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REVIEW

Development of the thyroid gland

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ABSTRACT

Thyroid hormones are crucial for organismal development and homeostasis. In humans, untreated congenital hypothyroidism due to thyroid agenesis inevitably leads to cretinism, which comprises irreversible brain dysfunction and dwarfism. Elucidating how the thyroid gland – the only source of thyroid hormones in the body – develops is thus key for understanding and treating thyroid dysgenesis, and for generating thyroid cells *in vitro* that might be used for cell-based therapies. Here, we review the principal mechanisms involved in thyroid organogenesis and functional differentiation, highlighting how the thyroid forerunner evolved from the endostyle in protochordates to the endocrine gland found in vertebrates. New findings on the specification and fate decisions of thyroid progenitors, and the morphogenesis of precursor cells into hormone-producing follicular units, are also discussed.

KEY WORDS: Thyroid, Endoderm, Pharyngeal, Neural crest, Evolution, Morphogenesis

Introduction

The thyroid gland (Fig. 1) and its hormones play multifaceted roles in organ development and in the homeostatic control of fundamental physiological mechanisms such as body growth and energy expenditure in all vertebrates (Maenhaut et al., 2015). The thyroid is formed from a midline anlage in the pharyngeal floor consisting of foregut endoderm cells that are committed to a thyroid fate (Fig. 2A). These thyroid progenitors then give rise specifically to the follicular cell lineage that eventually will form hormone-producing units – the thyroid follicles – that make up the thyroid gland (Fig. 1A,B). Differentiated cells within these follicles, known as thyrocytes, are strictly epithelial: they possess an apical surface that delimits the follicle lumen and a basal (or basolateral) surface that faces the extrafollicular space. It is these cells that produce the thyroid hormones triiodothyronine and thyroxine (T3 and T4), which are iodinated dipeptides that are synthesized, stored and secreted in a complex series of reactions (Fig. 1C) involving bidirectional transport to and from the lumen (Rousset et al., 2015). Thyroid hormone production thus requires that thyrocytes are both fully polarized and able to maintain a tight barrier between inside and outside; from this viewpoint, thyroid follicular cells share many properties with exocrine cells that distinguish the thyroid from other major endocrine glands. Thyroid-stimulating hormone (TSH or thyrotropin) produced by the pituitary is the main regulator of thyroid growth and function from late fetal life to adulthood (Maenhaut et al., 2015). However, thyroid organogenesis and *de novo* follicle formation occur independently of pituitary control

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(Hilfer, 1979; Postiglione et al., 2002), indicating that the embryonic thyroid relies entirely on locally derived inductive signals and morphogens for its proper development.

The thyroid also contains a second population of hormone-producing cells named parafollicular cells or C cells (Fig. 1B,C). These cells, which are also of endoderm origin and arise from the ultimobranchial bodies (UBBs; Fig. 2A), are neuroendocrine in nature and primarily synthesize calcitonin, which is a hypocalcemic hormone that serves as a natural antagonist to parathyroid hormone. Additionally, the thyroid gland contains a rich network of capillaries surrounding each follicle that provides systemic delivery of released hormones (Fig. 1B,C). The stromal compartment, which encapsulates and finely septates the follicular thyroid tissue, consists mainly of ectomesenchymal fibroblasts derived from the neural crest (Kameda et al., 2007). The thyroid also contains other interstitial cells such as macrophages and mast cells, which have attracted attention due to their functions in thyroid cancer (Visciano et al., 2015).

In its simplest form, as observed in most teleosts including zebrafish (Alt et al., 2006b), the follicular thyroid consists of rows of loosely associated follicles distributed ventrally along the anterior foregut (Fig. 2B). By contrast, tetrapods harbor an encapsulated thyroid gland that is located in the neck close to the trachea and of a size largely proportional to the adult body size of the species (Maenhaut et al., 2015). However, overall thyroid shape varies considerably among species (Fig. 2B). In most mammals, for example, the thyroid gland consists of two lobes connected by an isthmus portion crossing the upper trachea. By contrast, in cartilaginous fishes and some mammals, the thyroid is retained as a central single mass, whereas in amphibians and birds the isthmus is absent and the lobes are distinctly separated, thus forming bilateral glands (Gorbman, 1955). These various shapes are likely to represent different end stages of the same morphogenetic process. Notably, in mammals it is in this late stage of organogenesis that progenitors differentiate into follicular cells and begin to produce hormone; prior to this, thyroid-dependent embryonic and fetal development of the organism rely entirely on maternal supplies of thyroxine.

It appears that the shape and anatomical position of the thyroid have little if any functional role. Indeed, the severe and life-threatening hypothyroid state of mice made athyroid by an overdose of radioactive iodine (I^{131}), which accumulates in and destroys the gland, can be rescued by implanting thyroid follicles generated from mouse embryonic stem cells (ESCs) into the vascularized environment of the kidneys (Antonica et al., 2012). Nonetheless, the morphogenetic events that lead to the formation of sufficient amounts of functional, hormone-producing tissue are important to consider for understanding how thyroid developmental defects, which are the leading cause of congenital hypothyroidism (CH; Box 1) (reviewed by Wassner and Brown, 2015), may arise. Intriguingly, mouse studies have revealed that thyroid dysgenesis is a polygenic disease with variable penetrance that can present clinically with different phenotypes, even though the inactivating mutations are identical (Amendola et al., 2005).

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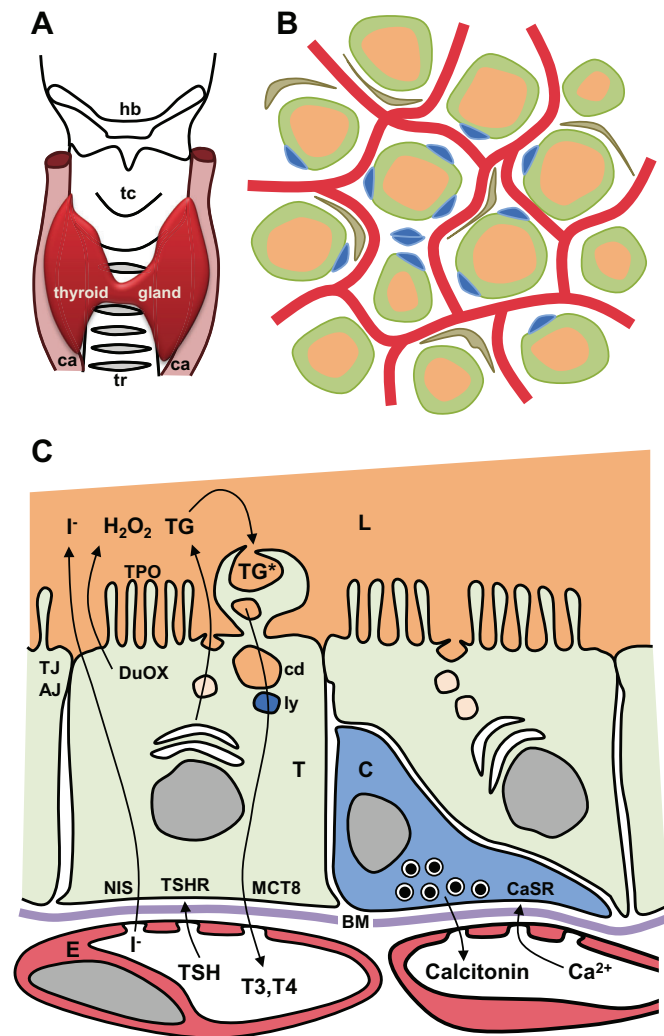
Fig. 1. Structure and function of the thyroid gland. (A) The human thyroid gland (red) consists of two lateral lobes and a connecting isthmus portion that crosses the midline at the level of the first or second tracheal cartilaginous rings. hb, hyoid bone; tc, thyroid cartilage; tr, trachea; ca, carotid artery. (B) Follicles, the functional units of the gland, vary in size and shape. Follicular cells (or thyrocytes), the major cell type in follicles, form a monolayered epithelium (green) that encloses a central cavity or lumen filled with colloid (orange), which constitutes a thyroid hormone reservoir. Each follicle is surrounded by a network of capillaries (red). Neuroendocrine cells (or C cells; blue), fibroblasts (gray) and other stromal cells (e.g. macrophages and mast cells; not shown) reside close to the follicles or interstitially. (C) The principal functions of thyrocytes and C cells are schematized. Iodide (I$^-$) is entrapped in the thyroid primarily by the sodium-iodine symporter (NIS) expressed in the basolateral plasma membrane of thyrocytes (T, green). Thyroglobulin (TG), a prohormone, is synthesized and secreted apically into the follicular lumen (L, orange), where it is iodinated by thyroid peroxidase (TPO) in the presence of hydrogen peroxide (H$_2$O$_2$) that is generated by an NADPH oxidase (DuOX). This series of reactions constitutes the mechanism by which TPO also couples iodotyrosines into iodothyronines (T3, T4) that remain covalently bound to the TG molecule. To release thyroid hormone into the circulation, iodinated TG (TG*) is internalized by endocytosis and degraded via the fusion of endosomes with lysosomes (ly). MCT8 (SLC16A2) mediates the transport of thyroid hormone out of the cell. Thyroid-stimulating hormone (TSH) from the pituitary stimulates, via its receptor (TSHR), most if not all steps involved in thyroid hormone biogenesis and release. Thyroid C cells (C, blue) typically exhibit a parafollicular or intrafollicular position. They respond to extracellular Ca^{2+} concentration by activating calcium-sensing receptors (CaSR), leading to release of calcitonin that is stored in dense-core granules. AJ, adherens junction; TJ, tight junction; cd, colloid droplet; E (red), endothelium (fenestrated); BM, basement membrane.

Here, we summarize what is currently known about the cellular and molecular mechanisms regulating thyroid development and differentiation, focusing on the mammalian gland but also providing an evolutionary perspective. We center our discussion around the follicular cells of the thyroid; recent advances in C cell development are summarized in Box 2 (reviewed by Kameda, 2016; Nilsson and Williams, 2016). We discuss pathogenetic mechanisms that are of relevance for understanding thyroid dysgenesis and CH. Recent attempts to generate functional thyroid tissue from ESCs for potential cell-based regenerative therapies for CH are also highlighted.

Evolution of the thyroid gland

Thyroid hormone regulates diverse functions in vertebrates but also in invertebrates devoid of a thyroid gland (Eales, 1997), emphasizing the importance of iodinated compounds throughout evolution. Hypothetically, a follicular thyroid evolved in vertebrates due to the participation of thyroid hormone in an ever-increasing number of biological processes and, hence, the requirement for an efficient mechanism of saving and storing iodine. But, exactly how and when did the thyroid gland evolve? Some clues may be found in protochordates (urochordates and cephalochordates) and larval lampreys (Fig. 2C,D), in which a thyroid equivalent constitutes a distinct part of the endostyle, a mucus-producing groove on the ventral wall of the pharynx primarily involved in filter-feeding (Fredriksson et al., 1988). Iodinating capacity and enrichment of thyroxine and mono- and di-iodotyrosines were first documented for the *Ciona* endostyle more than 50 years ago (Barrington and Thorpe, 1965a,b), supporting its role as a thyroid forerunner in invertebrates. The expression in the protochordate endostyle of orthologous genes known to be involved in thyroid differentiation and function in vertebrates further supports this role. However, epithelial cells in the endostyle capable of iodination do not exhibit a follicular structure but are zonally distributed dorsolaterally on both sides of the endostylar wall (Fredriksson et al., 1984, 1985, 1988) (Fig. 2C). Furthermore, whereas in vertebrates the follicular lineage originates from progenitors with identical expression patterns of thyroid developmental genes (Fernandez et al., 2015), in *Ciona* and amphioxus the corresponding gene orthologs are differentially expressed and the cells are, fully or partly, distributed in zones other than the iodine-binding domain (Hiruta et al., 2005) (Fig. 2C). From a phylogenetic viewpoint, this suggests that evolution of the vertebrate thyroid involved recruitment of distinct cell types from neighboring regions of the endostyle and the coalescence of their originally separated functions. Intriguingly, the larval endostyle in lampreys metamorphoses into a follicular thyroid (Fig. 2D), thus representing a transitional phase in thyroid evolution towards an endocrine gland (Marine, 1913; Wright and Youson, 1976; Kluge et al., 2005).

