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DEVELOPMENTAL BIOLOGY

Developmental Biology 263 (2003) 67–80

www.elsevier.com/locate/ydbio

Zebrafish *hhex, nk2.1a,* and *pax2.1* regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors

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Received for publication 15 January 2003, revised 23 June 2003, accepted 2 July 2003

Abstract

During zebrafish development, the thyroid primordium initiates expression of molecular markers such as *hhex* and *nk2.1a* in the endoderm prior to pharynx formation. As expected for an endodermally derived organ, initiation of thyroid development depends on Nodal signalling. We find that it also depends on three downstream effectors of Nodal activity, *casanova (cas), bonnie and clyde (bon),* and *faust (fau)/gata5*. Despite their early Nodal-dependent expression in the endoderm, both *hhex* and *nk2.1a* are only required relatively late during thyroid development. In *hhex* and *nk2.1a* loss-of-function phenotypes, thyroid development is initiated and arrests only after the primordium has evaginated from the pharyngeal epithelium. Thus, like *pax2.1,* both *hhex* and *nk2.1a* have similarly late roles in differentiation or growth of thyroid follicular cells, and here, we show that all three genes act in parallel rather than in a single pathway. Our functional analysis suggests that these genes have similar roles as in mammalian thyroid development, albeit in a different temporal mode of organogenesis.
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Keywords: *Pax2*; *cyclops*; *one-eyed pinhead*; Mouse; Hypothyroidism

Introduction

During vertebrate development, endodermal cells give rise to the digestive system with its derivatives such as lung, pancreas, and liver. Endoderm specification starts during gastrulation, and key factors involved in this process are regulated by the Nodal family of signalling molecules (Feldman et al., 1998). During the last decade, some of the genes have been identified that are required for further specification of the endoderm. Mutant analysis revealed that, in zebrafish, the transcription factors *casanova (cas), bonnie and clyde (bon),* and *faust/gata5 (fau)* form a network of interactions downstream of Nodal in specifying the endoderm (Kikuchi et al., 2000, 2001; Reiter et al., 2001). The steps of increasing commitment of endodermal cells to a later developmental fate, however, are still poorly understood.

The thyroid gland is considered to be an endoderm-derived organ, because it develops from precursor cells that evaginate from the ventral floor of the pharynx, in the same way as the lung primordium does at a slightly more posterior position. The predominant cell type of the thyroid gland is follicular cells that produce thyroid hormone (Gorbman and Bern, 1962), and these cells are traditionally considered to be of endodermal origin (reviewed in Macchia, 2000). In higher vertebrates, nonendodermal cell types, including neural crest-derived C cells and cells that give rise to blood vessels, merge with the population of follicular precursor cells (Manley and Capecchi, 1998) before the gland is encapsulated by connective tissue.

In contrast to higher vertebrates, the morphology of the mature gland is different in most bony fish (teleosts) in that the follicles are not encapsulated by connective tissue and do not form a compact gland. Instead, follicles are loosely distributed along the ventral aorta in the lower jaw area (Raine and Leatherland, 2000; Raine et al., 2001). This is also the case in zebrafish, in which differentiated follicles continue to appear during larval growth along the ventral aorta (Wendl et al., 2002). However, early steps of thyroid

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doi:10.1016/S0012-1606(03)00436-6

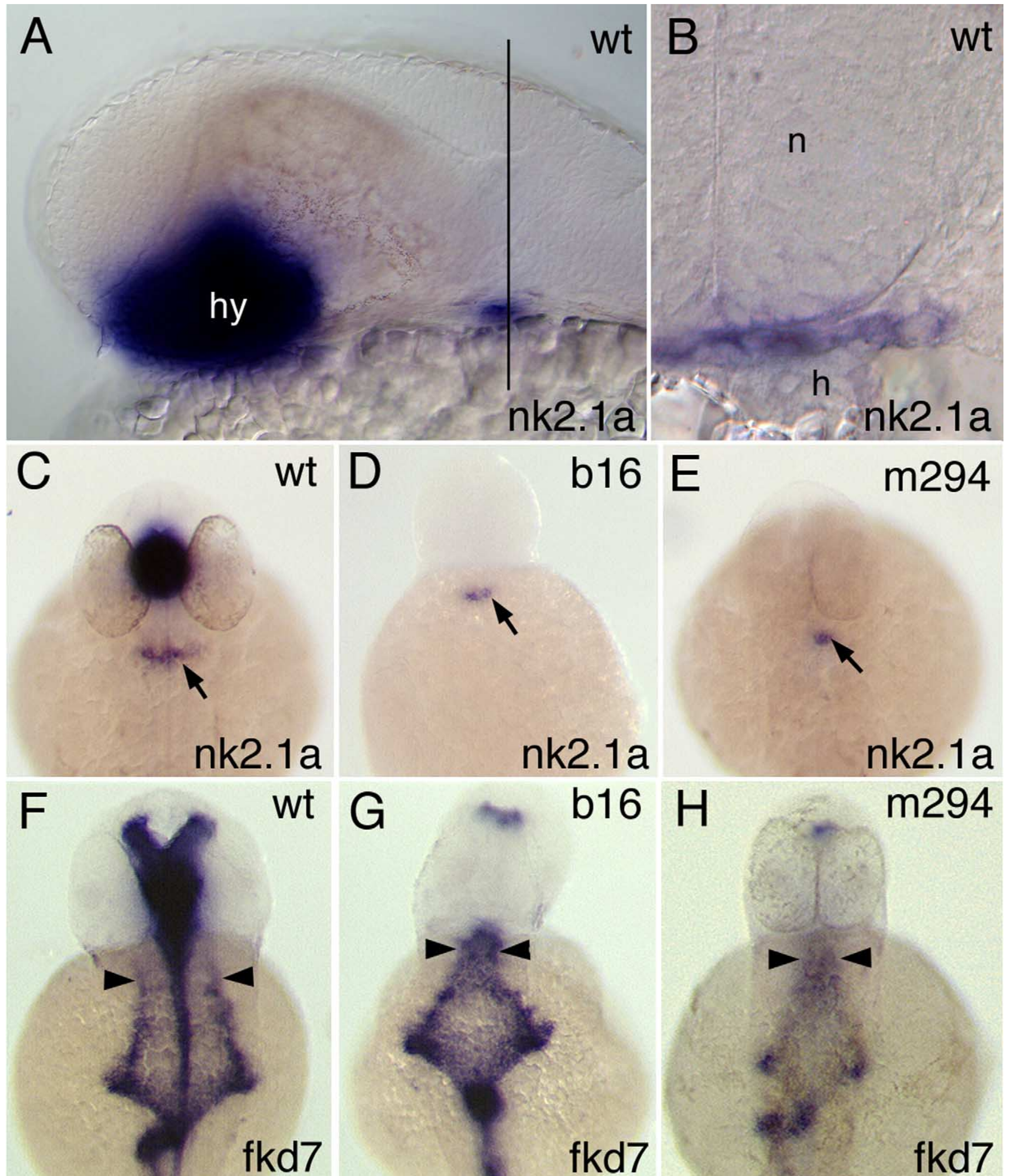

Fig. 1. Reduced endoderm coincides with a smaller thyroid primordium in $cyc^{-/-}$. Expression patterns of *nk2.1a* in thyroid precursor cells and *fkd7* in the endoderm (gene expression as indicated bottom right) at 26 hpf in wildtype or mutant embryos (indicated top right). Lateral view in (A); dorsal views in (C–H). (B) shows part of a section through a whole-mount stained embryo at a position as indicated by the black bar in (A). The arrows point to the thyroid primordium in (C–E). Arrowheads indicate the expression of *fkd7* in the pharyngeal endoderm on the level of the first to second branchial arch. Abbreviations: h, heart; hy, hypothalamus; n, neural tube.

