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An integrated regulatory network controlling survival and migration in thyroid organogenesis

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Abstract

The thyroid gland originates from the ventral floor of the foregut as a thickening of the endodermal cell layer. The molecular mechanisms underlying the early steps of thyroid morphogenesis are not known. Gene targeting experiments have contributed to the identification of several transcription factors, in general playing a role in the proliferation, survival, and migration of the thyroid cell precursors. The experiments reported here analyze the expression of the transcription factors Titf1, Hhex, Pax8, and Foxe1 in the thyroid primordium of null mutants of each of them. We found that most of these transcription factors are linked in an integrated regulatory network, each of them controlling the presence of other members of the network. The expression of Foxe1 is regulated in an intriguing fashion as it is strongly dependent on the presence of Pax8 in thyroid precursor cells, while the expression of the same gene in the pharyngeal endoderm surrounding the primordium is dependent on Sonic hedgehog (Shh)-derived signaling. Moreover, by the generation of mouse mutants expressing Foxe1 exclusively in the thyroid primordium, we provide a better understanding of the role of Foxe1 in these cells in order to acquire the competence to migrate into the underlying mesenchyme. In conclusion, we provide the first evidence of gene expression programs, controlled by a hierarchy of transcription factors expressed in the thyroid presumptive gut domain and directing the progression of thyroid morphogenesis.

Keywords: Morphogenesis; Organogenesis; Migration

Introduction

The gut tube of vertebrates appears, shortly after formation, as a rather amorphous cylindrical cavity lined, in its internal side, by the endoderm, a monotonous, single-layered sheet of epithelial-like cells. Subsequently, the gut adopts a variety of shapes along its length and, in addition, several buds evaginate from the tube. Each of these buds will carry out the formation of one of the gut-derived organs such as, in the most anterior part, the thyroid. It is likely that the morphological and functional differentiation along the gut tube is the consequence of the establishment of overlapping expression domains of transcription factors that will, through the activation of target genes, result in the formation of organ- or area-specific domains (regionalization phase) (Grapin-Botton and Melton, 2000; Shivdasani, 2002).

The thyroid bud is already visible in the foregut at embryonic day (E) 8.5 during mouse development. At E10,

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the bud deepens in the underlying mesenchyme; this process implies the progressive detachment of the thyroid bud from the floor of the primitive pharynx, which is completed by E10.5. The detached bud, clearly visible at E11, migrates caudally to reach the base of the neck, its final destination, at E13.5. There, it will give rise to thyroid follicular cells, the most abundant cellular population of the thyroid gland, responsible for the production of thyroid hormones. Starting at the thyroid anlage stage (specification phase), thyroid cells express a specific combination of transcription factors: Titf1 (formerly called TTF-1/Nkx2.1) (Lazzaro et al., 1991), Hhex (formerly called Hex) (Thomas et al., 1998), Pax8 (Lazzaro et al., 1991) and Foxe1 (formerly called TTF-2/Titf2) (Zannini et al., 1997). The combination of these proteins is a unique feature of thyroid precursor cells and their descendants, including the fully differentiated thyroid follicular cells producing thyroid hormones. There is little or no information on the signals that generate such a specific combination of transcription factors exclusively in the thyroid cell type. However, gene targeting experiments have demonstrated that in absence of either Titf1, Hhex, Pax8, or Foxe1, thyroid morphogenesis is severely impaired, thus suggesting that each of them plays an essential individual role in the organogenesis of this gland. In *Hhex*−/− mutants (Martinez Barbera et al., 2000), at E10, the thyroid primordium is either absent or severely reduced. In *Titf1*−/− mouse embryos (Kimura et al., 1996), the thyroid primordium forms in its correct position, but subsequently, the thyroid precursor cells undergo apoptosis (Kimura et al., 1999) and disappear at E10.5–11.5. A very similar picture occurs in *Pax8*−/− embryos (Mansouri et al., 1998) where initial phases of organogenesis are normal but, at E11.5, the thyroid primordium appears much smaller than in wild type embryos and at E12 the follicular cells are almost undetectable. In *Foxe1*−/− embryos (De Felice et al., 1998), thyroid precursor cells do not migrate and remain attached to the pharyngeal floor.

While it is clear that these transcription factors are all indispensable for normal thyroid morphogenesis, it is not known whether they are linked in a regulatory network. Another question of interest in thyroid organogenesis is the role of the Sonic hedgehog (Shh) signaling pathway, since it appears to be involved in most of the other endoderm-derived organs (Ingham and McMahon, 2001).

In this study, using several available mutant mouse lines and generating new ones, we have established that in thyroid cell precursors the transcription factors Titf1, Hhex, and Pax8 are linked in a regulatory network. We found that the expression of Foxe1 in thyroid cell precursors is strongly dependent on the presence of Pax8. We also show that the Foxe1 gene is subjected to a very precise spatial regulation since it depends on Pax8 for its expression in the thyroid bud and on Shh for its expression in the neighboring pharyngeal cells. Finally, we provide evidence strongly suggesting that the migration of the thyroid bud is a cell-autonomous event that requires the expression of Foxe1 in the migrating cells.

Materials and methods

Animals

Hhex (Martinez Barbera et al., 2000), *Titf1* (Kimura et al., 1996), *Pax8* (Mansouri et al., 1998), and *Foxe1* (De Felice et al., 1998) heterozygous mice were backcrossed to C57BL/6 for at least eight generations. To produce the homozygous embryos used in the experiments shown here, heterozygous mice were intercrossed. Wild type embryos from the same litter were used as control animals. Staged embryos were obtained by dissection of pregnant females. The day at which the vaginal plug was detected was designated as E0.5. Animals were killed by cervical dislocation.

Staged *Shh*−/− embryos (Chiang et al., 1996) were a generous gift of A. Ruiz i Altaba (NYU School of Medicine, New York).

Generation of Titf1$^{+/Foxe1}$ mice

Mouse *Titf1* gene has been isolated from a strain of 129/Sv mouse genomic library (Stratagene) using a probe corresponding to the 3′ UTR of rat *Titf1*. To prepare the targeting vector, a fragment extending from positions 4656 to 10443 of the reported mouse genomic sequence (Accession no. U19755), and containing the entire Titf1 coding sequence, was cloned in pBlueScript. A fragment spanning from the translation start site of *Titf1* (position 7957) to the end of the homeobox (position 9480) was removed and replaced by a sequence encoding the coding sequence of rat Foxe1 (extending from position 595 to position 1755 of the reported rat cDNA sequence, accession no. NM138909). The SV40 Poly A sequence was inserted downstream the *Foxe1* stop codon. The construct includes *HSV-tk* and *PGK-neo* cassette for selection of transfected ES cells. R1 ES cells were electroporated and selected as described. Genomic DNA from neomycin resistant clones was digested with *Bam*HI and analyzed by Southern blotting using a 500-bp fragment spanning from nucleotide 10512 to nucleotide 11042 of the mouse *Titf1* gene as a probe (Fig. 6A). Two ES cell clones in which the targeting vector had been properly integrated were injected into C57BL/6 blastocysts. Chimeric mice were bred with C57BL/6 mice for germline transmission of the modified allele. The heterozygous *Titf1*$^{+/Foxe1}$ mice were backcrossed for at least 8 generations on C57BL/6 genetic background.