It has been suggested that postmetamorphic animals required novel mechanisms to capture and store iodine following their invasion of freshwater, hence the evolution of a follicular thyroid (Youson and Sower, 2001). The shift to the generation of multiple follicles is likely to have facilitated iodide trapping by two processes: (1) the uptake of iodide from the circulation; and (2) the storage of iodine covalently bound to thyroglobulin, which is a giant glycoprotein that is secreted apically and sequestered in the follicle lumen, thus forming the luminal colloid (Fig. 1C). The importance of enriching prohormone in a secluded space as a means to iodinate more effectively is supported by observations that the necessary components for iodination are expressed in the endostylar zone of organic iodine binding in both urochordates and
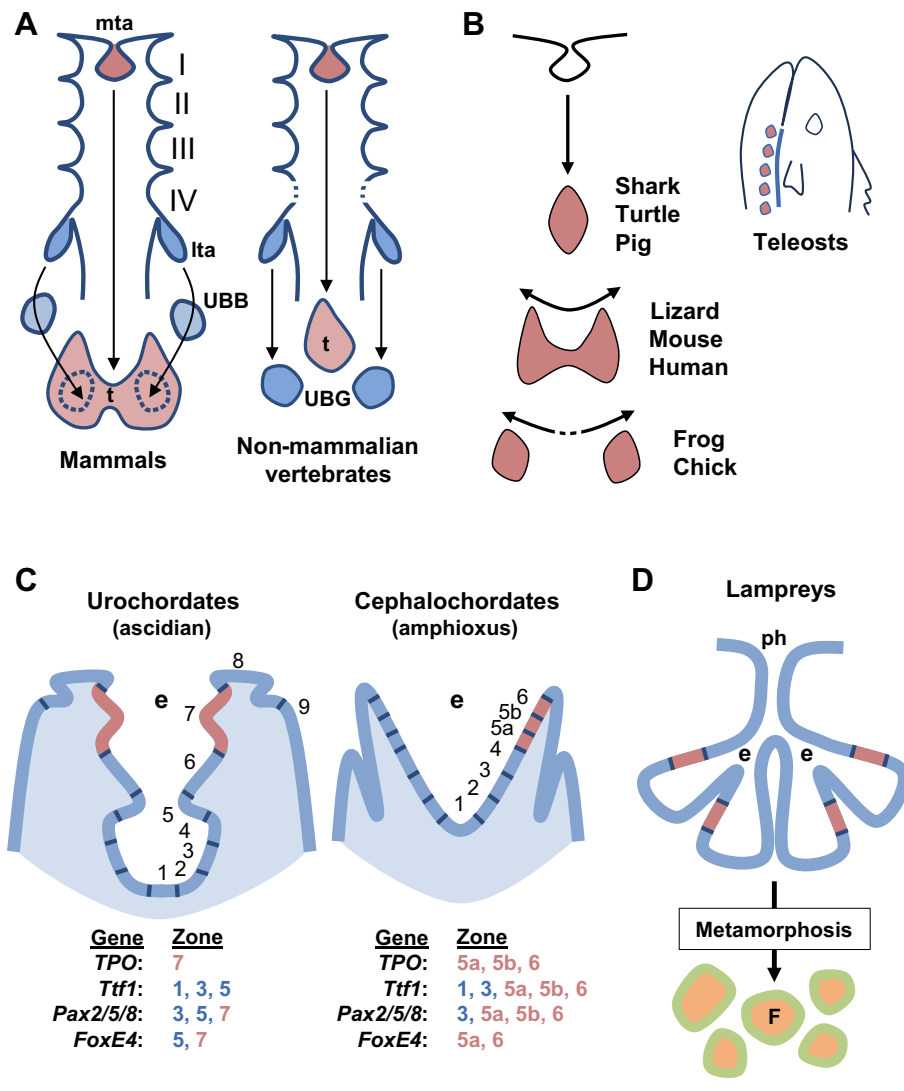
Fig. 2. Thyroid development from an evolutionary perspective. (A) The embryonic thyroid (t) with its follicular progenitors (red) develops from a central or median thyroid anlage (mta) in the pharyngeal floor. By contrast, the ultimobranchial bodies (UBBs) and C cell precursors (blue) develop from a paired lateral thyroid anlage (lta) confined to the most caudal of the pharyngeal pouches. Both the median and lateral primordia evaginate and migrate embedded in subpharyngeal mesoderm. In mammals, these primordia fuse to form a composite gland, whereas in all other vertebrates the UBBs develop into distinct organs: the ultimobranchial glands (UBGs). I-IV, pharyngeal arch numbers. (B) The vertebrate thyroid mostly appears as a compacted gland, although its shape varies from a single mass to two separated bodies, with the bilobed gland as a suggested intermediate. By contrast, most bony fish (teleosts) lack a compacted thyroid gland; instead, the loosely associated follicles are dispersed along the aorta close to their origins in the anterior endoderm. (C) Thyroid equivalents have been identified in invertebrates. The food-filtering endostyle (e) present in the pharyngeal wall of urochordates and cephalochordates exhibits iodine metabolizing zones (red) in distinct parts of the endostyle mucosal lining (blue). That these zones indeed represent a thyroid forerunner is supported by their co-expression of thyroid-specific genes such as thyroid peroxidase (*TPO*). Paralogs of other genes known to regulate thyroid development in vertebrates (*Tft1*, *Pax2/5/8* and *FoxE4*) show different expression patterns, being also expressed in other cellular zones of protochordate endostyles (numbered 1-9), suggesting divergent roles for these factors before the vertebrate thyroid evolved. Figure based on data published by Hiruta et al. (2005). (D) In lampreys, the multi-chambered larval endostyle (e) metamorphoses into a follicular thyroid (F), representing a transitional stage in thyroid evolution. ph, pharyngeal cavity of larvae.

cephalochordates (Fujita and Sawano, 1979; Tsuneki et al., 1983; Fredriksson et al., 1985). Moreover, sequencing the amphioxus genome has revealed homologs to all genes involved in thyroid hormone synthesis and metabolism in non-vertebrate chordates except for that encoding thyroglobulin (Paris et al., 2008). A recent comparative study confirmed that thyroglobulin is confined to the vertebrate series including lamprey (*Petromyzon marinus*) (Holzer et al., 2016), further supporting an evolutionary link between the evolution of a novel prohormone and the appearance of a follicular thyroid. Interestingly, thyroglobulin is expressed in the larval lamprey endostyle (Monaco et al., 1978), indicating that the basal machinery for prohormone synthesis and accumulation is already in place before the endostyle metamorphoses into a follicular thyroid. Folliculogenesis thus conveys an elegant solution to meet the increasing demands of hormone supply from a pre-existing factory.

Thyroid specification: *in vivo* and *in vitro*

The thyroid gland is the anteriormost organ that develops from foregut endoderm. In contrast to the requirements of other foregut derivatives, retinoic acid (RA) is not necessary for early thyroid specification and development (Bayha et al., 2009). In fact, exposure of *Xenopus* embryo explants to RA switches progenitors in the presumptive thyroid domain to a lung developmental program (Wang et al., 2011). In line with this, *in vitro* studies of mouse ESCs

have revealed similarities between thyroid and lung development. These, together with studies in mice, have identified key roles for fibroblast growth factors (Fgfs) and bone morphogenetic proteins (Bmps) in inducing thyroid fate.

Athyreosis has been reported for mice deficient for *Fgfr2b* (Revest et al., 2001) and *Fgf10* (Ohuchi et al., 2000), although a putative role of Fgf10 in thyroid specification is unlikely in light of recent observations of a small thyroid rudiment in *Fgf10* knockouts (Teshima et al., 2016). Additional indications for a pivotal role for Fgfs in triggering thyroid fate came from *ex vivo* studies of mouse endoderm focusing on the role of cardiogenic mesoderm in lung specification (Serls et al., 2005); signs of thyroid differentiation were reported after treating foregut explants with Fgf2 (Fig. 3A), but this preliminary observation was not further elaborated. In functional experiments on zebrafish (Wendl et al., 2007), Fgf1, Fgf2 and Fgf8 were found to redundantly rescue thyroid development in *han* (*hand2*) mutants lacking a thyroid (Fig. 3A). This study further suggested that cardiac mesoderm is the likely source of a thyroid inductive or instructive signal in zebrafish. Concurrently, proof of concept that differentiated thyroid cells can be generated from embryoid bodies derived from mouse ESCs was provided by a series of papers (Lin et al., 2003; Arufe et al., 2006; Ma et al., 2009); these studies also confirmed previous notions that thyroid follicle development is TSH independent (Hilfer, 1979;

Postiglione et al., 2002). More recently (Longmire et al., 2012), it was shown that Fgf2 and Bmp4 are necessary and sufficient for the directed differentiation of mouse and human ESCs and human induced pluripotent stem cells (iPSCs) into functional thyrocytes, provided that cells are pre-programmed to definitive endoderm (Kurmann et al., 2015) (Fig. 3B). Similarly, the addition of both Fgf2 and Bmp4 to *Xenopus* foregut explants successfully induced the expression of thyroid-specific genes, while inhibition of either the Fgf or Bmp signaling pathway prevented thyroid development in intact *Xenopus* embryos and in isolated mouse foreguts (Fig. 3A). Together, this indicates that Fgf2 and Bmp4 are key players of an evolutionarily conserved mechanism for thyroid specification. The cellular origins of Fgfs and Bmps in this context are as yet ill defined, although precardiac mesoderm is a plausible source (Wendl et al., 2007).

It has also been shown that, when conditioned in 3D culture and stimulated with TSH to generate follicles (Fig. 3C), fully functional thyroid follicular cells derived from mouse ESCs overexpressing two key thyroid transcription factors – Nkx2-1 and Pax8 – are capable of rescuing thyroid hormone levels in athyreotic mice (Antonica et al., 2012) . This exciting discovery opens the door to regenerative therapy for patients with CH. However, although several protocols are now at hand to generate thyroid progenitors and functional follicles from human ESCs or iPSCs (Kurmann et al., 2015; Ma et al., 2015), the field needs further advances (as reviewed by Hollenberg et al., 2016) to make this a realistic possibility. For example, the pool of Nkx2-1[+]/Pax8^{+} thyroid progenitors generated by these approaches is small compared with the number of co-induced lung progenitors, and sorting and enriching precursor cells currently requires genetic labeling (Kurmann et al., 2015). Evidently, additional factors that are of importance for the specification process are still to be identified. In addition, the instructive signals that delineate and distinguish thyroid and lung lineages are as yet unresolved, as is the fundamental question of whether these lineages have an ancestral and common endoderm progenitor. Of note, it was recently shown that Nkx2-1 alone can efficiently induce thyroid progenitors in a Foxa2-negative subpopulation of pre-patterned anterior endoderm derived from mouse ESCs (Dame et al., 2017). It is known that Foxa2, which is absolutely required for endoderm formation (Ang et al., 1993; Monaghan et al., 1993), is downregulated specifically in the mouse thyroid bud as compared with the adjacent endoderm and lung bud (Fagman et al., 2011). Moreover, Foxa2 is repressed in follicular progenitors throughout thyroid development, although lineage tracing shows that these cells are derived from Foxa2^{+} definitive endoderm (Johansson et al., 2015). Collectively, these findings suggest a novel Foxa2-independent transcriptional mechanism by which thyroid competence is acquired and that distinguishes thyroid development from other foregut derivatives that require Foxa2 (and Foxa1) (Kaestner, 2010). However, although this is an intriguing possibility, it remains unknown whether Foxa2 might actively repress a thyroid fate program in anterior endoderm.