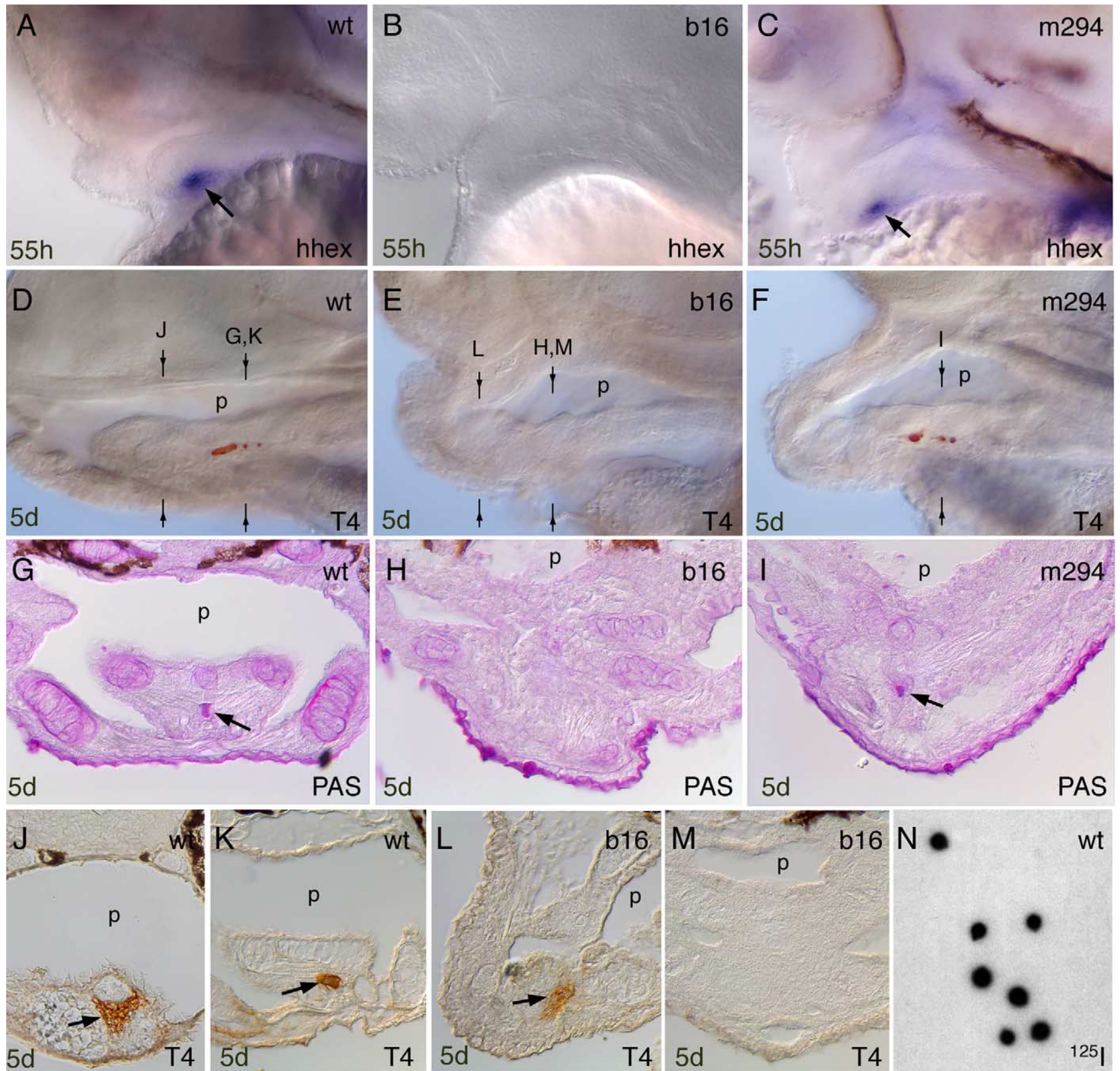

Fig. 2. Thyroid follicles are absent in *b16*$^{-/-}$ embryos but present in *cyc m294*$^{-/-}$ embryos/larvae. Whole-mount embryos (A–F) or paraffin sections (G–M). Genotype indicated at top right. Antibody staining (T4, brown staining), PAS staining (PAS), and gene expression are as indicated at bottom right; stages are indicated bottom left. Arrows point to the thyroid primordium (A, C), to thyroid follicles (G, I, K), or to the anterior domain of antibody staining (J, L). Arrow bars in (D–F) indicate level of sectioning as in (G–M). (A–C)*hhex* mRNA is absent in *b16*$^{-/-}$, but not in *m294*$^{-/-}$ embryos. (D–M) Thyroid follicles form in *m294*$^{-/-}$, but not in *b16*$^{-/-}$ larvae. (N) Radioactive iodide accumulates in wt zebrafish larvae. Autoradiography showing an X-ray film after exposure of seven fixed zebrafish larvae, blotted onto Whatman paper. Before fixation, larvae were incubated in ^{125}I. Abbreviation: p, pharynx.

development in zebrafish resemble what is known from higher vertebrates. The primordium evaginates from the pharynx and relocalises dorsocaudally to a position in close proximity to the aortic arches (Rohr and Concha, 2000).

Four transcription factors have been shown to play crucial roles in the development of thyroid follicular cell precursors in mammals. *Nkx2.1 (TTF1), TTF2, Hhex,* and *Pax8* are expressed during thyroid development in mice, and corresponding knock-out phenotypes show loss or reduction in size of the thyroid primordium soon after initial steps of thyroid development (De Felice et al., 1998; Kimura et al., 1996; Mansouri et al., 1998; Martinez Barbera et al., 2000). It has not yet been completely established, however, whether these genes act independently or depend on each other. In zebrafish, *nk2.1a, hhex,* and *pax8* are also expressed in the developing thyroid (Rohr and Concha, 2000;

Wendl et al., 2002). Additionally, *pax2.1* is expressed in these cells, and its loss-of-function phenotype in $noi^{-/-}$ mutant embryos shows that *pax2.1* is required for the development of thyroid follicles (Wendl et al., 2002). *pax8* appears to be downstream of *pax2.1* in zebrafish thyroid development, and it is rather *pax2.1* that has a role comparable to *Pax8* in mammalian thyroid development.

In the present study, we show that specification of endoderm by Nodal signalling and its downstream effectors is a prerequisite for development of all thyroid follicular cells in zebrafish. As *hhex* and *nk2.1a* are expressed in thyroid precursor cells even before a pharynx has differentiated from the endoderm, we analysed their role in thyroid development using loss- and gain-of-function studies. Surprisingly, we find that, like *pax2.1, hhex* and *nk2.1a* are required relatively late in thyroid development in zebrafish. Based on the functional comparison of these key transcription factors, we propose a model that distinguishes between two different modes of thyroid development in fish and mammals. Both modes imply that the homologous genes act in a comparable manner preceding a growth phase of thyroid development, albeit involving a different time scale of thyroid development in fish and mammals.

Materials and methods

Animals

Zebrafish work was carried out according to standard procedures (Westerfield, 2000). Staging in hours postfertilisation (hpf) or days postfertilisation (dpf) refers to development at 28.5°C. The following mutant fish lines were used: cyc^{b16} (Talbot et al., 1998), bon^{s9} (Kikuchi et al., 2000), cas^{ta56} (Kikuchi et al., 2001), fau^{s26} (Reiter et al., 1999), cyc^{m294} (Talbot et al., 1998), noi^{b21} (Lun and Brand, 1998), oep^{tz57} (Schier et al., 1997).

Embryonic manipulation

Synthetic *hhex* mRNA (Ho et al., 1999) was produced by using the message machine kit (Ambion), and approximately 100 pg were injected into one-cell-stage embryos. As a control, we injected synthetic *gfp* mRNA at similar or higher (approximately 150 pg) concentrations. If not given in the text or tables, numbers of *hhex* and *gfp* mRNA-injected embryos, respectively, were about 100 for each in situ hybridisation marker. Injection experiments were repeated at least once.

Morpholino RNA was purchased (Gene Tools) and dissolved as recommended by the provider. Morpholino sequences are: *hhex:* 5′-gcgtgcgggtgctggaattgcatga-3′; *nk2.1a*-1: 5′-gctcaaggacatggttcagcccgc-3′; *nk2.1a-2:* 5′-cgcgagcaggtttgctgaagctgcc-3′; *nk2.1b:* 5′-tcgtatgcttagggctcatcgacat-3′; unspecific control, fluorescein coupled: 5′-cctcttacctcagttacaatttata-3′. We always injected morpholinos into one-cell-stage embryos.

Rescue of morpholino-injected embryos was done by coinjecting mRNA that was mutated at third codon positions and in the 5′ UTR so that the deduced amino acid sequence remained unaltered, but the morpholino binding site contained at least three mismatches as compared with the wild type nucleotide sequence. Both for *hhex* and *nk2.1a* we cloned correspondingly modified cDNAs into a pCS2+ plasmid vector that was then used for making synthetic mRNA as described above.