Genotyping

Mutant embryos were identified by PCR analysis of embryonic DNA extracted from yolk sacs. The yolk sacs were incubated overnight at 60°C with lysis buffer (50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) and genomic DNA was extracted adding 0.3 volumes of 6 M NaCl and precipitated with isopropanol.

Samples were amplified for 33 cycles (94°C for 30 s, 57°C for 60 s, 72°C for 30 s).

To genotype *Titf1* mutants, the primers used for the amplification were the following: 5′ -GACGTGAGCAA-GAACATGG-3′ (in the exon II of *Titf1* gene); 5′ -GCC-GCCTTGTCCTTAGCC-3′ (in the exon II of *Titf1* gene); 5′ -CACCTTAATATGCGAAGTGGAC-3′ (in the neo coding sequence). PCR products were 233 bp for the wt allele and 324 bp for the mutated allele.

To genotype *Pax8* mutants, the primers used for the amplification were the following: 5′ -ATGCTAAGA-GAAGGTGGATGAG-3′ (in the intron III of *Pax8* gene); 5′ -GTTGATGGAGCTGACACTG-3′ (in the exon IV of *Pax8* gene); 5′ -TGCACGAGACTAGTGAGAC-3′ (in the promoter of the neo cassette). PCR products were 560 bp for the wt allele and 455 bp for the targeted allele.

Hhex embryos were genotyped as described (Martinez Barbera et al., 2000).

To genotype *Titf1*$^{+/Foxe1}$ mice or embryos, genomic DNA was digested with *Bam*HI and analyzed by Southern blotting using the probe described in Fig. 6A.

Histology, immunohistochemistry, in situ hybridization

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at pH 7.2, dehydrated through ethanol series, cleared in xylene, embedded in paraffin and 7 μm sections were cut.

Sections were dewaxed by standard techniques, and heat treatment to retrieve the antigen sites was performed. To quench endogenous peroxidases, the sections were treated with 1.5% hydrogen peroxide in methanol at room temperature. The sections were incubated for 1 h at room temperature with blocking solution (3% BSA, 5% goat serum, 20 mM $MgCl_2$, 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-rat Titf1 (Lazzaro et al., 1991), anti-rat Foxe1 (Dathan et al., 2002), anti-mouse Pax8 (Postiglione et al., 2002).

Non-radioactive in situ hybridization on paraffin sections was performed as described (Dathan et al., 2002). Hhex riboprobe is described in Martinez Barbera et al. (2000), Titf1 riboprobe is described in Lazzaro et al. (1991).

Results

Identification of a hierarchical regulatory network of transcription factors in thyroid cell precursors

To investigate the regulatory relationships among Titf1, Foxe1, Pax8, and Hhex in the thyroid primordium, we examined the expression of these genes in *Hhex* (Martinez Barbera et al., 2000), *Titf1* (Kimura et al., 1996), and *Pax8* (Mansouri et al., 1998) null mutants at the developmental stages E9 and E10. The gene expression in *Foxe1* null has already been studied (De Felice et al., 1998), except for that of Hhex, which is reported here. Our gene expression studies were performed by immunohistochemistry for Titf1, Pax8 and Foxe1 and by in situ hybridization for *Hhex*.

Hhex null

Previous studies have shown that the thyroid bud is visible in the majority of *Hhex* null E10 mouse embryos (classes II and III, 70% of the embryos). No expression of *Titf1* and *Foxe1* mRNA was detected by in situ hybridization experiments (Martinez Barbera et al., 2000). We too focused our attention only on classes II and III embryos and we decided to extend our studies to the corresponding proteins and to Pax8. Furthermore, we examined an earlier developmental stage (E9).

At E9 the thyroid anlage, identified as a restricted area of multilayered endoderm surrounded by a monolayer (Kaufman and Bard, 1999), is present in *Hhex* null mice and is comparable to the wild type. Furthermore, the expression of Titf1, Pax8, and Foxe1 (Figs. 1B, F and J, respectively) is not affected in *Hhex* null mice at this stage. Thus, specification of the thyroid cells does not require Hhex.

At E10 morphogenesis of the thyroid bud is already severely impaired in the absence of Hhex and only a few, non-migrating, cells can still be detected with specific antibodies. Concurring with the morphological alteration is the strong reduction in Pax8 (Fig. 1H) and Foxe1 proteins in the mutant embryos (Fig. 1L). Remarkably Foxe1 expression in the pharynx is not downregulated. Expression of Titf1 protein is not significantly reduced at this stage (Fig. 1D), in the Hhex mutants that we have analyzed. These data are not necessarily in contrast with the absence of *Titf1* mRNA in the developing thyroid of Hhex mutants previously reported (Martinez Barbera et al., 2000). The discrepancy could be explained assuming that the Titf1 protein is more stable than the corresponding mRNA. However, given the fact that phenotypes of varying severity have been described in Hhex mutants, we cannot rule out the possibility that the three mutant embryos examined from us all belonged to a class exhibiting a phenotype milder than that previously reported.

In conclusion, specification of the thyroid precursors does not require Hhex, as demonstrated by the onset of Titf1, Pax8, and Foxe1 expression in the absence of Hhex. At an immediately subsequent stage, the absence of Hhex causes profound alterations in the number of thyroid cell precursors and in the morphology of the bud. These alterations are consistent with a role of Hhex in proliferation, as observed already in pancreas organogenesis (Bort