Thyroid morphogenesis: a multistage process from bud to gland

Following its specification, the thyroid primordium undergoes a series of morphological changes (Fagman et al., 2006; Nilsson and Fagman, 2013) beginning at E20 in humans and E8.5 in mice (Fig. 4). (1) Formation of the thyroid placode. The first sign of the thyroid anlage becomes evident as it assembles close to the base of the tongue. (2) Conversion of the placode to the thyroid bud. This process takes place in close vicinity to the apical pole of the aortic sac. (3) Downward (caudal) migration of the thyroid primordium to a pretracheal position. The descent initially takes place with the primordium maintaining connection to pharyngeal endoderm via the thyroglossal duct, which then degenerates leaving the thyroid

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A Mouse, *Xenopus*, zebrafish (*in vivo*)
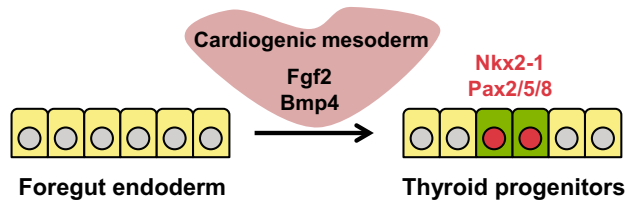
B Mouse, human (*in vitro*)

C Mouse (*in vitro*)

Fig. 3. Inducing thyroid fate *in vivo* and *in vitro*.
(A) Whole embryo and explant studies in mouse, *Xenopus* and zebrafish have shown that Fgf2 and Bmp4, presumably derived from the adjacent cardiogenic mesoderm, can induce thyroid fate in competent but ye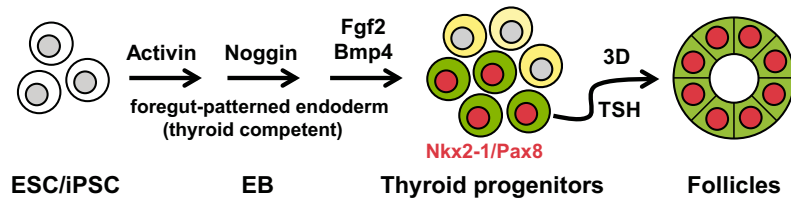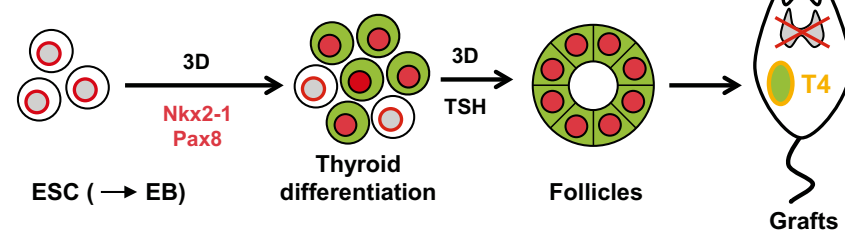t undifferentiated anterior endoderm cells. Thyroid progenitors are distinguished from other endoderm lineages by co-expression of Nkx2-1 and Pax8 (or Pax2 in *Xenopus* or Pax2/5/8 in zebrafish). (B) Co-stimulation with Fgf2 and Bmp4 recapitulates thyroid specification in mouse ESCs and human iPSCs after sequential induction of foregut-patterned endoderm. Further development into functional follicles requires 3D culture in Matrigel and the addition of TSH. (C) The overexpression of Nkx2-1 and Pax8 in mouse ESCs from a doxycycline-inducible transgene induces directed thyroid differentiation in embryoid bodies (EBs) without prior requirement of endoderm induction. Further development and follicle formation of these ESC-derived thyroid cells requires 3D culture and TSH stimulation. The transplantation of such *in vitro* generated thyroid follicles into athyreotic mice can rescue normal thyroid hormone (T4) levels.

primordium all surrounded by mesenchyme as migration proceeds until contact with the aortic sac is re-established. (4) Bifurcation of the primordium, during which time the thyroid tissue extends bilaterally along a pair of the pharyngeal arch arteries. During this stage, thyroid progenitors proliferate intensely. (5) Formation of the left and right thyroid lobes, which end up on either side of the larynx and proximal trachea, and are connected frontally by the thyroid isthmus. In vertebrates, thyroid bilobation also involves fusion with the UBB, which merges with each of the lateral thyroid lobes. (6) Folliculogenesis – the process by which the thyroid attains its final histoarchitecture and which constitutes the final morphogenetic stage. Follicle formation coincides with functional cell differentiation and the synthesis of thyroglobulin, although fetal thyroid hormone production does not begin until later, after the TSH-dependent expression of genes involved in iodide uptake and iodination (Szinnai et al., 2007). In mice, the entire process of thyroid organogenesis takes ~1 week (from E8.5 to E15.5); in humans, it extends over a much longer period and endogenous hormone synthesis is not evident before the eleventh week of gestation (Szinnai et al., 2007).

Developmental roles of key thyroid transcription factors
A number of intrinsic or cell-autonomous transcription factors have been shown to contribute to thyroid development (Fernandez et al., 2015) (summarized in Table 1). Four of them, namely Hhex, Nkx2-1, Pax8 and Foxe1, acting both individually and in concert, stand out as crucial and may thus be considered, collectively, as a thyroid signature within the anterior foregut endoderm. Below, we highlight

current knowledge of this quartet of transcription factors in the context of thyroid morphogenesis. It should be noted that, in humans, pathogenetic mutations in *NKX2-1*, *PAX8* and *FOXE1* are found only in a minority of patients with thyroid dysgenesis (Box 3), highlighting that, in most CH cases that arise due to developmental defects, the affected genes and molecular mechanisms of disease are still unknown.

An overview of the Hhex/Nkx2-1/Pax8/Foxe1 network
Although Hhex, Nkx2-1, Pax8 and Foxe1 are all expressed in several embryonic tissues (Table 1 lists those derived from anterior endoderm), it is only in the thyroid anlage that they are co-expressed and involved in a transcriptional network of interactions of mutual dependence (Parlato et al., 2004) (Fig. 5). Hence, deletion of any one of the genes encoding Hhex, Nkx2-1, Pax8 or Foxe1 inevitably confers athyreosis or severe thyroid hypoplasia (Kimura et al., 1996; Clifton-Bligh et al., 1998; De Felice et al., 1998; Mansouri et al., 1998; Martinez Barbera et al., 2000), despite the fact that the other factors are still being expressed in the thyroid placode (Parlato et al., 2004). At this early stage, Hhex, Nkx2-1 and Pax8 are expressed independently of each other, whereas diminishing levels of Foxe1 are evident in the absence of Pax8 (Fig. 5). As budding commences, Nkx2-1 promotes the expression of Hhex, Foxe1 and (weakly) Pax8, whereas Hhex and Pax8 regulate each other as well as Foxe1. Foxe1 is the only factor that does not regulate any of the others, indicting hierarchy within the network (Parlato et al., 2004) (Fig. 5). The functional relevance of this cross-regulatory network at the progenitor cell level is not yet fully understood. A downstream position and seemingly limited role of Foxe1 is somewhat
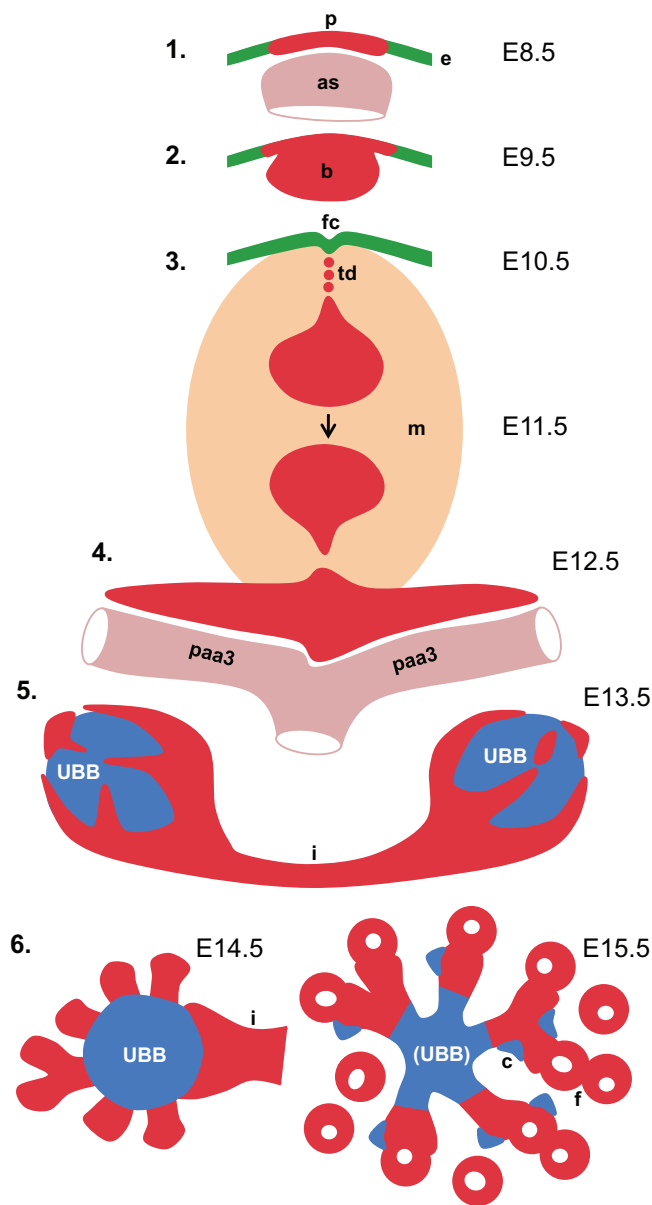
Fig. 4. Thyroid morphogenesis. Shown are six discernible stages of thyroid morphogenesis in the mouse. (1) Assembly of thyroid progenitors in a restricted area, termed the placode (p), in the pharyngeal floor of the endoderm (e). The placode abuts the cranial aspect of the aortic sac (as). (2) Emergence of the thyroid bud (b). (3) Detachment and migration of the thyroid primordium, leaving a residual pit, termed the foramen caecum (fc), in the mucosal lining of the presumptive pharyngeal cavity. The thyroglossal duct (td) transiently connects the descending thyroid to the pharyngeal endoderm. Mesenchyme (m) surrounds the migrating primordium. (4) Bifurcation of the thyroid primordium, which now extends bilaterally along the third pharyngeal arch arteries (paa3). (5) Bilobation, involving fusion of the midline thyroid primordium with the UBBs, which are derived from the lateral thyroid anlagen; in this process the UBB is initially enclosed by thyroid primordial tissue. An isthmus (i) portion crossing the upper trachea connects the two lobes. (6) Lobe growth, initially constituted by numerous parenchymal cords that project from the surface of the residual UBB, and folliculogenesis, which essentially is achieved by the conversion of solid cords into rows of microfollicles (f) that initially appose each other. Concurrently, C cell precursors (c) migrate centripetally along the cords. In mice, the entire process takes ~1 week, after which enlargement of the gland is attributed to the generation of new follicles and a gradual increase in the size of individual follicles. The morphogenetic stages of human thyroid development are nearly identical to those in mouse, although much prolonged.

