The role of Nodal-dependent transcription factors and blood vessels in the development of the zebrafish thyroid gland

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Berichterstatter:	PD Dr. Klaus Rohr
	Prof. Dr. Siegfried Roth
Tag der mündlichen Prüfung:	22.02.2007

To my Father

1. Introduction

1.1. *Zebrafish as a model organism*

The zebrafish is a teleost, the generic name of which is *Danio rerio*, a name designated since the 1993 Zebrafish Meeting at the Cold Spring Harbour Laboratory rather than Brachydanio rerio. It is a member of the family Cyprinidae and originated from the Ganges River, India.

Zebrafish are simple, rapidly developing animals that are amenable to detailed developmental analyses. Despite their relatively simple morphology, they share many developmental features with other commonly-studied vertebrates, such as frogs, chickens, and mice. Thus, studies of zebrafish will teach us about the development of other organisms, including humans. Several features make zebrafish an excellent animal to use in studies of genetics and development. First, zebrafish adults reach sexual maturity quickly and are very fecund. Thus, large numbers of embryos can be collected for study. Second, zebrafish embryos develop externally. Eggs that are laid into the surrounding water by adult females are immediately fertilized by sperm from adult males. Embryos are thus easily accessible for observation and experimentation. Third, zebrafish embryos are optically clear. Under a microscope, one can visualize tissues that lie deep within the embryo, follow individual cells during development, and recognize developmental mutants. Fourth, zebrafish embryos develop rapidly, as illustrated.

1.2. *Zebrafish embryonic development*

The newly fertilized egg or zygote (0 – ¾ h) (Fig.1A), is about 0.7mm in diameter at the time of fertilization. The embryo develops within a transparent eggshell, called the chorion. In the zygote, cytoplasmic streaming separates the zygote into two visibly different parts: a clear blastodisc at the animal pole and a yolky cytoplasm at the vegetal pole.

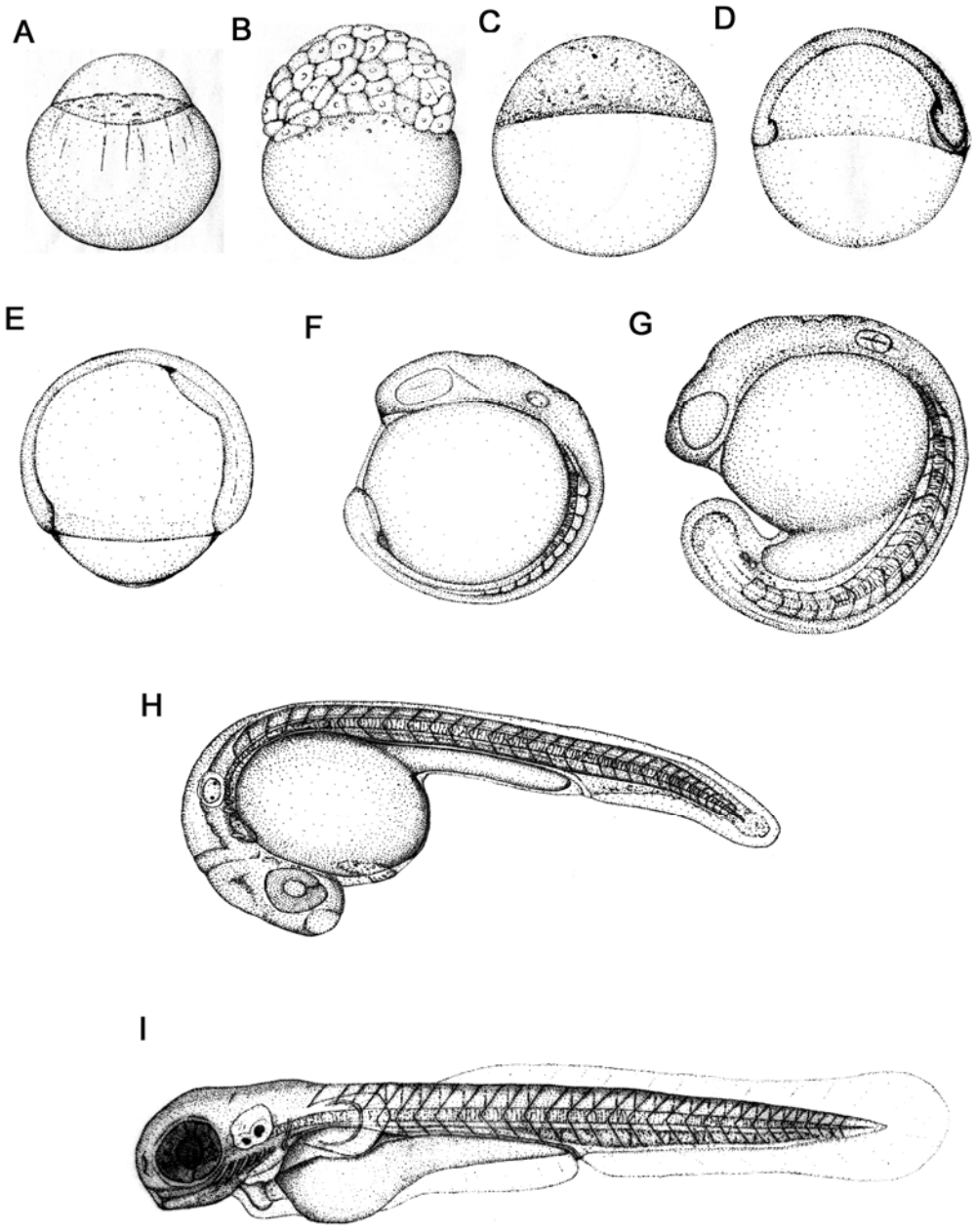

Figure 1: Zebrafish developmental series. The animal pole is to the top for the early stages(A-E), anterior is up (F) or to the left (G-I) at later stages. (From: Kimmel et al.,1995. developmental Dynamics 203:253-310).

The blastodisc is the part of the zygote that undergoes cleavage and gives rise to blastomeres, the cells that later form the embryo. Cleavage period (¾ – 2 ¼ h) (Fig. 1B) includes the first six regular, rapid, and synchronous divisions of the embryo. At the end of the cleavage period, the blastomeres sit as a mound of cells on top of the large yolk. Up to midblastula stages the embryo has utilized proteins and RNA that the mother deposited in the egg (Amacher, 2001).

During midblastula stages, the embryo begins to transcribe its own genes. Another major event that begins during the blastula period is epiboly, which describes the thinning and spreading of the cellular blastodisc over the yolk. Near the end of this period, the blastomeres are referred to collectively as the blastoderm, a sheet-like array of cells that sits like a cap on top of the large yolk. Epiboly continues during the gastrula period (5¼ –10 h) (Fig. 1D, E). During this period, cell movements dramatically reorganize the embryo, so that by the end of this period, three embryonic axes are clearly visible. That is, the anterior head of the embryo is clearly distinguished from the posterior tail bud, dorsal tissues are distinct from ventral ones, and medial tissues are easily discernible from lateral ones. Many embryonic genes are expressed in region-specific patterns. Cell movements during gastrulation also produce the germ layers (ectoderm, mesoderm and endoderm) of the embryo. Finally, near the end of the gastrula period, the neural plate forms; this is the first morphological sign of central nervous system development.

