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Early thyroid development requires a *Tbx1-Fgf8* pathway

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Abstract

The thyroid develops within the pharyngeal apparatus from endodermally-derived cells. The many derivatives of the pharyngeal apparatus develop at similar times and sometimes from common cell types, explaining why many syndromic disorders express multiple birth defects affecting different structures that share a common pharyngeal origin. Thus, different derivatives may share common genetic networks during their development. *Tbx1*, the major gene associated with DiGeorge syndrome, is a key player in the global development of the pharyngeal apparatus, being required for virtually all its derivatives, including the thyroid. Here we show that *Tbx1* regulates the size of the early thyroid primordium through its expression in the adjacent mesoderm. Because Tbx1 regulates the expression of *Fgf8* in the mesoderm, we postulated that Fgf8 mediates critical *Tbx1*-dependent interactions between mesodermal cells and endodermal thyrocyte progenitors. Indeed, conditional ablation of *Fgf8* in *Tbx1*-expressing cells caused an early thyroid phenotype similar to that of *Tbx1* mutant mice. In addition, expression of an *Fgf8* cDNA in the *Tbx1* domain rescued the early size defect of the thyroid primordium in *Tbx1* mutants. Thus, we have established that a Tbx1->*Fgf8* pathway in the pharyngeal mesoderm is a key size regulator of mammalian thyroid.

INTRODUCTION

The thyroid is an endocrine gland that secretes two types of hormones, thyroxin and calcitonin, which are produced by two distinct cell types, the thyroid follicular cells (TFC) and the para-follicular or C-cells, respectively. These two cell types derive from distinct regions of the pharyngeal endoderm, specifically the ventral the pharyngeal endoderm provides the TFC progenitors, while the endoderm of the 4th pharyngeal pouches provides the C-cell progenitors. The TFC progenitor population constitutes the early primordium at approximately embryonic day (E) 8.5, when it appears as thickened epithelium on the ventral-medial wall of the pharynx, just caudal to the 1st pharyngeal arch. The early primordium then grows without further cell proliferation (Fagman et al., 2006) presumably by recruiting cells from the adjacent endoderm,

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and forms a pit by invaginating into the pharyngeal mesenchyme. Between E11.5 and E13.5, the primordium grows deeper into the mesenchyme, detaches from the pharyngeal endoderm, and begins to expand laterally. Later, the thyroid primordium reaches the trachea where it fuses with the 4th pouch-derived ultimobranchial bodies, which provide the C-cells of the mature organ. At around E15–16, the thyroid gland acquires its final shape, i.e. two lobes connected by a narrow isthmus. From this stage, the organ grows and begins to express functional markers such as thyroglobulin (tg), thyroperoxidase (TPO) and Tshr (De Felice, 2004).

The signals that induce the initial events of specification and migration of thyroid precursor cells are still unknown. It has been postulated that the invagination process involves epithelial-mesenchyme transition. However, recently it has been demonstrated that the epithelial phenotype is maintained by thyroid progenitor cells throughout organogenesis (Fagman et al., 2003). Indeed, thyroid precursors express high levels of E-cadherin, which is epithelial cell-specific, throughout development (Fagman et al., 2003), suggesting that the invagination is not associated with transition and active migration, but rather it might be due to passive movement of the primordium along with remodeling of the surrounding mesenchyme. In addition, it has been shown a cell autonomous role of *Foxe2* in the migration process (De Felice et al., 1998). The disruption of thyroid morphogenesis associated with mutations of genes expressed in tissues surrounding the primordium, e.g. *Shh* and *Hoxa5*, demonstrates the importance of tissue interactions during thyroid development (Fagman et al., 2004; Meunier et al., 2003).

DiGeorge syndrome (DGS) is associated with developmental defects of the derivatives of the pharyngeal apparatus that result in many birth defects, including cardiovascular, craniofacial and ear defects (Scambler, 1993; Shprintzen et al., 2005). DGS patients also show developmental abnormalities of pharyngeal-derived glands such as the thymus, parathyroids and, in some cases, thyroid (Bassett and Thakker, 1995; Scuccimarri and Rodd, 1998). Consistent with this, mice mutated for *Tbx1*, a key gene in the pathogenesis of DGS, recapitulate most, if not all, the above mentioned abnormalities (Baldini, 2005), including thyroid abnormalities (Fagman et al., 2007; Liao et al., 2004).

Tbx1 encodes a T-box transcription factor that is expressed regionally and dynamically in the ectoderm, endoderm and mesoderm of the developing pharyngeal apparatus. The endodermal expression domain encompasses the dorsal and lateral aspects of the pharynx but does not include the thyroid domain, while the mesodermal expression domain is adjacent to and partially surrounds the thyroid domain.

The thyroid primordium and the ultimobranchial bodies, which derive from the 4th pharyngeal pouches, fuse at E13 and contribute to the mature gland. *Tbx1*-null mutants do not have 4th pharyngeal pouches, thus thyroid hypoplasia in these mutants could be explained in part by the absence of the ultiomobranchial bodies-derived component of the mature organ (Liao et al., 2004). However, loss of *Tbx1* already affects thyroid development at E11.5 (Fagman et al., 2007), indicating that loss of the 4th pharyngeal pouch is insufficient to explain the phenotype.

In this study, we show that *Tbx1*-dependent signals from the mesoderm are required to ensure that the proper number of endodermally-derived thyroid precursors populate the primordium. We provide genetic evidence that *Fgf8* is a critical intermediary of this function because 1) Tbx1 positively regulates *Fgf8* expression in the mesoderm, 2) removal of *Fgf8* from *Tbx1*-expressing cells causes thyroid hypoplasia, 3) forced expression of *Fgf8* from the endogenous *Tbx1* locus partially rescues the thyroid phenotype in *Tbx1* mutants.

It has been shown that *Fgf10* (also expressed in mesoderm) and its receptor Fgfr2 are required for thyroid development (Celli et al., 1998; De Felice, 2004; Ohuchi et al., 2000). However, *Fgf8* has not been implicated in mammalian thyroid morphogenesis until now. Our data support a *Tbx1*-dependent role for *Fgf8* in thyroid development, and they are consistent with a recent

report showing a critical role of this gene in Zebrafish thyroid development (Wendl et al., 2007). In addition, our data show that pharyngeal mesoderm (including the cardiogenic mesoderm of the secondary heart field) supports thyroid development, thus providing a possible explanation for the frequent association between congenital heart disease and thyroid dysmorphogenesis (Olivieri et al., 2002).