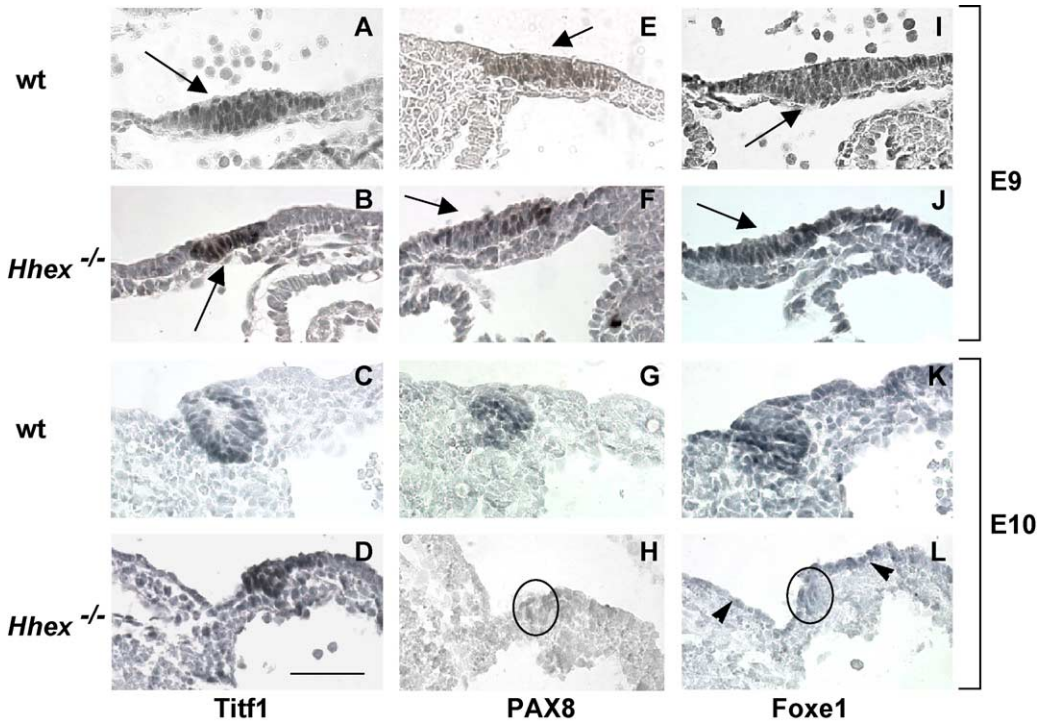

Fig. 1. Gene expression in the *Hhex*−/− developing thyroid. Expression of Titf1, Pax8, and Foxe1 is shown at different developmental stages in *Hhex*−/− embryos. For each stage and genotype, adjacent sagittal sections were immunostained with anti-Titf1 (panels A to D), anti-Pax8 (panels E to H), or anti-Foxe1 (panels I to L) specific antibodies. In the panels from A to J arrows point to the thyroid primordium. At E10 both Pax8 (panel H) and Foxe1 (panel L) are strongly downregulated in the thyroid rudiment (encircled); Foxe1 expression in the endodermal cells of the pharynx is preserved (panel L, arrowheads). (In all sections, anterior is up, ventral is left; scale bar = 50 μm).

et al., 2004). In the thyroid, such an effect correlates with, and could be at least in part explained by, an essential role of Hhex in the maintenance of *Titf1*, *Pax8*, and *Foxe1* expression in the developing thyroid.

Titf1 null

Previous studies have shown that the thyroid bud is visible in *Titf1* null E10 mouse embryos. However, the thyroid cell precursors disappear around E10.5-E11 in these embryos (Kimura et al., 1996), and it has been suggested that apoptosis is involved in this disappearance (Kimura et al., 1999). The expression of other thyroid-specific markers was not studied and is reported below.

At E9, both the morphology of the thyroid bud deprived of Titf1 and the levels of Pax8, Foxe1, and *Hhex* are comparable to those of the wild-type bud (Figs. 2B and A, F and E, J and I respectively). On the contrary, at E10, the developing thyroid of the mutants is smaller than that of the wild type. Pax8 shows a slightly reduced expression (Fig. 2D), while Foxe1 and *Hhex* are strongly downregulated (Figs. 2H and L). As in the case of Hhex, the absence of Titf1 only affects Foxe1 expression in the thyroid precursors and not in the neighboring pharyngeal cells (Figs. 2G and H), stressing the presence of different mechanisms of regulation of Foxe1 expression in the two abutting cell populations.

The findings reported above support a role of Titf1 only subsequent to specification that consists in allowing the survival of the thyroid precursors. Such a role might be mediated, at least in part, by the control that Titf1 exerts on the maintenance of Hhex and Foxe1 expression. This hypothesis is consistent with the reduced survival of thyroid cell precursors observed also in Hhex (Martinez Barbera et al., 2000 and this study) and Foxe1 (De Felice et al., 1998) mutants.

Pax8 null

Previous studies have shown that the thyroid bud is present in *Pax8* null mouse embryos but the thyroid precursors disappear at around E11.5–E12 in this mutant (Mansouri et al., 1998), probably as a result of an apoptotic mechanism (A. Mansouri, unpublished data). Only the expression of Titf1 mRNA was studied. Here, we extend these studies to Titf1 and Foxe1 proteins and to *Hhex* mRNA.

At E9, the *Pax8*−/− mouse shows a thyroid anlage whose morphology cannot be distinguished from that of the wild type (Fig. 3, compare A with B, E with F or I with J). No differences were detected in the levels of the Titf1 protein either (Figs. 3A, B). *Hhex* expression, detected by in situ hybridization, is also unchanged in the Pax8−/− mutants compared to the wild type (Figs. 3J