The embryo elongates considerably during the segmentation period (10–24 h, Fig. 1F-H). A complete complement of about 30 bilateral somite pairs forms in an anterior to posterior sequence. The rudiments of primary organs, such as notochord, kidney, and blood, become visible. The central nervous system undergoes dramatic changes; the neural plate forms a solid neural keel, which then hollows out to form a neural tube. Additionally, the brain becomes regionalized, with forebrain, midbrain and hindbrain subdivisions becoming distinct from one another. From around 24 hpf, muscular contractions occur, and

the embryo moves within the chorion. In the pharyngula period (24–48 h, Fig. 1H) the seven pharyngeal arches rapidly form. Pharyngeal arch development is common to all vertebrates, and in zebrafish, pharyngeal arches later essentially form the jaw and gills. Also during the second day, the head straightens, fins form, pigment cells differentiate, the circulatory system forms, the heart starts to beat, and coordinated swimming movements begin. In addition, embryos become responsive to touch, revealing the development of sensory-motor reflexive circuits.

Embryos hatch from their chorions during hatching period (48–72 h, Fig. 1I). By this time, many of the organ rudiments have formed; however, the gut and its associated organs are still developing. In addition, this period is marked by rapid development of pectoral fins, jaws and gills. During the two days after hatching (72–120 h), the zebrafish juvenile starts to swim and feed. The embryonic yolk, which has sustained the embryo until this time, is almost depleted. The zebrafish embryo has rapidly become a small version of the adult.

1.3. *Thyroid gland*

The thyroid gland is a member of the endocrine system. The English medical term derives from the Greek words “thyreos” meaning “shield”, and “eidos” meaning “form”. In humans, the thyroid gland is located at the front of the neck (Fig.2 A). It is slightly heavier in women than in men and enlarges in pregnancy.

The thyroid gland is responsible for producing thyroid hormone in all vertebrates. Although thyroid hormone is best known for its role in regulating metabolism in the adult organism, it is also required during development for many processes.

The thyroid gland in humans is a brownish-red organ, in most cases having two lobes connected by an isthmus; it normally weighs about 28g and consists of cuboidal cells arranged to form epithelial follicles (Fig.2 B). These follicles are

supported by connective tissue that forms a framework for the entire gland. In the normal thyroid gland, the follicles are usually filled with a colloid substance containing the protein thyroglobulin in combination with the main thyroid hormone thyroxine, tetraiodothyronine (T_4), and a secondary metabolistic form, triiodothyronine (T_3). These hormones are composed of the amino acid tyrosine, containing four and three iodine atoms, respectively. Thyroxin is an iodine-containing compound. In fact, the thyroid gland itself has a high content of iodine, which is necessary for optimal thyroid health and function. Thyroid hormone production starts with the synthesis of thyroglobulin. Thyroglobulin is then secreted into the colloidal lumen of the follicle where tyrosine residues are iodinated and where it is condensed to produce tri- (T3) and tetra-iodinated thyronine (T4, thyroxine) (Frieden and Lipner, 1971). T3 and T4 remain covalently bound to thyroglobulin as long as they are stored in the colloid. The bound forms of T3 and T4 are eventually taken up by the follicular cells and prototypically separated from the thyroglobulin. Free T3 and T4 are then released and act as thyroid hormone.

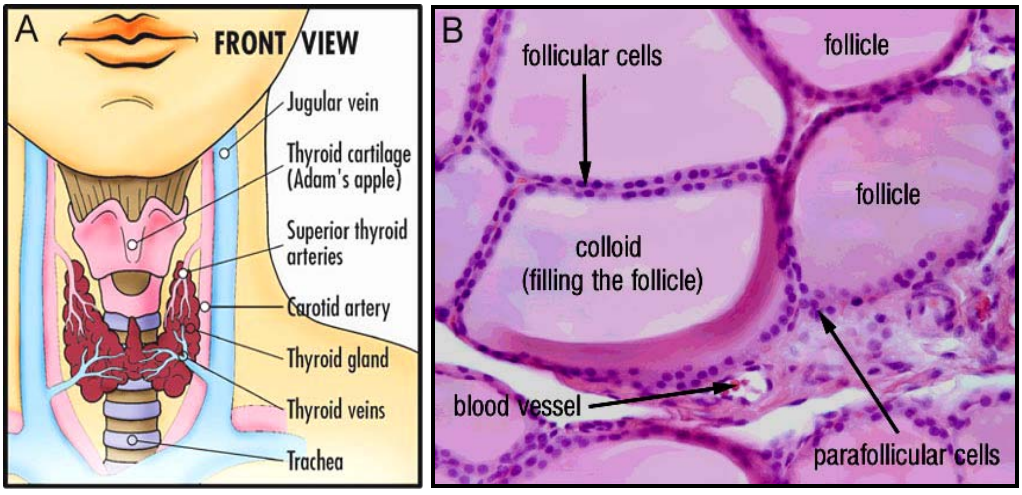

Figure 2: The thyroid gland in human. (A) Drawing showing the thyroid position and related structures in the neck region. (B) Cross section in the thyroid showing the structure of the thyroid follicles. (From University of Michigan webpage, http://www.um-endocrine-surgery.org).

Although the thyroid gland constitutes about 0.5 percent of the total human body weight, it holds about 25 percent of the total iodine in the body, which is obtained from food and water in the diet. Iodine usually circulates in the blood as an inorganic iodide and is concentrated in the thyroid to as much as 500 times the iodide level of the blood.

The amount of T4 and T3 secreted by the thyroid is controlled by the thyroid-stimulating hormone (TSH) of the pituitary gland. TSH, in turn, is regulated by a substance called thyroid-stimulating hormone releasing factor (TRF), which is secreted by the hypothalamus.

The functions of the thyroid gland include: regulation of normal body growth in infancy and childhood, regulation of metabolism, regulation of body temperature, maintenance of skeletal maturation and regulation of protein, fat and carbohydrate metabolism. These functions are dependent upon serum T4 levels.

Any disorder of the thyroid that leads to reduced thyroxine production at birth is called Congenital Hypothyroidism (CH) (Macchia, 2000). On the one hand, defects in genes responsible for thyroxine production can account for congenital hypothyroidism. On the other hand, such a phenotype can also be the result of earlier defects in development, such as mis-specification or mis-localisation of thyroid primordial cells.

1.4. *Thyroid gland in zebrafish*

The thyroid of teleost fishes is organised as thyroid follicles as in other vertebrates, and these follicles are presumed to produce thyroid hormone in the same way (Leatherland, 1994; Rauther, 1940). In contrast to higher vertebrates, however, the thyroid of many teleosts is not a compact single organ. Most teleost species that have been investigated have thyroid follicles loosely distributed

within the mesenchyme of the ventral head area, mainly in the vicinity of the anterior aorta. Nevertheless, comparative data are scarce and there is wide variation in teleost thyroid morphology from one set of bilateral nuclei of follicles in medaka (Raine et al., 2001) to dense groups of follicles throughout the gill region in trout (Raine and Leatherland, 2000).