MATERIALS AND METHODS

Mouse lines

The following mouse lines have been described: *Tbx1*$^{+/-}$ (Lindsay et al., 2001), *Tbx1*$^{Cre/+}$ (Huynh et al., 2007), *Tbx1*$^{flox/flox}$ (Xu et al., 2004), *Tbx1* $^{\Delta E5/+}$ (a null allele, (Xu et al., 2004)), *Tbx1*$^{Fgf8/+}$ (Vitelli et al., 2006), *Mesp1*$^{Cre/+}$ (Saga et al., 1999), *Fgf8*$^{flox/flox}$ (Meyers et al., 1998), Tie2Cre (Kisanuki et al., 2001), *Foxa2m*$^{cm/+}$ (Park et al., 2008), *Fgfr1*$^{flox/flox}$ (Xu et al., 2002), *Fgfr2*$^{flox/flox}$ (Yu et al., 2003), and R26R (Soriano, 1999). All lines were backcrossed into the C57Bl/6 genetic background for at least two generations. PCR strategies for mouse genotyping have been described in the original reports. For ablation of *FgfR1* and *FgfR2* in the pharyngeal endoderm by E8.0 , pregnant females were orally gavaged with 0.05mg/gm body weight tamoxifen at E6.75 as described in (Park et al., 2008). We have examined at least 3–5 embryos per experimental point and developmental stage.

Immunohistochemistry and in situ hybridizatiion

Mouse embryos or dissected thyroids were collected in phosphate buffered saline (PBS), and fixed in 4% paraformaldehyde overnight. Following fixation, embryos were dehydrated through graded ethanols, embedded in paraffin wax and sectioned (7μm). Sections were dewaxed by standard techniques, and heat-treated for the antigen retrieval. To quench endogenous peroxidases, sections were treated with hydrogen peroxide in methanol at room temperature. Sections were incubated for 1 hr at room temperature with blocking solution (3% BSA, 5% goat serum, 20 mM MgCl2, 0.3% Tween 20 in PBS) and then with primary antibodies overnight at 4°C. Staining procedures and chromogenic reactions were carried out according to the protocols of the Vectastain ABC kit protocol (Vector Laboratories). The primary antibodies used were: anti Titf1/Nkx2.1 (kindly provided by Dr R. Di Lauro), anti-human Thyroglobulin (Tg, Dako), anti phosphorylated histone H3 (Upstate), anti-Fgf8 (MAB323, R&D Systems), and anti-Fgfr4 (H-121, Santa Cruz). For morphological analyses, thyroid sections were dewaxed and stained with Hematoxylin and Eosin. Digital images from serial histological sections were used for 3-dimensional reconstructions using the softtware WinSURF (provided by SurfDriver, http://www.surfdriver.com/).

Cell counts of thyroid primordia (E9.0-E11.0) were carried out using high magnification microscopy on consecutive tissue sections and counting all the cells of the primordia.

Non radioactive in situ hybridization experiments were carried out using standard methods and probes for *Tbx1, Fgf8, Fgfr1, Fgfr2, Fgfr3* and *Fgfr4.*

Beta-galactosidase detection

Mouse embryos were fixed in 4% paraformaldehyde and processed for X-gal staining according to standard procedure. Embryos were embedded in paraffin wax and sectioned (10μm). Sections were counterstained with nuclear fast red.

RESULTS

Tbx1 is required in early thyroid development

The thyroid of E17.5 *Tbx1*$^{-/-}$ embryos is hypoplastic (Fagman et al., 2007; Liao et al., 2004). However, the primary developmental defects of thyroidogenesis caused by *Tbx1* loss of function are unknown. To define the mutant phenotype during development, we carried out a morphological analysis of the thyroid and thyroid primordium at different embryonic stages. Consistent with previously reported data (Fagman et al., 2007), we found that at E18.5, *Tbx1*$^{-/-}$ embryos had hypoplastic thyroid that was sometimes correctly located close to the cricoid cartilage, and was formed by very small left and right lobes (Fig. 1a–a') or, in most cases, by a single lobe (Fig. 5b'). In addition, we observed that the lumen of thyroid follicles of mutant embryos appeared slightly enlarged (Fig. 1a',b'). Immunohistochemistry revealed that *Nkx2-1* is normally expressed and that thyroglobulin is normally produced by mutant follicles (Fig. 1c–c').

The thyroid primordium was clearly identifiable from E9.0, when it appeared as a well-organized bi-layer of cells in the ventral endoderm of the primitive pharynx, that stained with anti Nkx2-1 immunohistochemistry (Fig. 2a). In *Tbx1*$^{-/-}$ embryos, we observed that Nkx2-1 + cells are not organized in a cell bi-layer, but appeared clustered (Fig. 2a'). At E9.5, thyroid precursor cells of wild type embryos invaded the surrounding mesenchyme to form the thyroid primordium. In *Tbx1*$^{-/-}$ embryos, the thyroid primordium was flatter than in controls (Fig. 2b'). At E10, the migration of the thyroid primordium toward the aortic sac was delayed in mutants (Fig. 2c'), and we observed persistence of the thyroglossal duct, which constitutes a continuity between the primordium and the pharyngeal endoderm (arrowhead in Fig. 2c'). Because the mutant primordium was smaller at all stages observed, we quantified its size by counting the number of Nkx2-1+ cells at E9.0-E10.5 (14–40 somites). Results showed a significant reduction of Nkx2-1+ cells at all stages tested (Tab. 1).

These data show that the mutant thyroid primordium is abnormal from the earliest stages of its development (14 somites), in terms of morphology and cell number, but known differentiation markers of the primordium, such as Tg, *Nkx2*-1, *Foxe1*, *Pax8*, and *Hhex1* were expressed normally (Fig. 2 and supplementary Fig. 1). In some experiments (for example see supplementary Fig. 1), Foxe1 immunostaining appeared slightly increased in the *Tbx1*$^{-/-}$ samples. However, repeated experiments could not confirm this difference.