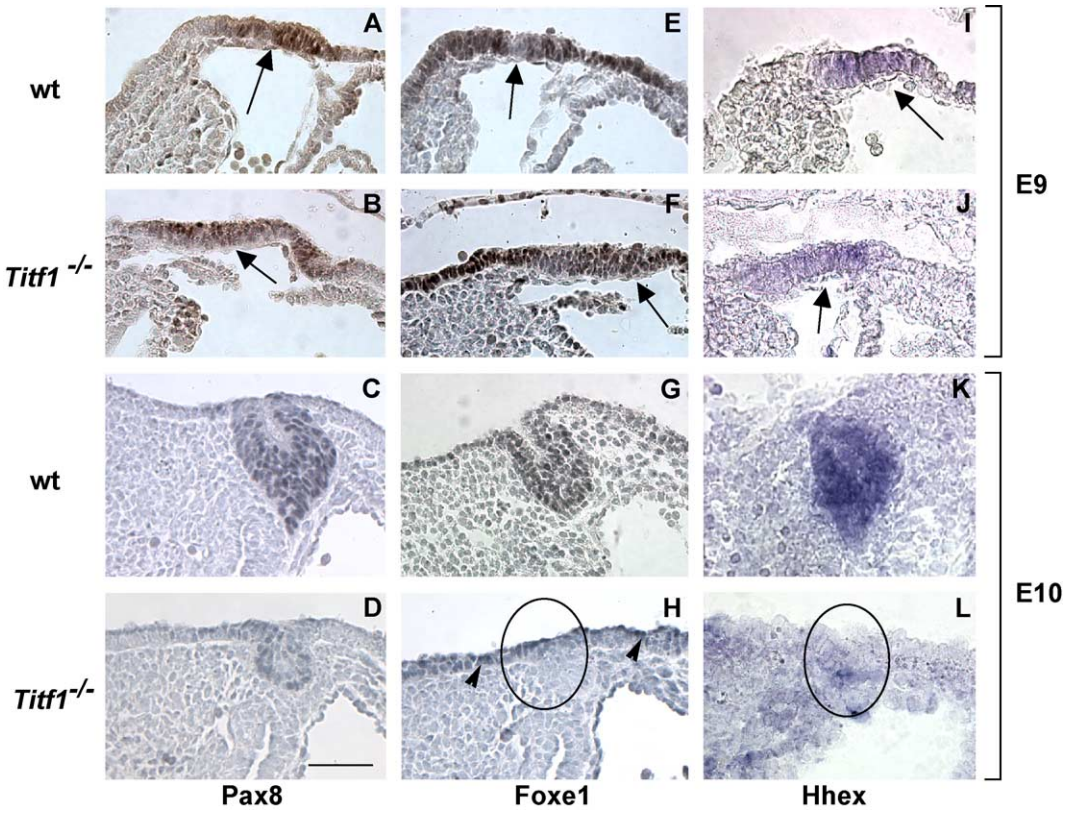

Fig. 2. Gene expression in the *Titf1−/−* developing thyroid. Expression of Pax8, Foxe1 and *Hhex* in wild type and *Titf1−/−* mouse embryos is shown at different stages of development. For each stage and genotype, adjacent sagittal sections were immunostained with anti-Pax8 (panels from A to D) or anti-Foxe1 (panels from E to H) specific antibodies. *Hhex* mRNA expression was followed by in situ hybridization (panels I to L). In the panels from A to J arrows point to the thyroid primordium. At E10 Foxe1 expression is strongly downregulated in the *Titf1−/−* developing thyroid (panel H, encircled) but not in pharyngeal cells (panel H, arrowheads). At the same stage *Hhex* mRNA is hardly detectable in the *Titf1−/−* thyroid primordium (panel L, encircled), while the level of Pax8 expression is only slightly reduced (panel D). In panel H and L, the thyroid primordium is identified because it shows an increased cellular density in comparison to the surrounding mesenchyme. (In all sections, anterior is up, ventral is left; scale bar = 50 μm).

and I). In contrast, a dramatic decrease of Foxe1 can be observed in the mutant thyroid anlage with an evident persistence of Foxe1 in the neighboring foregut cells (Figs. 3E, F).

At E10, the thyroid primordium migrates into the underlying mesenchyme. At this stage, no relevant changes occur in the expression pattern of Titf1, as already reported by (Mansouri et al., 1998) and as shown in Fig. 3D. The downregulation of Foxe1 expression becomes even more severe (Fig. 3H). *Hhex* mRNA levels, which are not affected at E9, are almost undetectable in Pax8 null (Fig. 3L) revealing that the maintenance, and not the initiation, of Hhex expression requires Pax8.

Our data show that Pax8 is required for the expression of Foxe1 in the thyroid cell lineage and suggest Foxe1 as a downstream target of Pax8. In agreement with this proposal, Pax8 is expressed normally in Foxe1 null embryos (De Felice et al., 1998).

Foxe1 null

Previous studies have shown that the thyroid bud is formed in *Foxe1* null mouse embryos but the thyroid precursors do not migrate and the precursors disappear at around E11.5 in only 50% of the embryos. Titf1 and Pax8 proteins are expressed in the mutant thyroid precursors at E10 (De Felice et al., 1998). *Hhex* mRNA, whose expression was not studied previously, is also not affected at E10 (Fig. 4).

Migration of thyroid primordium is a cell autonomous process

We have previously shown that Foxe1 plays an essential role in thyroid cell migration (De Felice et al., 1998). Since Pax8 exerts a tight control on Foxe1 expression, we asked whether in these mice the Foxe1-deprived thyroid precursors would migrate. Surprisingly, we discovered that the thyroid of *Pax8−/−* mice migrates normally even though it contains no detectable Foxe1 (Fig. 5D).

A hypothesis that could explain the migration of the Foxe1-deprived thyroid bud in *Pax8−/−* mice is that the expression of Foxe1 in the pharyngeal cells surrounding the bud can promote migration. We thus decided to ask if in the reciprocal arrangement (Foxe1 present in the thyroid bud and absent in the pharyngeal endoderm) migration would

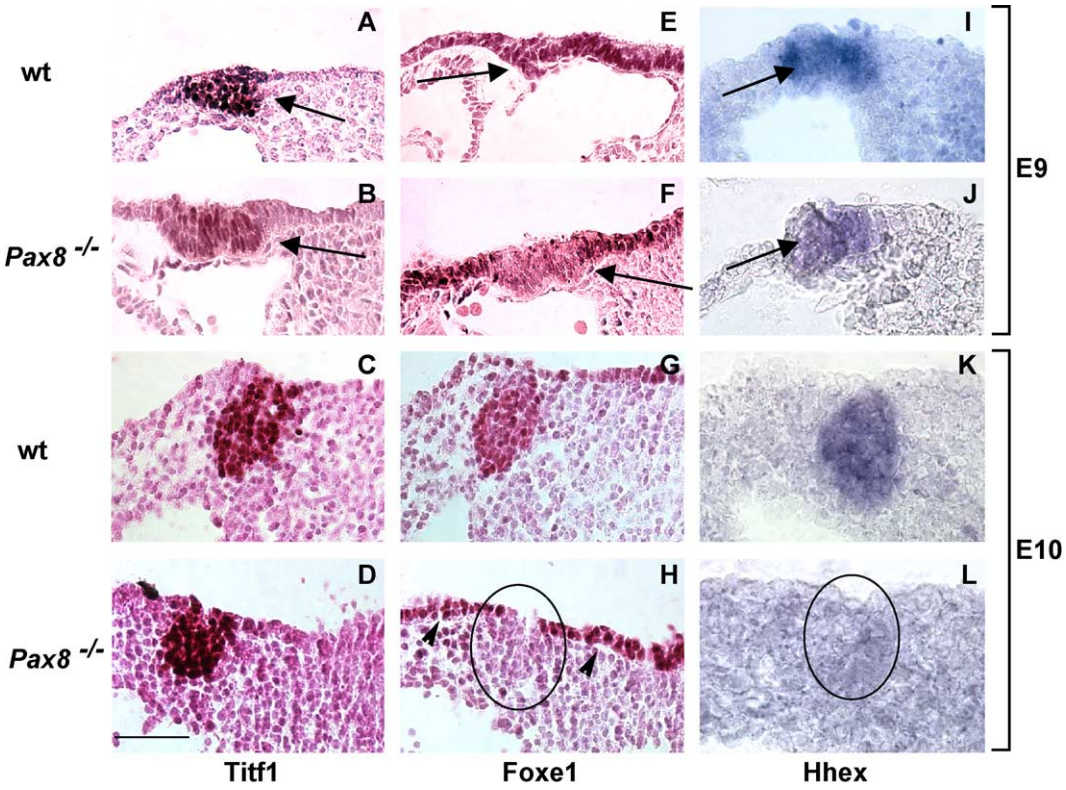

Fig. 3. Gene expression in the *Pax8−/−* developing thyroid. Expression of Titf1, Foxe1, and Hhex is shown at different developmental stages of the thyroid gland in wild type and *Pax8−/−* mouse embryos. For each stage and genotype, adjacent sagittal sections were immunostained with anti-Titf1 (panels from A to D) or anti-Foxe1 (panels from E to H) specific antibodies and counterstained with eosin. *Hhex* mRNA expression was examined by in situ hybridization (panels I to L). In the panels from A to J, arrows point to the thyroid primordium. At E9 in thyroid cell precursors (arrows) the levels of Foxe1 are greatly diminished in *Pax8−/−* embryos (panel F) in comparison to those of wild type (panel E). At E10, the level of Foxe1 expression is lost in the thyroid primordium (panel H, encircled), but not in the adjacent pharyngeal cells (panel H, arrowheads). The levels of *Hhex* mRNA appear strongly reduced in the developing *Pax8−/−* thyroid (panel L, encircled). (In all sections, anterior is up, dorsal is left; scale bar = 50 μm).