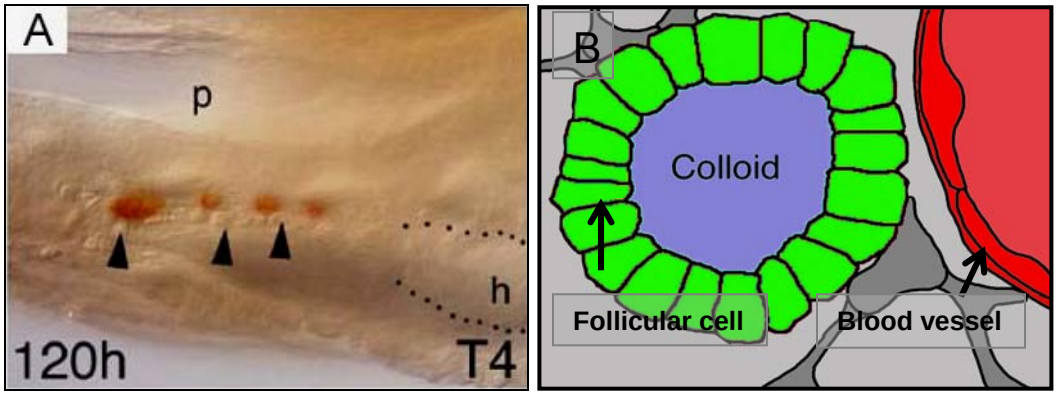

Figure 3: (A) The normal position and distribution of thyroid gland in adult zebrafish and (B) the structure of thyroid follicular cell in zebrafish. P. pharynx, h. heart, and the arrow head indicate the ventral aorta. (A) lateral veiw +T4 immunostaining in 120h zebrafish embryo.

The thyroid gland in adult zebrafish is not a compact structure encapsulated in connective tissue; it's rather a loose aggregation of follicles, close to the ventral aorta, distributed between the first pair of gills and the heart (Fig.3A) (Wendl, et al., 2002). Colloid filled follicles are detected along the ventral aorta, in the ventral midline of the gill chamber (Fig.3A). The follicles appear alone or in loose aggregations of two or three embedded in connective tissue (Wendl, et al. 2002). Their shape is irregular and varies in diameter from 14 mm to 140 mm. In summary, the zebrafish thyroid gland is composed of follicles that are dispersed as described in other teleosts. In zebrafish, all follicles are found close to the ventral aorta, from the first gill arch to the bulbus arteriosus. These cells form colloid-filled follicles (Fig.3B), produce thyroid hormone, store it in the colloid-filled lumen of the follicle and control the release of the hormone from the colloid into the blood stream (Leatherland, 1994). In zebrafish development, immunostaining of thyroid hormone reveals a first follicle at around 60 hour's postfertilization (hpf), when the embryo hatches from the egg shell. An increasing

number of further follicles is generated throughout larval life (Wendl et al., 2002; Elsalini et al., 2003).

1.5. *Development of the thyroid gland*

In humans and mice, the thyroid gland develops from two types of primordia (De Felice and Di Lauro, 2004). One primordium, the so called midline diverticulum, evaginates from the midline of the pharyngeal floor, on the level between the first and second pharyngeal pouch. This midline diverticulum relocates and reaches a position deep in the cervical mesenchyme. During relocation it fuses with a second type of paired, bilateral primordial, the ultimobranchial bodies. After fusion of both types of primordia, the gland's cells differentiate into follicles and C-cells (Kusakabe et al., 2005; Fagman et al., 2006). The endoderm-derived midline diverticulum gives rise to follicle cells and the bilateral ultimobranchial bodies to C-cells. C-cells produce the peptide hormone calcitonin-related polypeptide alpha (Calca), also known as calcitonin. Calca is thought to be involved in calcium regulation, however, its detailed role in humans is unclear (Inzerillo et al., 2002).

In non-mammalian vertebrates such as fish, amphibia, and birds, thyroid follicle cells and C-cells are found in separate organs. Thyroid follicle cells form the thyroid gland and do not fuse with the calcitonin producing ultimobranchial bodies that can be found as distinct glands elsewhere in the body (Le Douarin et al., 1974; Le Lievre and Le Douarin, 1975; Noden, 1991; Walker and Liem, 1994).

In zebrafish, the thyroid develops from the same tissue as the thyroid in mammals, the endodermal tissue in the pharynx (Wendl et al. 2002; Alt et al., 2006a), indicating they are homologous structures. Thyroid development is classically subdivided into a few distinguishable steps, primarily based on observations in mammals: first, a group of cells buds off the floor of the primitive

pharynx. Second, these cells reposition dorsocaudally to reach the anterior wall of the trachea. Third, the precursor cells proliferate and, fourth, differentiate into thyroid follicular cells (Macchia, 2000) (Fig.4).

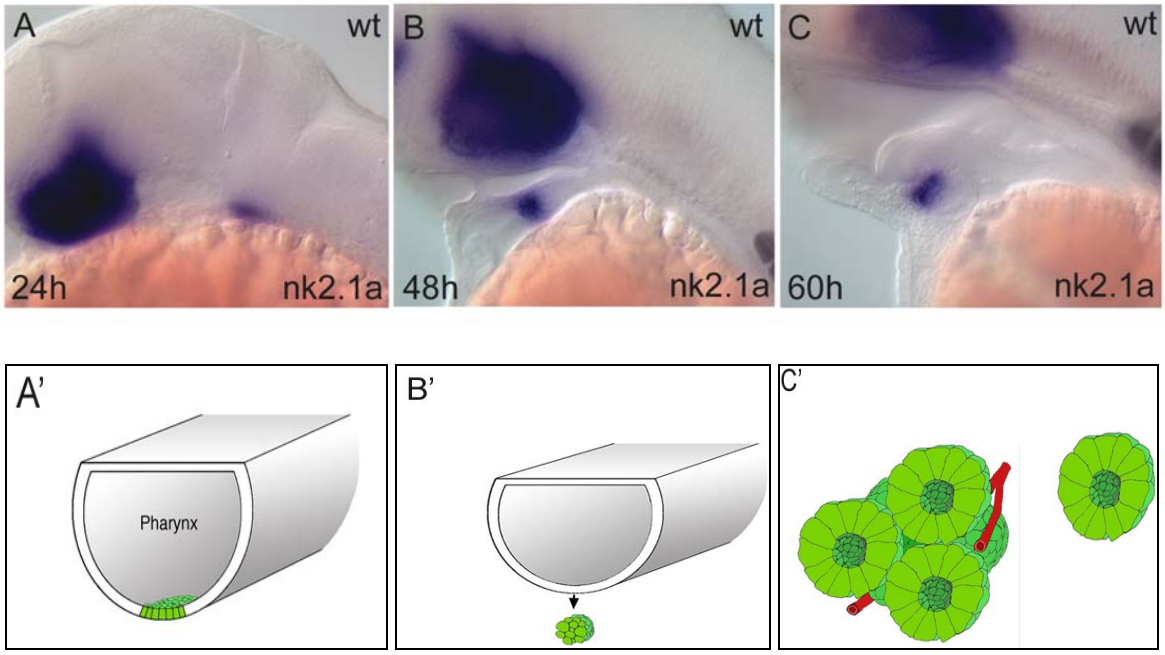

Figure 4: The thyroid follicular cells derive from the endodermal tissue in the pharynx in zebrafish. (A+A') Induction, (B+B') Relocalisation and (C+C') Growth/Differentiation of thyroid primodia. The first three pictures are: lateral views in three different stages (indicated bottom left) of wildtype zebrafish embryos with thyroid marker *nk2.1a* in situ staining.