Mesodermal expression of *Tbx1* regulates the size of the thyroid primordium

Tbx1 is not expressed in the thyroid primordium (Fig. 3a) but it is strongly expressed in the surrounding mesoderm and in the pharyngeal endoderm lateral to the primordium. In order to test whether *Tbx1* is expressed in endodermal precursors of the thyroid primordium, we used a *Tbx1*^{Cre} knock-in line (Huynh et al., 2007) in combination with the R26R reporter (Soriano, 1999), to label the progeny of *Tbx1*-expressing cells. We examined E9.5 *Tbx1*$^{Cre/+}$;R26R embryos and found very few (1–3) β-gal+ cells in the thyroid primordium (Fig. 3a'), indicating that *Tbx1*-expressing cells do not represent an important source of thyroid cells. Thus, the role of *Tbx1* in the thyroid primordium must be cell non-autonomous. Because *Tbx1* is expressed in neighboring mesodermal cells ((Fagman et al., 2007) and Fig. 3a), we hypothesized that there may be a *Tbx1*-dependent signal in this tissue. To address this hypothesis, we eliminated *Tbx1* in mesodermal cells using the *Mesp1*^{Cre} driver (Saga et al., 1999). The ability of *Mesp1*^{cre} to delete *Tbx1* exclusively in the mesoderm has been demonstrated previously (Zhang et al., 2006). We examined the thyroid size in *Mesp1*$^{cre/+}$;*Tbx1*$^{\Delta E5/fl}$ embryos (M-ko mutants) at two embryonic stage, E9.5–10 and E18.5, using Nkx2-1 as a marker. At both stages, we found a strong reduction of organ size, similar to *Tbx1*$^{-/-}$ embryos (Fig. 3b',c'). Thus, mesodermal *Tbx1* is required for early thyroid development. We postulated that *Tbx1* may have

a positive, cell non-autonomous effect on endodermal cell proliferation, thus indirectly controlling the number of endodermal cells destined to become thyroid cells. To test this, we evaluated cell proliferation in the ventral endoderm of M-ko mutants at E8.5. Results showed a significant reduction of cell proliferation in M-ko embryos (Tab. 2, an example of data is shown on supplementary Fig. 2) suggesting that the reduced size of the primordium may be due to a reduced number of thyroid precursors or to their reduced proliferation at early stages of development.

Thyroid morphogenesis requires expression of *Fgf8* in *Tbx1*-expressing cells

Tbx1 regulates *Fgf8* expression in the secondary heart field (Hu et al., 2004; Zhang et al., 2006), a mesodermal population located lateral and ventral to the pharyngeal endoderm. This expression domain is located just caudal to the thyroid primordium at E9.0 (Fig. 4a–a') and is abolished in M-ko embryos (Fig. 4b–b'). At later stages we could not identify any mesenchymal expression domain near the thyroid primordium (stages tested: E10.5, E11.5, and E14.5, data not shown). It has been shown that mesodermal *Fgf8* is important for endodermal cell proliferation (Park et al., 2006). Therefore, we tested whether Fgf8 may function as the *Tbx1*-dependent extra cellular signal critical for thyroid growth. To establish whether *Fgf8* expression in *Tbx1*-expressing cells is important for thyroid development, we conditionally deleted the gene using a $Tbx1^{Cre}$ driver with a conditional (floxed) allele of *Fgf8* indicated as $Fgf8^{fl}$ (Meyers et al., 1998). We observed that the thyroid of $Tbx1^{Cre/+};Fgf8^{fl/-}$ embryos at E9.5 (Fig. 4c–c') and E18.5 (Fig. 4d,d') is smaller than in controls. Complete cell counts of thyroid primordia confirmed the significant reduction of primordium size at E9.5 (Tab. 1). Three dimensional reconstruction of histological sections from E18.5 organs illustrates these differences (compare Fig. 5a', b' and c').

Thus, *Tbx1*-expressing cells represent a source of Fgf8 important for thyroid morphogenesis.

Fgf8 expression in the *Tbx1* domain partially rescues the $Tbx1^{-/-}$ thyroid phenotype

If loss of *Fgf8* expression in the *Tbx1* domain is part of the pathogenetic mechanism leading to thyroid hypoplasia in $Tbx1^{-/-}$ embryos, then forced expression of *Fgf8* in the *Tbx1* domain should ameliorate the $Tbx1^{-/-}$ thyroid phenotype. To test this idea, we used the $Tbx1^{fgf8}$ allele in which an *Fgf8* cDNA has been knocked into the *Tbx1* locus (Vitelli et al., 2006). In these animals, the *Fgf8* cDNA is expressed in the *Tbx1* domain and the *Tbx1* gene in which the cDNA has been inserted is not functional. Thus, $Tbx1^{Fgf8/-}$ embryos are null for *Tbx1*, but express *Fgf8*. We harvested $Tbx1^{Fgf8/-}$ and $Tbx1^{Fgf8/Fgf8}$ embryos at E10.5 and carried out cell counts of thyroid primordia. Results showed that the cell number of knock-in embryos was not statistically different from those of wild type embryos (Tab. 3). We also carried out histological analyses of $Tbx1^{Fgf8/-}$ E18.5 embryos. Results indicated that $Tbx1^{Fgf8/-}$ thyroids are substantially larger than in $Tbx1^{-/-}$ embryos (compare Fig. 5 b–b' and d–d'), although they have abnormal morphology, mostly limited to a large lobe (compare with control, Fig. 5a'). These data demonstrate a partial rescue of the $Tbx1^{-/-}$ thyroid size phenotype and support a pathogenetic role of *Fgf8* dosage reduction in the thyroid phenotype of $Tbx1^{-/-}$ embryos.