occur. To create such a case, we constructed a mouse line, indicated as *Titf1*$^{+/Foxe1}$, where the Foxe1 coding sequence is inserted in the *Titf1* locus (Figs. 6A and B). We first showed that in these mice Foxe1 is ectopically expressed in Titf1 expression domains, such as the lung and the diencephalon (data not shown). Subsequently we obtained *Foxe1−/−, Titf1*$^{+/Foxe1}$ embryos by appropriate crossings. These mutants are characterized by the expression of Foxe1 only in thyroid cell precursors and not in pharyngeal cells. The phenotype of these embryos at E10.5 (Fig. 6D) was compared with a *Foxe1−/−, Titf1+/+* from the same litter (Fig. 6C). Even though the level of expression of Foxe1 in the thyroid precursors is low (not shown), we observed a clear rescue of the thyroid bud migration (Fig. 6D). Thus, expression of Foxe1 in the thyroid bud is sufficient for migration to occur, even in the absence of Foxe1 in the neighboring pharyngeal endoderm, demonstrating that migration of the thyroid bud depends on Foxe1-controlled, intrinsic features of the thyroid cell precursors. We suggest that the migration observed in the *Pax8−/−* thyroid cell precursors could be due to either to the presence of low levels Foxe1 undetectable by our antibodies or other compensatory mechanisms, possibly induced by the absence of Pax8, that might relieve the dependence on Foxe1 for migration.

Shh expression is excluded from the thyroid presumptive endoderm

In all the mutants studied here, and also in the Foxe1 knock-out (De Felice et al., 1998) specification of thyroid cells is not impaired. The signaling molecule Shh has been reported to be expressed in most of the foregut epithelium and its distribution in a regional-specific pattern along the anterior–posterior axis of the endoderm has been suggested to be involved in the determination of cell fates (Bitgood and McMahon, 1995; Ingham and McMahon, 2001). Thus, we analyzed *Shh* expression in the developing thyroid. At E9, *Shh* mRNA is expressed in the endoderm of the pharyngeal floor but is excluded from cells of the thyroid anlage (Fig. 7A), clearly identified by *Titf1* expression (Fig. 7B) in an adjacent section. This finding is in accordance with data recently published (Fagman et al., 2004).

Given the observation that both Foxe1(Perrone et al., 2000) and Hhex (Brickman et al., 2000) may act as

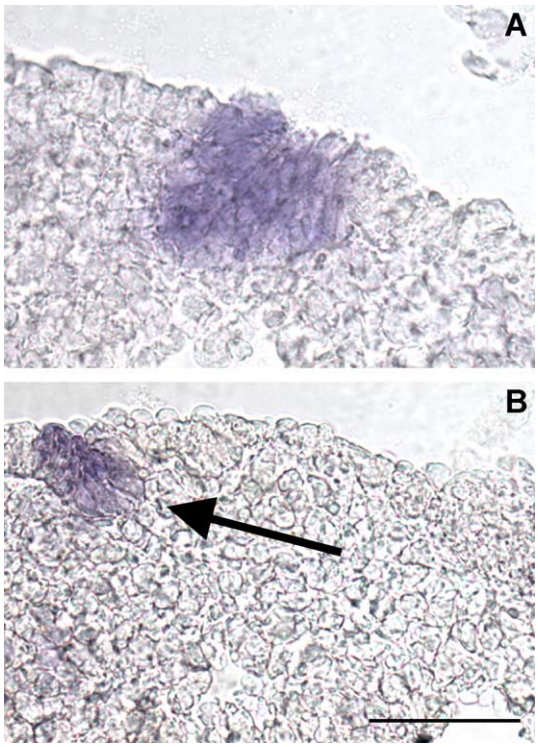

Fig. 4. Hhex expression in the Foxe1−/− developing thyroid. Sagittal sections of E10 wild type (panel A) and *Foxe1−/−* (panel B) embryos. The levels of *Hhex* mRNA in the mutant developing thyroid (panel B, arrow) appear to be comparable to those of wild type developing thyroid. (In all sections, anterior is up, ventral is left; scale bar = 50 μm).

transcriptional repressors, we tested whether each of these transcription factors plays a role in repressing *Shh* expression specifically in the thyroid primordium. Therefore, we analyzed whether *Shh* mRNA expression is altered in *Foxe1−/−* and *Hhex−/−* embryos. Figs. 7B and D show that the expression pattern of *Shh* is not significantly different in the thyroid primordium of both these mutant embryos in comparison with the wild type embryos. Thus, the signal responsible for the exclusion of Shh from the thyroid anlage is not known and it may be the same as the one responsible for the specification of the anlage cells.

Shh signaling in the pharyngeal endoderm controls Foxe1 expression but is not required for thyroid specification

The existence of a boundary established at the transition between Shh-producing and non-producing cells is the prerequisite for patterning and morphogenesis in other systems (Apelqvist et al., 1997; Treier et al., 2001). To investigate whether Shh exclusion from the thyroid anlage could have a similar relevance in thyroid morphogenesis, we analyzed *Shh* null embryos at E10. In these mutant embryos, the thyroid bud was properly formed and displayed a normal pattern of gene expression, as the expression of Titf1, Pax8, and Foxe1 in the thyroid anlage (Figs. 7H, I and J respectively) does not show any difference in comparison to the wild type one (Figs. 7E, F and G respectively).