1.6. *Development of endoderm*

Gastrulating vertebrate embryos generate the three germ layers, known as ectoderm, mesoderm and endoderm, during early development. The induction and differentiation of endodermal cells and the formation of organs derived from the gut tube have been poorly analyzed in comparison to ectoderm or mesoderm. Zebrafish fate map studies began in the 1990s and these fate maps showed that both endoderm and mesoderm originate from a common progenitor (Fig.5). Both germ layers derive from cells near the blastoderm margin and both involute into the forming hypoblast during gastrulation (Warga and Kimmel, 1990). At 40% epiboly, just prior to gastrulation, endoderm progenitor cells are

located in a narrow field of cells along the margin. Furthermore, there is asymmetric distribution of the endoderm progenitor in the margin, and more endoderm progenitor is found dorsally than ventrally (Warga and Nüsslein-Volhard, 1999) (reviewed in Fukuda and Kikuchi, 2005).

Detailed fate mapping studies have shown that at the late blastula stage the position of the endodermal progenitors in the marginal zone resembles a topographic arrangement of the presumptive digestive system. The position a cell occupies along the dorsoventral axis before gastrulation reflects its future location with respect to the anteroposterior (AP) axis: dorsal-most cells will give rise to the most anterior endoderm, the pharynx; lateral cells will give rise to the digestive organs, and ventral cells to the posterior part of the alimentary canal (Warga and Nüsslein-Volhard, 1999).

Analyses of zebrafish mutants as well as overexpression studies have identified key components of the pathway leading to endoderm formation in zebrafish. At the top of this pathway lies Nodal signalling. The first evidence that members of the Nodal family were essential for endoderm formation in zebrafish came from the analysis of mutants deficient for two Nodal-related factors, Cyclops (Cyc) and Squint (Sqt), which lack endoderm and the majority of mesoderm (Feldman et al., 1998; Ober et al., 2003).

Nodal signals are received by EGF-CFC coreceptors and type I and II Activin receptors, which function as serine/threonine kinases. Receptor activation leads to phosphorylation of the transcription factors Smad2 and Smad3. This results in their binding to Smad4, nuclear translocation, and association with additional transcription factors such as FoxH1 and Mixer to regulate target genes. Nodal signalling is antagonized by feedback inhibitors such as Lefty proteins, which are divergent members of the TGFβ family and block EGF-CFC coreceptors, and Dapper2, which enhances the degradation of type I Activin receptors (Schier and Talbot, 2005).

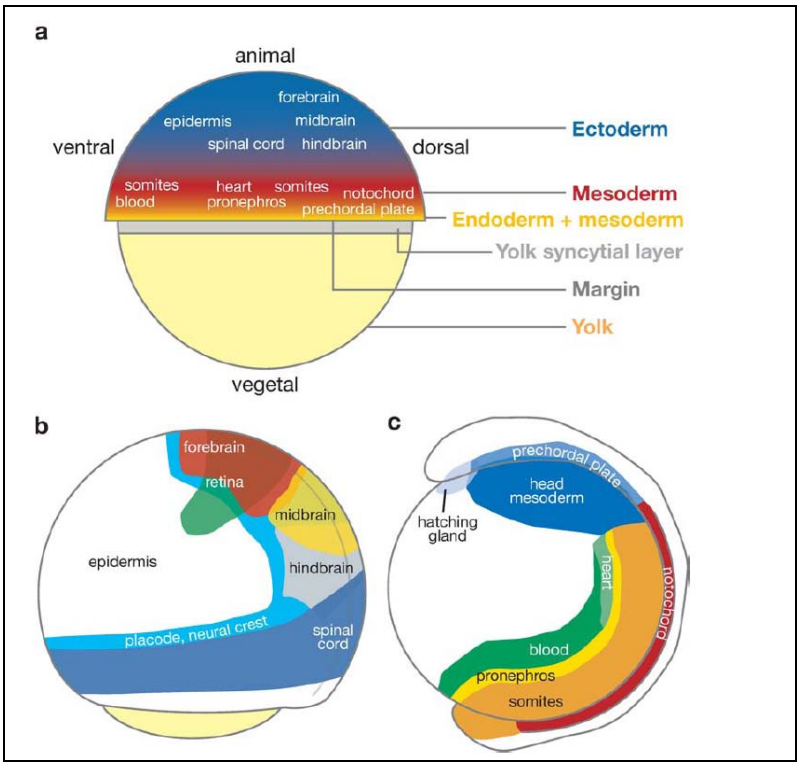

Figure 5: Zebrafish fate maps. (*a*) Fate map at 50% epiboly stage, the onset of gastrulation. Lateral view, dorsal to the right, animal pole to the top. Future germ layers are arranged along the animal-vegetal axis. Different mesodermal and ectodermal fates are arranged along the dorsal-ventral axis. (*b*) Fate map of ectoderm at 90% epiboly. Lateral view, dorsal to the right, animal pole and anterior to the top. (*c*) Model fate map of mesoderm at early somite stage. Lateral view, dorsal to the right, animal pole and anterior to the top. Regions shown here are approximations derived in part from the expression patterns of marker genes (ZFIN.org). The posterior region of the tail bud will continue to extend and give rise to different mesodermal and ectodermal fates. (From: Schier and Talbot, 2005).

Mutant screens in zebrafish have identified several components of the Nodal signalling pathway. These include the Nodal signals Cyclops (Cyc) and Sqt, the EGF-CFC coreceptor One-eyed pinhead, and FoxH1 (*schmalspur (sur)*) and Mixer (*bonnie* and *clyde (bon)*) (See: Schier and Talbot, 2005).

Absence of Nodal signaling in *cyc;sqt* double mutants or maternal-zygotic *one-eyed pinhead* mutants results in embryos that lack all endoderm and mesoderm, with the exception of a few somites in the tail. Mutants also lack trunk spinal cord, but develop forebrain, midbrain, hindbrain, and tail spinal cord (Schier et al., 1996). Conversely, increasing Nodal signaling by loss of Lefty1 and Lefty2 or

overexpression of Cyc or Sqt results in the fate transformation of ectodermal cells into mesoderm or endoderm. Several genes have been identified that are regulated by Nodal signalling and mediate its endoderm-inducing activity, including the Sox gene *casanova*, the GATA gene *faust*, and the homeobox genes *bonnie*, *clyde* and *mezzo* (reviewed in Schier and Talbot, 2005). These four genes encode transcription factors and appear to be direct targets of the Nodal signalling pathway.

1.7. *Development of the vascular system*

The formation of a functional vascular system is essential for the proper development of vertebrate embryos, as well as for the survival of adults. The vascular system provides oxygen, carries away metabolic waste products, serves as the conduit for hormones and provides space for the immune response.

During embryogenesis, the vascular system is constructed by two distinct processes. The first is vasculogenesis, which forms a primary capillary network from hemangioblasts, which are putative precursors specified from mesoderm. The mature vascular structures are formed by a second process, called angiogenesis, which is a remodeling of the endothelial cells from the existing capillary network into mature blood vessels (Yamada et al., 2000). During embryogenesis, blood and endothelial cell development are closely associated with each other. In mammalian embryos, the blood islands on the yolk sac consist of both endothelial and blood cells. Both cell types are thought to emerge from a common precursor, the hemangioblast (Fig.6) (Choi et al., 1998).

The zebrafish has recently emerged as an advantageous model organism to study how the network of vertebrate blood vessels arises during development. In zebrafish embryos, both endothelial and blood cell precursors originate in the lateral plate mesoderm (Fig.7) and during the middle and late somitogenesis stages reside in the region known as the intermediate cell mass (ICM) (Fig.7).