Next, we addressed the question as to which FGF receptor may mediate the response of the pharyngeal endoderm/thyroid primordium to Fgf8. Because it has been shown that *Fgfr1* and *Fgfr2* are expressed in this tissue, and because conditional alleles are available for these genes, we have deleted both these genes in the pharyngeal endoderm using the $Foxa2^{mcm}$ cre driver (Park et al., 2008). To this end, we have examined the thyroid gland in $Fgfr1^{flox/-};Fgfr2^{flox/-};Foxa2^{mcm/+}$ E18.5 embryos. Results showed that the thyroids of these mutants were not significantly different from those of control littermates (Supplementary Fig. 3). Thus, the expression of these two receptor genes in the pharyngeal endoderm appears to be dispensable for thyroid development. Next, we have tested the pharyngeal endoderm

expression of the other two receptors, *Fgfr3* (by in situ hybridization) and *Fgfr4* (by in situ hybridization and immunohistochemistry). Results showed that at E8.5 and E9.5, *Fgfr3* is very weakly expressed (Supplementary Fig. 4) while *Fgfr4* is not expressed at this stage (although it has been shown to be expressed in the pharyngeal endoderm at earlier stages (Serls et al., 2005)) (data not shown).

DISCUSSION

Thyroid organogenesis is associated with the expression of a set of transcription factor-encoding genes, *Nkx2-1*, *Foxe1*, *Pax8* and *Hhex1* (Parlato et al., 2004). Although these factors are also expressed in other embryonic tissues, they are co-expressed only in the endodermal cells fated to become TFC. Mouse knockout data have demonstrated their key role in thyroid organogenesis. In *Nkx2-1*$^{-/-}$ or *Pax8*$^{-/-}$ embryos, the thyroid primordium forms but by E11.5 it degenerates (Mansouri et al., 1998; Minoo et al., 1999). In *Hhex1*$^{-/-}$ embryos, the thyroid primordium forms and expresses *Nkx2-1*, *Foxe1* and *Pax8*, but at later stages their expression is downregulated and the thyroid is small (Parlato et al., 2004). In *Foxe1*$^{-/-}$ embryos, the thyroid primordium forms but does not migrate properly. Thus, none of these transcription factors is individually required for thyroid specification or primordium formation. There are also examples of genes that are not expressed in the thyroid but that affect its development. For example, *Hoxa3*$^{-/-}$ and *Eya1*$^{-/-}$ mutants have thyroid hypoplasia. In both cases, thyroid defects have been interpreted as being secondary to failed development of the ultimobranchial bodies (Manley and Capecchi, 1995; Xu et al., 2002)).

Congenital hypothyroidism has been reported in several cases of 22q11DS patients, although it is not a common feature of the syndrome (Weinzimer et al., 1998). *TBX1* is the major candidate gene in this syndrome, and *Tbx1* mouse mutants present with a small thyroid, suggesting that the gene contributes, directly or indirectly, to thyroid development (Liao et al., 2004). Recently, it has been suggested that the bilobation defect of the thyroid in *Tbx1*-null mice could be due to failure of the thyroid primordium to establish a contact with the aortic sac, which normally occurs at around E11.5–12 (Fagman et al., 2007), possibly through failed signaling between endothelial cells and primordium. Our data presented here revealed that *Tbx1* has a much earlier role in thyroid size, as the primordium is smaller from E9. In order to exclude a role of *Tbx1* in the endothelium adjacent to the primordium, we ablated the gene in endothelial cells using the Tie2-Cre driver. Results showed that the thyroid of Tie2-Cre; *Tbx1*$^{flox/-}$ embryos at E18.5 was of normal size (Supplementary Fig. 5).

The role of *Tbx1* in the thyroid appears to be limited to regulating the number of cells of the primordium and does not appear to be involved in differentiation of thyroid precursors, as the expression of thyroid specific markers was not affected in *Tbx1* mutants. In addition, the mutant thyroid primordium does not regress and is still capable of growing, although it will never reach the normal, final size. Suggesting that the primary defect is at or precedes primordium formation. Conditional ablation of *Tbx1* in the mesoderm is sufficient to recapitulate the null thyroid phenotype, thus indicating that the mesoderm plays a critical role in signaling to the developing thyroid or their progenitors. We show that loss of *Tbx1* in the mesoderm is associated with reduced cell proliferation in the pharyngeal endoderm of early embryos, thus providing a possible explanation as to why the primordium is small. We have tested the hypothesis that one of the possible *Tbx1*-dependent signals critical for thyroid growth is *Fgf8*. Indeed, conditional ablation of *Fgf8* in *Tbx1*-expressing cells causes severe early and late thyroid hypoplasia, similarly to *Tbx1* ablation. Conversely, forced expression of *Fgf8* in the *Tbx1* expression domain, partially rescues the early and late *Tbx1*$^{-/-}$ thyroid phenotype. Endogenous *Fgf8* expression in the mesoderm near the thyroid primordium is only detectable in early embryogenesis (up to approx. E9.0), consistent with the early appearance of the *Tbx1* mutant phenotype. In an attempt to determine whether the tissue targeted by Fgf8 is the

pharyngeal endoderm, we have tested the requirement of *Fgfr1* and *Fgfr2* in this tissue using conditional deletion. *Fgfr1* and *Fgfr2* are expressed in the pharyngeal endoderm (Moon et al., 2006; Wright et al., 2003). Results indicated that compound deletion of these two receptors does not cause thyroid abnormalities suggesting that the relevant receptors may be *Fgfr3*, weakly expressed in the pharyngeal endoderm, and/or *Fgfr4*, expressed in the pharyngeal endoderm at the 7 somite stage (Serls et al., 2005) but not at E9.0. An alternative possibility is that the target tissue is a non-endodermal tissue that, in turn, signals to thyroid precursors.

The Fgf8-rescued thyroids are misshapen as they do not show distinct bilobation. This may be due to at least four reasons. First, *Tbx1* ablation affects a number of genes, including other members of the FGF family, most notably *Fgf10*, which is also required for thyroid development (Ohuchi et al., 2000) and is expressed in the mesoderm. Because deletion of *Fgf8* is sufficient to cause thyroid hypoplasia, it is possible that the two ligands have distinct roles in thyroid development. Second, forced expression of *Fgf8* from the *Tbx1* locus does not rescue any of the major $Tbx1^{-/-}$ phenotypic findings in $Tbx1^{Fgf8/-}$ embryos (Vitelli et al., 2006), including the profound dysmorphogenesis of the pharyngeal apparatus as a whole. Thus, the "rescued" thyroid grows within a highly abnormal anatomical environment, which could explain the abnormal shape. Third, $Tbx1^{Fgf8/-}$ embryos, like $Tbx1^{-/-}$embryos, do not develop 4th pharyngeal pouches, thus the rescued thyroid would not have the ultimobranchial body-derived component. Fourth, size and bilobation of the thyroid may be regulated by different mechanisms. For example, bilobation may be achieved through physical interactions with arteries (which are abnormal in $Tbx1^{-/-}$ embryos and not rescued by forced expression of *Fgf8*) , as previously suggested (Alt et al., 2006; Fagman et al., 2007; Liao et al., 2004).

