Thyroid transcription factors in development, differentiation and disease

Lara P. Fernández, Arístides López-Márquez and Pilar Santisteban

Abstract | Identification of the thyroid transcription factors (TTFs), NKX2-1, FOXE1, PAX8 and HHEX, has considerably advanced our understanding of thyroid development, congenital thyroid disorders and thyroid cancer. The TTFs are fundamental to proper formation of the thyroid gland and for maintaining the functional differentiated state of the adult thyroid; however, they are not individually required for precursor cell commitment to a thyroid fate. Although knowledge of the mechanisms involved in thyroid development has increased, the full complement of genes involved in thyroid gland specification and the signals that trigger expression of the genes that encode the TTFs remain unknown. The mechanisms involved in thyroid organogenesis and differentiation have provided clues to identifying the genes that are involved in human congenital thyroid disorders and thyroid cancer. Mutations in the genes that encode the TTFs, as well as polymorphisms and epigenetic modifications, have been associated with thyroid pathologies. Here, we summarize the roles of the TTFs in thyroid development and the mechanisms by which they regulate expression of the genes involved in thyroid differentiation. We also address the implications of mutations in TTFs in thyroid diseases and in diseases not related to the thyroid gland.


Introduction

Thyroid tissue cells simultaneously express four genes that encode the transcription factors homeobox protein Nkx-2.1 (NKX2-1, also known as thyroid transcription factor 1; encoded by NKX2-1), forkhead box protein E1 (FOXE1, also known as thyroid transcription factor 2; encoded by FOXE1), paired box protein Pax8 (PAX8; encoded by PAX8) and haematopoietically-expressed homeobox protein Hhex (HHEX; encoded by HHEX), which are collectively known as the thyroid transcription factors (TTFs).1–3 Each of the TTFs is expressed in several other tissues in adult humans (Table 1), but they are only expressed together in epithelial thyroid follicular cells. These endoderm-derived cells are the most predominant cell type in thyroid tissue and are dedicated to the synthesis of thyroid hormones.4 Thyroid follicular cells are organized in follicular structures, which constitute the structural and functional units of the adult thyroid gland (which also contains a minority population of calcitonin-producing C-cells).1,2

Although NKX2-1, FOXE1, PAX8 and HHEX are involved in thyroid differentiation, they belong to different families of transcription factors. NKX2-1 and HHEX are both members of the homeodomain family, FOXE1 is a forkhead domain protein and PAX8 is a paired domain family member (Table 1). These four transcription factors present a high degree of conservation across different species.3

Perturbations in levels of expression of the TTFs that result from mutations and/or epigenetic modifications can lead to development of several clinically relevant conditions. Disruptions to thyroid morphogenesis in humans result in phenotypes—such as thyroid agenesis or athyreosis (complete absence of thyroid tissue), ectopia (thyroid displacement), hypoplasia (small thyroid) and hemiagenesis (presence of one-lobe thyroid)—that together are known as thyroid dysgenesis,4,5 which in some cases is accompanied by thyroid dysfunction.6,7 Findings from several studies suggest that defects in the functions of TTFs contribute to thyroid dysgenesis; however, the molecular pathways and mechanisms associated with these phenotypes remain unknown.1

Normal developmental processes and cancers share multiple pathways that are related to cell proliferation and differentiation. Therefore, unsurprisingly, transcription factors that have a role in organogenesis have also been implicated in tumorigenesis. Changes in the expression of genes that encode TTFs and/or sequence variations in these genes have been associated with a number of thyroid and non-thyroid tumours, although the precise mechanisms that drive each of these processes are largely unknown.8 This Review summarizes current knowledge of the functions of TTFs in thyroid development, differentiation and pathogenesis, as well as their involvement in development of other organs, such as the lung and kidney, and their contribution to diseases not related to the thyroid gland.

TTFs

Over the past two decades, seminal work has led to the identification of TTFs and characterization of their functions in thyroid biology. In 1989, NKX2-1 was identified...
as a thyroid-specific transcription factor in a differentiated rat thyroid cell line. The gene encoding this transcription factor was independently cloned shortly thereafter by two groups, who termed this gene Ttf1 and T/ebp. The human NKX2-1 protein contains a 17-amino-acid motif that is conserved among NK2.1 transcription factor family members, and the NKX2-1 gene is expressed in the adult thyroid gland, as well as in lung tissue, basal ganglia neurons, cortical interneurons and the hypothalamus.

The FOXE1 transcription factor was identified in the same study as NKX2-1 and was originally termed TTF2. In 1997, the genes Ttf2 and Fkhhl15, which encode rat and human FOXE1, respectively, were identified. FOXE1 is a single-exon gene encoding a 38–42 kDa protein, which contains a forkhead DNA-binding domain, two putative nuclear localization signals flanking the DNA-binding domain and a polyalanine stretch that ranges from 11 to 19 residues in length. FOXE1 is a pioneer factor, as it has an intrinsic capacity to bind to and open chromatin structures via its winged-helix DNA-binding domain, which functions to facilitate binding of transcription factors to DNA.

In humans, FOXE1 is expressed in a number of tissues, including the thyroid gland, testis, epidermis and hair follicles. Additionally, FOXE1 expression has been detected in human thymus, brain, heart, pancreas, placenta, lung, liver, skeletal muscle, kidney, colon and small intestine.