During vasculogenesis, the endothelial cell progenitors (angioblasts) give rise to the major blood vessels of the trunk, the dorsal aorta, and axial vein, as well as the endocardium of the heart (Fig.6).

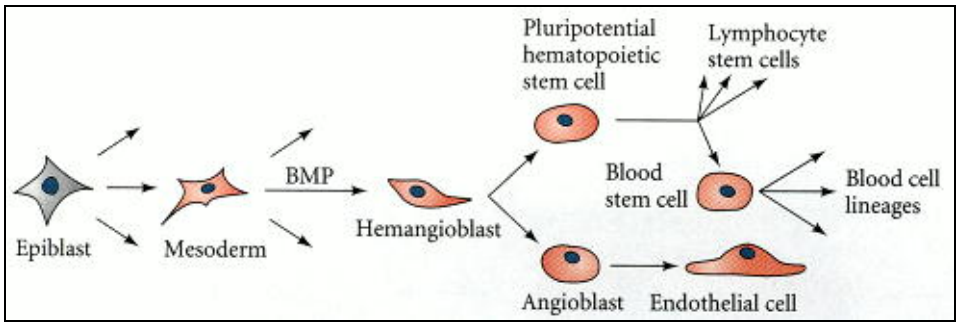

Figure 6: Blood and endothelial cell development. During vasculogenesis, the endothelial cell progenitors (angioblasts) give rise to the major blood vessels of the trunk, the dorsal aorta, and axial vein, as well as the endocardium of the heart. (From Gilbert, 2000).

During subsequent angiogenesis, the axial vessels sprout to form secondary vessels in the trunk region of a zebrafish embryo (for a review, see Sumanas et al., 2005). The molecular mechanisms of development of the vascular system in vertebrate embryos remain relatively unexplored. Several signaling molecules and transcription factor genes have been implicated in the development of the vertebrate vasculature: *vegf*, *vegfr1*, *vegfr2*, *vegfr3*, *tie1*, *tie2*, *angiopoietin1*, *angiopoietin2*, *ephrinB2*, *ephB4*, *scl*, *fli1*, *ets1*, *runx*, semaphorins and plexins have been analyzed in zebrafish, amphibians, birds and mammals, and found to display similar temporal and spatial expression patterns (for a review, see Jin et al., 2005).

During the last decade, the molecular mechanisms of blood and blood vessel formation started to be elucidated. The transcription factor Scl/tal1 functions at the level of the hemangioblast, affecting both hematopoietic and vascular development in both mouse and zebrafish (Liao et al.,1998; Liao et al., 2000). Transcription factors including Gata2, Gata1, C-myb, and transcription factor–interacting proteins such as Lmo2 gradually restrict the subpopulation of blood-

cell precursors to adopt the erythroid fate (for a review, see Sumanas et al., 2005).

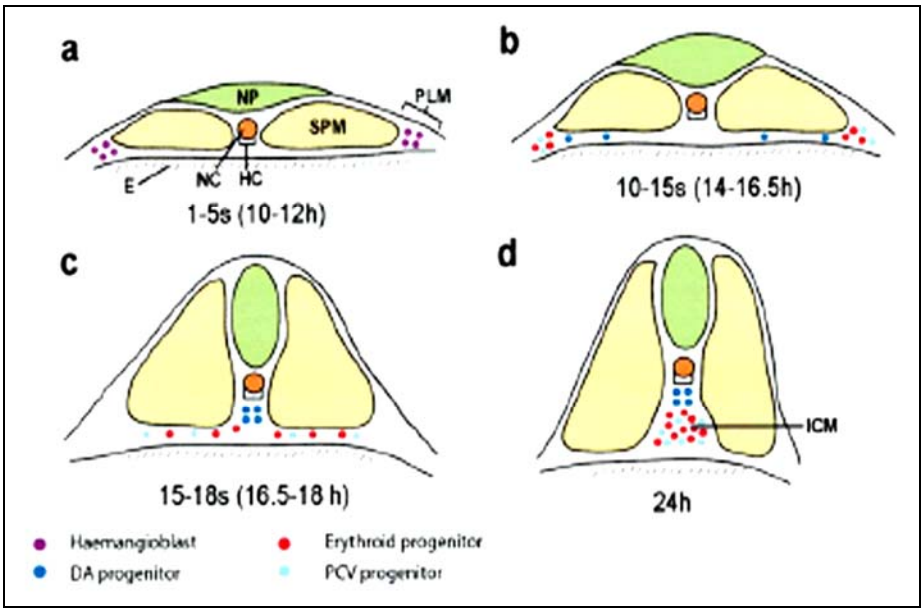

Figure 7: Schematic diagram of early blood and endothelial development in the trunk of zebrafish embryos. Both endothelial and blood cell precursors originate in the lateral plate mesoderm and during the middle and late somitogenesis stages reside in the region known as the intermediate cell mass (ICM). E, endoderm; HC, hypochord; ICM, intermediate cell mass; NC, notochord; NP, neural plate; PCV, posterior cardinal vein; PLM, posterior lateral mesoderm; SPM, somitic paraxial mesoderm; DA, dorsal aorta. (From: Gering and Patient, 2005).

Vascular endothelial growth factor (Vegf) is required for vascular endothelial development in both mouse and zebrafish and functions through tyrosine kinase receptors Vegfr1/Flt1 and Vegfr2/Kdr (Carmeliet et al., 1996). Angiopoietin receptor tyrosine kinase Tie2 is important in angiogenesis, while Tie1 is critical for endothelial cell integrity and survival (Puri et al., 1995). Zebrafish homologs of tie1 and tie2 are also expressed in vasculature (Lyons et al., 1998). Vegf regulates the migration and survival of endothelial cells. Mice lacking a functional Vegf signal show various developmental defects, including a reduced number of endothelial cells and a failure to form a functional vasculature (Carmeliet et al., 1996). Similar phenotypes have been reported in *Xenopus* and chick embryos with compromised Vegf signaling (Cleaver and Krieg, 1998). In zebrafish, Vegf

appears to be crucial for angioblast formation as well as for the subsequent differentiation into arterial endothelial cells (Lawson et al., 2002).

Another example of functional conservation in vascular development is Hh. In zebrafish embryos, Shh in the notochord appears to regulate *vegf* expression in the somites, which in turn regulates vascular development (Lawson et al., 2002). In chick and mice, Hh signaling also appears to be important in vascular development (Vokes et al., 2004). However, complete signal transduction pathways involved in hematopoiesis, vasculogenesis, and angiogenesis are still unknown.

1.8. *Hedgehog (Hh)*

During animal development, gradients of signaling molecules play an important role in cell type specification. Members of the Hedgehog (Hh) family of intercellular signaling molecules control a variety of developmental processes, ranging from segment patterning in *Drosophila* to organogenesis, left-right asymmetry and dorsoventral patterning of the spinal cord and forebrain in vertebrates (reviewed by Ingham and McMahon, 2001). Aberrant regulation of Hh signaling in humans causes developmental defects such as holoprosencephaly (HPE) and postaxial polydactyly, and can also lead to various types of cancers, including basal cell carcinoma and medulloblastoma (for a review, see Sekimizu et al., 2004).