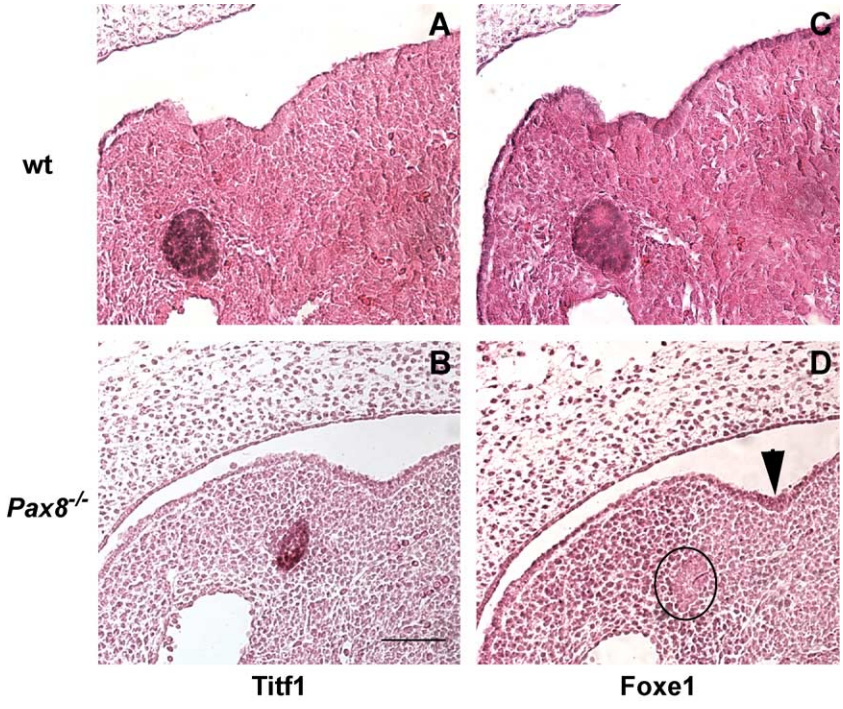

Fig. 5. *Pax8−/−* thyroid precursor cells are able to migrate. Sagittal sections of E11 wild type (panels A and C) and *Pax8−/−* (panels B and D) embryos were immunostained with anti-Titf1 (panels A and B) or anti-Foxe1 (panels C and D) specific antibodies and counterstained with eosin. In *Pax8−/−* embryos, thyroid cell precursors (panel D, encircled) do not express Foxe1, which is present in the pharyngeal cells (panel D, arrowhead). However, thyroid cells are able to migrate into the underlying mesenchyme. The expression of Titf1 (panel B) is used as a molecular marker to confirm the presence of migrating thyroid cell precursors. (In all sections, anterior is up, dorsal is left; scale bar = 50 μm).

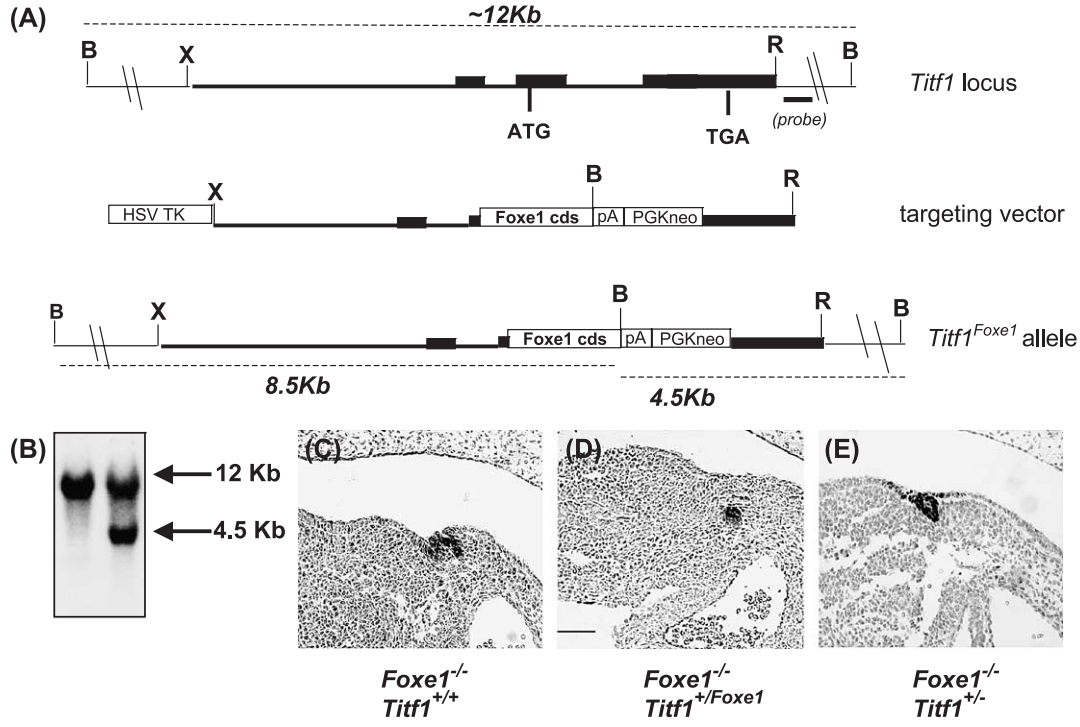

Fig. 6. Generation of *Titf1*$^{+/Foxe1}$ mice. (Panel A): Structure of the *Titf1* locus modified by homologous recombination. Black boxes represent exons; ATG and TGA codons are indicated. The position of the probe used to genotype the ES cell clones and the mice is indicated by a black bar. Foxe1 cds: Foxe1 coding sequence; HSV TK and PGKneo: selection markers; pA: SV40 Poly A sequence; B: *Bam*HI. X: *Xba*I; R: *Eco*RI. (Panel B): Southern blot analysis of genomic DNA from mouse tails digested with *Bam*HI and probed with the sequence indicated in the panel A. The lower band corresponds to the targeted allele (4.5 Kb), the upper band to the wild type allele (12 Kb). Sagittal sections of E10.5 *Foxe1*−/− (panel C), *Foxe1*−/− in *Titf1*$^{+/Foxe1}$ background (panel D) and *Foxe1*−/− in *Titf1*+/− background (panel E) stained with anti-Titf1 antibody. Foxe1 expression only in the thyroid primordium is sufficient to rescue proper development as shown in *Foxe1*−/− in *Titf1*$^{+/Foxe1}$ background (panel D). (In all sections anterior is up, ventral is left; scale bar = 50 μm).

Interestingly, in the *Shh*−/− embryos, lack of Shh is accompanied by loss of Foxe1 expression in the anterior part of the foregut and the presumptive oral cavity, but not in the presumptive thyroid (Fig. 7J).

Our observations exclude a relevant role of Shh in the specification of thyroid precursors. In contrast, we demonstrate that Shh is required for Foxe1 expression in the surrounding pharyngeal endoderm.