Table 1. Expression and established functions of thyroid transcription factors in organ primordia derived from foregut endoderm

Gene	Expression	Transcriptional roles
Hhex	Thyroid bud	Survival
	Follicle cells	n.d.
	Lung bud	n.d.
	Liver bud	Specification, budding
	Pancreatic bud	Positioning
	Endocrine pancreas	Insulin secretion
Nkx2-1	Thyroid bud	Survival
	Follicle cells	Differentiation
	Ultimobranchial body	Survival
	C cells	Differentiation
	Lung bud	Branching
	Club cells (Clara cells)	Specification
	Type II alveolar cells	Differentiation
Pax8	Thyroid bud	Survival
	Follicle cells	Differentiation
Foxe1	Thyroid bud	Survival, migration
	Follicle cells	Hormone synthesis
	Pharyngeal endoderm	n.d.

Data are from mouse. n.d., not determined.

surprising given its proposed pioneer activity in regulating thyroid hormone production (Cuesta et al., 2007) and the prominent role of Fox proteins as pioneer transcription factors (Iwafuchi-Doi and Zaret, 2016). However, Foxe1, similar to Nkx2-1 and Pax8, has fundamentally different functions in embryonic versus adult thyroid cells (Fernandez et al., 2015), and before and after functional differentiation of the gland, suggesting that its transcriptional activity is determined by tissue context.

Hhex: distinguishing thyroid budding from the budding of other organs in the ventral endoderm

Hhex is ubiquitously expressed in the foregut endoderm (Fig. 5). Aside from its prominent role in the transcriptional network regulating early thyroid development (Parlato et al., 2004), it is not known if Hhex directly influences specific steps in thyroid morphogenesis, although it might play a role acting indirectly through Pax8 (discussed below) in thyroid progenitor cell survival (Table 1). Furthermore, the role of Hhex in the embryonic thyroid appears to differ from its role in other midline foregut derivatives. For example, earlier studies have shown that Hhex is important for specification of the ventral pancreas (Bort et al., 2004) and liver bud formation (Bort et al., 2006). In these processes, Hhex determines the positioning of progenitors in the appropriate endoderm domain for organ induction and promotes transition to a pseudostratified budding epithelium. This is not so in the embryonic thyroid, as although total progenitor cell number is decreased and the bud is hypoplastic in $Hhex^{-/-}$ thyroid primordium, its central position in the pharyngeal floor overlaying the roof of the aortic sac is not different from that of the wild-type bud, and multilayering of budding cells is evident (Parlato et al., 2004). Furthermore, the nascent hepatic endoderm in $Hhex^{-/-}$ embryos shows re-expression of Shh and, presumably directed by Shh, conversion of this domain into a duodenal gut phenotype (Bort et al., 2006). By contrast, in the absence of Hhex, the thyroid placode and bud rudiment remain Shh negative (Parlato et al., 2004). These observations argue against the hypothesis that *Shh* repression by Hhex might be a general mechanism necessary for budding from gut endoderm, as previously suggested (Bort et al., 2006). However, further studies are clearly needed to characterize how Hhex functions at this early stage of thyroid development and to identify other factors that may drive the budding process.

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Although thyroid dysgenesis is causal in 85% of children with congenital hypothyroidism (CH), mutations in known developmental genes account for only a minority of cases. For example, *NKX2-1* mutations give rise to brain-thyroid-lung syndrome, which is characterized by benign hereditary chorea, respiratory distress and CH (Krude et al., 2002), reflecting the established roles of NKX2-1 in brain, lung and thyroid development. The syndrome is inherited as a dominant trait with variable penetrance of thyroid and lung phenotypes; neurological symptoms are always present. Bamforth-Lazarus syndrome, which is caused by homozygous *FOXE1* mutations, also reflects the embryonic expression of FOXE1 in affected tissues leading to, aside from thyroid dysgenesis, cleft palate, bifid epiglottis, choanal atresia and spiky hair (Clifton-Bligh et al., 1998). Interestingly, a consanguineously inherited gain-of-function mutation in *FOXE1* reproduces athyreosis and craniofacial malformations (Carre et al., 2014), suggesting that FOXE1-mediated developmental regulation is critically dependent on gene dosage. It was also recently discovered that polymorphism within a *FOXE1* non-coding enhancer element confers increased risk of developing CH, cleft palate and thyroid cancer (Lidral et al., 2015). Autosomal dominant *PAX8* mutations can also lead to isolated thyroid hypoplasia, although associations with urogenital tract abnormalities, including unilateral kidney agenesis, have been reported (Fernandez et al., 2015). Again, this is expected considering that the embryonic tissue expression of PAX8 is limited to the thyroid and kidney.

Nkx2-1: a multi-organ regulator of morphogenesis from pharyngeal endoderm

Nkx2-1, formerly known as thyroid transcription factor-1 (TTF-1), plays a key role in organogenesis from the pharyngeal endoderm, acting within three separate but closely located domains: the thyroid bud, the lung bud and the last pair of the pharyngeal pouches (Table 1). Besides leading to athyreosis, an absence of Nkx2-1 causes arrested lung development at an early stage, characterized by diminished branching (Minoo et al., 1999), while the UBBs are retained as rudimentary appendages that eventually regress in *Nkx2-1* null mutants (Kusakabe et al., 2006a). Thus, although lineage determination and terminal differentiation differ among these primordial tissues, it can be speculated that the requirement for Nkx2-1 might reflect shared mechanisms in early morphogenesis. This possibility is supported by observations that the Nkx2-1 ortholog in lampreys is ubiquitously expressed throughout the entire endostyle (Kluge et al., 2005), i.e. also outside the protothyroid zone, suggesting a broader field of action for Nkx2-1 in the anterior endoderm than currently recognized. On this basis, it is assumed that a major role of Nkx2-1 is to determine ventral fates in the endoderm. Notably, the endostylar epithelium of ascidians (*Styela clava* and *Ciona intestinalis*) and lamprey consists of groups of ciliated and granular, presumably neuroendocrine, cells (Thorndyke and Probert, 1979; Nilsson et al., 1988; Kluge et al., 2005), which are also encountered in the respiratory airways and UBB. It can thus be hypothesized that the protochordate endostyle might be the predecessor to all Nkx2-1-dependent endodermal organs, having divided into three separate domains with distinct fates (first thyroid and UBB, later lung) as vertebrates evolved. In support of this, it has been shown that calcitonin is expressed in the ascidian endostyle (Thorndyke and Probert, 1979) and that Nkx2-1 regulates calcitonin expression in thyroid C cells (Suzuki et al., 2007). The vertebrate lung always develops from the ventral foregut but whether its ancestral cells can be traced back to the common Nkx2-1-positive endoderm domain present in the closest invertebrate relatives has not been investigated. Notably, in hemichordates, which do not

possess a true endostyle and lack the iodide-metabolizing cells conspicuous of a protothyroid, the paralogous Nk2.1 is widely expressed in anterior endoderm, further arguing for an ancient role for Nkx2-1 in pharyngeal evolution and development (Takacs et al., 2002; Simakov et al., 2015).

Nkx2-1 also appears to play pleiotropic roles in the embryonic thyroid, acting in both early and late stages of morphogenesis. Its phosphorylation on multiple serine residues distinguishes these roles (Silberschmidt et al., 2011). Accordingly, knock-in of a phosphorylation-deficient but transcriptionally active Nkx2-1 protein has no effect on the earlier morphogenetic stages but leads to impaired follicular development associated with loss of cadherin 16 (Ksp-cadherin) and disarranged localization of other junctional proteins [E-cadherin (cadherin 1) and ZO-1 (Tjp1)], suggesting that Nkx2-1 phosphorylation specifically regulates glandular differentiation into a follicular thyroid (Silberschmidt et al., 2011). A similar additional role for post-translationally modified Nkx2-1 was previously shown for the developing lung (DeFelice et al., 2003). The kinase(s) and phosphatase(s) involved in this phosphorylation have not been identified. Although transcriptional profiling has identified a number of target genes that are downregulated in the absence of phosphorylated Nkx2-1 (Silberschmidt et al., 2011), their functions in thyroid development remain to be established. Nkx2-1, presumably in its phosphorylated form, also maintains thyroid histoarchitecture postnatally (Kusakabe et al., 2006b). Finally, it should be noted that Nkx2-1 is regularly employed as a marker for identifying follicular progenitors in tissue sections or genetically for lineage tracing, with a cautionary note that Nkx2-1 is also expressed in the UBB epithelium and embryonic C cells (Kusakabe et al., 2006a).

Pax8: a master regulator of thyroid progenitor survival

The thyroid gland is the only endoderm-derived organ that expresses Pax8 (Table 1, Fig. 5). Placode formation and budding of the thyroid primordium do not differ between wild-type and *Pax8* null mutant mice (Parlato et al., 2004). However, subsequent regression of the primordium in a high percentage of animals lacking Pax8 or any one of the other key thyroid transcription factors strongly suggests that a functioning network ensures progenitor cell survival (Table 1). Indeed, morpholino-mediated knockdown experiments in zebrafish have confirmed that *nkx2.1a*, *pax2a* and probably also *hhex* are required for thyroid progenitor cell survival (Elsalini et al., 2003; Porreca et al., 2012). In both mice and zebrafish, the anti-apoptotic factor Bcl2 strongly accumulates specifically in the thyroid bud, suggesting that anti-apoptotic signals are important in early thyroid development and that the mechanism is evolutionarily conserved (Fagman et al., 2011; Porreca et al., 2012). Pax8 governs this process, as evidenced by the observation that Bcl2 is downregulated in $Pax8^{-/-}$ thyroids, which show increased apoptosis, while the expression of Nkx2-1 is maintained (Fagman et al., 2011). The susceptibility of the thyroid primordium to undergo regression in the absence of such a strict control mechanism of progenitor cell survival is puzzling. Notably, in wild-type mice, Nkx2-1$^+$ cells present in the regressing thyroglossal duct undergo apoptosis (Inoue et al., 2015), suggesting that keeping developing thyroid cells tightly assembled is important for cell survival. Indeed, Pax8 is essential for the expression of cadherin 16 in thyroid cells (de Cristofaro et al., 2012), and it has also been shown that the co-expression of multiple cadherins is likely to secure cohesiveness in the developing thyroid (Fagman et al., 2003; Cali et al., 2007). By stimulating apical polarization through cadherin 16, Pax8 was recently shown to promote folliculogenesis in cultured adult thyrocytes (Koumarianou et al., 2017), suggesting that Pax8 might promote *de novo* follicle formation by a similar mechanism in
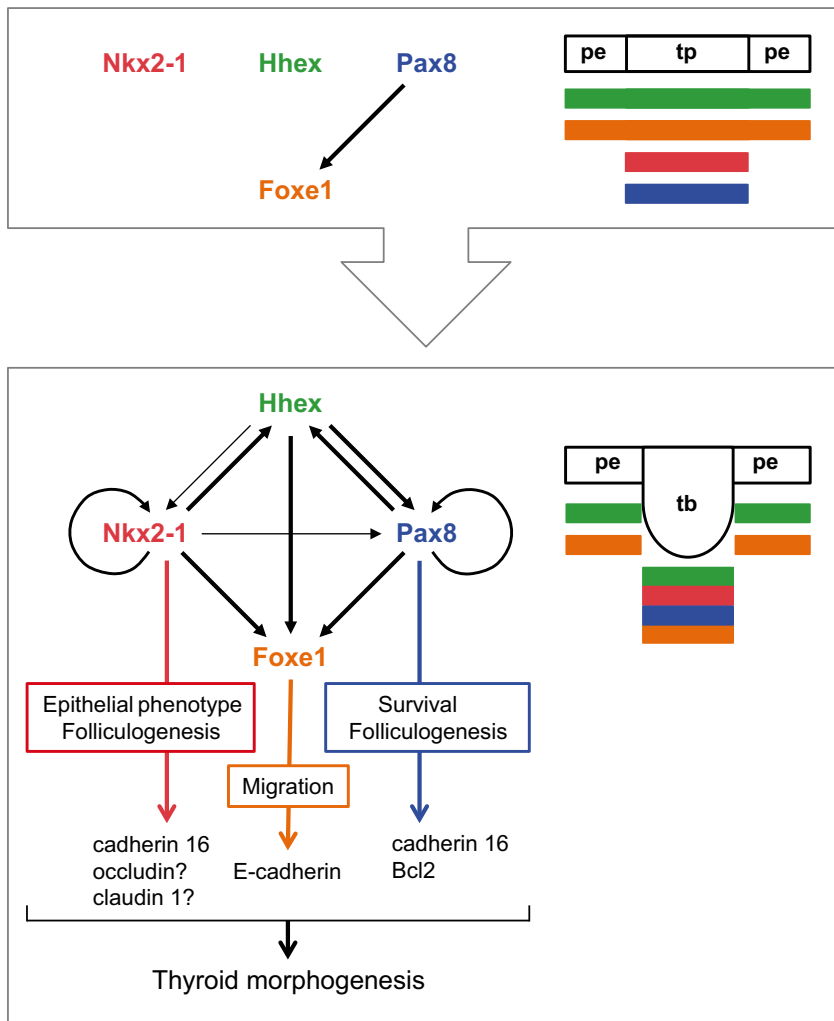
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Fig. 5. Transcription factors involved in early thyroid development. During thyroid placode (tp) formation (top), Hhex, Nkx2-1, Pax8 and Foxe1 are co-expressed in thyroid progenitors. With the exception of Foxe1, which requires Pax8 to be expressed, these transcription factors do not cross-regulate each other at this stage. As the thyroid bud (tb) forms (bottom), each factor except Foxe1 transactivates or by other means regulates the expression of the others (arrows). Autoregulation of Nkx2-1 and Pax8 expression has been shown for cultured thyroid cells, suggesting that a similar feed-forward transcriptional mechanism might also be operating in development, contributing to propagation of the thyroid lineage. Additionally, all but Hhex have been shown to differentially control the expression of adhesion and junctional proteins that are likely to mediate the distinct functions of Nkx2-1, Pax8 and Foxe1; the regulation of occludin and claudin 1 by Nkx2-1 has so far only been shown for lung cells (Runkle et al., 2012). pe, pharyngeal endoderm.