PAX8 was originally described in the developing excretory system and thyroid gland of mice. As with other members of the paired domain family, PAX8, a 48 kDa protein, contains two DNA-binding domains: a paired domain and a homeodomain. In addition to the thyroid gland, human PAX8 is expressed in the renal excretory system, epithelial cells of the endocervix, endometrium, ovary, Fallopian tube and seminal vesicle, as well as in the epidermis, pancreatic islet cells and in lymphoid cells.

The HHEX gene is comprised of four exons and encodes a 30 kDa protein containing an evolutionarily conserved 60-amino-acid DNA-binding homeodomain. Although HHEX expression was first detected in human haematopoietic cells, the thyroid gland and the liver also express high levels of HHEX.

During mouse organogenesis, Nkx2-1, FoxE1, Pax8 and Hhex are expressed in thyroid cell precursors. Expression of these genes persists in adult thyroid cells to establish and maintain the differentiated thyroid phenotype. The roles of NKX2-1, FOXE1 and PAX8 in binding to DNA and regulating genes that drive thyroid hormone synthesis, such as Tg, Tpo, Slc5a5 and Tshr, have been extensively characterized. However, the number of studies that have addressed the capacity of HHEX to bind and regulate thyroid gene promoters is limited.

### TTFs in thyroid gland development
At embryonic day (E)8.5 of mouse development, cells in the floor of the primitive pharynx coexpress Nkx2-1, Pax8, Foxe1 and Hhex, driving commitment towards a differentiated thyroid fate. At E9.5, these committed cells form a bud, which expands and dissociates from the pharyngeal floor at E10.5–E11.5 and begins a caudal descent, ultimately forming two lobes on either side of the larynx and the upper trachea by E14.5.

### Table 1 | General characteristics of thyroid transcription factors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NKX2-1</th>
<th>FOXE1</th>
<th>PAX8 (Pax8)</th>
<th>HHEX (Hhex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human gene (mouse gene)</td>
<td>NKX2-1 (Nkx2-1)</td>
<td>FOXE1 (Foxe1)</td>
<td>PAX8</td>
<td>HHEX</td>
</tr>
<tr>
<td>Protein aliases</td>
<td>NKX2A, TITF1,</td>
<td>TTF2, TITF2,</td>
<td>None</td>
<td>HEX, PRH, PRHX</td>
</tr>
<tr>
<td></td>
<td>TTF1, T/EBP</td>
<td>FKHHL15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcription factor family</td>
<td>Homeodomain</td>
<td>Forkhead domain</td>
<td>Paired domain</td>
<td>Homeodomain</td>
</tr>
<tr>
<td>Location</td>
<td>14q13.3</td>
<td>9q22.33</td>
<td>2q12-q14</td>
<td>10q23.33</td>
</tr>
<tr>
<td>Number of exons</td>
<td>3</td>
<td>1</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Protein length (amino acids)</td>
<td>371</td>
<td>373</td>
<td>450</td>
<td>270</td>
</tr>
<tr>
<td>Protein weight (kDa)</td>
<td>38</td>
<td>38</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>Protein identity between human,</td>
<td>98</td>
<td>89</td>
<td>95</td>
<td>95</td>
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<tr>
<td>mouse and rat (%)</td>
<td></td>
<td></td>
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<tr>
<td>Non-thyroid-tissue expression</td>
<td>Lung, nervous</td>
<td>Testis, epidermis, hair follicles, thymus, brain, heart, pancreas, placenta, lung, liver, skeletal muscle, kidney, colon, small intestine</td>
<td>Excretory system, endocervix, endometrium, ovary, Fallopian tube, seminal vesicle, epididymis, pancreatic islet cells, lymphoid cells</td>
<td>Liver, haematopoietic cells</td>
</tr>
<tr>
<td>in adult (ref)</td>
<td>system (14–16)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At this time, the thyroid precursor cells have reached their definitive position and Tg expression is activated. Folliculogenesis is complete at E16 and expression of Tshr and Tpo drives the final stages of functional differentiation, resulting in expression of sodium/iodide cotransporter (encoded by Slc5a5) and thyroid hormone biosynthesis at E16.5 (Figure 1).

Small variations in the exact times of the expression of these proteins during thyroid gland development have been reported, and these are likely to be a result of the use of the term ‘embryonic day’ instead of the number of somites in a given study. Several studies have examined the role of TTFs in mouse development (Table 2). Here, we will briefly review important aspects of the contributions of TTFs to thyroid development and highlight unanswered questions and controversial issues.

**NKX2-1**

During development NKX2-1 is expressed in multiple tissues and cell types in addition to the thyroid gland (including the fourth branchial pouch, ultimobranchial body, lung, trachea, posterior pituitary, hypothalamus and medial ganglionic eminence, as well as the parathyroid and C-cells). NKX2-1−/− mice fail to survive after birth as a result of having severe lung malformations, thyroid and pituitary agenesis and anomalies in the ventral forebrain. Specification in NKX2-1−/− mice occurs normally and cells that comprise the primordial thyroid are present at E8.5. However, expression of Tpo and Tshr is not activated until folliculogenesis begins at E16 and day 60 post-fertilization in mice and humans, respectively. At E16.5 in mice and day 70 post-fertilization in humans, the thyroid gland is completely formed and Slc5a5 expression takes place. Synthesis of thyroid hormones T3 and T4 is initiated when the thyroid gland is completely functional. Abbreviation: E, embryonic. Adapted with permission from Elsevier Limited © Macchia, P. E. Recent advances in understanding the molecular basis of primary congenital hypothyroidism. Mol. Med. Today 6, 36–42 (2000).
**FOXE1**