Because Hh signaling plays such a central role in development and disease, the Hh signaling pathway has been investigated in considerable detail (Fig.8). Genetic and in vitro studies in *Drosophila* have revealed that Hh signals are transduced by binding of Hh ligands to the Patched (Ptc) cell-surface receptor, resulting in the activation of the transmembrane protein Smoothened (Smo). In *Drosophila*, the intracellular regulation of Hh signaling is mediated by post-translational modifications of Cubitus interruptus (Ci), a zinc-finger-containing

transcription factor of the Gli family that can be both an activator and a repressor of Hh target genes. In the absence of Hh signal, proteolytic cleavage converts Ci to a transcriptional repressor. In the presence of Hh signals, cleavage of Ci is inhibited and a full-length activator isoform predominates. In vertebrates, at least three Gli genes, *Gli1*, *Gli2* and *Gli3*, mediate the transcriptional response to Hh signals. The functions of these different Gli genes have been analyzed in mouse, *Xenopus*, zebrafish and cultured cells (reviewed by Ingham and McMahon. 2001).Thus, in both vertebrates and invertebrates, Hh signaling controls the expression of target genes by modulating the activity of the downstream Gli/Ci transcription factors. Studies in *Drosophila* have identified a large number of proteins that are involved in the regulation of this Gli/Ci activity (for a review, see: Sekimizu et al., 2004).

Genetic studies of Hh signaling in zebrafish complement the analyses in fly and other vertebrate species, and provide an approach to look into the regulation of Hh signaling in vertebrates. In vertebrates, Sonic hedgehog (Shh) is expressed in the notochord and floor plate of the neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ekker et al., 1995), and is essential for the induction of floor plate, motoneurons and a class of ventral interneurons in the neural tube (Chiang et al., 1996; Ericson et al., 1996). Shh signaling is also required for the induction of muscle and sclerotome cell types in somites (reviewed by Bumcrot and McMahon, 1995). A large number of zebrafish mutations, collectively called the midline mutants, have been identified that lead to ventral neural tube defects, absence of an optic chiasm and defects in slow muscle fiber formation (Brand et al., 1996; Chen et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996). Many of these midline mutants have now been shown to encode components of the Hh signal cascade.

shh is disrupted in *sonic-you* (*syu*) mutants (Schauerte et al., 1998), *smo* is disrupted in *slow muscleomitted* (*smu*) (Chen et al., 2001; Varga et al., 2001), *gli2* is disrupted in *you-too* (*yot*) (Karlstrom et al., 1999), *gli1* is disrupted in

detour (*dtr*) (Karlstrom et al., 2003) and *dispatched1* is disrupted in *chameleon* (*con*) (Nakano et al., 2004).

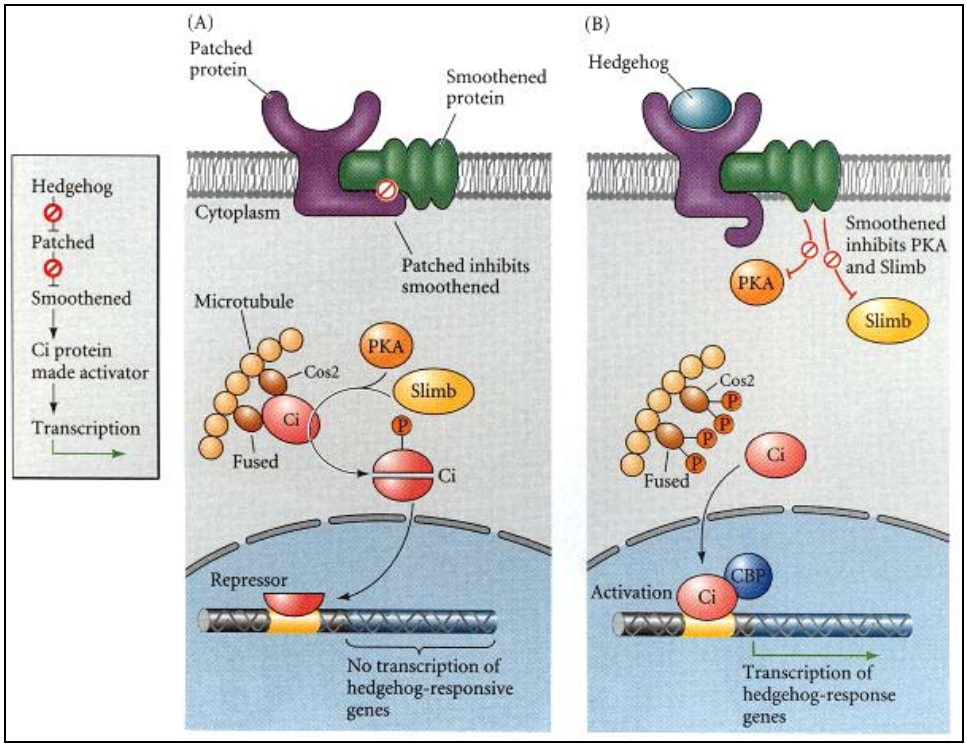

Figure 8: The Hedgehog signal transduction pathway. The Patched protein in the cell membrane is an inhibitor of the Smoothened protein. (A) In the absence of Hedgehog binding to Patched, the Ci protein is tethered to the microtubules (by the Cos2 and Fused proteins). This allows the PKA and Slimb proteins to cleave Ci into a transcriptional repressor that blocks the transcription of particular genes. (B) When Hedgehog binds to Patched, its conformation changes, releasing the inhibition of the Smoothened protein. Smoothened releases Ci from the microtubules (probably by adding more phosphates to the Cos2 and Fused proteins) and inactivates the cleavage proteins PKA and Slimb. The Ci protein enters the nucleus, binds a CBP protein and acts as a transcriptional activator of particular genes.(From Gilbert, 2000).

One remarkable feature of the Hedgehog signal transduction pathway is the importance of cholesterol. Cholesterol is critical for the catalytic cleavage of sonic hedgehog protein, only the amino-terminal portion of the protein is functional and secreted, and the Patched protein that binds the Sonic hedgehog protein also needs cholesterol in order to function.

Environmental factors that cause developmental anomalies are called teratogens (from the Greek, meaning "monster-former"), two teratogens known to cause

cyclopia in vertebrates are jervine and cyclopamine. Both substances are found in the plant *Veratrum californicum*. Plants of the genus *Veratrum* have a long history of use in the folk remedies of many cultures, and the jervine family of alkaloids, which constitute a majority of *Veratrum* secondary metabolites, have been used for the treatment of hypertension and cardiac disease (for a review, see Chen et al., 2002). The association of *Veratrum californicum* with an epidemic of sheep congenital deformities during the 1950s (Binns et al., 1962) raised the possibility that jervine alkaloids are also potent teratogens. Jervine and cyclopamine (11- deoxojervine) given during gestation can directly induce cephalic defects in lambs, including cyclopia in the most severe cases (Keeler and Binns 1965). Cyclopamine inhibits Hh pathway activation by binding directly to Smo (Chen et al., 2002). This binding interaction is localized to the heptahelical bundle and likely influences the Smo protein conformation. Cyclopamine binding is also sensitive to Ptch function, providing biochemical evidence for an effect of Ptch action on Smo structure (Chen et al., 2002).

1.9. *Aim of the work*

Normal thyroid function is essential for development, growth, and metabolic homeostasis. Defects in any step of thyroid development (such as specification, proliferation, migration, growth, organization, differentiation, and survival) may result in a congenital anomaly and/or impaired hormonogenesis, leading to variable degrees of hypothyroidism (Trueba et al. 2005).