thyroid development. Notably, in humans with inactivating *PAX8* mutations, the thyroid phenotype is not as severe as after NKX2-1 inactivation, and the gland may even be of normal size, although in the absence of one *PAX8* allele patients are overtly hypothyroid due to dyshormonogenesis (Meeus et al., 2004). This further supports the notion that Pax8 is likely to regulate both early thyroid morphogenesis and the thyroid differentiation that takes place later but with distinct mechanisms.

Foxe1: promoting migration of the thyroid primordium
Foxe1, formerly known as thyroid transcription factor-2 (TTF-2), is ubiquitously expressed in the pharyngeal endoderm (Table 1, Fig. 5) but appears to be regulated differently in the thyroid domain than in other endoderm-derived tissues (Parlato et al., 2004). Studies in mice indicate that Foxe1 promotes the migration of thyroid precursor cells (De Felice et al., 1998; Parlato et al., 2004). However, the mechanism by which this occurs *in vivo* is unknown; migration of the bud still occurs, albeit incompletely, in *Pax8*$^{-/-}$ embryos in which Foxe1 expression is undetectable, so it is likely that other, presumably non-cell-autonomous, factors contribute to migration (for a more detailed discussion of this, see Fagman and Nilsson, 2010). The expression profile of Foxe1-regulated genes has been studied in a rat thyroid cell line (Fernandez et al., 2013). This analysis confirmed the proposed role of Foxe1 as a pioneer factor in thyroid cell differentiation (Cuesta et al., 2007) but, unfortunately, did not provide insight into its promigratory function in thyroid development. In palatogenesis, which

is defective in patients harboring *FOXE1* mutations that confer both thyroid and craniofacial malformations (Clifton-Bligh et al., 1998; De Felice et al., 1998), Foxe1 was recently shown to directly transactivate two developmental genes, namely *Msx1* and *Tgfb3*, which are known to drive epithelial-to-mesenchymal transition (EMT) and cell migration (Venza et al., 2011). Whether Foxe1 also targets these genes in the embryonic thyroid has not been investigated. The fact that all migrating thyroid bud progenitor cells stick together as a single body until bilobation and fusion with the UBB (Fagman et al., 2006) and that E-cadherin expression is maintained throughout this entire process (Fagman et al., 2003) argue against a role for EMT in thyroid development (although partial EMT cannot be excluded). Notably, E-cadherin is a target gene of Foxe1 (Fernandez et al., 2013). Altogether, these findings suggest that Foxe1 promotes collective rather than single-cell migration. Maintenance of the epithelial phenotype of progenitor cells during migration might function to prevent precocious dissolution of the thyroid primordium. Additionally, Foxe1 appears to be important for progenitor cell survival, presumably acting downstream of Pax8. This is likely to explain the different phenotypes observed in *Foxe1*-deficient mice, in which the thyroid rudiment may either be retained sublingually due to inhibited migration (corresponding to the most common ectopic location in humans), or completely regresses, as observed in 50% of embryos (Parlato et al., 2004).

Studies in amphioxus suggest that the ancestral gene to *Foxe1* duplicated late in vertebrate evolution to attain divergent functions of

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paralogs, but *Foxe1* remained involved in pharyngeal development (Yu et al., 2002). In the larval stage, the Foxe1 ortholog *AmphiFoxE4* is expressed not in the endostyle but in the club-shaped gland that also derives from pharyngeal endoderm. Since a homologous structure is missing in the vertebrate line, it was proposed that the genetic program responsible for evagination of the club-shaped organ from endoderm and possibly involving FoxA4 might have been transferred to the nearby endostyle before its disappearance (Yu et al., 2002), thus suggesting a mechanism for the evolutionary switch to a follicular thyroid prefiguring morphogenesis of the vertebrate thyroid. However, *FoxE4* is expressed specifically in the iodine-binding zone of the adult endostyle in both amphioxus and tunicates (Hiruta et al., 2005) (Fig. 2), suggesting that the established regulatory role of Foxe1 in thyroid hormone biosynthesis (Fernandez et al., 2015) might have already been accomplished by the orthologous factor in protochordates. Moreover, knockdown of *foxe1* does not influence the embryonic thyroid in zebrafish (Nakada et al., 2009). Together, these observations argue against the idea that Foxe1 regulates thyroid development by an evolutionarily conserved mechanism. It is likely, therefore, that a morphogenetic role is ascribed to Foxe1 only in animals in which the thyroid primordium migrates a considerable distance to reach its final orthotopic position.

Extrinsic factors that regulate thyroid morphogenesis

The development of the thyroid, similar to that of other foregut derivatives, is regulated by extrinsic factors that are derived from adjacent or more distant embryonic tissues. The fact that thyroid progenitors assembling in the pharyngeal floor are in immediate proximity to the cardiac outflow tract (Fig. 4) suggests that cardiac mesoderm and possibly also vessel-derived signals contribute to the regulation of thyroid morphogenesis. Indeed, there is a significant coincidence of thyroid dysgenesis and cardiovascular malformations in humans (Devos et al., 1999), indicating that normal development of the thyroid and heart are in some way linked. The fact that both processes are influenced by transcription factors such as Nkx2-5 (Searcy et al., 1998; Dentice et al., 2006), Isl1 (Cai et al., 2003; Westerlund et al., 2008) and Tbx1 (Fagman et al., 2007; Guo et al., 2011) in mice further supports the possibility of shared developmental traits. It should be noted that, although important for morphogenesis, embryonic vessels do not have an inductive role for the thyroid, as shown in the zebrafish *cloche* mutant which is devoid of any vascularization (Opitz et al., 2012).

Classical ablation studies in chick embryos have also revealed a role for cranial neural crest in thyroid development (Bockman and Kirby, 1984), and more recent findings indicate that subpopulations of neural crest cells differentially influence the distinct stages of chick thyroid morphogenesis (Maeda et al., 2016). This again highlights that the embryonic thyroid shifts microenvironments more than once before reaching its final position in the neck, and thus is likely – as we indeed highlight below and as summarized in Fig. 6 – to be regulated by various factors produced by these microenvironments and tissues. This also emphasizes the importance of considering spatiotemporal patterns of gene expression. Additional phenotypes might thus be revealed in knockout experiments if the gene of interest is inactivated at a later developmental stage, i.e. after it is first expressed in thyroid-associated mesenchyme.

Regulation of thyroid size and positioning: roles for mesodermal Tbx1 and Fgfs

Tbx1, a member of the T-box family of transcription factors and the prime candidate gene missing in 22q11.2 deletion or DiGeorge syndrome (DGS) (Liao et al., 2004), profoundly influences thyroid

morphogenesis (Fagman et al., 2007; Lania et al., 2009). In mice, Tbx1 is expressed in both the pharyngeal endoderm and subpharyngeal mesoderm but only its mesodermal activity promotes the generation of Nkx2-1$^+$ progenitors in the thyroid placode (Lania et al., 2009). This is similar to its role in cardiovascular development (Zhang et al., 2006) but differs from its effects on other parts of the pharyngeal apparatus, which preferentially depend on Tbx1 that is expressed in the endoderm (Arnold et al., 2006). The effect of Tbx1 on the embryonic thyroid is mediated by Fgf8 that is also produced in the mesoderm (Lania et al., 2009). Although a similar Fgf8-dependent pathway governed by *han* and *ace* (*fgf8a*) probably operates at the level of thyroid specification in zebrafish (Wendl et al., 2007), it is conceivable that in the mammalian thyroid the Tbx1-Fgf8 pathway promotes progenitor propagation rather than specification per se. Indeed, a thyroid bud is formed in *Tbx1*$^{-/-}$ mice but does not develop properly beyond this stage, leading to a hypoplastic single-lobed gland located close to the midline (Fagman et al., 2007). Although the molecular mechanism underlying this phenotype is not clear, studies suggest that vasculature might be involved. Accordingly, thyroid bilobation occurs initially along the third pharyngeal arch arteries (Fagman et al., 2006), and *Tbx1* null embryos exhibit severe malformations of the vascular tree emerging from the outflow tract (Zhang et al., 2005), suggesting a possible link. However, careful imaging shows that thyroid budding is delayed in *Tbx1* mutants such that contact with the aortic sac never takes place, possibly owing to diminished numbers of Tbx1$^+$ mesenchymal cells that might otherwise promote detachment and migration of the primordium (Fagman et al., 2007). Thyroid hypoplasia in *Tbx1*-deficient mice is therefore likely to be due to a primary loss of vessel contact anatomically, which in turn might impact access to growth signals that promote bilateral expansion of the primordium along embryonic vessels at a later stage. This probably explains the increased risk of individuals with DGS to develop thyroid dysgenesis and overt hypothyroidism (Stagi et al., 2010), and provides a mechanistic understanding of the occasional clinical reports of thyroid displacement associated with abnormal routing of aortic branches (Konno and Kanaya, 1988; de Almeida et al., 2009). Thyroid bilobation defects have also been reported for mice deficient for *Frs2a*, which encodes a docking protein in the Fgf receptor signaling pathway that also diminishes growth of the mesenchyme surrounding the thyroid primordium (Kameda et al., 2009). It is likely that the thyroid phenotype in *Frs2a* mutants depends, at least in part, on impaired regulation of thyroid development by Tbx1-Fgf8.