Our data demonstrate an important role for *Fgf8* in mammalian thyroid development and are consistent with recent findings in the Zebrafish model (Wendl et al., 2007). In addition, we show that *Tbx1* is a critical regulator of *Fgf8* expression relevant to thyroid development. A *Tbx1*-responsive enhancer has been previously demonstrated in the *Fgf8* gene (Hu et al., 2004). Thus, Tbx1 regulates, via Fgf8, the size of the early thyroid primordium. We hypothesize that this function is operated by regulating the proliferation of endodermal progenitors of thyrocytes. Direct demonstration of this mechanism will require positive identification of these progenitors, which is not currently possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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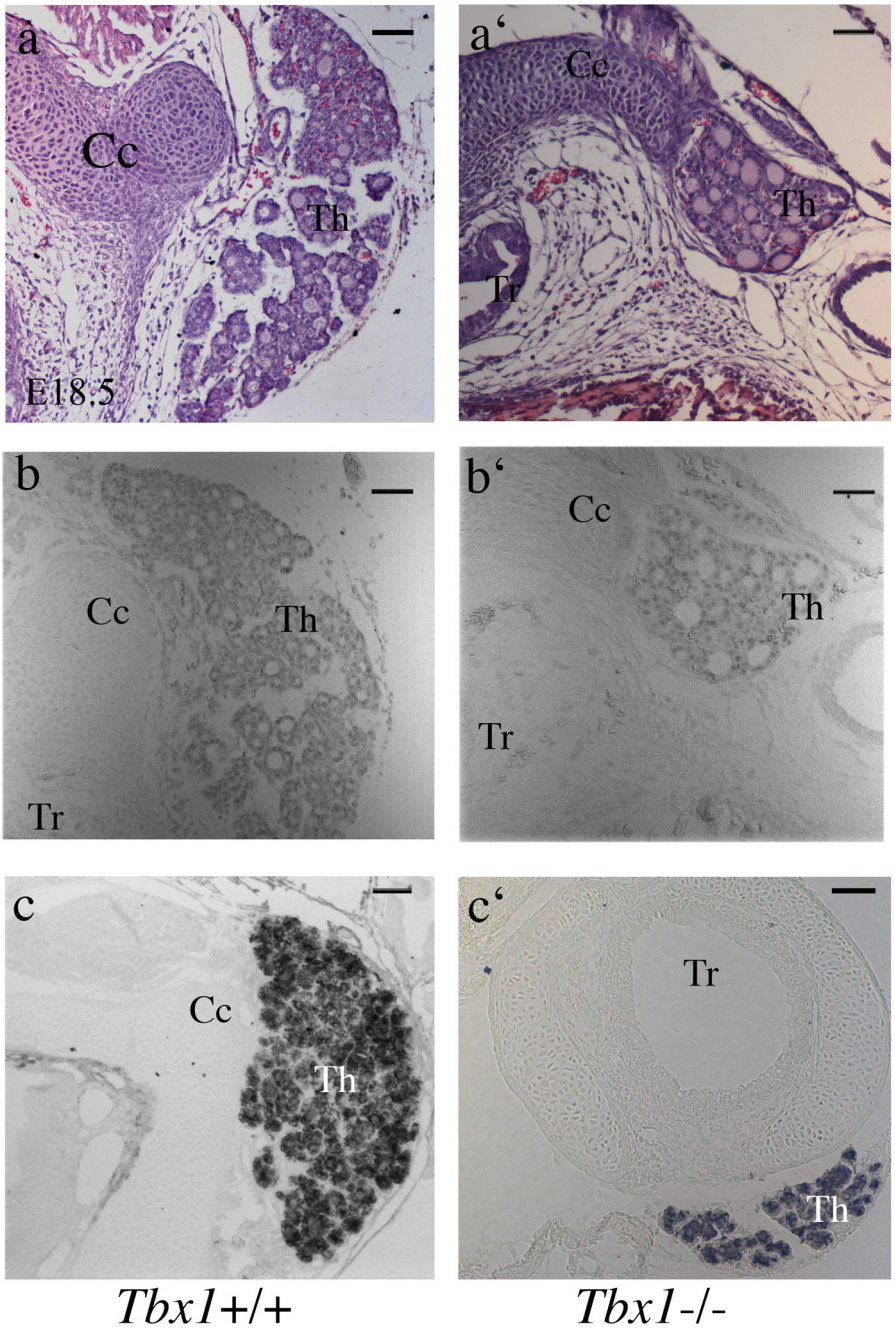

Figure 1. Hypoplasia and dysmorphogenesis of the thyroid in Tbx1$^{-/-}$ embryos at E18.5

Hematoxylin and eosin staining of histological sections from wild type and mutant thyroids (a-a'). Immunohistochemistry with anti-Nkx2-1(b-b') and anti-Tg (c-c') antibodies demonstrates the presence of differentiated and functional thyroid follicular cells. *Cc* cricoid cartilage, *Th* thyroid, *Tr* trachea. The scale bar is 50μm.