Immunohistochemistry and histology

T4 antibody staining and periodic acid/Schiff (PAS) staining on sections was performed as described previously (Wendl et al., 2002). For whole-mount antibody staining, larvae from 4 to 6 dpf were fixed in paraformaldehyde (PFA) at 4°C overnight or for 1 h at room temperature, washed in phosphate-buffered saline containing 0.3% Tween (PBT), then washed and stored in methanol at −20°C. For bleaching, and to block endogenous peroxidases, embryos were incubated in 3 ml 10% H_2O_2 in methanol overnight at room temperature, then 10 ml PBT were added, mixed, and incubated for a further 16 to 24 h at room temperature.

Larvae were washed in PBT, blocked in normal goat serum for 2 h, incubated with a polyclonal antibody (1:4000 rabbit anti-thyroxine BSA serum, ICN Biochemicals) that detects thyroid hormone at its location of production in thyroid follicles (Raine et al., 2001) for 2 h, then washed in PBT for 3 h. Incubation with a biotinylated secondary antibody was for 2 h, washing in PBT for 3 h. Incubation with the ABC kit (Vectastain) was for 2 h according to the instructions of the manufacturers. Larvae were washed again in PBT for 3 h, then once in PBS, and incubated in DAB (0.2 mg/ml PBS) for 30 min. To stain, 1 μl of a 0.3% aqueous H_2O_2 solution was added under observation by using a dissection scope. All procedures were carried out at room temperature, washing steps can be extended at 4°C. For detailed analysis, larvae were postfixed in PFA for 15 min at room temperature, washed in PBT, and gradually transferred to 70% glycerol.

^{125}I Assay

Larvae at 3 and 4 dpf were incubated in 4.5 ml zebrafish medium containing ≈2 μc [^{125}I]-NaI as used for similar studies before (Brown, 1997) for 3–7 h at room temperature. Larvae were then fixed at 75 and 102 hpf, respectively, in 4% PFA overnight, then washed several times in PBS for one day and one night until radioactivity was not detectable any more in supernatant PBS. Fixed embryos were placed on Whatman paper, air dried for 10 min, sealed in Saran Wrap, and exposed to X-ray film for 2 days at −70°C.

For phenylthiourea (PTU) treatment, embryos/larvae

Table 1
T4 immunostaining of follicles correlates with autoradiography signal after incubation with ^{125}I

	T4 immunostaining: anterior domain	T4 immunostaining: follicles	^{125}I signal in autoradiography at 75 hpf	^{125}I signal in autoradiography at 102 hpf
Wild-type	+ from 60 hpf	+ from 72 hpf	−	+
bonnie and clyde$^{-/-}$	−	−	Not tested	−
b16$^{-/-}$	+ from 60 hpf	−	−	−
noi$^{tu29-/-}$	+ from 60 hpf	−	Not tesed	−
0.003% PTU-treated	+ from 60 hpf	−	Not tested	−

Note. T4 immunostaining on sections is either present (+) or absent (−) in various mutants and goitrogen (PTU)-treated embryos. An assay of incorporated radioactive iodide stains X-ray film autoradiographically (+; see also Fig. 2N) or not (−).

were incubated from 12 hpf throughout development in 0.003% PTU. This dose is generally used to reduce pigmentation in zebrafish research (Westerfield, 2000) and has been shown to abolish T4 immunoreactivity of thyroid follicles (Elsalini and Rohr, 2003). For incubation of larvae with radioiodide, [^{125}I]-NaI was added to PTU-containing medium.

Results

Nodal signalling and subsequent steps of endoderm specification are required for thyroid development

In zebrafish, the first specific markers of the thyroid primordium are expressed early during endoderm formation, even prior to formation of the pharyngeal lumen (Fig. 1A and B). Thus, marker gene expression suggests that, in zebrafish, a domain of undifferentiated endodermal cells defines the thyroid primordium prior to morphogenesis of the pharyngeal tube. We wondered how this observation is reflected on the genetic level and analysed mutants that show different degrees of compromised endoderm specification.

The Nodal pathway is required for endoderm formation during gastrulation (Feldman et al., 1998). In *one-eyed pinhead,* a mutant that lacks a cofactor required for Nodal signalling, the endoderm is not specified (Schier et al., 1997), and, as predicted for an organ of endodermal origin, the thyroid primordium is absent (data not shown).

cyc encodes one of the zebrafish Nodal ligands (Sampath et al., 1998), and *b16*$^{-/-}$ and *m294*$^{-/-}$ mutant alleles disrupt Cyc function. *b16*$^{-/-}$ is a gamma ray-induced mutation (Hatta et al., 1991) that lacks the lower telomeric region of LG12, including cyc (Talbot et al., 1998), whereas *m294*$^{-/-}$ is an ENU-induced point mutation in the *cyc* gene (Sampath et al., 1998; Schier et al., 1996). In *cyc*$^{-/-}$ mutants, Nodal signalling is less compromised than in *oep*$^{-/-}$, as another *nodal*-related gene, *squint,* can partially compensate for the loss of *cyc*. Expression of *nk2.1a* indicates that the thyroid primordium is smaller in *b16*$^{-/-}$ and *m294*$^{-/-}$ compared with wild-type (Fig. 1C–E), consistent with an overall reduction in pharyngeal endoderm in the vicinity of the thyroid primordium (Fig. 1F–H; Warga and Nüsslein-Volhard, 1999). Thus, *cyc* is required for normal numbers of endodermal cells, and reduction in the extent of endoderm correlates with reduced size of the thyroid primordium.

The three genes *casanova (cas), bonnie and clyde (bon),* and *faust/gata5 (fau)* have been shown to be required downstream of Nodal signalling for the specification of endoderm (Kikuchi et al., 2000, 2001; Reiter et al., 1999). We find that the thyroid primordium is completely absent in *cas*$^{-/-}$, *bon*$^{-/-}$, and *fau*$^{-/-}$ mutants (data not shown; for *bon*$^{-/-}$, see Elsalini and Rohr, 2003). Thus, thyroid development is not only dependent on Nodal signalling, but also on subsequent steps of endoderm specification.

A functional thyroid gland is absent in b16$^{-/-}$ *embryos that lack both cyc and hhex activity, but not in cyc m294*$^{-/-}$ *mutant embryos*

The *hhex* gene is expressed in the thyroid primordium of developing zebrafish (Fig. 2A). The *hhex* locus maps close to the *cyc* locus and both are deleted in *b16*$^{-/-}$ mutant embryos (Liao et al., 2000). Accordingly, *hhex* mRNA is missing from *b16*$^{-/-}$ embryos (Fig. 2B). In *m294*$^{-/-}$ embryos, however, *hhex* is expressed (Fig. 2C), demonstrating that Cyc activity is not required for *hhex* expression.

Using an antibody that labels thyroid hormone (T4; Raine et al., 2001; Wendl et al., 2002), we fail to detect functional thyroid follicles at 3–5 dpf in *b16*$^{-/-}$ whole-mount embryos (Fig. 2D and E). In contrast, in *m294*$^{-/-}$ embryos, a row of thyroid follicles forms in the midline of the ventral pharynx as in wild-type siblings (Fig. 2F). We counted the number of follicles of one clutch of 5-dpf *m294*$^{-/-}$ larvae and found that homozygous *m294*$^{-/-}$ larvae have an average of 3.6 follicles (n = 5), as compared with 4.5 follicles in siblings (n = 14). Hence, a smaller primordium in *m294*$^{-/-}$ allows normal development of a reduced number of thyroid follicles.