In addition to being expressed in the thyroid gland, FOXE1 is expressed during development of other tissues that are derived from the pharyngeal arches and pharyngeal wall, such as the tongue, palate and oesophagus, as well as in ectoderm-derived organs, such as anterior pituitary, choanae and hair follicles. Although Foxe1 knockout mice are born alive, they fail to survive beyond 2 days post-birth as a result of having a severe cleft palate. These mice can develop with one of two thyroid phenotypes: either thyroid agenesis or thyroid ectopia (usually with a sublingual location of the thyroid). These differences in phenotypes have been proposed to be caused by stochastic events during organogenesis or by the variations in the genetic backgrounds of the mice (Table 2). **Reintroduction of wild-type Foxe1 exclusively into thyroid precursor cells of mice deprived of the endogenous gene restores proper gland positioning, which suggests that FOXE1 functions to regulate migration of these cells.** The expression pattern of FOXE1 throughout pharyngeal floor also suggests that this factor has a minor role in specification of thyroid follicular cell precursors.

**PAX8**

The TTF PAX8 is expressed in the developing thyroid gland, kidneys and myelencephalon. The formation of the primordial thyroid and budding from the primitive pharynx occurs normally in Pax8−/− mice. However, at E11.5, the primitive thyroid in these mice is smaller than in normal mice, and at E12.5 thyroid follicular structures are absent from these animals. Consequently, Pax8−/− mice have thyroid hypoplasia, low birth weight and growth retardation, infertility and only 20% of these animals survive up to 3 weeks of age owing to the effects of severe hypothyroidism. Treatment with levothyroxine increased length of survival of the mice, however, fertility was not recovered in male Pax8−/− mice, as a result of a primary impairment in development of the reproductive system. These phenotypes demonstrate that PAX8 is essential to survival of thyroid precursor cells in the later stages of thyroid development (Table 2). Similar to NKX2-1, PAX8 might contribute to the maintenance of survival of thyroid precursor cells by inhibition of apoptotic mechanisms, as demonstrated by Pax8−/− mice expressing lower levels of the anti-apoptotic gene Bcl2 in the thyroid bud at E10 than wild-type counterparts. Furthermore, the proportion of apoptotic cells in the primitive thyroid of Pax8−/− mice is increased. Although PAX8 is expressed in tissues other than the thyroid, Pax8−/− mice do not have defects in nervous system or kidney development. This seeming lack of phenotype might be due to the redundant activity of other PAX family proteins, such as PAX2 and PAX5, in these tissues. Interestingly, Pax2−/−;Pax8−/− embryos fail to form a metanephros and other structures, such as ureter and genital tracts, as a result of defects in mesenchymal–epithelial transitions required for nephric duct formation. However, mice with either a Pax2+/− or Pax8+/− genotype alone do not have renal abnormalities. Furthermore, expression of Hhex and Foxe1 genes is downregulated in the thyroid primordium of Pax8−/− mice, demonstrating a role for PAX8 as regulator of expression of other TTFs during organogenesis and in maintenance of adult tissues.

**HHEX**

During embryogenesis, HHEX is expressed in the developing thyroid gland, as well as in the primitive thymus, liver, lungs and pancreas. Embryos of Hhex−/− mice fail to survive beyond E15.5; the embryonic phenotype of these mice varies from severe to mild, according to the degree of malformation of organs such as the liver and brain. Severely affected embryos do not express Foxe1 and Nkx2-1 at E8.5 and these mice have thyroid agenesis in later developmental stages (Table 2). Although this phenotype might suggest that HHEX plays a critical part in thyroid specification, the general defects in the anterior endoderm of these mice, demonstrate that this developmental impairment is not thyroid-specific. Conversely, embryos with moderate to mild malformations do show some degree of thyroid specification and develop a hypoplastic thyroid that remains connected to the pharynx. At E9, mildly affected mice express normal levels of Pax8, Foxe1 and Nkx2-2; however, by E10, Pax8 and Foxe1 expression is dramatically downregulated, which might account for the small and ectopic thyroid gland phenotype seen at E15.5.

These data demonstrate that HHEX is not required for either specification or budding of the primordial thyroid, but that this TTF regulates expression of Pax8 and Foxe1, which control bud formation and survival of thyroid precursor cells in the later stages of thyroid development. The role of HHEX in thyroid organogenesis might be to regulate cell movement in the anterior foregut endoderm via a non-cell-autonomous mechanism, as has been described in liver.

**Hierarchy of TTF expression**

Although all four TTFs are expressed in thyroid precursor cells, the question of whether all of these factors are necessary to commit an undifferentiated cell to a thyroid fate has arisen. Experiments in mouse embryonic stem cells showed that transient expression of only Pax8 and Nkx2-1 is sufficient to drive differentiation of an epithelial

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**Table 2 | Phenotypes of mice in which TTF-encoding genes have been knocked out**

<table>
<thead>
<tr>
<th>TTF gene</th>
<th>Thyroid gland</th>
<th>Other tissues and organ systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nkx2-1</td>
<td>Complete absence of thyroid follicles</td>
<td>Malformations in lung and pituitary glands, Abnormalities in ventral forebrain, No septation between trachea and esophagus</td>
</tr>
<tr>
<td>Foxe1</td>
<td>Ectopy or agenesis of thyroid gland</td>
<td>Cleft palate</td>
</tr>
<tr>
<td>Pax8</td>
<td>Complete absence of thyroid follicles</td>
<td>Infertility</td>
</tr>
<tr>
<td>Hhex</td>
<td>No specification of thyroid precursors or thyroid agenesis at later stages</td>
<td>Brain and liver malformations</td>
</tr>
</tbody>
</table>