Discussion

In this paper, we demonstrate that the transcription factors known to be essential for normal organogenesis of the thyroid gland are linked in an integrated regulatory network. The regulatory relationships found are summarized in Table 1 and Fig. 8. The first observation is that none of the factors appear to be involved in the specification process, as in the absence of each one of them thyroid specification occurs regularly at E9. We define thyroid specification the formation of the thyroid anlage, a multilayered endodermal button that can be molecularly identified by the expression of *Titf1*, *Pax8*, and *Hhex*. We do not include in the definition of specification the expression of *Foxe1* since this gene is expressed throughout the pharyngeal floor (Zannini et al., 1997) and hence it is not a marker of thyroid specification in this region. As Table 1 summarizes, at E9 in each knockout mouse, the remaining markers of specification remain unaltered. Thus, the events responsible for the onset of their expression, and hence of thyroid specification, remain unidentified. The only detectable effect at E9 on gene expression is the tight control exerted by Pax8 on Foxe1. Surprisingly, 1 day later, the scenario is completely changed and a complex network of reciprocal regulatory interaction is revealed. Interestingly, this network includes only the genes that, in the pharyngeal floor, are specific to the thyroid anlage, that is, *Titf1*, *Hhex*, and *Pax8*, while *Foxe1* senses the regulatory signals from the network but does not participate in it (Table 1), (De Felice et al., 1998). It has been recently observed that in zebrafish (Elsalini et al., 2003), the expression of homologues of *Titf1* and *Pax8*, *nk2.1a* and *pax2.1*, respectively, is extinguished in the thyroid primordium of *Hhex* morphants, in agreement with our observations in mice. We suggest that these regulatory interactions might function as a fail-safe mechanism that will lead to the disappearance of thyroid cell precursors if any one of the genes, early in development, malfunctions, since each of them will initiate a chain of events leading to the complete removal of thyroid cells.

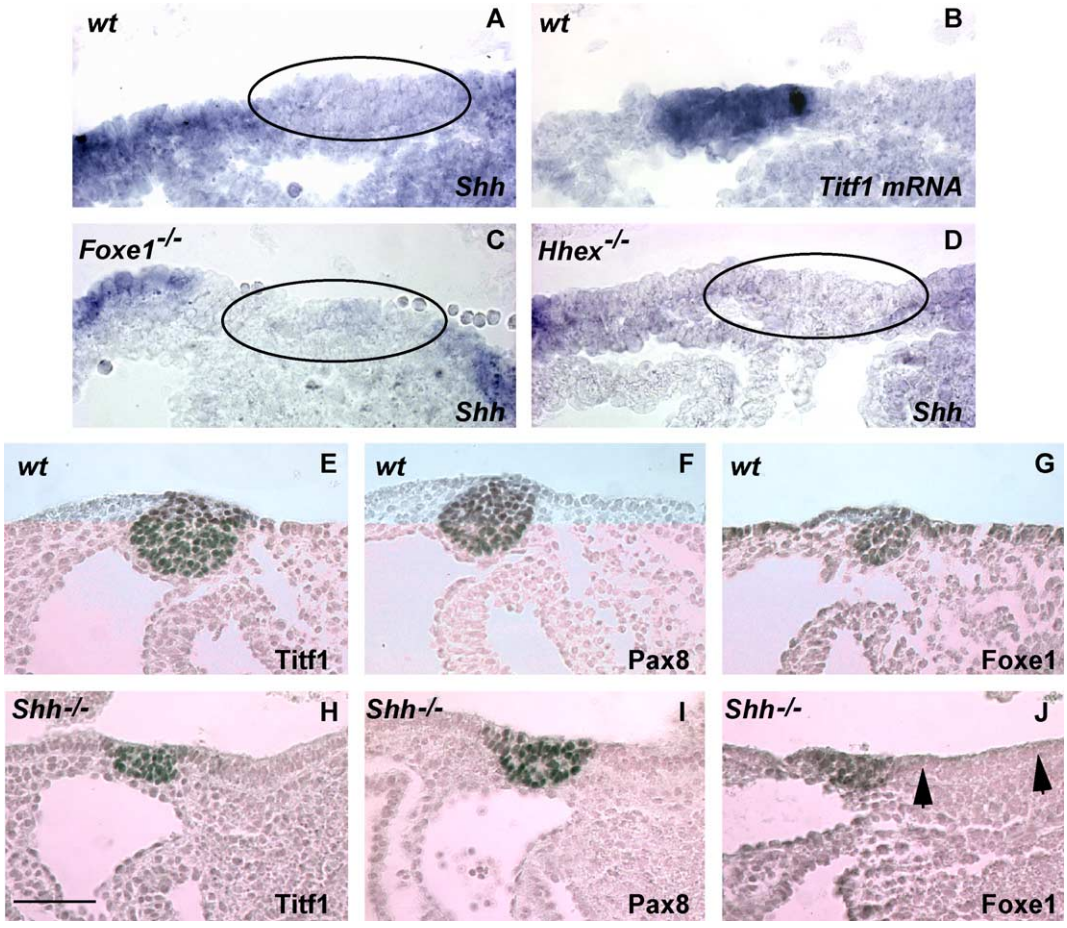

Fig. 7. Shh is absent in the developing thyroid while regulating Foxe1 expression in the developing pharynx. Sagittal sections of E9 (panels A to D) and 10 (panels E to J) embryos. For each section the genotype (left side up) and the molecular marker probed (right side down) are indicated. Expression of Shh mRNA in wild type (panel A), *Foxe1*−/− (panel C) and *Hhex*−/− (panel D) embryos was analyzed by in situ hybridization. *Titf1* (panel D) mRNA expression was used to identify the thyroid primordium on an adjacent section. Shh is selectively excluded from the thyroid primordium (encircled) of wild type (panel A), *Foxe1*−/− (panel B) and *Hhex*−/− (panel C). In Shh null embryos (panels H to J), the developing thyroid is smaller than in the wild type ones (panels E to G) but expresses Titf1, Pax8 and Foxe1. However, Foxe1 is downregulated in the floor of the foregut (panel J, arrowheads). (In all sections anterior is up, dorsal is left; scale bar = 50 μm).

Furthermore, the severe phenotype observed in each of these mutants should now be re-interpreted as the consequence of the removal of the entire regulatory network. Such an integrated network might also explain why malformations of the thyroid gland are frequent in humans (Macchia et al., 1999), since the pool of genes that can be affected is rather large and the consequences of mutation in one of them might have catastrophic consequences.

Foxe1, clearly located downstream of the thyroid regulatory network, shows a remarkable regulation, since it depends on two diverse regulatory pathways in adjacent cells. In the thyroid primordium it is tightly regulated by Pax8, which is necessary for the onset of its expression, thus making *Foxe1* a likely candidate as a transcriptional target of Pax8. However, in the surrounding endodermal cells of the pharynx, Foxe1 is expressed in a Pax8 independent

Table 1
Summary of the genetic analysis in the developing mutant thyroid of *Hhex*, *Titf1*, *Pax8*, and *Foxe1* null mice

	E9				E10			
	Hhex mRNA	Titf1 protein	Pax8 protein	Foxe1 protein	*Hhex* mRNA	Titf1 protein	Pax8 protein	Foxe1 protein
Hhex−/−	−	+	+	+	−	+	−	−
Titf1−/−	+	−	+	+	−	−	+	−
Pax8−/−	+	+	−	−	−	+	−	−
Foxe1−/−	+*	+*	+*	−	+	+*	+*	−

The "−" and "+" symbolize respectively the absence and the presence of the considered molecular marker (protein for Titf1, Pax8, and Foxe1, mRNA for Hhex) in the various null mice.
* Data not shown.