We next tested whether lack of T4 immunostaining in *b16*$^{-/-}$ larvae reflects the absence of the thyroid gland. PAS staining visualises glycoproteins of the colloid in thyroid follicles in both wild-type (Fig. 2G) and *m294*$^{-/-}$ (Fig. 2I) larvae but not *b16*$^{-/-}$ larvae (Fig. 2H). In zebrafish larvae treated with drugs that interrupt thyroid hormone synthesis

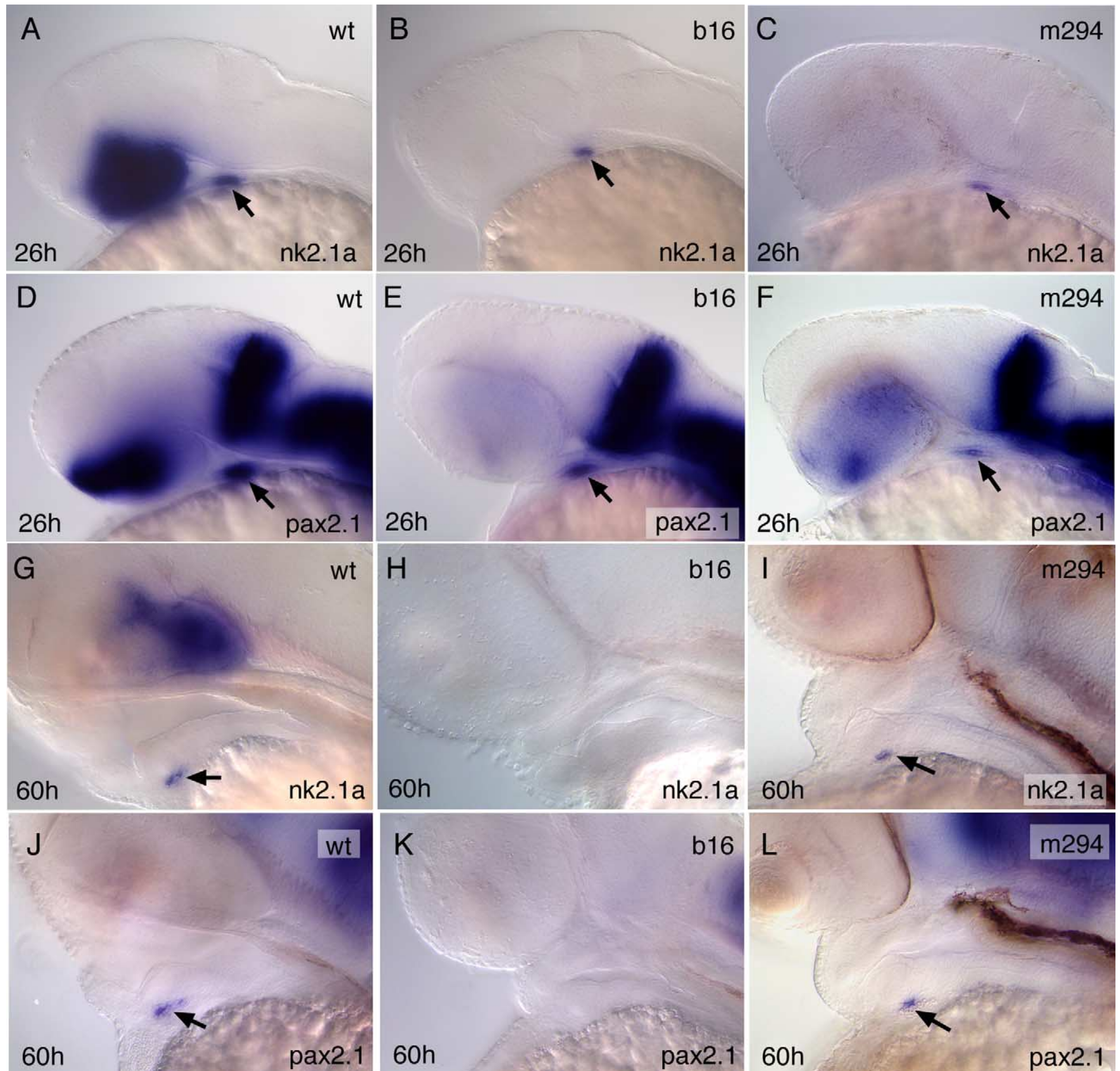

Fig. 3. A thyroid is induced in both *b16*$^{-/-}$ and *m294*$^{-/-}$ embryos. Expression patterns of thyroid markers (indicated at bottom right) at different stages of development (indicated bottom left) in mutant embryos (indicated at top right). Arrows point to the thyroid primordium.

but do not interfere with thyroid follicle development, T4 immunostaining is absent, but PAS still stains colloid in the follicles (Elsalini and Rohr, 2003). Thus, absence of both T4 immunostaining and PAS staining suggests that thyroid follicles are missing in *b16*$^{-/-}$.

In previous work, we have found that on sections (but not in whole mounts, presumably due to sensitivity), the T4 antibody detects not only thyroid follicles (Fig. 2K), but also a nonfollicular anterior domain of immunostaining at the level of the hyoid arch (the second branchial arch; Fig. 2J). This anterior domain is not only present in wild-type larvae, but also in the *pax2.1/noi*$^{-/-}$ mutant that lacks thyroid follicles (Wendl et al., 2002). We found that, although follicles are absent (Fig. 2M), an anterior domain of T4 immunostaining is detectable in sections of *b16*$^{-/-}$ mutant larvae (Fig. 2L).

We next asked whether the anterior domain of immunostaining in *b16*$^{-/-}$ and *pax2.1/noi*$^{-/-}$ mutants reflects thyroid-specific physiological activity. In an assay of iodide uptake using radioactive ^{125}I (Brown, 1997), we found that 4- or 5-dpf wildtype larvae incorporate sufficient ^{125}I to stain X-ray film autoradiographically (Fig. 2N). Mutant

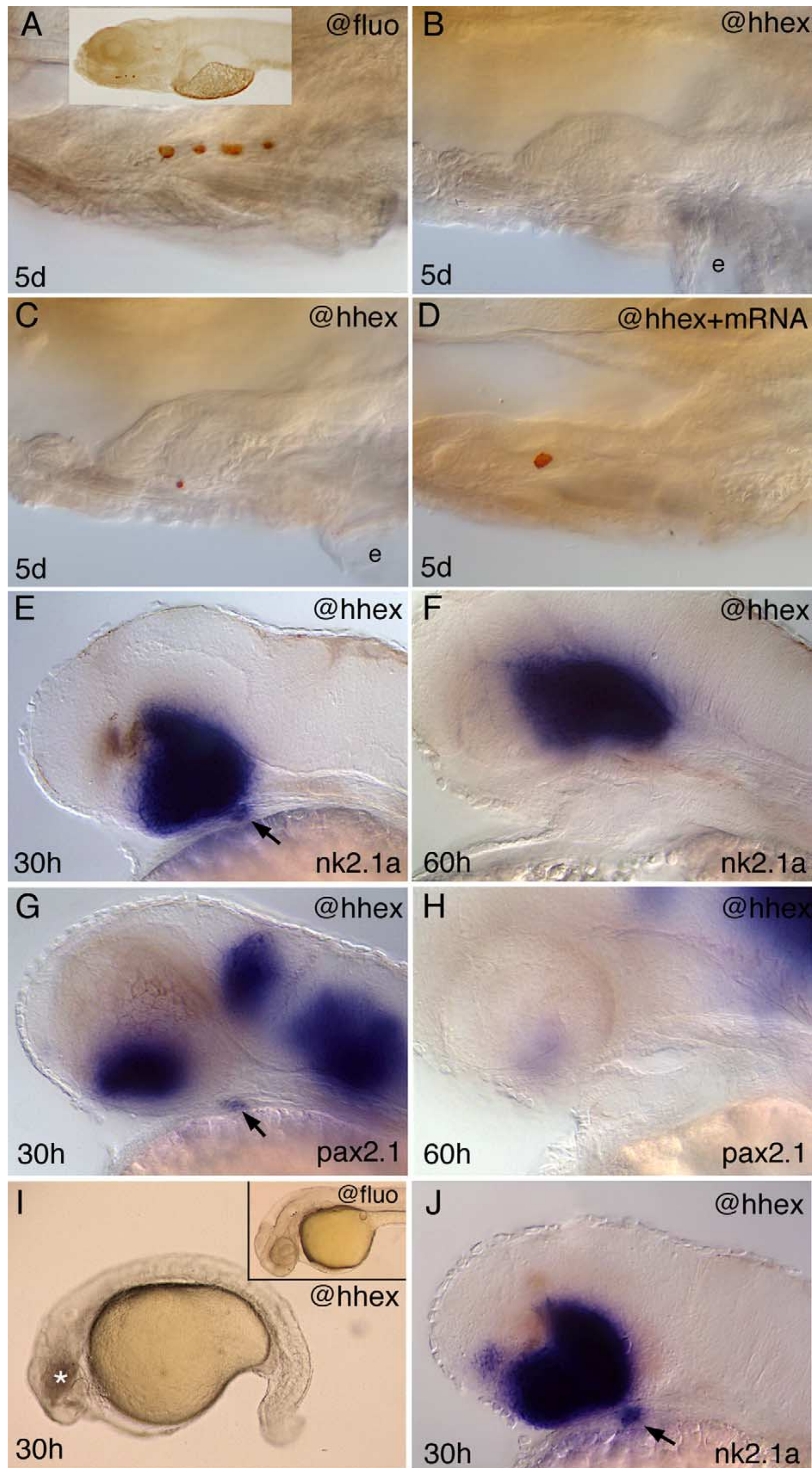

Fig. 4. Abrogation of *hhex* function causes absence of thyroid follicles. (A–D) T4 immunostaining in different morpholino-injected (A–C) and morpholino plus mRNA coinjected (D) embryos. (E–H, J) Expression patterns of thyroid markers (indicated at bottom right) in different morpholino-injected embryos. (I) Morphology of high concentration *hhex* morpholino (0.3 mM)-injected embryo (insert: 0.3 mM control morpholino). The asterisk marks cell death in the head. Morpholino injection indicated at top right; @fluo, control morpholino fluorescein labelled; @hhex, *hhex* morpholino alone; @hhex + mRNA, *hhex* morpholino plus mutated *hhex* mRNA in (D). Concentrations of morpholinos are 0.17 mM in all cases except (C) (0.07 mM), (I and J) (0.3 mM). Arrows in (E, G, and J) point to the thyroid primordium; stages at bottom left. Abbreviation: e, heart edema.