**Abbreviation:** TTF, thyroid transcription factor.
thyroid lineage.66 Additionally, overexpression of Nkx2-1 in embryonic stem cells was sufficient to induce expression of FoxE1.69 Studies in developing mice showed that in embryos at E10, Hhex expression was regulated by Nkx2-1 and Pax8 and, as a part of an autoregulatory loop, HHEX controlled expression of Pax8 and Foxe1.46 Thus, expression of Pax8 and Nkx2-1 induces expression of Hhex and Foxe1 in pluripotent cells, which drives thyroid differentiation.

These findings support a previous postulation that a hierarchy exists in the functional network of interactions between the TTFs.48 At the very beginning of thyroid development (E8.5–9), expression of Nkx2-1, Pax8 and Hhex is independent, as the absence of any one of these genes does not affect the expression of the others. By contrast, the expression of Foxe1 is regulated by Pax8. The genes Nkx2-1, Pax8 and Hhex are linked by reciprocal regulatory interactions. At E10, each of the transcription factors encoded by these three genes influences the expression of the genes that encode the other TTFs, and together these proteins control the expression of Foxe1, which is downstream in the thyroid regulatory network.48

Of note, the primordial thyroid forms normally in all TTF-mutant mice, which suggests that each of these transcription factors is not individually necessary for development of this structure, but that they are required for the emergence and survival of the thyroid bud (Figure 1).43,55,57,64 Thus, the transcription factors upstream of Nkx2-1, Pax8, Foxe1 and Hhex will need to be identified to fully understand the initial events that control thyroid specification in thyroid progenitor cells.32 Embryonic stem cells or induced pluripotent stem cells will provide important tools for identifying these upstream factors and other contributors to thyroid development.

**TTFs in the differentiated thyroid**

Differentiated thyroid follicular cells express proteins that are critical for biosynthesis, storage and secretion of the thyroid hormones T₃ and T₄.1 These proteins include thyroglobulin (Tg), thyroid peroxidase (TPO), sodium/iodide cotransporter (SLC5A5, also known as NIS), thyrotropin receptor (TSHR), dual oxidase 1 and 2 (DUOX1 and DUOX2, respectively), solute carrier family 26 member 4 (SLC26A4, also known as pendrin), iodothyrosine dehalogenase 1 (IYD-1) and monocarboxylate transporter 8 (MCT8). TTFs regulate the expression of genes that encode several of these proteins; thus, the coordinated expression of TTFs is essential for maintaining the function of the differentiated thyroid (Figure 2).36,40 The actions of Nkx2-1, FOXE1, Pax8 and HHEX in thyroid differentiation have been extensively studied; however, few investigations have addressed the identification of the genomic targets of the TTFs in the differentiated thyroid. Emerging research using genomic approaches has provided some insight into the functions of TTFs in mature thyroid tissues. For example, genomic targets of Pax8 (such as enhancers, silencers and boundary elements) that potentially regulate transcription of downstream genes in a differentiated thyroid cell line have been described.67

**Nkx2-1**

The gene that encodes thyroglobulin (Tg) was the first identified transcriptional target of Nkx2-1,11,68,69 which interacts with co-activators, such as WWTR1 (also known as TAZ),70 at the Tg promoter to regulate expression. Other genes regulated by Nkx2-1 in the adult rodent thyroid include Tpo, Tshr, Slc5a5, Hhex and Slc26a4.13,71–76 Microarray analysis of thyroid cells has revealed both known and new putative Nkx2-1 target genes, including the genes that encode other TTFs, Foxe1, Pax8 and Hhex, as well as Duox1, Duox1l, Cdh1, Vim and genes encoding proteins involved in cell cycle arrest (Figure 2).77 Moreover, Nkx2-1 regulates its own expression via an autoregulatory loop that is driven by the presence of Nkx2-1 binding sequences in the promoter of the Nkx2-1 gene (Figure 2).78–80

The Nkx2-1 core binding site is CAAG (Figure 3),11,12,81 which shares elements with the consensus binding nucleotide sequence T(T/C)AAGTG(G/C) of the NK-2 homeodomain family.62 Additionally, a genomic analysis in a human lung cancer cell line identified the motif sequence c/gTg/tGAGa/tGg/c as the most significant
**Figure 3** | Consensus binding motifs of TTFs. Known binding motifs of TTFs and the protein families to which they belong. The core binding motif for PAX8 was determined by ChIP-seq analysis in differentiated rat thyroid cell lines; NKX2-1, FOXE1 and HHEX binding sequences were defined by individual gene-based approaches in thyroid cell systems.11,12,37,67,71,101 Abbreviations: ChIP-seq, chromatin immunoprecipitation sequencing; TTF, thyroid transcription factor.