Congenital hypothyroidism (CH) affects one in about 4000 newborns in western countries, and thyroid dysgenesis (TD) accounts for about 85% of the cases; the other 10–15% result from functional disorders in hormone synthesis. TD includes absence of thyroid tissue (athyreosis), presence of ectopic tissue, as well as hypoplasia of an orthotopic gland. Ectopic thyroid can be the cause of CH, followed by athyreosis. In this study, we introduce zebrafish as a model to investigate the molecular and genetic mechanisms that control thyroid development.

2. Materials and methods

2.1. *Used chemicals and materials*

Inorganic and organic chemicals were usually purchased in the highest quality available from Biomol (Hamburg), Biozym (Hameln), Fluka (Neu-Ulm), Life Technologies (Karlsruhe), Merck-Eurolab (Darmstadt), Pharmacia (Freiburg), Roche Diagnostics GmbH (Mannheim), Roth (Karlsruhe), Serva (Heidelberg), and Sigma (Taufkirchen).

Zebrafish –Ringer (Stock-solutions)

Stock 1 (25x): 169, 5 g/L Sodium Chloride NaCl **+** 5, 4 g/L Potassium Chloride KCl **+** 29, 75 g/L HEPES $C_8H_{18}N_2O_4S$

Stock 2 (100x): 26, 4 g/L Calcium Chloride $CaCl_2$ **+** 34, 96 g/L Calcium Chloride Dihydrat $CaCl_2$**.**2 H_2O **+** Autoclaving

1/3 ZFR: 12 ml Stock 1 **+** 9 ml Stock 2 **+** 879 ml D.H_2O. Adjust the PH to 7,2 by using 1N Sodium hydroxide NaOH.

Tricaine

3 -Aminobenzoic acid ethyl ester $C_9H_{11}O_2$**.**CH_4SO_3 (Sigma).
800 mg tricaine powder +200 ml D.H2O.

Bouin's solution

75ml Picric acid + 25ml Formalin 40% + 5ml acetic acid glacial

4% Paraformaldehyde (PFA)

- 40g PFA powder (Sigma) dissolved in 1L PBS. Warming up slowly in fume hood. Don't boil.
- Aliquot and Store at -20°C.

10xPBS (phosphate buffered saline)

- 80 g Sodium Chloride NaCl.
- 2 g Potassium Chloride KCl.
- 14.4 g Sodium Phosphate Na2HPO4.
- 2.4 g Potassium Phosphate KH2PO4.
- ~900 ml D.H2O
- PH ~7.2 – 7.6
- adjust to 1 L
- autoclaving

1xPBT (phosphate buffered saline containing tween)

1L 1xPBS **+** 5ml 20%Tween 20 **=** 1xPBT

Hyb -

50% Formamid CH_3NO (ROTH) **+** 5x SSC **+** 0,1% Tween 20 (Sigma) **or** Triton X-100 ($C_{38}H_{70}O_{13}$).
Set pH to 6 with 92 µl/10ml of 1M citric acid and Store at -20°C.

Hyb +

Hyb- **+** 5mg/ml torula yeast RNA (Sigma) **+** 50µg/ml Heparin (Roth)
Store at -20°C.

20XSSC

- 175.3 g Sodium Chloride NaCl.
- 88.2 g Sodium Citrate $C_6H_5Na_3O_7.2H_2O$.
- 800 ml $D.H_2O$
- adjust the pH to 7.0 with few drops of a 14N solution of HCl
- adjust to 1 L with $D.H_2O$

1 ml 20XSSC + 9 ml $D.H_2O$ = 2XSSC
1 ml 20XSSC + 99 ml $D.H_2O$ = 0.2XSSC
1 ml 2XSSC + 9 ml $D.H_2O$ = 0.2XSSC

Staining buffer (X-pho buffer)

0,1M Tris $C_4H_{11}NO_3$ pH 9,5 (ROTH) **+** 50mM Magnesium Chloride MgCl2 (ROTH) **+** 0,1% Tween **or** Triton X -100 ($C_{38}H_{70}O_{13}$) **+** 0,1M Sodium Chloride NaCl.

Glycerol

$C_3H_8O_3$ (ROTH)
50%, 70%, 90% in PBS diluted

2.2. *Methods*

Animals

Zebrafish work was carried out according to standard procedures (Westerfield, 2000), and staging in hours post fertilisation (hpf) or days post fertilisation (dpf) refers to development at 28.5-29°C. Embryos or larvae were dechorionated manually and anaesthetized in tricaine before fixation. Some zebrafish embryos used for insitu hybridisation were treated with PTU to prevent pigmentation at young stages. As PTU belongs to a class of chemicals that have the potential to interfere with the function of the thyroid (Elsalini and Rohr, 2003), we did not treat embryos used for immunohistochemistry with PTU and confirmed all in-situ hybridization experiments with non-treated embryos.

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), *smu*^{b641} (Varga.et al, 2001), *syu*^{t4} (Schauerte et al 1998), and homozygous *cloche* mutant *clo*^{s5} (Stainier et al., 1995). *kdr*^{y17} (Covassin et al., 2006) is allelic to the *kdr* (*kdr*) lines described previously (Habeck et al., 2002).

Microinjection

Synthetic, capped mRNA prepared according to standard procedures (see below) was injected into fertilized zebrafish eggs prior to first cleavage. Injections were carried out under sight control using a dissecting microscope and with the aid of a micromanipulator. I did not dechorionate the fertilized eggs prior to injection. Chorions were removed about 24 hours later, at which time zebrafish embryos are well developed and crucial steps in embryogenesis have been completed. Micropipettes were made on a horizontal puller using 1mm Hilgenberg glass capillary tubing with an inner filament for backfilling the pipettes with DNA solution. Injection solution contained 0.5% phenol red to allow estimation of the injected volume.

Embryo manipulation

The synthetic mRNA was produced by using the message machine kit (Ambion), and was injected into one-cell-stage embryos. As a control, I injected synthetic *gfp* mRNA at similar concentrations. Numbers of mRNA injected embryos were about 100 for each insitu hybridisation marker, and the 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'

vegf: 5'-taagaaagcgaagctgctgggtatg-3'

scl: 5'-aatgctcttaccatcgttgatttca-3'

tnnt2: 5′-catgtttgctctgatctgacacgca-3′

Unspecific control, fluorescein coupled: 5'-cctcttacctcagttacaatttata-3'

Preparation of specimens

Fixation of fish embryos was done overnight in 4% paraformaldehyde in PBS at 4°C for whole-mount in situ hybridization and histochemistry, or in Bouin's solution at room temperature for histology and histochemistry. Paraformaldehyde fixed embryos were then washed in PBT and stored in methanol at –20°C. Bouin fixed tissue was transferred to 70% ethanol and stored at room temperature.

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. Antibody staining was subsequently performed as described below.

2.3. *Molecular biology techniques*

Standard molecular biology techniques were carried out according to Sambrook, et al 2001 for DNA preparation; Plasmids were transformed into the *E.coli* k12 strain and cultured in 100ml LB medium containing appropriate antibiotics. The DNA was purified using Qiagen Midi prep kits (Qiagen).