Table 2
hhex morphants fail to develop a functional thyroid gland, but coinjection of *hhex* mRNA restores follicle development in part of the morphants

Injection	(1) Normal row of 2–6 follicles	(2) Embryos with one thyroid follicle	(3) Embryos with no thyroid follicles
0.17 mM *hhex* MO	8 (18%)	0 (0%)	37 (82%)
0.1 mM *hhex* MO	4 (18%)	3 (14%)	15 (68%)
0.07 mM *hhex* MO	6 (10%)	10 (17%)	43 (73%)
0.05 mM *hhex* MO	10 (13%)	11 (15%)	54 (72%)
0.3 mM control MO	67* (100%)	0 (0 %)	0 (0 %)
0.17 mM *hhex* MO + 100 pg *hhex* mRNA (mutated)	3 (7%)	14 (33%)	26 (60%)
0.17 mM *hhex* MO + 150 pg *hhex* mRNA (mutated)	15 (29%)	21 (41%)	15 (29%)

Note. Presence of T4 immunostaining in morpholino-injected zebrafish larvae at 5 dpf. After *hhex* morpholino injection, three different phenotypes can be observed: (1) Embryos with two to six thyroid follicles and without heart edema or any other visible defects, some of them presumably representing embryos that escaped efficient injection; (2) Embryos with heart edema, small eyes, and one single thyroid follicle; (3) Embryos with heart edema, small eyes, and no thyroid follicles. *, Some of the control morpholino-injected embryos show heart edema, but have a normal row of three to six follicles.

b16$^{-/-}$, *noi*$^{-/-}$, and *bon*$^{-/-}$ larvae, however, do not stain X-ray film, indicating that little or no iodide is incorporated in these mutants (Table 1). Goitrogens are drugs that block the uptake of iodide into the thyroid, the biosynthesis or the release of thyroid hormone. In goitrogen-treated larvae, the anterior domain still stains positive with the T4 antibody, and again, these larvae do not stain X-ray film after incubation with ^{125}I (Table 1). In conclusion, the anterior domain of T4 immunoreactivity does not reflect biosynthesis of thyroid hormone. A possible explanation is that the anterior domain represents localised maternal thyroid hormone. Follicles labelled with T4 immunostaining in whole-mount larvae therefore most likely reflect the only site of thyroid hormone production.

Both *nk2.1a* and *pax2.1* are expressed from 24 hpf in the thyroid primordium of developing zebrafish, and we have demonstrated previously that *pax2.1* is required for thyroid follicle development (Wendl et al., 2002). Both genes are initially expressed in *b16*$^{-/-}$ embryos (Fig. 3A–F) and so a thyroid primordium initially forms in the absence of the *hhex* gene, and neither *nk2.1a* nor *pax2.1* is, at these early stages, dependent on *hhex*. At around 60 hpf, however, both markers (and *pax8*; see Wendl et al., 2002) disappear in the thyroid primordium of *b16*$^{-/-}$, but not in *m294*$^{-/-}$ (Fig. 3G–L). Altogether, these results show that, although a primordium is present, a functional thyroid gland fails to form in *b16*$^{-/-}$ larvae.

hhex is required for differentiation of thyroid follicles

The failure of the thyroid gland to develop in *b16*$^{-/-}$ embryos might be due to the deletion of the *hhex* gene, but could also be due to deletion of other unknown genes in *b16*$^{-/-}$. We therefore used *hhex* morpholino antisense RNA to test whether *hhex* itself is required for thyroid development. *hhex* morpholino RNA in zebrafish disrupts hepatocyte development in the liver (Wallace et al., 2001; H. Field and D. Stainier, personal communication), resembling the phenotype of *Hhex*$^{-/-}$ mouse embryos (Martinez Barbera et al., 2000). *hhex* morphants develop heart edema (Fig. 4B), but about 50% of injected embryos survive to at least 6 dpf. In contrast to control injected embryos, *hhex* morphants lack T4 immunostaining (Fig. 4A and B), and so with regard to the thyroid follicles, the late phenotype of *hhex* morphants resembles the *b16*$^{-/-}$ phenotype.

Varying concentration of morpholino injection allows generation of hypomorphic phenotypes. Injection of lower concentrations of *hhex* morpholino results in a higher percentage of larvae that show some follicle differentiation at 5 dpf (Fig. 4C, Table 2). This result suggests that reduced *hhex* function results in reduced growth of the thyroid primordium or the follicles. Coinjection of 100 pg *hhex* mRNA that has a mutated binding site for the hhex morpholino rescued follicle development in 33% of larvae (Fig. 4D, Table 2). Coinjection of 150 pg mutated *hhex* mRNA results in even higher numbers of larvae with follicles (Table 2). We assume that the ratio of rescue is relatively low due to a late and continued requirement of *hhex* gene function as expected from the mutant phenotype. Thus, the activity of the stable *hhex* morpholino RNA can only be partially compensated by comparably instable *hhex* mRNA, nevertheless indicating specificity of the *hhex* morpholino.

hhex is not required for formation or migration of a thyroid primordium

Analysis of *nk2.1a* and *pax2.1* expression showed that although follicles are absent, a thyroid primordium is present in *hhex* morphants (Fig. 4E and G). However, expression of these genes is extinguished in the thyroid primordium of morphants by around 60 hpf (Fig. 4F and H). In order to test whether high concentrations of *hhex* morpholino affect the induction or size of the thyroid primordium, we injected 0.3 mM *hhex* morpholino. Even at these high concentrations that interfere with gastrulation processes (Fig. 4I), we do not observe a visibly reduced thyroid primordium (Fig. 4J). Thus, *hhex* morpholino injection phenocopies *b16*$^{-/-}$ mutant embryos with respect to the loss of the primordium at around 60 hpf. On the contrary, the smaller thyroid primordium in *b16*$^{-/-}$ mutants cannot be

caused by *hhex* deficiency and is therefore presumably due to the *cyc* deficiency alone.

As in mammals, the zebrafish thyroid primordium moves from the pharyngeal epithelium to its final position at the base of the lower jaw (Rohr and Concha, 2000). In *b16*$^{-/-}$ mutants, relocalisation of the thyroid primordium does take place during the period when expression of *nk2.1a* and *pax2.1* fades and disappears (Fig. 5A and B). Analysis of cell death using acridine orange did not reveal increased cell death in the lower jaw area of either *b16*$^{-/-}$ or *hhex* morphants (data not shown), and so the fate of thyroid precursors remains unclear.

We also compared the *hhex* deficiency phenotype with the *pax2.1/noi*$^{-/-}$ phenotype. In the presumed null allele *noi*$^{tu29-/-}$, *hhex* and *nk2.1a* expression disappears at around 30 hpf (Wendl et al., 2002). In the weak allele *noi*$^{tb21-/-}$, however, both markers disappear more slowly and are weakly detectable until 55 hpf. As in *b16*$^{-/-}$ mutants and *hhex* morphants, we observe that the thyroid primordium relocalises (Fig. 5C and D). In conclusion, the *hhex* gene is not involved in relocalisation of the primordium, and absence of *hhex* leads to a relatively late thyroid phenotype similar to the weak *noi* allele.