**FOXE1**

Maintenance of the differentiated thyroid is reliant on expression of Foxe1, which mediates TSH-driven expression of the Tg and Tpo genes (Figure 2).99–101 The promoter regions of both these FOXE1 target genes contain the AAACA core (Figure 3),71 where FOXE1 generally functions as a transcriptional activator, although it can also act as a transcriptional repressor.96 Additionally, FOXE1 can bind with the transcription factor nuclear factor 1 (also known as NFI/CTF) to form a complex that regulates Tpo expression in response to external hormonal stimuli, such as TSH and insulin-like growth factor 1, in adult rat thyroid cells.19,97 A genomic analysis performed in a rat thyroid follicular cell line identified two thyroid-specific genes, Duox2 and Slc5a5, as novel direct FOXE1−NF1/CTF targets (Figure 2).98 Other genes, such as Cdh1 and Nrd1a2, were also identified as transcriptional targets of FOXE1 (Figures 2 and 4).96 Furthermore, in an established human kidney cell line, novel FOXE1 target genes, such as MSX1 and TGFB3, have been identified (Figure 4).39,106 Together, these results expand upon the classical FOXE1-associated functions and provide new insights about FOXE1 transcriptional networks in differentiated thyroid cells.

**PAX8**

PAX8 is the master regulator of the differentiated thyroid phenotype,63 and is required in thyroid cells for transcriptional activation of Tg, Tpo, and Slc5a5 (Figure 2).35,101 Initial analyses performed in a rat thyroid follicular cell line revealed a PAX8 core binding sequence of eight nucleotides (TGCCCAg/cT) at the Tg and Tpo promoters.101 Next-generation sequencing approaches in thyroid cells have extended these findings and the PAX8 consensus binding motif has since been redefined to GNNCAgCCTGCGTACCC (Figure 3).36,67,101–104 The PAX8 core binding sequence overlaps with the NKX2-1 binding sequence in the Tg and Tpo promoters,101 and PAX8 synergizes with NKX2-1 to drive transcriptional activation of the Tg gene.105 Other PAX8 binding partners have been described: PAX8 cooperates with TAZ26 to regulate expression of Tg; with histone acetyltransferase p300 to mediate Tg and Tpo transcription;106,107 and with CTCF, SP1 and β-catenin at the Slc5a5 promoter.67,108

PAX8 has been demonstrated to regulate expression of Hhex100 and Foxe1.80,110 Regulation of other thyroid-specific genes, for example Dio157 and Duox2, by PAX8 has also been reported; however, the reports regarding the contribution of PAX8 to transcriptional regulation of Duox2 are conflicting.80,111 Finally, similar to Nkx2-1, PAX8 expression is also autoregulated owing to the presence of PAX8 binding sites in the regulatory regions of the PAX8 promoter (Figure 2).108,112

Given that PAX8 is considered a master regulator transcription factor, it is possible that it regulates expression of genes other than thyroid-specific genes. In fact, several well-known tumour suppressor genes, including TP53113 and WT1,114 have been identified as transcriptional targets of PAX8 in human astrocytoma cells and in an in vitro system, respectively. Furthermore, findings from studies using whole-genome sequencing have expanded knowledge of PAX8 functions in thyroid cancer and thyroid biology with the identification of several target genes involved in carcinogenesis (Bracl), thyroid malignancies (phosphatidylinositol/insulin and MAPK pathways) and cell-cycle processes (Cdkn2B, Ccnb1 and Ccnb2, among others).67 PAX8 has been shown to preferentially bind in non-promoter CpG-rich genomic regions, and direct involvement of PAX8 in the regulation of genes involved in cell proliferation and differentiation (Cited2, Taz, Runx2, Trib1), signal transduction (Wnt4), apoptosis, cell polarity and transport (Myo5b, Rab17, Kcnj16), cell motility and adhesion (Rab11a, Rab8a, Ncam and Cdh16) and a plethora of DNA–protein-related processes has been described.67 PAX8 has also been shown to be involved in tumour cell proliferation through regulation of E2F1,115 as well as in proliferation and apoptosis of differentiated thyroid epithelial cells via direct transcriptional regulation of Tp53inp1116 and Bcl2 (Figure 4).39

**HHEX**

The role of HHEX in the adult thyroid and its function as a transcriptional regulator of genes that encode thyroid differentiation markers is poorly understood. In thyroid cells, HHEX represses transcription of Tg by inhibiting the activating effects of PAX8 and NKX2-1 via binding to the core DNA sequences 5′-TAAT-3′ or 5′-CAAG-3′ in the Tg promoter (Figure 3).37 Interestingly, however,
HHEX positively regulates the transcription of its own gene via an autoregulatory loop similar to that of NKX2-1 and PAX8.\(^{75,78–80}\) In contrast to the few studies that have addressed the role of HHEX in differentiated thyroid cells, the functions of HHEX as regulator of development processes is well characterized. For example, HHEX regulates Vegfa expression in cardiac development;\(^{117}\) interacts with the transcription factor TAL-1 during haematopoiesis in zebrafish;\(^{118}\) and regulates expression of Tle4 and nodal in early development, together with β-catenin.\(^{119}\) In immortalized prostate and breast cell lines, HHEX was shown to directly regulate transcription of ENG and influence cell migration.\(^{120}\)

**Mutations in TTFs**

Thyroid pathologies are common among endocrine diseases and range from severe congenital disorders to different types of highly aggressive tumours. Given the important role of TTFs in development, cell proliferation and differentiation, it is unsurprising that mutations in the genes that encode these proteins are also associated with thyroid disorders.