Blood vessel-mediated control of thyroid morphogenesis

The importance of embryonic large vessels for thyroid development was first demonstrated in *Shh*$^{-/-}$ mice in which the thyroid in late embryos is hypoplastic, resembling the clinical malformation hemiagenesis (Fagman et al., 2004). Subsequent tomographic analyses revealed abnormal pharyngeal arch artery development leading to asymmetrically positioned carotid arteries and ipsilateral deviation of the thyroid rudiment in *Shh* null mutants (Alt et al., 2006a). Notably, segments of the carotids, which are eventually located just lateral to the thyroid lobes (Fig. 1A), derive from those pharyngeal arch arteries along which the thyroid primordium extends during bilateral growth (Fagman et al., 2006). Together, this suggests that symmetric arch arteries define thyroid positioning in an intermediate stage of thyroid development and that this process is Shh dependent (Alt et al., 2006a). However, the thyroid itself does not seem to have a direct role in this process. Earlier in development,
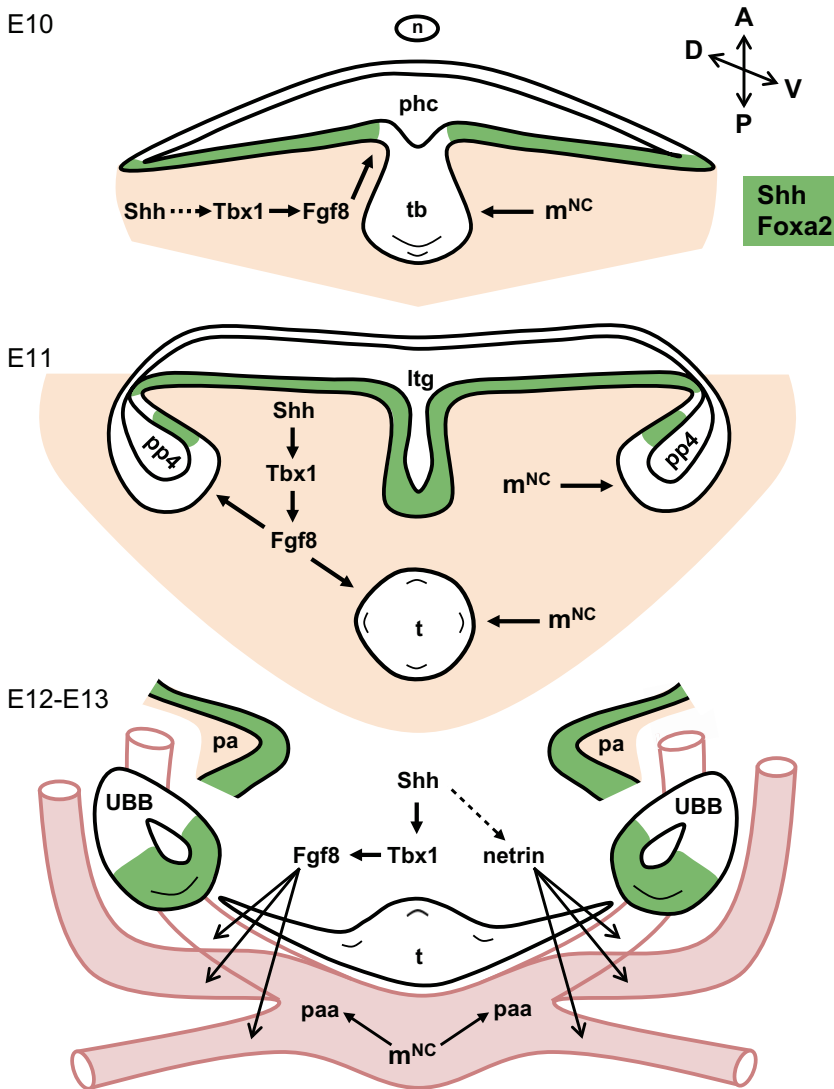
Fig. 6. Non-cell-autonomous signals that regulate thyroid development. Fgf8 promotes mouse thyroid morphogenesis in at least three distinct stages of development: recruitment of Nkx2-1$^+$ progenitors to the thyroid placode (top), detachment and migration of the thyroid bud (middle), and transverse elongation of the thyroid primordium after its descent (bottom). These effects are governed by Tbx1 transcriptional activity, which might be triggered by Shh derived from the pharyngeal endoderm. Notably, however, Shh and Foxa2, a potential target of the Shh/Smo signaling pathway, are selectively repressed in progenitors committed to a thyroid or UBB fate in early development; approaching fusion with the thyroid primordium, a subpopulation of UBB cells express Shh (the same accounts for few thyroid follicular progenitors; not shown), which may have a role in the fusion process. Thyroid morphogenesis is also linked to blood vessel development (as highlighted in the bottom panel). Netrin might be involved in the effector mechanism that links these developmental processes. Distinct subpopulations of neural crest-derived mesenchyme (m^{NC}) also influence both thyroid and UBB development. Shh is also necessary for the fusion of thyroid and UBB. n, notochord; phc, pharyngeal cavity; ltg, laryngotracheal groove; pp4, fourth pharyngeal pouch; pa, pharyngeal arch; paa, pharyngeal arch arteries; tb, thyroid bud; t, thyroid primordium.

Shh is ubiquitously expressed in the ventral endoderm except in the domain that forms the thyroid bud (Fagman et al., 2004; Parlato et al., 2004). This is similar to the situation observed during the development of the pancreas, in which repression of Shh specifically in the dorsal anlage is necessary for fate determination of pancreatic progenitors (Hebrok et al., 2000); if not repressed, Shh cell-autonomously switches dorsal pancreas to an intestinal fate while, conversely, the pancreatic domain expands at the expense of gastroduodenal endoderm if Shh is not expressed there (Kim and Melton, 1998). By contrast, the endoderm territory destined to a thyroid fate does not increase if Shh is globally deleted (Parlato et al., 2004). It is also worth noting that Foxe1 expression in the pharyngeal endoderm adjacent to the thyroid anlage requires Shh, whereas thyroidal Foxe1 does not (Parlato et al., 2004). This indicates that Shh has no direct role in Foxe1-mediated migration of the thyroid primordium. Surprisingly, lineage tracing of Shh$^+$ progeny indicates that no thyroid progenitors express Shh (Westerlund et al., 2013), suggesting that the follicular cell lineage, contrary to what might be expected, originates in a dorsal domain of the anterior foregut rather than in the endoderm immediately surrounding the thyroid placode. It is therefore likely that Shh influences thyroid development indirectly by shaping the

pharyngeal apparatus and its vasculature. Notably, in $Shh^{-/-}$ mice the thyroid is not only hypoplastic but also fails to fuse with the UBB (Westerlund et al., 2013). Misguidance due to vessel aberrations might contribute to this phenotype, although a budding defect in the absence of Shh, causing the retention of the UBB in the pharyngeal pouch, is a more likely explanation for this phenotype (Westerlund et al., 2013).

In zebrafish, live imaging employing the mCherry red fluorescent reporter has elegantly illustrated the coordinated development of the thyroid and the vasculature emerging from the apical pole of the heart (Opitz et al., 2012). In this study, mCherry was targeted to the thyroid through the thyroglobulin reporter and thus the cells detected were about or had already started to form follicles. This suggests that, in zebrafish, it is the differentiated thyroid cells rather than progenitors residing in the pharyngeal endoderm that interact with endothelial cells. In the mouse, by contrast, thyroid-vessel interactions occur in two distinct phases: first, precursor cells associate with the aortic sac and pharyngeal arch arteries as the undifferentiated primordium buds, migrates and lobulates (Fagman et al., 2006); and, second, newly differentiated cells establish so-called angiofollicular units as microvessels sprout into the prospective thyroid lobes (Hick et al., 2013). However, the precise

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mechanisms by which blood vessels influence thyroid morphogenesis are unknown. A trivial explanation, supported by the asymmetric, ipsilateral colocation of the thyroid and carotid arteries in *Shh*-deficient mice (Fagman et al., 2004; Alt et al., 2006a), is that the pharyngeal arteries serve as guiding tracks for directed growth, which in the case of normal symmetry results in a bilobed gland. Direct evidence of guidance by embryonic vessels has indeed been provided for the zebrafish thyroid by live imaging (Opitz et al., 2012). More recent studies in zebrafish infer an important role for netrin 1 expressed in pharyngeal arch mesenchyme in the conjoined development of thyroid and vasculature (Opitz et al., 2015). Netrins are members of the laminin superfamily that possess both chemoattractant and chemorepellent functions in embryonic development (Lai Wing Sun et al., 2011) and have been found to collaborate with Shh signaling as guidance cues (Sloan et al., 2015). A recent study of the transcriptional co-regulator Taz in zebrafish (Pappalardo et al., 2015) suggests that the Hippo pathway might govern thyroid size also via effects on the vasculature; although Taz expression is observed in thyroid cells, the prevalence of aberrant vessels in Taz-deficient fish argues in favor of a vascular patterning defect that indirectly affects thyroid development.

Finally, it should be noted that ablation studies in chick embryos have shown that neural crest-derived mesenchyme regulates thyroid lobe formation independently of embryonic vessels (Maeda et al., 2016). A thyroid lobe defect may thus appear even though the pharyngeal arch arteries seemingly develop normally. Although the mechanism for this is unclear, this finding supports the notion that thyroid bilobation relies on multiple factors that do not necessarily involve vessel contact.

The proliferation of thyroid progenitors and follicular precursor cells

Unlike adult thyroid cells, embryonic thyroid cells multiply independently of TSH (Peter et al., 1988; Postiglione et al., 2002). The switch to TSH-dependent growth does not occur until morphogenesis is completed and the cells have differentiated and produce thyroid hormone; in mice, this occurs postnatally (Postiglione et al., 2002) whereas in humans TSH regulates fetal thyroid growth in the third trimester. Interestingly, TSH resistance prior to functional differentiation is inherent to the progenitor phenotype, as evidenced by the lack of effect of a constitutively active TSH receptor when it is targeted to an earlier developmental stage than when the native receptor is expressed (Postiglione et al., 2002).

BrdU labeling in mouse embryos has revealed that proliferation rates vary spatiotemporally throughout thyroid morphogenesis (Fagman et al., 2006) (Fig. 7, top). An essential lack of mitotic Nkx2-1$^+$ progenitors until E11 implicates that initial enlargement of the thyroid bud most likely occurs by cell recruitment from outside the placode domain. Similar observations in chick embryos (Smuts et al., 1978) suggest that recruitment is a general mechanism of thyroid bud growth (Fig. 8A). It is possible that Fgf- and Bmp-mediated signals generated in the cardiogenic and pharyngeal mesoderm (Serls et al., 2005; Wendl et al., 2007; Lania et al., 2009; Kurmann et al., 2015) have a role in this process, although a direct mitogenic effect has not been demonstrated. Interestingly, in mice deficient for *Hes1*, a Notch target and transcriptional repressor, the thyroid bud is smaller and the final thyroid phenotype is hypoplastic, yet the number of BrdU$^+$/Nkx2-1$^+$ cells in the bud is paradoxically increased (Carre et al., 2011). This suggests the intriguing possibility that cell proliferation is actively repressed to enhance the influx of progenitors. In support of this, it has been

shown that thyroid progenitors express cyclin-kinase inhibitors when present in the bud but not at later developmental stages (Carre et al., 2011). It is conceivable that these features also are of importance for the thyroid budding process (Fig. 8A). Recent observations indicate that multilayering of the thyroid bud requires Cdc42-mediated apical constriction of progenitors centrally located in the thyroid placode (Loebel et al., 2016). By contrast, knockdown of *Rhou*, which encodes a Cdc42-related atypical Rho GTPase, paradoxically increases the size of the thyroid bud by a mechanism that appears to involve loss of apical polarity of budding cells (Loebel et al., 2011). Progression of thyroid budding by recruitment from adjacent endoderm might thus be facilitated by directed displacement of progenitors that acquire an unpolarized phenotype as they lose contact with the pharyngeal cavity (Fig. 8A). Collectively, these findings in mouse essentially confirm the cellular dynamics of thyroid budding as originally observed in chick embryos (Kinebrew and Hilfer, 2001).

