proposed that prevention of the active mark histone H3 lysine 27 acetylation (H3K27Ac) by PRC2 is a mechanism by which the complex mediates repression of transcription (Pasini *et al.*, 2010). Hence, the observed partial repression of *FOXE1* expression in WRO cells is attributed to the partial conservation of the H3K27Ac mark. Unmethylayed CGIs play a key role in the recruitment of PRCs and most PRC2 target genes remain constitutively unmethylated throughout development (Cedar and Bergman, 2009; Lynch *et al.*, 2012). In cancer, *de novo* methylation of these CGIs is triggered, probably via mechanisms involving the interaction between enhancer of zeste homolog 2 (EZH2), the histone methyltransferase component of PRC2, and DNA methyltransferases (Viré *et al.*, 2006).

Our *in vitro* results demonstrated the impact of global DNA methylation of different *FOXE1* promoter constructs on the activity of the reporter gene, where strong transcriptional repression of the luciferase activity was seen (Chapter 2, section 2, Figure 5). Moreover, our data indicate that regional (patch) methylation of the wild type T-DMR (encompassing CpG island 1 with wild type CpG_{14} and CpG_{15}) resulted in a significant decrease in reporter gene activity in comparison to mock-methylated control. In contrast, no significant difference in the activity was observed upon point mutating the two CpG dinucleotides (Chapter 2, section 2, Figure 6), thus indicating their involvement in modulating the expression of *FOXE1*. This finding is in consistent with the current model depicting the relationship between DNA methylation and gene expression. According to this model, CpG dinucleotides residing close to the TSS (<2 kb upstream or downstream of TSS) i.e. within the promoter region, whether or not they are located in CGIs, correlate negatively with gene expression, hence being hypermethylated leads to lower levels of gene expression (Varley *et al.*, 2013). Of note, several studies have pointed to the correlation between site-specific CpG methylation and either the repression or reduction of tissue or cell-specific gene expression (Ben-Hattar and Jiricny, 1988; Wicki *et al.*, 1997; Gonzalgo *et al.*, 1998; Robertson and Jones, 1998; Grant *et al.*, 1999; Boatright *et al.*, 2000; Pogribny *et al.*, 2000; Sato *et al.*, 2011).

Generally, two different mechanisms by which DNA methylation represses gene expression have been identified. First, CpG methylation contributes to transcriptional repression by directly preventing ubiquitous transcriptional regulators (such as E2F or CREB) from binding to their target gene promoters (Iguchi-Ariga and Schaffner, 1989; Campanero *et al.*, 2000; Yoo and Jones, 2006). A second mechanism involves the binding of methyl-CpG-binding proteins

and the subsequent recruitment of repressive complexes such as histone deacetylases (HDACs) that lead to chromatin compaction and in turn transcriptional repression of the gene is an alternative indirect mechanism (Jones *et al.*, 1998; Nan *et al.*, 1998; Wade, 2001; Fuks *et al.*, 2003). Among the methyl-CpG-binding protein, MeCP2 has been shown to bind to as few as one to three methylated cytosines (Nan *et al.*, 1993), thus supporting the notion that site-specific methylation of CpG dinucleotides is involved in transcriptional repression. The methylation of CpGs14-15 of *FOXE1*, could sterically prevent the binding of a transcription factor/complex to a putative regulatory element(s) needed for fine-tuning *FOXE1* gene expression. Hence, identification of possible cis-regulatory elements in CpG island 1 of *FOXE1* will be an interesting subject for future research.

In summary, the results obtained in this thesis provided evidence on the unprecedented plausible association between the Wnt signalling pathway with CHTD secondary to thyroid ectopy. Such an association was attained via the first integrative analysis of ectopic thyroid tissues, which provides a prototype approach for studying other congenital disorders currently unexplained by classical genetics. Moreover, our results have demonstrated the presence of a T-DMR in the promoter of *FOXE1*, the gene encoding the only thyroid-specific transcription factor shown to be associated with thyroid ectopy in animal models. Such a T-DMR is an ideal candidate region for variants associated with CHTD.