Gain of Hhex function supports a late role for hhex in thyroid development

We next analysed the gain-of-function phenotype of *hhex* by injecting *hhex* mRNA. Ectopic *hhex* expression dorsalises the embryo at epiboly stages (Ho et al., 1999), and later, *hhex*-injected embryos have a shorter trunk and tail, whereas head morphology appears normal (Fig. 6A–H). At 28 hpf, we did not observe any visible differences in size or strength of marker gene expression in the thyroid primordium of *hhex* mRNA-injected embryos (Fig. 6A, B, E, and F). However, by 40 hpf, about 30% of *hhex* mRNA-injected embryos showed a visibly enlarged thyroid primordium. In these cases, the primordium is anterior–posteriorly expanded and comprises about two to three times more cells than normal (Fig. 6C, D, G, and H). This suggests that additional Hhex activity does not affect formation of the thyroid primordium but can increase its later growth.

hhex acts in parallel to nk2.1a during initial development of the zebrafish thyroid primordium

In the absence of the *hhex* gene, *nk2.1a* is expressed during early development of the thyroid primordium. We wanted to know whether *hhex* in turn is expressed in the absence of *nk2.1a* function. As there is no *nk2.1a* mutant available, we used two different *nk2.1a* morpholinos to knock down Nk2.1a protein. As a control, we also injected *nk2.1b* morpholino RNA. *nk2.1b* is a paralogue of *nk2.1a* that is not expressed in the thyroid (Rohr et al., 2001). At a concentration of 0.3 mM, *nk2.1a* and *nk2.1b* morphants appear to develop normally, but in about 50% of injected embryos, small areas of cell death are apparent in the preoptic area of the forebrain (Fig. 7A and B). As both genes are expressed in the ventral forebrain, then this is likely to be a specific phenotype.

Thyroid follicles fail to develop in more than 80% of 5-dpf-old *nk2.1a* morphants (Fig. 7C, Table 3), whereas they are unaffected in *nk2.1b* morphants (Table 3, and data not shown). Coinjection of *nk2.1a* mRNA-containing mismatches in the morpholino target sequence leads to normal thyroid follicles in about 40% of the embryos (Fig. 7D, Table 3), a frequency much higher than the usual 5–20% of embryos that might escape efficient morpholino injection. Thus, *nk2.1a* mRNA rescues about a third of *nk2.1a* morphants and therefore further confirms the specificity of the morpholinos. Again, as with the *hhex* gene, the rescue might not be very efficient because of a relatively late requirement for *nk2.1a* mRNA in the thyroid.

We find that both *hhex* and *pax2.1* are initially expressed in the thyroid primordium of *nk2.1a* morphants (Fig. 7E–H; 100% of $n = 72$), but expression fades around 35 hpf and is absent by 60 hpf (Fig. 7I–L; 50 [88%] of $n = 57$ for *hhex* and 58 [94%] of $n = 62$ for *pax2.1*). The initial expression of *hhex* and *pax2.1* in *nk2.1a* morphants further suggests that induction of *hhex* expression is independent of *nk2.1a*.

Discussion

Establishment of endoderm by Nodal signalling and its downstream effectors is a prerequisite for thyroid development

The assumption that the thyroid gland is of endodermal origin is based on the observation that the early primordium of the thyroid becomes visible as a bud at the ventral floor of the pharynx (Macchia, 2000). We show that Nodal signalling is required for thyroid development, and the absence of a thyroid primordium in *oep*$^{-/-}$ and *cas*$^{-/-}$ mutants can be explained by missing endoderm. In *bon*$^{-/-}$ and *fau*$^{-/-}$ mutants, numbers of early endodermal precursor cells are reduced, but remaining cells eventually form some reduced gut tissue in *bon*$^{-/-}$ (Kikuchi et al., 2000), or a nearly complete, but misshaped and abnormal gut in *gata5/fau*$^{-/-}$ (Reiter et al., 2001). We never detected any thyroid tissue in *bon*$^{-/-}$ and *gata5/fau*$^{-/-}$, suggesting that endodermal precursor cells cannot contribute to the thyroid in the absence of Bon or Gata5 function. *cyc m294*$^{-/-}$ mutant embryos show that, if a smaller number of primordial cells is specified, these are still able to form functional, but less follicles. This suggests that the loss of thyroid in *bon*$^{-/-}$ and *gata5/fau*$^{-/-}$ embryos is due to a requirement for these genes in a specific early step in thyroid development rather than due to an indirect consequence of reduced endoderm development.

Fig. 5. Migration of thyroid primordium occurs in *b16*$^{-/-}$, and *noi tb21*$^{-/-}$ embryos. Expression patterns of thyroid markers (indicated at bottom right) in different mutants (indicated at top right) at stages when migration of the thyroid is taking place (indicated at bottom left). Note the distance of the thyroid primordium to the pharyngeal epithelium and also the smaller size of the primordium in *b16*$^{-/-}$ (B) compared with wild-type (A). Arrows point to the pharyngeal opening (p); e marks the ventral pharyngeal epithelium. Arrowheads indicate expression in the thyroid primordium.

A late role of hhex in zebrafish thyroid development

In mice, *Hhex* deficiency leads to an arrest in thyroid development at around 10.5 dpc (Martinez Barbera et al., 2000), just after evagination from the pharyngeal epithelium, and long before onset of thyroid function that takes place around 15.5 dpc (Lazzaro et al., 1991). In zebrafish, we find that *hhex* deficiency results in the disappearance of

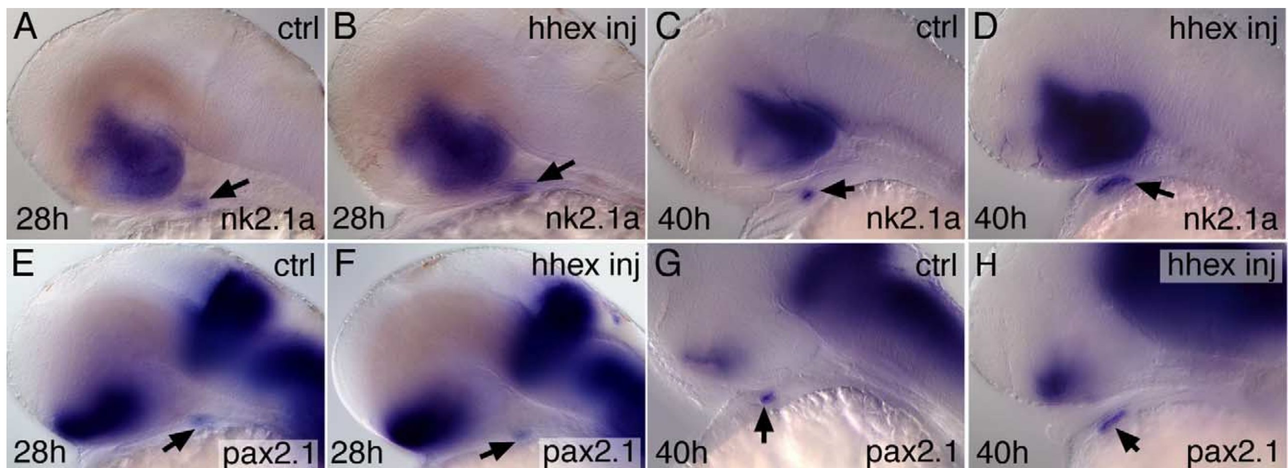

Fig. 6. Overexpression of *hhex* leads to late expansion of the thyroid primordium. Embryos are injected with *hhex* mRNA or *gfp* mRNA as a control (ctr1; indicated top right). Marker gene expression as indicated bottom right; stages as indicated bottom left.