**Thyroid dysgenesis**

The anatomical position of the thyroid gland is determined by a complex signalling network, and disruptions in this pathway can result in a spectrum of malformations that are collectively termed thyroid dysgenesis.\(^{111}\) Many of the phenotypes of animal models in which either Nkx2-1, FoxE1, Pax8 or Hhex have been knocked out (Table 2) accurately reflect the phenotypes of congenital thyroid disorders in human patients (Table 3). Among these disorders, primary congenital hypothyroidism is highly prevalent, with an incidence of approximately 1 in 3,500 newborn individuals.\(^{5,6}\) Congenital hypothyroidism can be consequent to disruptions in thyroid hormone biosynthesis (a condition known as dyshormonogenesis), but in many cases results from thyroid dysgenesis.\(^{8}\) The spectrum of phenotypes included within human thyroid
Brain–lung–thyroid syndrome is characterized by congenital hypothyroidism, respiratory distress and benign hereditary chorea (a movement disorder); however, all three major phenotypes are not always present in a given individual and the severity of each phenotype can vary. All patients with NKK2-1 mutations have a neurological phenotype; however, only a portion of these individuals have concomitant thyroid and/or lung disease, possibly as a result of certain mutations in NKK2-1 having a deleterious effect in lung development. Most mutations in NKK2-1 result in haploinsufficiency, but a few mutations result in generation of a protein that has a dominant-negative effect on wild-type NKK2-1. Notably, the most prevalent cause of brain–lung–thyroid syndrome is the existence of mutations in NKK2-1, which is why routine screening for mutations in this gene is recommended for patients who present with benign hereditary chorea alone or together with congenital hypothyroidism and/or lung problems.

FOXE1
Despite intensive research, only a few mutations have been described so far in FOXE1, many of which are in the forkhead domain, resulting in a protein that partially or completely lost its capacity to bind DNA and that is consequently unable to activate transcription. Interestingly, however, an emerging study has identified a novel gain-of-function mutation in FOXE1 in a patient with Bamforth–Lazarus syndrome.

The FOXE1 phenotypes are not restricted exclusively to the thyroid gland and mutations are often associated with craniofacial alterations. For example, the first homozygous missense mutation identified in the FOXE1 gene was seen in two brothers with Bamforth–Lazarus syndrome, who presented with athyreosis, cleft palate, spiky hair, bilateral choanal atresia and a bifid epiglottis.

The major thyroid phenotypes associated with mutations in FOXE1 are athyreosis and/or hypoplasia but never ectopia (as has been described in Foxe1–/– mice). However, a few cases of ectopic thyroid gland have been described in individuals with polymorphisms that affect the length of the polyalanine tract of the FOXE1 protein. Analysis of the region encoding the polyalanine tract provided the first evidence that FOXE1 might be a susceptibility gene involved in thyroid dysgenesis. Analysis of different haplotype distributions in patients with athyreosis, thyroid ectopy and hemiagenesis showed an association between homozygous mutations in the FOXE1 gene that resulted in an alanine tract length of either 14 or 16 residues and risk of thyroid dysgenesis. These polymorphisms were exclusively associated with thyroid phenotypes and suggest that even in the absence of mutations, polymorphisms in FOXE1 can influence the thyroid gland development. The function of the polyalanine tract in FOXE1 is not known; however, changes in the length of the alanine stretch in other transcription factors can result in either a loss or a gain of function and have been associated with several congenital malformation syndromes.

### Table 3 | Human phenotypes and syndromes associated with mutations in TTF-encoding genes

<table>
<thead>
<tr>
<th>TTF gene</th>
<th>Mutation location</th>
<th>Phenotype</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKK2-1</td>
<td>Homeobox or region encoding the transactivation domain</td>
<td>Normal thyroid, thyroid agenesis, athyreosis, hypoplasia, hemiagenesis and/or benign hereditary chorea and respiratory distress</td>
<td>Brain—lung—thyroid syndrome</td>
</tr>
<tr>
<td>FOXE1</td>
<td>Forkhead box</td>
<td>Thyroid hypoplasia, athyreosis and/or cleft palate, choanal atresia, bifid epiglottis, spiky hair and tongue-tie</td>
<td>Bamforth–Lazarus syndrome</td>
</tr>
<tr>
<td></td>
<td>Polymorphisms determining the length of the polyalanine tract</td>
<td>Thyroid ectopia and hemiagenesis</td>
<td>Thyroid dysgenesis</td>
</tr>
<tr>
<td>PAX8</td>
<td>Paired box, region encoding the transactivation domain, or promoter region</td>
<td>Thyroid hypoplasia, athyreosis, thyroid ectopia and, rarely, unilateral kidney and problems in urogenital tract</td>
<td>Congenital hypothyroidism due to thyroid dysgenesis</td>
</tr>
<tr>
<td>HHEX</td>
<td>No mutations identified in humans</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviation: TTF, thyroid transcription factor.
PAX8
A number of PAX8 mutations have been found in patients with thyroid dysgenesis, most of which are localized to the paired box domain, with only a few in the region encoding the transactivation domain and three in the promoter region. These mutations can affect different functions of the protein, such as DNA binding, gene activation, protein stability and cooperation with the co-activator p300. Affected individuals have heterogeneous PAX8 autosomal dominant mutations, although in mice both alleles must be affected to produce a congenital hypothyroidism phenotype. The thyroid phenotypes seen in patients are mainly thyroid gland hypoplasia and, to a lesser extent, athyreosis and ectopy. The phenotype of individuals with PAX8 mutations is usually restricted to the thyroid gland and is characterized by congenital hypothyroidism as a result of dysgenesis. Nevertheless, associations with unilateral kidney agenesis and abnormalities in the urogenital tract have been described.