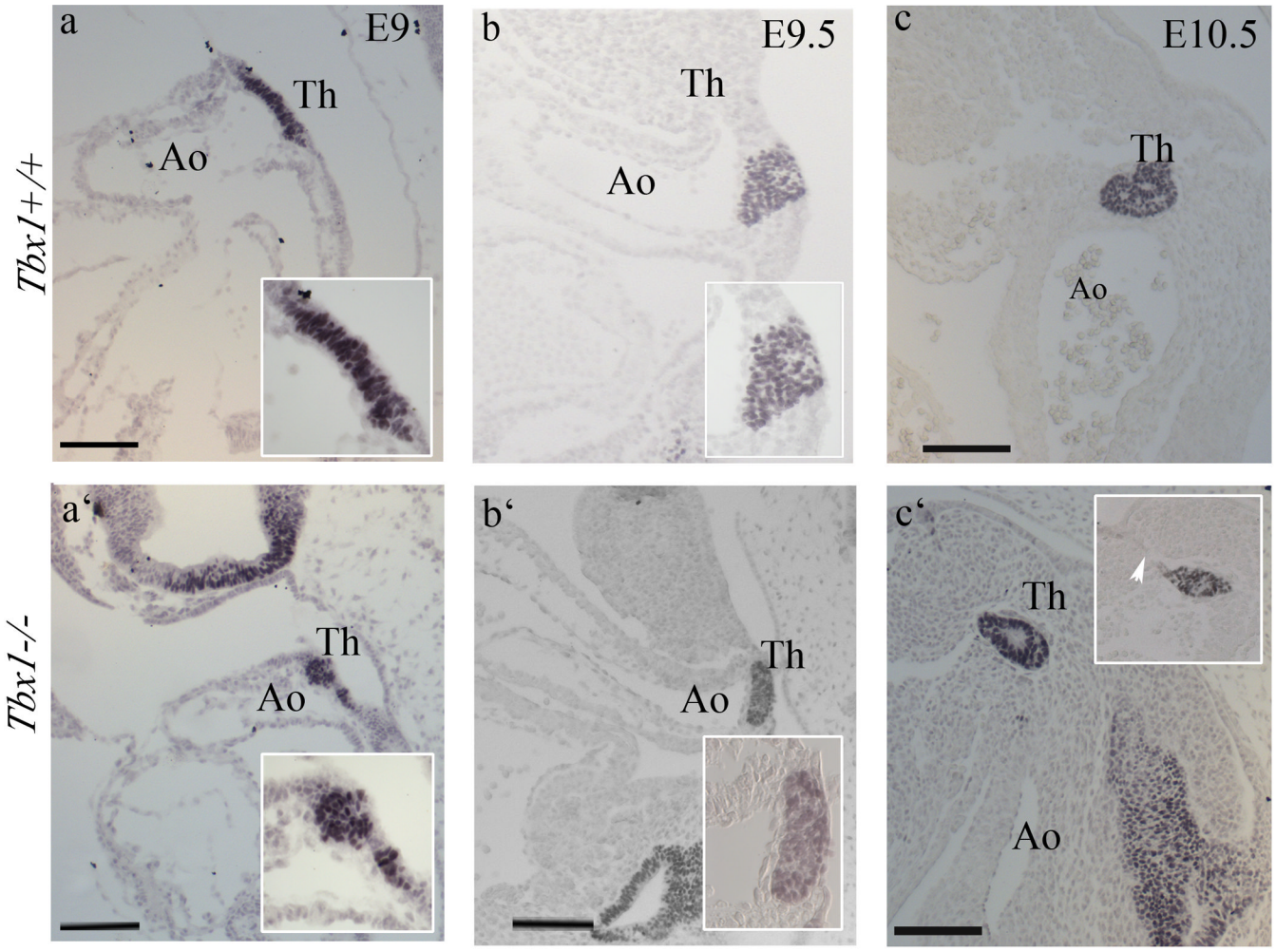

Figure 2. Early abnormalities of the thyroid primordium in Tbx1 mutants

(a–c') Nkx2-1immunohistochemistry on sagittal sections of wild type (a–c) and *Tbx1*-null (a'-c') embryos at E9, E9.5 and E10.5. The mutant thyroid primordium (14 somites) has lost the characteristic bi-layer cell morphology (compare insets in a' and a). At E9.5, the mutant thyroid primordium is flatter than in wild type (b,b'), while at E10.5 it is clearly smaller than wild type and shows a persistent thyroglossal duct (white arrow). *Ao* aortic arch. The scale bar is 100μm.