CHAPTER 4: CONCLUSIONS AND PERSPECTIVES

Congenital hypothyroidism from thyroid dysgenesis (CHTD) is a common congenital disorder with a prevalence of 1 case in 4,000 live births (Deladoëy *et al.*, 2007b). CHTD occurs consequent to a complete absence of thyroid (athyreosis) or a failure of the thyroid to migrate to its anatomical location (anterior part of the neck), which results in thyroid ectopy (lingual or sub-lingual, the most common form). The majority of CHTD cases has no known cause, but is associated with a severe deficit in thyroid hormones (hypothyroidism), which can lead to severe mental retardation if left untreated. The major contribution of the work presented in this thesis was the identification of novel candidate genes and signalling pathways as plausible mechanisms underlying the pathogenesis of CHTD (Abu-Khudir *et al.*, 2010). In addition, the identification of a T-DMR in the promoter of *FOXE1*, a thyroid transcription factor known to be involved in regulating the migration of the developing thyroid, as it represents an ideal spot for variants that might be associated with CHTD (Abu-Khudir *et al.*, 2014). Moreover, we proposed evidence that contradicts the current knowledge on the normal development of the thyroid gland (Vandernoot *et al.*, 2012). **Finally, we believe that the body of work presented in this doctoral thesis has paved the way for many future avenues of research that will aid in better understanding both the normal and pathogenic development of the thyroid gland. These are briefly summarized below.**

Regarding the **genome-wide approach**, the results of our integrative molecular profiling of human ectopic thyroids revealed the association between genes involved in the Wnt signalling pathway and congenital hypothyroidism due to thyroid ectopy. Such an association was attributed to the differential expression profile of ectopic thyroids, though the molecular basis could not be identified (i.e. independent of promoter methylation and CNVs). This was the motivation behind considering higher definition methodologies aiming to search for rare **genetic variants** associated with defective thyroid migration during embryogenesis. In this respect, we performed the whole-exome of (i) three ectopic thyroids with matched normal tissue (leukocytes) from the same individual (looking for somatic mutations), (ii) of six sporadic cases and one familial case of direct transmission of thyroid ectopy from mother to daughter (looking for rare susceptibility variants). This approach is consistent with the probable multigenic cause of CHTD,

where first hits could be rare inherited or *de novo* germline mutations, while the additional hits could also be a germline or else somatic mutations in a different gene.

Whole-exome sequencing (WES) is an application of the next-generation sequencing (NGS) technologies that focuses on the protein-coding regions, or exons, which constitute 1.5–1.7% of the human genome (Rabbani *et al.*, 2012). These regions of the genome encompass the most interpretable disease-causing genetic variations associated with Mendelian (monogenic) disorders as well as many disease-predisposing single nucleotide polymorphisms (SNPs). In addition, WES is applied in the identification of the genetic bases of various disorders among which are intellectual disorders (Shoubridge *et al.*, 2010; Vissers *et al.*, 2010; Abou Jamra *et al.*, 2011; Caliskan *et al.*, 2011), immunological disorders (Bolze *et al.*, 2010; Byun *et al.*, 2010), somatic mutations and predisposing variants in various cancers (Link *et al.*, 2011; Nikolaev *et al.*, 2012), and others (Bilgüvar *et al.*, 2010; Gilissen *et al.*, 2010; Lupski *et al.*, 2010; O'Roak *et al.*, 2011; Zuchner *et al.*, 2011). With the regard of congenital disorders, novel disease genes have been identified via whole-exome studies in congenital heart disease (Arrington *et al.*, 2012; Zaidi *et al.*, 2013). Of note, mutations in the Pendrin (*SLC26A4*) gene have been identified using exome sequencing in patients with structural thyroid defect (a secondary loss of normal thyroid follicles in the sense of a thyroid atrophy) from consanguineous families, thus extending the variable clinical spectrum of patients with *SLC26A4* mutations (Kuhnen *et al.*, 2014).

As mentioned above, to identify putative genetic variants, we performed WES on ectopic thyroid tissues as well as on leukocytes from sporadic and familial cases. Subsequently, WES data were processed via two approaches, that used different alignment algorithms for single nucleotide variants (SNVs) calling. In order to identify genes of potential interest, data were analyzed with considering gene ontology (GO), possible thyroid expression, and biological relevance. Moreover, results were compared with the transcriptome, methylome, and structural genomic variants of ectopic thyroids from our integrative analysis (Abu-Khudir *et al.*, 2010). **Our preliminary results revealed the occurrence of a recurrent SNV in a novel gene (we called it CH1, a gene involved in cell migration)** in three affected individuals (over 8 individual WES data). Once this potential variant will have been validated, its impact on migration of the human normal Nthy-ori thyroid cells will be determined using the xCELLigence Real-Time Cell Analyzer (RTCA) DP instrument. The xCELLigence system provides

continuous measurement and quantification of cellular migration by measuring electrical resistance.