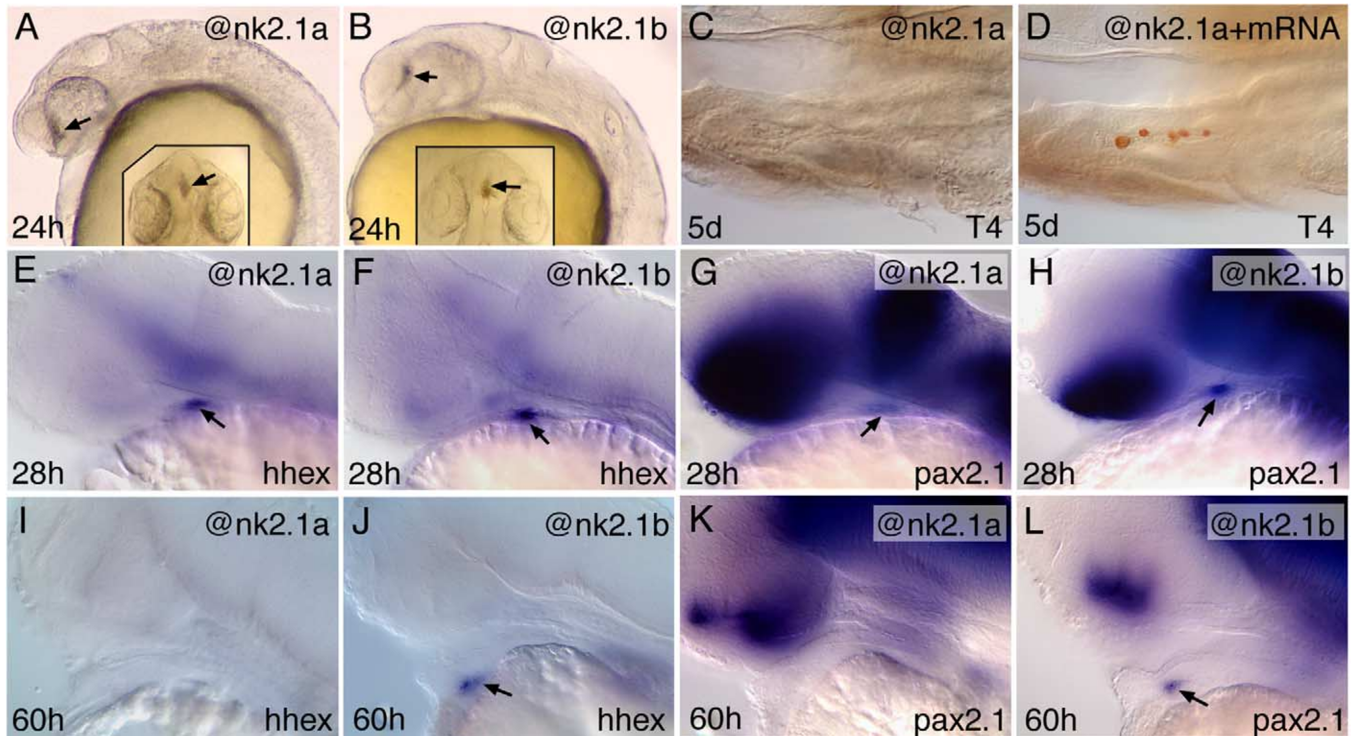

Fig. 7. Abrogation of *nk2.1a* function causes failure of thyroid differentiation. (A, B) Morphology of injected embryo heads, lateral views (inserts: dorsal views). The arrows point to cell death in the area of the optic recess. (C, D) T4 immunostaining. (E–I) Marker gene expression. Morpholino injection indicated at top right; @nk2.1a, *nk2.1a* morpholino; @nk2.1b, *nk2.1b* morpholino; @nk2.1a + mRNA, *nk2.1a* morpholino and mutated *nk2.1a* mRNA. Arrows point to the thyroid primordium; stages at bottom left. Marker genes are indicated at bottom right.

the thyroid primordium just before the onset of thyroid hormone production. Nevertheless, both phenotypes are similar in that, in both species, induction and evagination from the pharynx is independent of *hhex/Hhex*. In mice, the thyroid primordium grows after evagination for 5 days before follicles differentiate (Fig. 8A; Lazzaro et al., 1991), and then many follicles differentiate simultaneously. In zebrafish, the thyroid primordium does not undergo this extended phase of early growth. From 24 to 72 hpf, the primordium consists of a relatively constant number of 30–40 cells (K.B.R., unpublished observations). At 72 hpf, a very small first follicle expressing thyroid hormone forms, and during subsequent larval development, the number of follicles then gradually increases (Fig. 8B; Wendl et al., 2002). Thus, in both species, *hhex/Hhex* is required at a comparable step of thyroid development prior to significant tissue growth.

Table 3
A functional thyroid is missing in *nk2.1a* morphants

Injection	2–6 Follicles present	Follicles absent
0.3 mM *nk2.1a* MO-1	4 (8%)	45 (92%)
0.3 mM *nk2.1a* MO-2	7 (13%)	48 (87%)
0.3 mM *nk2.1a* MO-1 + 75 pg *nk2.1a* mRNA (mutated)	21 (40%)	32 (60%)
0.3 mM *nk2.1a* MO-2 + 75 pg *nk2.1a* mRNA (mutated)	34 (44%)	44 (56%)
0.3 mM *nk2.1b* MO	63 (100%)	0 (0%)

Note. Presence or absence of T4 immunostaining in *nk2.1a* and *nk2.1b* morpholino-injected embryos, as judged by visible follicles in 5-dpf whole-mount larvae. Similar results were obtained with both different nk2.1a morpholinos (MO1 and MO2). Coinjection of morpholino and *nk2.1a* mRNA restored follicle development in part of the embryos.

The enlarged thyroid primordium following *hhex* overexpression suggests that *hhex* can promote growth of the thyroid primordium. Given that the zebrafish thyroid primordium does not grow significantly under wild-type conditions, we think that *hhex* initially fulfils a role in maintenance or modest growth of thyroid primordial cells, and that increasing doses of transcript might be able to promote increased thyroid tissue growth. In particular, it might be possible that *hhex* is normally involved in growth of follicles after 72 hpf, as *hhex* continuous to be expressed in thyroid follicles during later stages (T. Wendl and K.B.R., unpublished observations).

Developmental genes can play crucial roles in tumorigenesis. For instance, in humans, a chromosomal translocation involving the *PAX8* gene is a possible cause for thyroid cancer (Kroll et al., 2000). Differentiated thyroid tumors maintain *TTF1(NKX2.1)* expression, form follicles, and often express *PAX8,* a prerequisite for the production of thy-