Epigenetic factors and noncoding regions
In all cases of thyroid dysgenesis, mutations in the genes that encode the TTFs have variable penetrance, supporting the notion that thyroid dysgenesis is a complex disease and suggesting that additional genetic elements might contribute to the wide spectrum of observed phenotypes. The effects of TTFs can be modulated by other, as yet unidentified, factors as this disease has a mutigenic origin. In fact, the molecular aetiology of most patients with thyroid dysgenesis remains unclear as mutations in TTFs have been identified in only a few cases and discrepancies exist between observed genotypes and phenotypes.

Thus, new mutations that contribute to thyroid dysgenesis should be sought in introns and regulatory regions, such as the 3′ untranslated region, where microRNAs (miRNAs) bind, in addition to the coding regions of genes. Supporting a role for miRNAs in thyroid biology, inactivation of the miRNA-processing enzyme, Dicer, leads to severe hypothyroidism in mice. Moreover, many of the genes downstream of the TTFs that ultimately control thyroid organogenesis are still unknown, and it is possible that mutations in one or more of these genes are involved in thyroid dysgenesis. In this respect, next-generation sequencing and other genomic approaches are promising technologies, as studies using these tools have already identified new target genes of TTFs.

Finally, the discordance for thyroid dysgenesis in monozygotic twins also supports a role for the involvement of epigenetic mechanisms in these disorders. Transcriptome, methylome and genomic variations in ectopic thyroid glands have been reported, and although the molecular underpinnings of the variations could not be defined, these results offer new possibilities for determining the causes of defective thyroid migration. Indeed, an emerging study has reported tissue-dependent differential methylation of the FOXE1 promoter and identified two consecutive CpG dinucleotides in the promoter that act as epigenetic modifiers of FOXE1 expression.

Associations with thyroid cancer
Thyroid cancer is the most frequently occurring neoplasia of endocrine organs, and over 90% of thyroid tumours arise from follicular thyroid cells. Follicular-cell-derived tumours can be subclassified into three groups: well-differentiated carcinomas (including papillary thyroid carcinoma [PTC], which accounts for around 85% of cases, and follicular thyroid carcinoma [FTC], which represents ~5–10% of cases); poorly differentiated carcinomas (2%); and anaplastic or undifferentiated carcinomas (ATC), which represent the most infrequently occurring thyroid neoplasias. Patients with well-differentiated follicular carcinomas generally have a better prognosis than patients with poorly differentiated carcinomas or ATC.

NKX2-1
In lung cancer, expression levels of NKX2-1 have been widely studied and are used as a diagnostic and prognostic factor. Either absent expression or amplified NKX2-1 levels of expression are indicative of a worse prognosis than normal expression levels. Nevertheless, no mutations in NKX2-1 have been found in lung adenocarcinomas. Interestingly, opposing functions for NKX2-1 in tumours—oncogenic or tumour-suppressive—have been described, which constitutes the as yet unresolved NKX2-1 paradox. The dual roles of this transcription factor might be explained by different cell contexts in which NKX2-1 interacts with either co-activators or co-repressors to regulate the expression of oncogenes and/or tumour suppressor genes; however, this explanation is speculative and remains to be substantiated.

Although the role of NKX2-1 as a tumour suppressor in thyroid cancer has not been studied in detail, NKX2-1 expression decreases with increasing dedifferentiation states in thyroid cancers, with anaplastic tumours having very low levels of NKX2-1 expression (Table 4). Furthermore, a single nucleotide polymorphism (SNP) at 14q13.3, a genomic region close to the NKX2-1 gene, has been associated with PTC and FTC. and a germline g.1016C>T missense mutation that leads to the Ala339Val mutation in the transactivation domain of NKX2-1 has been found in PTC and in multinodular goitre patients. Finally, NKX2-1 rearrangements have also been described in T-cell acute lymphoblastic leukaemia.

FOXE1
The role of FOXE1 as a susceptibility gene for thyroid cancer has been extensively characterized (Table 4). Several association studies have been performed and the findings replicated, revealing two SNPs that are clearly associated with PTC and FTC in multiple populations. Both rs955113 and rs1867277 are located in the same disequilibrium block (meaning that these alleles co-occur on the same haplotype more often than is expected by chance), and are situated 5 kbp and 238 bp away from the FOXE1 promoter.

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upstream of the FOXE1 transcription start site, respectively. Additionally, rs955113 has been associated with radiation-induced PTC.\(^{168}\) When rs1867277 is present, binding of the transcription factors upstream stimulatory factor 1 and upstream stimulatory factor 2 to the FOXE1 promoter in thyroid cells is increased, supporting the hypothesis that increased binding of these factors leads to increased FOXE1 expression and confers a motile advantage to cancer cells.\(^{167}\) Moreover, FOXE1 expression follows the same pattern as NKX2-1 expression, and shows an inverse correlation with dedifferentiation, with anaplastic tumours having low levels of FOXE1 expression.\(^{169,170}\) An additional study demonstrated that a gradient of levels of FOXE1 expression in PTC tumour borders correlates with FOXE1 mislocalization in the cytoplasm, unless the FOXE1 SNP rs1867277 is present.\(^{171}\)

Alterations in FOXE1 such as loss of heterozygosity at the FOXE1 chromosomal area,\(^{172}\) FOXE1 promoter methylation\(^{173}\) and the presence of a rare variant of FOXE1, which results in a modified alanine tract length,\(^{174}\) have been also associated with development of squamous cell carcinoma. Moreover, methylation of the FOXE1 promoter has been linked to pancreatic and breast cancers.\(^{175,176}\)