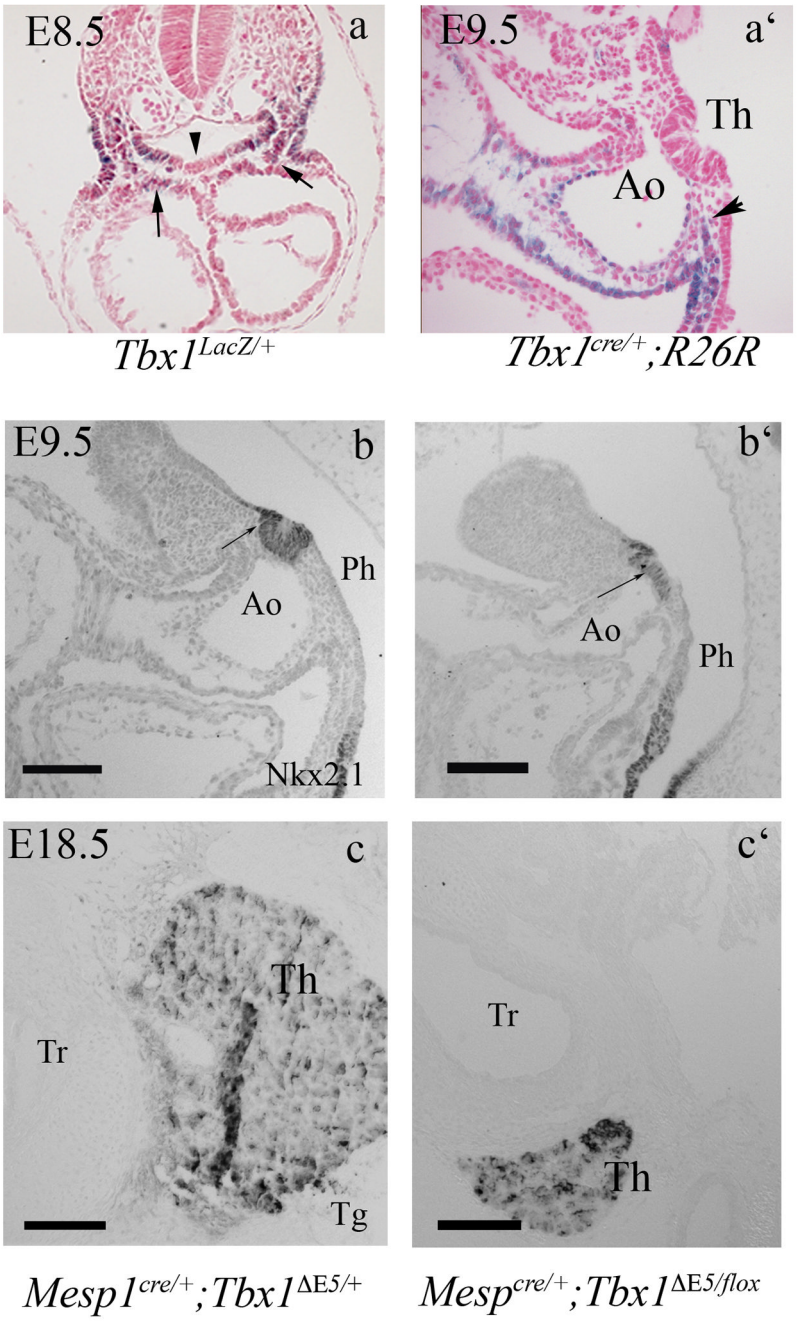

Figure 3. Thyroid development requires Tbx1 expression in the mesoderm

(a) Transverse section of an X-gal stained, *Tbx1*$^{lacZ/+}$ E8.5 embryo showing *Tbx1* expression in the mesenchyme adjacent to the thyroid primordium (arrows) but not in the primordium itself (arrowhead). (a') cell fate analysis in *Tbx1*$^{Cre/+}$; R26R mice. A group of β-gal+ cells surrounds the thyroid primordium (black arrow) but no labeling is visible within the primordium. (b–c) The thyroid phenotype in *Mesp1*$^{cre/+}$;*Tbx1*$^{flox/-}$ embryos at E9.5 and E18.5. Nkx2-1immunohistochemistry on sagittal sections of mutant embryos at E9.5 (b') reveals a differentiated thyroid primordium that is smaller and flatter than in controls (b). (c–c') Tg immunohistochemistry of *Mesp1*^{Cre}/+ ;*Tbx1*$^{flox/-}$ mutant embryos at E18.5. *Ao* aortic arch, *Tr* trachea. The scale bar is 100μm.

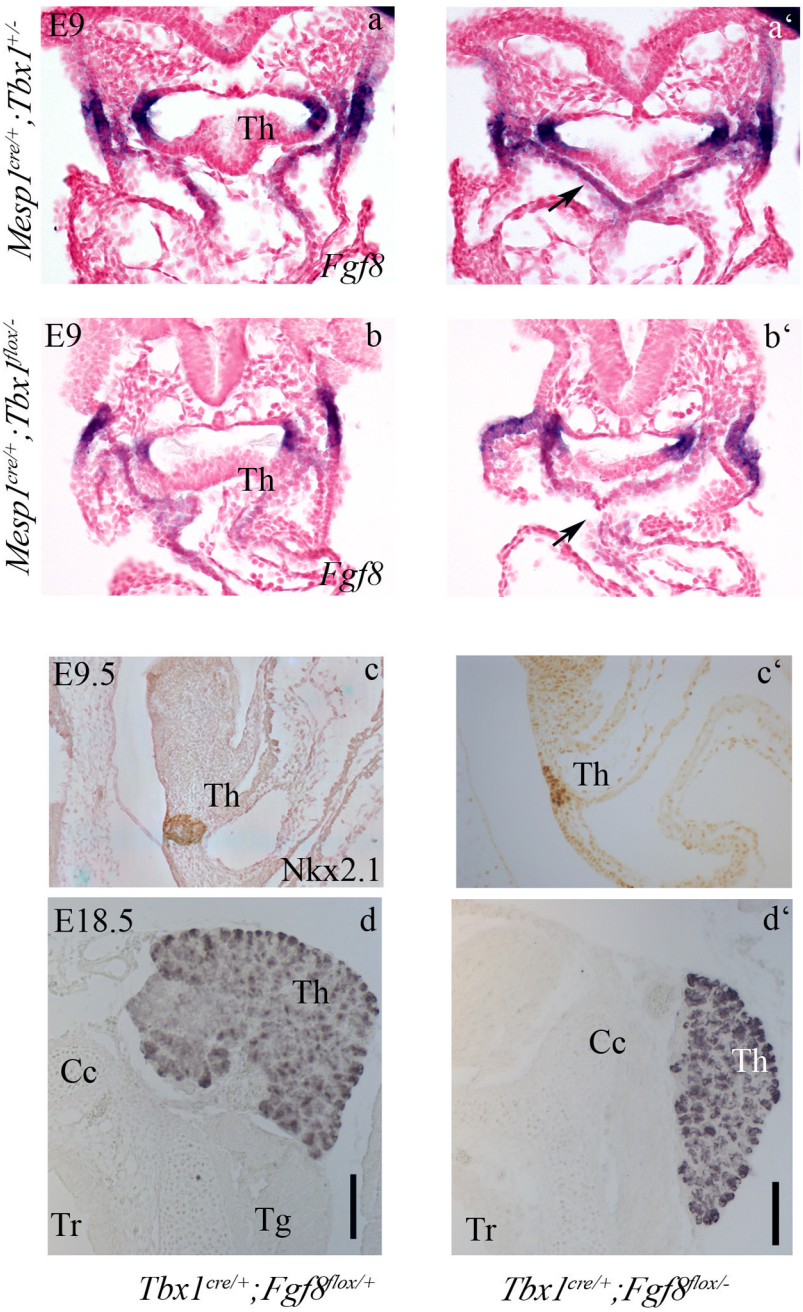

Figure 4. Ablation of Fgf8 in Tbx1-expressing cells causes thyroid hypoplasia

Transverse sections of E9.0 embryos show *Fgf8* expression in the mesoderm (black arrow in a') caudal to the thyroid primordium, indicated as Th in the more cranial sections, a and b, in control and Mko embryos (a,a', and b, b', respectively). Note the down regulation of the mesodermal domain in the M-ko embryo.