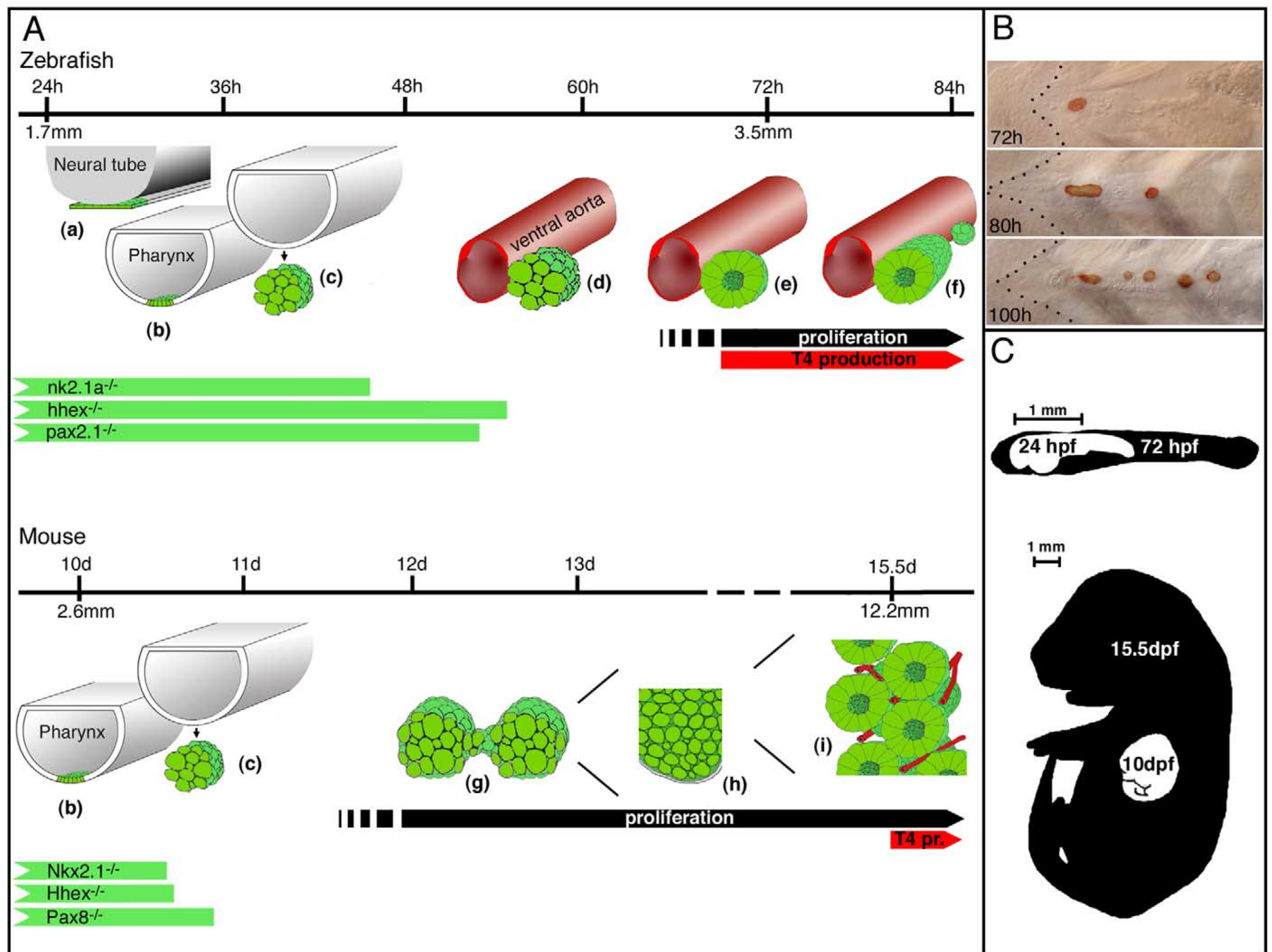

Fig. 8. Comparison between thyroid development in zebrafish and mice. (A) Schematic illustrations comparing thyroid development in zebrafish and mice. The scale bar shows time of development (h, hpf for zebrafish; d, dpc for mice) and size of the embryo/foetus/larvae at beginning of thyroid development and beginning of hormone production. Green colour indicates developing thyroid tissue. (a) Early marker gene expression in endoderm; (b) primordium at ventral midline of pharynx; (c, d) evagination and relocalisation; (e) differentiation into one first follicle; (f) growth of follicles; (g) bifurcation of primordium; (h) growth of primordial cells, surrounded by connective tissue; (i) differentiation into many follicles at the same time. Black arrows indicate the growth phase of thyroid development; red arrows onset of thyroid function as judged by T4 production. Green bars symbolise how far the thyroid primordium develops in the mutants/morphants/knock-outs of the indicated genes. This information is based on Kimura et al., 1996 and Macchia et al., 1999 (*Nkx2.1*), Martinez Barbera et al., 2000 (*Hhex*), and Mansouri et al., 1998 (*Pax8*). The bar for *pax2.1* reflects thyroid development in the weak allele *noi*^{tb21}. (B) In zebrafish, an increasing number of thyroid follicles appear after onset of thyroid hormone (T4) production. Ventral views of pharyngeal area, the dotted line marks the posterior margin of the mandibular arch. (C) Comparison of overall size of developing zebrafish and mouse, respectively, during the time of thyroid development. The white outline indicates the embryos at onset of thyroid marker gene expression, the black outline the larva/foetus at onset of thyroid hormone production. For each species, the stages are drawn to scale. Size after Westerfield, 2000 and Kaufman, 1992.

roglobulin and other thyroid-specific proteins in this tumor type (Fabbro et al., 1998; Pasca di Magliano et al., 2000). Undifferentiated tumors in contrast switch off *TTF1* and *PAX8* expression. *HHEX* expression is maintained in all differentiated thyroid tumors investigated and also in the majority of undifferentiated thyroid tumors (D'Elia et al., 2002). *HHEX* expression is therefore more widespread in thyroid tumors than *TTF1* or *PAX8* (D'Elia et al., 2002). Given that zebrafish *hhex* functions like human *HHEX,* our overexpression data suggest that *HHEX* might be part of the machinery that maintains growth in thyroid tumors.

hhex acts in a regulatory genetic network of thyroid differentiation

In mice it has been shown that *Nkx2.1* is not expressed in the thyroid primordium of *Hhex*$^{-/-}$ mice, leading to the assumption that *Hhex* has a particularly early requirement during thyroid development (Martinez Barbera et al., 2000). In zebrafish, *nk2.1a, hhex,* and *pax2.1* are induced and subsequently expressed independently of each other from their onset of expression at 24 hpf to approximately 60 hpf. Thus, in embryos deficient for *hhex,* both *nk2.1a* and *pax2.1*

are initially expressed; in *nk2.1a* morphants, both *hhex* and *pax2.1* are initially expressed; and in *pax2.1/noi*$^{-/-}$ mutant embryos, both *hhex* and *nk2.1a* are initially present (Wendl et al., 2002). However, as not all of the markers have been tested in each of the mouse mutations, we cannot be sure of the extent to which the genetic network of thyroid differentiation is conserved between mammals and fish.

cas$^{-/-}$, *bon*$^{-/-}$, and *gata5/fau*$^{-/-}$ mutants suggest that Cas, Bon, and GATA5 are likely to have relatively widespread roles in endoderm specification (Kikuchi et al., 2000, 2001; Reiter et al., 2001) while *hhex, nk2.1a,* and *pax2.1* are involved much later, in maintenance, differentiation, and/or growth of the thyroid. Similarly, in *Hhex*$^{-/-}$, *Nkx2.1*$^{-/-}$, and *Pax8*$^{-/-}$ mutant mice, the primordium develops at least until relocalisation from the pharynx. Thus, between endoderm specification and thyroid differentiation, we expect as yet undiscovered genes to be responsible for induction of the thyroid primordium.

A different time scale of thyroid organogenesis in zebrafish and mice is reflected by temporal differences in gene expression

Comparing zebrafish and mouse reveals a different temporal progression of thyroid development in these species. The early appearance of thyroid markers before pharynx formation may be adaptation to fast development of zebrafish embryos. After 4–6 days, when larvae have used up their yolk sac containing maternal thyroid hormone, they rely on a functional thyroid gland. At this stage, the follicles are proliferating as the larva and juvenile fish grows (Fig. 8B). Mouse embryos, in contrast, are supplied with maternal thyroid hormone up to birth, and after initial specification, 5 days of cell proliferation occur prior to final thyroid differentiation and onset of function (Fig. 8A and C).

Despite a different time scale of thyroid development in zebrafish and mice, our work reveals that *hhex/Hhex, nk2.1a/Nkx2.1,* and *pax2.1/Pax8* act presumably in the same manner during differentiation of the thyroid gland. The zebrafish genome contains a high number of duplicated genes that derive from teleost-specific genome duplication (Force et al., 1999). However, in the case of these three transcription factors, it seems that duplicated paralogous genes have not maintained a redundant role in thyroid development. For *hhex,* only this single gene can be found in the zebrafish genome sequence that is in the process of final assembly (version 2; for access see Acknowledgments). The *Nkx2.1* gene is duplicated in zebrafish, but only *nk2.1a,* not *nk2.1b* is expressed in the thyroid (Rohr et al., 2001). In the case of *pax2.1/pax8,* we showed that *pax8* acts downstream of *pax2.1,* and *pax2.2* is not expressed in the thyroid (Wendl et al., 2002).

Three additional aspects of thyroid development in mice imply differences in the genetic networks that direct thyroid development compared with fish. First, the thyroid primordium in mice and humans bifurcates during growth. Second, in all vertebrates apart from most teleosts, the primordium becomes encapsuled by connective tissue. Third, in mammals neural crest-derived C-cells merge with the developing follicular cells, and indeed, these cells can form a thyroid rudiment, even in the absence of thyroid follicular cells (Mansouri et al., 1998). C-cells are not known to be present in teleosts. In conclusion, zebrafish present a simple, although in some aspects presumably derived, mode of thyroid development. It is likely that the zebrafish will be a very fruitful system for identifying genes functioning in thyroid development, and it can be expected that at least the basic key players of thyroid development act comparably to mammals. Further it will be particularly interesting to investigate how species-specific evolutionary differences in thyroid organogenesis are realised at the molecular level.

Acknowledgments

The genome sequence data for zebrafish *hhex* were produced by the Zebrafish Sequencing group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/Danio rerio. We are grateful to Steve Wilson (UCL) for supporting early aspects of this work (with funding from The Wellcome Trust) and corrections on the manuscript. We are also grateful to Didier Stainier (UCSF) and his lab for support, embryos, and morpholino samples. We further wish to thank José Campos-Ortega (Cologne) and his lab for discussion and support, Heiko Krude (Berlin) for discussion and comments on the manuscript, and Donald Brown (Baltimore) for advice on the ^{125}I assay. This work was mainly funded by an Emmy-Noether fellowship of the Deutsche Forschungsgemeinschaft (to K.B.R), and O.A.E. is supported by the Libyan Ministry of Education/Garyounis University.

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