Bilateral elongation of the thyroid primordium is characterized by a burst of progenitor cell proliferation (Fig. 7, top). This encompasses the majority of cells without any obvious regional differences (Fagman et al., 2006), suggesting that there is no gradient of growth stimulation. By contrast, after fusion with the UBB, further enlargement of the lateral lobes involves regionalized proliferation of leading cells present at the tip of radially oriented parenchymal cords that will later transform into arrays of follicles (Fagman et al., 2006) (see also Fig. 4). The key local factors that govern distinct growth patterns during bilobation and subsequent lobe enlargement have not been identified, although several have been implicated. Cell-autonomous transcription factors are likely to act permissively, as evidenced by the variable phenotypes of thyroid hypoplasia of a correctly positioned gland in familiar forms of CH due to *PAX8* mutations (Congdon et al., 2001) and in *Nkx2-1;Pax8* double-heterozygous mice (Amendola et al., 2005). Interestingly, a recent study indicates that Fgf10 influences thyroid shape and size differently depending on whether the *Fgf10* gene is globally inactivated or deleted in neural crest-derived mesenchyme (Teshima et al., 2016), contradicting previous notions that Fgf10 might influence early thyroid development (Ohuchi et al., 2000), although the precise roles of Fgf10 of different cellular origins were not further investigated. The fact that the embryonic thyroid is severely hypoplastic in *Shh*-deficient mice (Fagman et al., 2004) suggests that Shh, which is expressed in a minority of parenchymal cells in the mouse thyroid gland in late development (Westerlund et al., 2013), might act also as an intrinsic growth regulator, possibly reciprocally interacting with intrathyroidal stromal cells. The scattered distribution of these cells is suggestive of a putative stem cell niche. However, although the adult thyroid contains a population of cells with stem properties that appears to be activated upon tissue regeneration after partial thyroidectomy (Hoshi et al., 2007; Okamoto et al., 2013), a stem cell concept for the thyroid gland is controversial given the very slow replacement of individual follicular cells; indeed, the expected turnover of adult thyrocytes is more than 8 years (Coclet et al., 1989), in contrast to the high mitotic rate of thyroid cells in fetal life and infancy (Williams, 2015).

The need for tight control of thyroid cell proliferation is emphasized by recent observations in a mouse model of Down syndrome in which overexpression of *Dyrk1a*, the candidate gene for the trisomy 21 phenotype, leads to fetal thyroid hyperplasia and increased final thyroid size (Kariyawasam et al., 2015). Dyrk1a is a multifaceted kinase involved in growth control (Fernandez-Martinez et al., 2015) and is a central player in the Hippo pathway regulating organ size (Dick and Mymryk, 2011). In this
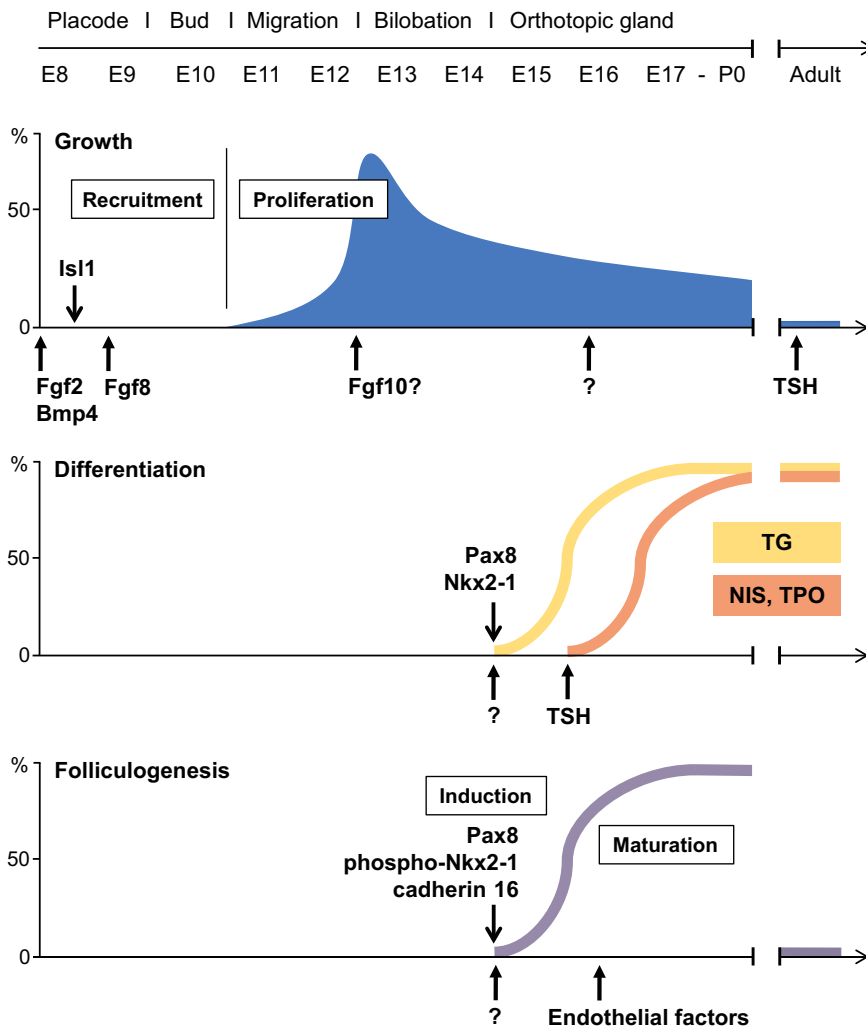
Fig. 7. The timing of primordial growth and functional differentiation in the embryonic thyroid. In mouse embryos, the proliferation of Nkx2-1[+] progenitors (top) is low until downward migration of the thyroid primordium ends and bilobation starts. Prior to this, the thyroid bud conceivably grows in size due to recruitment of cells from adjacent parts of the anterior endoderm. It is thus likely, but not formally proven, that thyroid specification (induced by the concerted action of Fgf2 and Bmp4) originally takes place outside the physical domain of the thyroid placode as defined by Nkx2-1 expression. Isl1 and Fgf8 promote the recruitment of thyroid progenitors into the thyroid placode, while Fgf10 secreted from neural crest-derived mesoderm promotes thyroid bilobation, although when and where Fgf10 exerts this effect is unknown. In late development, thyroid progenitors continue to proliferate, leading to enlargement of the embryonic thyroid in its final position. TSH does not influence thyroid growth until postnatally. The differentiation of thyroid progenitors (middle) is accompanied by the TSH-independent expression of thyroglobulin (TG) and, 1-2 days later, the TSH-dependent expression of sodium-iodide symporter (NIS) and thyroid peroxidase (TPO), after which point the embryonic gland is competent to synthesize thyroid hormone. Pax8 and post-translationally modified Nkx2-1 (which targets cadherin 16) are required for differentiation of progenitors and for follicle formation (bottom). Folliculogenesis is also initiated concomitantly with the expression of TG. Initiating factors synchronizing these processes are as yet unidentified. Further maturation of developing follicles depends on reciprocal interactions with microvessels and hence endothelial-derived factors (see also Fig. 8).

process, Dyrk1a interacts with Dream (Kcnip3), a transcriptional repressor that is also known to suppress thyroid function by downregulating thyroid-specific gene expression (Rivas et al., 2004; D'Andrea et al., 2005). This suggests a putative role for Dream in thyroid developmental growth and differentiation that warrants further investigation. Since Dyrk1a overexpression also negatively affects thyroglobulin expression and follicle formation and paradoxically increases Nkx2-1, Pax8 and Foxe1 expression in the prospective thyroid lobes (Kariyawasam et al., 2015), it can be speculated that Dyrk1a, with Dream as a putative partner, might be important for maintaining the balance between progenitors and differentiated follicular cells at a critical stage of organogenesis when fetal production of thyroid hormone starts and yet a fairly high growth rate is required to ensure thyroid enlargement until the final size of the gland is obtained.
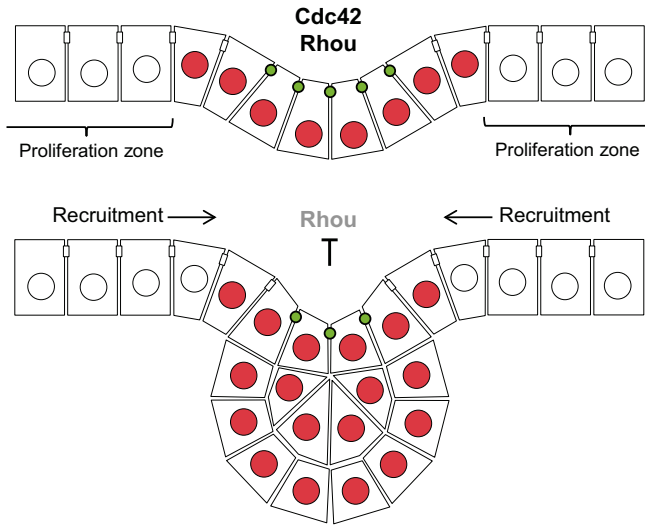
Folliculogenesis and thyroid differentiation: from progenitor to functional thyrocyte

In mice, follicle formation – the final stage in thyroid morphogenesis – involves the assembly of progenitors into a reticular network of solid parenchymal chords, just before differentiation takes place (Fagman et al., 2006). Numerous microfollicles exhibiting a small lumen can be discerned throughout the gland at E15, suggesting that folliculogenesis is a synchronous process at this stage; new follicles indeed form later but this is likely to relate to the generation of more

follicular cells as the gland enlarges postnatally (Nathaniel, 1986). Ultrastructural studies of the thyroid primordium in chick embryos have revealed that primitive follicle lumina secluded by tight junctions first appear in doublets of adjacent epithelial cells (Hilfer, 1979; Ishimura and Fujita, 1979). This indicates that embryonic thyroid follicles are first formed by hollowing rather than cavitation (Fig. 8B). However, this mechanism might not account for all species with a follicular thyroid. In teleosts, individual follicles develop sequentially by budding from the anterior endoderm and differentiate almost instantaneously, i.e. an intermediate stage of proliferation of undifferentiated progenitors forming a solid primordium is lacking (Wendl et al., 2002; Alt et al., 2006b). In the metamorphosing lamprey, as the endostyle proper degenerates, the initial thyroid follicles appear to develop by evagination of the remaining endostyle chambers (Fig. 2D) (Marine, 1913; Kluge et al., 2005).