Nkx2.1 immunohistochemistry in control (c) and $Tbx1^{Cre/+};Fgf8^{flox/-}$ E9.5 embryos (c') reveals a differentiated but smaller thyroid in embryos with conditional ablation of *Fgf8*. Similarly, at E18.5, Tg immunohistochemistry reveals a small thyroid in a $Tbx1^{Cre/+};Fgf8^{flox/-}$ embryo (d') compared to wild type (d). The scale bar is 50μm

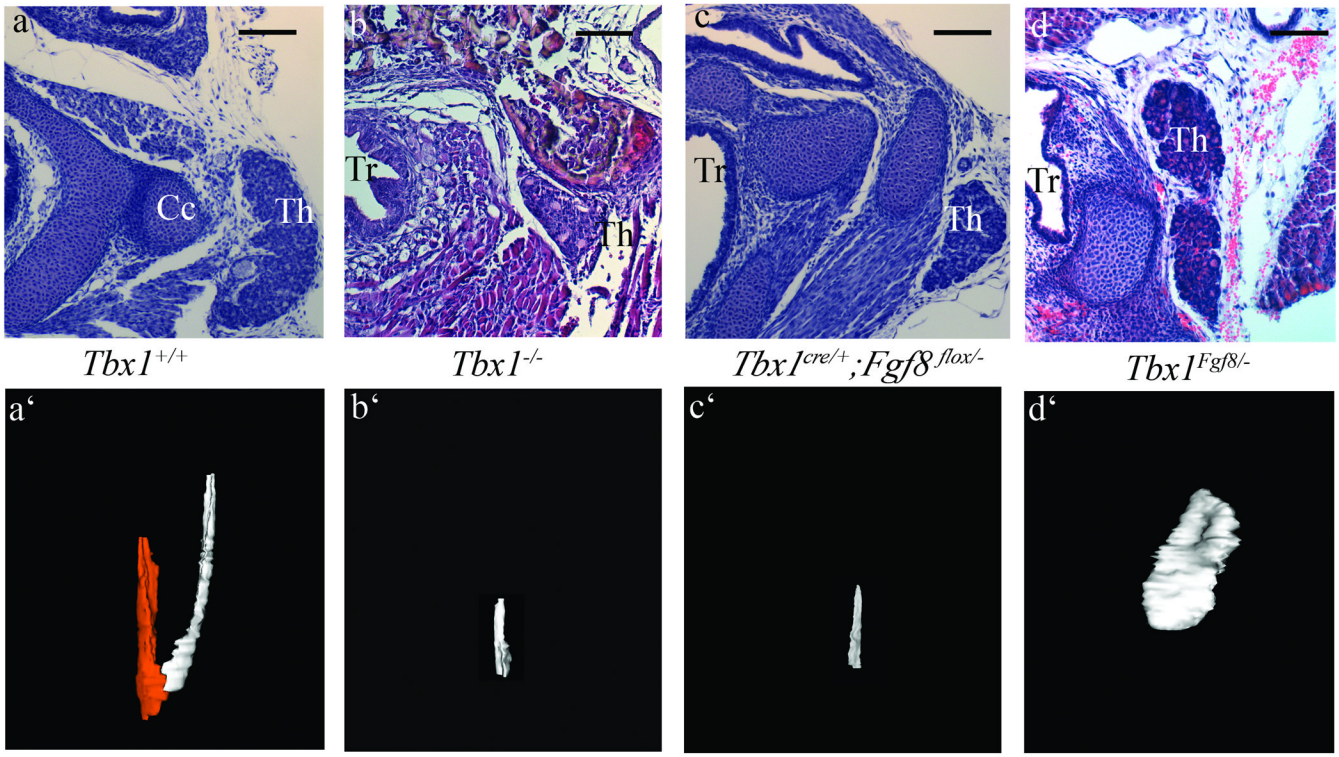

Figure 5. Fgf8 expression in the Tbx1 domain is able to rescue the Tbx1$^{-/-}$ thyroid phenotype

Histological analyses and corresponding 3D reconstructions of wild type (a,a’), *Tbx1*$^{-/-}$ (b,b’), *Tbx1*$^{cre/+}$;*Fgf8*$^{flox/-}$ (c,c’) and *Tbx1*$^{Fgf8/-}$ thyroids at E18.5. The two colors in the a’ panel indicate the left (white) and right (red) lobes. Note the increased size but abnormal shape of the rescued thyroid of Tbx1 mutant embryos expressing *Fgf8* from the *Tbx1* domain (d’). The scale bar is 50μm.

Table 1

	Tbx1$^{+/+}$		*Tbx1*$^{-/-}$		*Tbx1*$^{Cre/+}$;*Fgf8*$^{fl/-}$	
Age	Som.	n cells	Som.	n cells	Som.	n cells
E9	16 s	369	14 s	152		
	14 s	360	14 s	144		
				P=0.001		
E9.5	24 s	438	25 s	127	24–26 s	271
	22 s	515	21 s	306		250
	22 s	615	22 s	392		170
				P=0.008		*P*=0.03
E10.5-11	40 s	540	40 s	479		
	39 s	600	39 s	390		
	39 s	635	39 s	320		
	38 s	696	38 s	190		
				P=0.03		

Cell count (n cells) of thyroid primordia in embryos with the genotype and developmental stages indicated. *P* values are referred to comparisons with wild type counts. Som: somite stage.

Table 2

Mesp1$^{cre/+}$; *Tbx1*$^{\Delta E5/+}$		*Mesp1*$^{cre/+}$;*Tbx1*$^{\Delta E5/flox}$	
Total cell number	**Mitotic index**	**Total cell number**	**Mitotic index**
828	0.11	644	0.08
542	0.09	680	0.03
839	0.11	737	0.07
			$P<0.05$

Mitotic index (number of PH3+ cells over the total) in the ventral region of the pharyngeal endoderm of E8.5 embryos (3 controls and 3 M-ko, somite-matched).

Table 3

	Tbx1$^{+/+}$		*Tbx1*$^{Fgf8/-}$		*Tbx1*$^{Fgf8/Fgf8}$	
Age	**Som.**	**n cells**	**Som.**	**n cells**	**Som.**	**n cells**
E10	34 s	395	34 s	539	34 s	434
	34 s	417	34 s	331	34 s	564
			34 s	303		
				P=0.42		*P*=0.17

Cell count of thyroid primordia of *Fgf8*-rescued embryos compared to wild type. *P* values indicate that the size of primordia in rescued embryos is not significantly different from that of wild type embryos.