Considering the **candidate-gene approach**, our results showed a tissue-specific differentially methylated region (T-DMR) in the promoter of *FOXE1*. In light of the previously proposed association between differentially methylated regions (DMRs) and genetic and/or epigenetic variations and hence their consideration as hotspots for disease-associated mutations (Beaudet and Jiang, 2002; Cooper *et al.*, 2010), we decided to screen this DMR in our cohort of CHTD patients using Sanger sequencing searching for a functionally relevant genetic variation. Our results revealed a 1 bp deletion c. −1277delC in the promoter region of *FOXE1* (Figure 14) that is recurrent in our cohort of patients **(27/81; 33,33%)** than controls **(2/84; 2,38%)** and these results have been validated in an independent replication cohort from France (in collaboration with Pr Michel Polak, Hôpital Necker, Paris). Seven independent luciferase reporter gene assays were carried out to assess the functional characteristics of this deletion, which showed a **20%** decrease in promoter activity of the variant construct compared with that of the wild-type construct in Nthy-Ori 3-1 cells (Figure 15). For future work, we will determine the impact of this deletion on DNA-protein interactions using *in vitro* (electrophoretic mobility shift assay or EMSA) and *in vivo* (chromatin immunoprecipitation or ChIP assay).

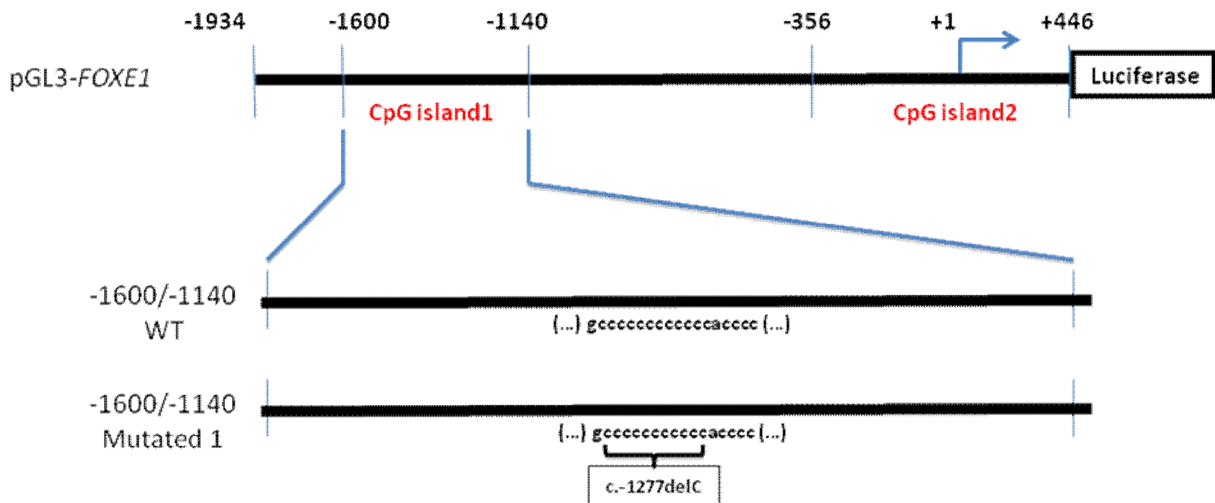

Figure 14: A single base pair deletion in the DMR of *FOXE1* promoter (Magne *et al.*, unpublished data).

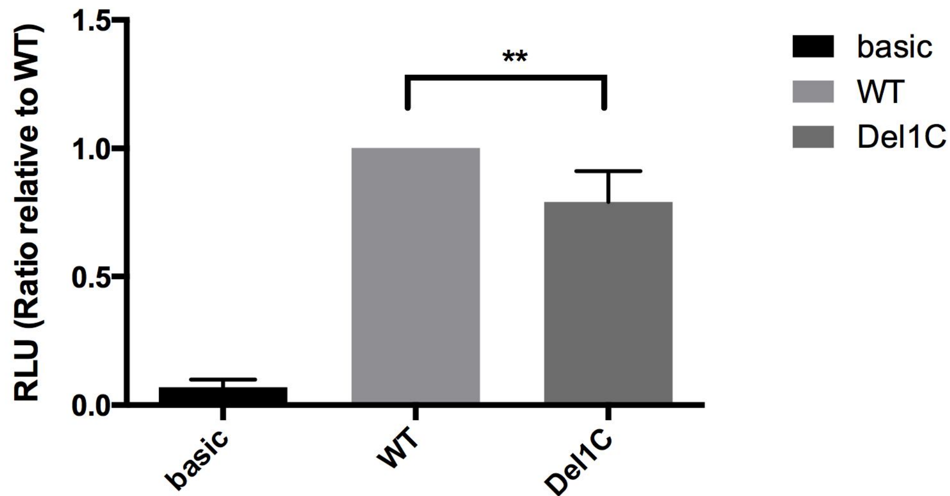

Figure 15: Luciferase assay testing wild type and variant *FOXE1* promoter constructs (Magne *et al.*, unpublished data).

In conclusion, the work in this thesis has helped to further our understanding of the molecular mechanisms involved in thyroid dysgeneis. It has already paved the way for additional studies to explore new genes and pathways involved not only in thyroid embryogenesis, but also potentially in other malformations.

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