A longstanding question has been whether terminal histogenesis of the thyroid gland and primordial follicle formation solely depend on intrinsic properties of cells committed to a thyroid fate or whether extrinsic factors, systemic or locally produced, might contribute. Evidently, dissociated thyroid cells in suspension cultures self-assemble into cysts (Nitsch et al., 1984) and, if embedded into an inert matrix of collagen type I, form stable follicles without other requirements (Ingeson-Carlsson and Nilsson, 2014). Studies in mice, chick and zebrafish also confirm that TSH has no role in *de novo* follicle formation (Hilfer, 1979; Postiglione et al., 2002; Alt

DEVELOPMENT

A Bud formation

Cdc42
Rhou

Proliferation zone   Proliferation zone

Recruitment ⟶   Rhou   ⟵ Recruitment
                          T

B Lumen formation

av ○   ○
TJ L TJ
av ○   ○

C Follicle maturation

L

laminin
collagen IV

Bmp/Smad
signaling

Vegfa

laminin-111

ec
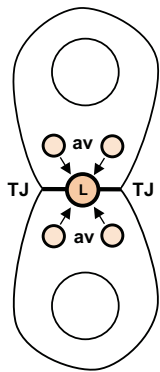
Fig. 8. Cellular dynamics during thyroid bud formation and folliculogenesis. (A) The thyroid bud grows via progenitor cell recruitment from the adjacent endoderm; cell cycle progression is transiently repressed in Nkx2-1[+] (red) budding cells only. Initial budding, which involves a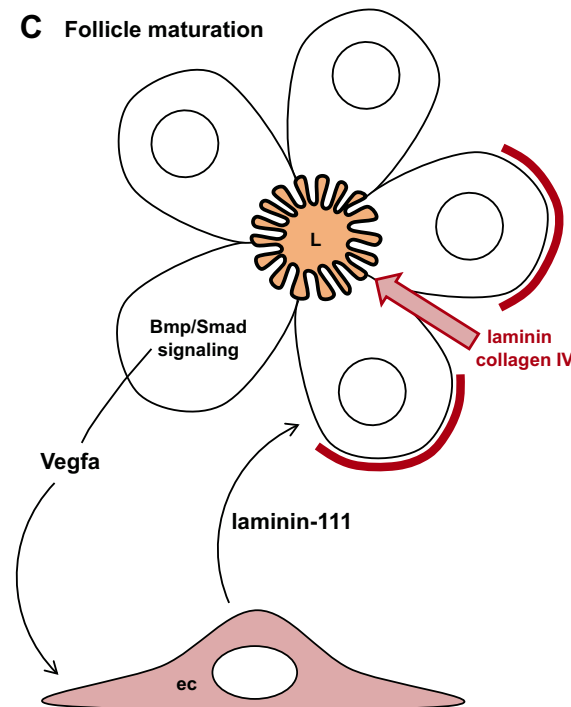pical constriction of the junction-associated cytoskeleton (green) and the transition of cells to an unpolarized phenotype, sequentially depends on Cdc42 and Rhou; the inhibition of Rhou is then required for budding to progress. (B) Initiation of follicle formation requires only two progenitor cells that adhere and polarize with their apical poles facing each other. A microlumen (L) is formed by coalescence of apical vesicles or vacuoles (av) that accumulate in the cytoplasm of both cells. Such preformed vacuoles exhibit apical markers (e.g. ezrin) and are likely to also contain thyroglobulin that is conjointly secreted as the lumen forms. Concurrently, tight junctions (TJs) are established, secluding the luminal compartment from the extrafollicular space. (C) The maturation of follicles to functional units requires that the embryonic thyroid is properly vascularized. Endothelial ingrowth is governed by the embryonic thyroid cells themselves, which secrete Vegfa. Local factors, such as laminin-111, released by adjacent endothelial cells (ec) in turn promote further development of follicles. Deposition of laminin and collagen type IV into a nascent basement membrane is necessary for follicles to mature and increase in size. The crosstalk with endothelial cells leading to follicle maturation depends on Bmp/Smad signaling in thyroid cells, possibly achieved by autocrine stimulation. The model is mainly based on observations by Hick et al. (2013) and Villacorte et al. (2016).

et al., 2006b). On the other hand, although forced co-expression of Nkx2-1 and Pax8 in ESCs is sufficient to induce thyroid differentiation, subsequent 3D culture in Matrigel in the presence of TSH is required to generate follicles *in vitro* (Antonica et al., 2012; Kurmann et al., 2015) (Fig. 3). Early studies of embryonic chick thyroid explants indicated that the mesenchymal component is necessary for follicles to appear (Hilfer and Stern, 1971). More recently, *ex vivo* studies on mouse thyroid primordia have shown that microvessels contribute to folliculogenesis, involving reciprocal interactions that are probably elicited by the thyroid cells themselves via vascular endothelial growth factor A (Vegfa) secretion (Hick et al., 2013). Interestingly, proper follicle development in mouse embryos also requires the formation of an epithelial basement membrane, which is governed by epithelial-endothelial crosstalk mediated by Bmp/Smad signaling and

laminins (Villacorte et al., 2016). It thus appears that the angiofollicular units of the gland not only serve an endocrine function but are also already established during development and fuel the conversion of an undifferentiated primordium to a follicular thyroid (Fig. 8C).

There are also other features that link folliculogenesis and the functional differentiation of thyroid cells (Fig. 7, middle and bottom). In human fetuses, thyroglobulin is expressed before any signs of follicle formation and endogenous hormone synthesis (Szinnai et al., 2007). This is in line with the hypothesis that thyroglobulin evolved before follicles in primitive vertebrates. Moreover, so-called intracellular lumina have been observed in dispersed thyroid cells (Ekholm and Bjorkman, 1984; Rousset et al., 1986) and, although these have been considered by some as an artifact of cell culture, similar structures have been observed

ultrastructurally in the neonatal rat thyroid (Nathaniel, 1986) and more recently in the embryonic mouse thyroid (Villacorte et al., 2016), suggesting that apical vacuolar compartments corresponding to intracellular lumina also probably have a developmental role. Notably, in chick embryos thyroglobulin is stored within the lumen at the first appearance of nascent follicles (Ishimura and Fujita, 1979). These findings collectively favor a mechanism of *de novo* follicle formation by fusion of intracellular lumina and synchronized secretion of thyroglobulin (Fig. 8B), thus linking the last step of thyroid morphogenesis to functional differentiation of the gland. Studies also suggest that the three-dimensional features of thyroid follicles not only confer a storage place for prohormone but also promote iodinating capacity and hence thyroid hormone biosynthesis (Bernier-Valentin et al., 2006).

A key question is what prevents premature thyroid differentiation despite the fact that both Nkx2-1 and Pax8, the only transcription factors necessary and sufficient to induce thyroglobulin expression (Antonica et al., 2012), are transcriptionally active as early as thyroid progenitors can be discerned in the thyroid bud (Fig. 5). Interestingly, enforced phosphorylation of Nkx2-1 at an earlier developmental stage does not induce precocious follicle formation (Silberschmidt et al., 2011), supporting the notion that folliculogenesis is spatiotemporally linked to thyroid differentiation in late development (Fig. 7, middle and bottom). Accordingly, it is perhaps not surprising that microvessels invading the thyroid primordium long before terminal differentiation (Fagman et al., 2006) are unable to promote follicle maturation until follicle formation is naturally initiated (Hick et al., 2013). In contrast to the zebrafish thyroid, in which progenitors differentiate and form follicles almost instantly after budding from anterior endoderm (Alt et al., 2006b), it is conceivable that mouse thyroid progenitors enrolled in growth and migration during a prolonged phase of morphogenesis must remain in an undifferentiated state. Thyroid progenitors maintain expression of E-cadherin throughout development, indicating that their epithelial phenotype is preserved (Fagman et al., 2003). However, as soon as they enter the thyroid bud and lose contact with the pharyngeal cavity the tight junction protein ZO-1 is downregulated (Loebel et al., 2016) and does not reappear until microfollicles are formed (Fagman et al., 2006; Villacorte et al., 2016), suggesting that signals governing apical-basal polarity in embryonic thyroid cells are repressed until this point.

Concluding remarks

As highlighted above, we are slowly beginning to gain a better understanding of the molecular and cellular mechanisms involved in generating a functional thyroid gland. The factors involved in triggering thyroid fate in the pharyngeal endoderm have been revealed, although their target genes and fine-tuning at each developmental stage remain to be identified and characterized. We also have a clearer view of the growth and migration of the thyroid primordium, and of the differentiation of committed progenitors. However, there are still several missing pieces in the puzzle. For example, the actual site in anterior endoderm where the thyroid lineage is specified and how thyroid and lung progenitors separate *in vivo* into distinct lineages despite sharing inductive mechanisms remain to be identified and characterized. The morphogenetic process that foregoes follicle formation and instead keeps proliferating progenitors in a dedifferentiated state even though all the necessary factors for thyroid differentiation are transcriptionally active is also unclear. This is reminiscent of the reverse process – dedifferentiation accompanied by accelerated growth – that characterizes thyroid cancer cells undergoing tumor progression.

Box 4. Development of thyroid cancer phenotypes

The most frequent malignant tumors in endocrine organs besides ovarian cancer arise from thyroid follicular cells. Papillary thyroid cancer (PTC), of which there are several subtypes, accounts for 85% of cases. The most prominent oncogenic drivers in PTC are $BRAF^{V600E}$ (60%) and *RET* rearrangements (15%), the latter being mainly associated with radiation exposure (Cancer Genome Atlas Research Network, 2014). Notably, *FOXE1* is a cancer susceptibility gene for PTC (Fernandez et al., 2015). Whether this has a developmental underpinning, based on the cell-autonomous role of Foxe1 in embryonic thyroid migration, is an open question. Follicular thyroid cancer (FTC), which retains a follicular phenotype and functional differentiation even in metastatic disease, accounts for less than 10% of thyroid malignancies. Interestingly, *PAX8*, the master gene of thyroid differentiation, is involved in tumor development in one-third of FTCs; its rearrangement with *PPARG*, which encodes a peroxisome proliferator-activated receptor, generates an oncogenic PAX8-PPARγ fusion protein (Raman and Koenig, 2014). A putative role of mutant PAX8 in FTC tumor development is, however, unknown. *RAS* mutations are also found in both FTC and PTC. Anaplastic thyroid cancer (ATC) is a rare but highly malignant tumor with a very rapid and invasive growth characterized by completely undifferentiated pleomorphic cells. A follicular cell origin of ATC is indicated by the presence of dedifferentiated PTC foci in some tumors, although it is suggested that ATC might also arise *de novo* from a putative thyroid stem cell (Guo et al., 2014). Medullary thyroid cancer (MTC), a neuroendocrine tumor originating from C cells, is mostly caused by oncogenic *RET* mutations. MTC is early invasive, reflecting the natural migratory behavior of the ancestral cells. Familiar MTC may occur already in childhood due to a germline *RET* mutation; this also confers the most serious tumor phenotype of multiple endocrine neoplasia syndromes (Moline and Eng, 2011).

Given that thyroid carcinoma is a common malignancy within the endocrine system, with both follicular cells and C cells being implicated (see Box 4), learning more about the natural process of thyroid morphogenesis will aid our understanding of tumor cell dedifferentiation potentially driven by reactivation of thyroid developmental programs.

With model organisms, in particular transgenic mice, it is possible to reproduce the major phenotypes of thyroid dysgenesis in humans, thereby proving that this can be a genetic disease. However, known mutations in developmental genes are causal for only a minority of patients with congenital hypothyroidism from thyroid dysgenesis (CHTD), which is the most common neonatal metabolic disorder (Van Vliet and Deladoëy, 2014). Notably, the familiar form of CHTD accounts for only 2% of cases (Castanet et al., 2000) (see Box 3), and affected monozygotic twins are mostly discordant, indicating that thyroid dysgenesis generally results from as yet poorly characterized epigenetic phenomena, early somatic mutations and postzygotic stochastic events (Perry et al., 2002; Deladoey et al., 2007).

Prominent among future challenges is the possibility to safely implement reconstituted functional thyroid follicles from autologous PSCs in humans with a view to rescuing hypothyroidism. Although this cell-based therapy has been demonstrated using murine models (Antonica et al., 2012; Kurmann et al., 2015), major obstacles still remain, notably the necessary high-yield generation of cells that are only committed to a thyroid fate. A recent study on human ESCs suggests some means by which this might be feasible (Ma et al., 2017). However, it is fair to say that there is still a long way to go before we can make a follicular thyroid in a test tube that can be used to treat patients. Nonetheless, recent progress in the field is certainly paving the way for this exciting prospect.

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