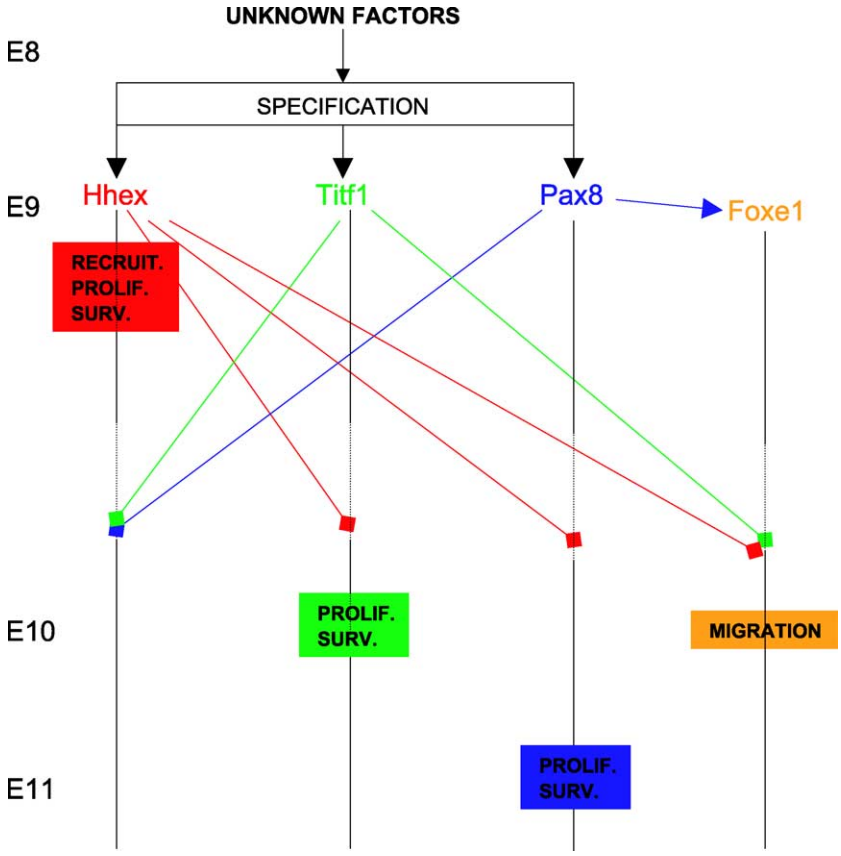

Fig. 8. Hypothetical regulatory network among transcription factors regulating thyroid morphogenesis. A model for the regulatory pathway involved in the morphogenesis of the thyroid primordium can be assembled from the data we have reported. We show the relationships among the transcription factors, Hhex, Titf1, Pax8, and Foxe1, in thyroid precursors. The transcription factor and the functions controlled by this are shown in different colors. A transcription factor can be involved in the initiation (arrow) or maintenance (square) of other factors. The dashed vertical lines indicate that the expression of the considered transcription factor is lost unless signals from other factors are provided. The embryonic day corresponding to the blocking of the developmental cascade in absence of the factor is also reported.

manner but is exquisitely sensitive to the Shh signaling pathway, since its expression is abolished in *Shh* mutants. Our data point to a regionalized regulation of Foxe1 expression along the anterior–posterior axis of the embryonic endoderm that could be explained by the presence of two separate enhancers in the *Foxe1* regulatory region, one Pax8- and the other Shh-dependent.

Our previous analysis of *Foxe1* null mutants demonstrated that Foxe1 is required for the migration of the thyroid precursors (De Felice et al., 1998). However, Foxe1 is expressed in both thyroid and pharynx. To study the role, if any, of the pharyngeal cells expressing Foxe1 in the migration process, we generated a mouse mutant where the expression of Foxe1 is restricted to the developing thyroid and is removed from the pharynx. In these mutants, the thyroid precursor cells do migrate, demonstrating that migration of the thyroid bud is a cell autonomous event. We favor the hypothesis that the migration observed in *Pax8* null embryos is due to low levels of Foxe1 in the thyroid bud itself, undetectable by our antibodies.

It has been speculated that during the evolution of the chordates, *Foxe1* has been co-opted in the genetic network that controls thyroid specification to contribute to the program enabling the thyroid progenitors to migrate from the endodermal epithelium (Mazet, 2002; Yu et al., 2002). Indeed, the invertebrate Chordate Amphioxus does not express any gene homologous to *Foxe1* in the endostyle, the pharyngeal structure homologous to the vertebrate thyroid. On the contrary, *AmphiFoxE4*, the *Foxe1* homologous in amphioxus, is expressed in another endodermal region (Yu et al., 2002). Interestingly, the endostyle does not migrate from the pharyngeal endoderm, like the thyroid primordium does. Therefore, the low position that Foxe1 holds in the regulatory hierarchy controlling thyroid development could be a reminiscence of an ancient evolutionary story.

In an attempt to identify the signaling pathways regulating the patterning of the presumptive thyroid endoderm, we analyzed *Shh* expression in the developing thyroid. The finding of the exclusion of *Shh* in thyroid precursor cells suggests the possibility of a specific role of this absence in thyroid morphogenesis as already described in other organs of endodermal origin (Apelqvist et al., 1997; Treier et al., 2001). While our data demonstrate that Shh is not required for thyroid budding, we do not have any information on whether the boundary of *Shh* expression in the foregut is required for thyroid morphogenesis.

In conclusion, our work reveals that *Titf1*, *Pax8*, *Foxe1*, and *Hhex* could be considered "selector genes" required for thyroid morphogenesis. How these factors and others signaling effectors collaborate to promote appropriate organ development remains unresolved. The possibility of a direct control of the expression at transcriptional level has to be taken into account. Indeed, it has recently been shown that Titf1 regulates the activity of *Hhex* promoter in vitro (Puppin et al., 2003).

We have demonstrated that in thyroid precursors the maintenance of the transcription factors Titf1, Foxe1, Pax8, and Hhex requires a cross-regulatory network within them, while the onset of their expression seems to be independent of each other's expression. A scenario not unlike this is observed during heart development. *Nkx2-5* is a cardiac selector gene, whose expression is essential for heart morphogenesis (Olson and Srivastava, 1996). It has been shown that its expression is initiated properly but not sufficiently sustained in *Rae28* null embryos. *Rae28* belongs to the Polycomb group of genes, known to maintain transcription states, once initiated, probably by regulating chromatin structure (Shirai et al., 2002).

The study of cis-regulatory elements of thyroid specific selector genes would be the first step in the elucidation of the transactivating factors establishing the thyroid-specific molecular network. The knowledge of these factors will provide information on how thyroid precursor cells differentiate themselves from their neighbors in the floor of the primitive pharynx.

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