**PAX8**

Expression of PAX8 is increased in neoplastic renal tissues, Wilms tumours, ovarian cancer and Müllerian carcinomas.\(^{27,28,169,177–179}\) However, in thyroid cancers, the pattern of PAX8 expression remains unclear and two studies have reported conflicting results. One study described an expression pattern similar to that of NKX2-1 and FOXE1 expression,\(^{161}\) however, another research group proposed that PAX8, but not NKX2-1, could be a good anaplastic carcinoma marker.\(^{169}\) The PAX8–PPARG fusion gene is widely accepted to be an early follicular-thyroid-carcinoma-specific oncogene.\(^{180,181}\) The PAX8–PPARY fusion protein has been found in ~36% of FTCs, 16% of the follicular variant of PTCs, ~11% of the follicular adenomas and 2% of Hürthle cell carcinomas.\(^{182–184}\) Expression of a fusion protein that contains the PAX8 DNA binding domain has been hypothesized to increase cell-cycle transition, reduce apoptosis and enhance cell growth.\(^{181}\) Finally, an epistatic interaction between PAX8 and STK17B (a gene encoding a serine-threonine kinase involved in apoptosis regulation) has been reported, further expanding known PAX8 functions (Table 4).\(^{185}\)

**HHEX**

HHEX is expressed in anaplastic carcinomas; however, the protein is mislocalized in the cytoplasm (Table 4).\(^{166}\) This aberrant localization might represent a mechanism by which the nuclear functions of HHEX are regulated. The HHEX transcription factor contains a homeodomain, and it has been proposed that several, as yet unknown, HHEX functions might be related to cytoplasmic signal transduction through this domain, as has been described for engrailed proteins.\(^{166}\) Loss of nuclear localization and/or loss of functional HHEX in AML and CML\(^{187}\) suggests a role in the development of thyroid gland and for maintaining the functional differentiated state of the adult thyroid. Divergent roles of TTFs have been described in the thyroid differentiation programme, as well as in cell proliferation and apoptosis. Importantly, TTF-regulated targets and functions are not restricted to genes in the thyroid gland, revealing new and fascinating perspectives to understanding the role of each of the TTFs in other tissues. Numerous studies indicate that TTFs are not individually required for the specification of thyroid precursor cells. Nevertheless, signals involved in the induction

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**Table 4 | Expression patterns and genetic alterations of TTFs in thyroid cancer**

<table>
<thead>
<tr>
<th>TTF</th>
<th>Protein expression levels or localization</th>
<th>Genetic alterations</th>
<th>Predisposition</th>
<th>Other cancers (alterations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKX2-1</td>
<td>PTC &gt; FTC &gt; anaplastic(^{162–163})</td>
<td>rs944289(^{164}) Mis sense germ line mutation g1016C&gt;T (A1a339Val)(^{165})</td>
<td>PTC and FTC PTC</td>
<td>Increased expression in lung adenocarcinomas and small-cell carcinomas(^{158}) Amplifications but no mutations detected(^{9})</td>
</tr>
<tr>
<td>FOXE1</td>
<td>PTC &gt; FTC &gt; anaplastic(^{168,170})</td>
<td>rs95512(^{164}) rs1867277(^{167})</td>
<td>PTC</td>
<td>Loss of heterozygosity, promoter methylation and presence of 16-alanine stretch allele in SCC(^{173})</td>
</tr>
<tr>
<td>PAX8</td>
<td>Conflicting reports (either PTC &gt; FTC &gt; anaplastic(^{165}) or PAX8 is expressed in a high proportion of anaplastic carcinomas(^{169}))</td>
<td>PAX8–PPARG(^{160,161,165}) Epistasis with STK17B</td>
<td>FTC</td>
<td>Increased expression in neoplastic renal tissues,(^{172}) Wilms tumour,(^{27}) Müllerian carcinoma(^{179}) and ovarian cancer(^{168})</td>
</tr>
<tr>
<td>HHEX</td>
<td>PTC, FTC and anaplastic (anaplastic expression mislocalised in cytoplasm)(^{166})</td>
<td>None</td>
<td>None</td>
<td>Loss of nuclear localization(^{166}) and/or loss of functional HHEX in AML and CML(^{187})</td>
</tr>
</tbody>
</table>

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; FTC, follicular thyroid cancer; PTC, papillary thyroid cancer; SCC, squamous cell carcinoma; TTF, thyroid transcription factor.
of expression of the TTFs remain unidentified. In particular, signals derived from cardiogenic mesoderm might have critical functions in thyroid development and need to be studied in depth. The molecular pathways and mechanisms that connect TTFs to thyroid dysgenesis and thyroid cancer are largely unknown and require further study. The study of the involvement of TTFs in non-thyroid cancers is also of particular interest. Emerging genomic analyses have provided some clues, but additional studies are needed. Functional analyses of susceptibility, the contribution of miRNAs, stem cells and of differential gene expression patterns within tumours and between tumour subtypes are of utmost importance.

Review criteria

A PubMed search of articles published between 1981 and 2014 was done. Keywords included “Nkx2-1”, “TTF1”, “FoxE1”, “TTF2”, “Pax8”, “Hex”, “thyroid”, “development”, “differentiation”, “thyroptroism”, “thyroid dysgenesis” and “thyroid cancer”.

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De Felice, M. 
Butt, S. J. 
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Author contributions

The authors contributed equally to all aspects of the article.