Restriction enzyme digests were carried out in a volume ~50µl using an appropriate 10x enzyme buffer and 20 units of enzyme per 5-10µg DNA (table 1). Enzyme digests were carried out at 37°C and checked on 1% agarose gel by electrophoresis. Purification of DNA was carried out by 1x phenol:chloroform extraction, 1x chloroform extraction followed by precipitation. DNA was precipitated with 0.1 volume 3M Na acetate and 0.7 volume isopropanol, centrifuged for 20 minutes and the DNA pellet washed in 70% ethanol, air dried and taken up in RNAse free H_2O.

Plasmid name	Stock number	Polymerase		Restriction enzyme	
		In situ probe	RNA injection	Anti sense	sense
Fkd7	K13	T7		Bam H1	
gfp	K20		Sp6		Xba1
Hex	K21	Sp6	Apa1	Nco1	Sp6
Kdr	K17	T7		ECO R1	
Nk2.1a	K27	T3		Sac II	
Nk2.1b	K28		T7		BamH1
Pax2.1	K33	T7		Bam H1	
Scl	K122		T3		Eco R1
Shh	K39		Sp6		Bam H1
Tie1	K61	T7		Spe I	
Vegf 121 &165	K50 & K51	T7	Sp6	Bam H1	Not1

Table 1: The plasmid and the enzymes used to prepare the in situ probe or mRNA injections.

0.5-1µg of the linear DNA was used in the in vitro transcription reaction for synthesis for antisense RNA labeled with digoxigenin or for mRNA for injection. For generation of capped mRNA for injections see above. For synthesis of in situ probe a 20µl reaction was set up in 2µl 10x transcription buffer, 2µl DiG-labelling mix; 0.5µl RNasin, 1µl RNA polymerase (table 1) and RNAse free H_2O. Transcription mix was incubated at 37°C for 2-4hours. The RNA was washed in D.H_2O by using Millipore filter tubes.

2.4. *Histological methods*

Fixation in Bouin's solution, embedding in paraffin, sectioning,T4 immunostaining on sections, and PAS staining were carried out as described previously (Wendl et al. 2002). Paraffin embedding was carried out according to standard procedures (see below), and 8 µm sections were cut. For histology, sections were dewaxed by washing 2x in rotihistol 5min each and 3x in 100% ethanol 5min each, than dried and stained.

Sections of adult and larval zebrafish were stained with Giemsa, Haematoxylin and Eosin, and PAS staining in order to visualise the thyroid follicles. Giemsa stain involved 5 minutes incubation in Giemsa solution as used for blood stain, followed by two washes in tap water. Haematoxylin and Eosin staining was carried out according to Ehrlich (Fluka, catalogue number 03972): 5 minutes incubation, followed by 10 minutes washing in running tap water. All stained slides were dehydrated and mounted in Entellan neu (Merck).

Paraffin embedding

- After fixation in Bouin's solution, replace Bouin's by 70% ethanol.
- Wash 4X in 100% ethanol for 2h.
- In glass vial, replace by a mixture of 50% ethanol and 50% Rotihistol for 1h.

- Replace by 100% Rotihistol.
- Incubate in 65°C.
- Add 50% paraffin.
- Replace paraffin several times during a day.
- Make paraffin block containing the embryos.
- Sectioning by using microtome, 8 mm sections cut.
- Receive the sections on slides.
- And incubate the slides on warm plate.

PAS staining

Periodic acid/Schiff (PAS) staining was carried out according to the manufacturer's instructions (Merck, catalogue number 1.01646). Staining on sections was performed as described previously (Wendl et al., 2002).

- After Dewaxing and drying
- Wash in D.H_2O for 2min
- Incubate in periodic acid (Merck) for 5min
- Wash in tap H_2O for 3min and 1min in D.H_2O
- Incubate in Schiff's reagent (Merck) for 15min
- Wash in tap H_2O for 3min and 1min in D.H_2O
- Dehydration: Incubate the stained sections in 30%, 50%, 70%, and 100% ethanol for 5min each.
- Incubate 2x in 100% xylol for 5min each.

Whole Mount T4 Antibody Staining

The staining procedure followed general protocols using the Vectastain elite ABC kit (Westerfield, 2000). Whole-mount immunohistochemistry with antibodies against the thyroid hormone T4 (polyclonal rabbit anti T4, ICN Biochemicals; Wendl et al., 2002) or thyroglobulin (polyclonal rabbit anti human thyroglobulin, Dako) in zebrafish larvae was performed as described elsewhere. 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, and then incubated in DAB (0.2 mg/ml PBS) for 30 min. To stain, 1µl 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.

In Situ Hybridization

Whole-mount in situ hybridisation for zebrafish embryos was carried out according to standard procedures (Westerfield, 2000). The experimental embryos grow in PTU to prevent pigmentation and dechorinated by using the Pronase.

Embryos were fixed in 4% paraformaldehyde/ PBS (PFA) overnight and stored in 100% methanol at -20°C. Specimens were washed twice with PBST and then digested with 5 mg/ml proteinase-K in PBST for several minutes depending on the stage (table 2). They were washed and fixed again in 4% PFA for 20 min. After the proteinase-K was washed off, embryos were transferred into HYB+ solution and prehybridized for 2-4h at 60°C. Approximately 5ng/ml DIG-labeled RNA probe was added and hybridized overnight at 60°C. Embryos were washed at 60°C in Hyb-*/*2XSSC (75:25) for 15 min; in Hyb-*/*2XSSC (50:50) for 15 min and in Hyb-*/*2XSSC (25:75) for 15 min. For detection, embryos were blocked at least for 2 h at room temperature in blocking reagent (Roche). Embryos were incubated for 2h in antibody Anti-Digoxigenin-AP (Roche) diluted 1:6000 in blocking reagent, then wash in PBT overnight.

The following day specimens were washed in staining buffer (X-pho buffer) (4ml 1M tris pH 9.5, 2ml 1M MgCl2, 800µl 5 M NaCl, 200µl 20% Tween 20, and fill up to 40ml with D.H_2O). Embryos were then incubated in BM purple AP substrate, precipitating (Roche).To stop the color reaction, PBST was added, followed by fixation in 4% PFA. For detailed analysis, larvae were washed in PBT, and gradually transferred to 90% glycerol.

Proteinase K concentration

stage in hpf	*Concentration in PBT*	*time in min*
24	**1:1000**	**6**
26	**1:1000**	**8**
28	**1:1000**	**10**
30	**1:1000**	**30**
32-33	**1:500**	**25**
35	**1:250**	**10**
40	**1:250**	**15**
45	**1:250**	**30**
48	**1:250**	**35**
55	**1:250**	**45**
60	**1:100**	**30**
72-75	**1:100**	**60**

Table 2: The concentration of Proteinase K and the time of incubation for different zebrafish stages.

Double staining (insitu + Tg antibody staining)

- Stop the in situ in PBT.
- Wash 5x in PBT
- Bleach : (o.5 ml 30%H_2O_2 + 1ml PBS) for15 min in dark.
- Rinse 2x in PBT.
- Postfix in PFA for 15 min at R.T.
- Wash 5x in PBT for 5min each.
- Incubate in 3% NGS for 2h.
- Incubate in TG 1:6000 in 3% NGS for 2h.

- Wash several times in PBT for 3h or overnight.
- Incubate in secondary antibody (LINARIS) for 2h.
- Wash several times in PBT for 3h or overnight.
- Incubate in AB kit for 2h (1ml PBT + 20µl A + 20µl B).
- Wash several times in PBT for 3h.
- Incubate in DAB 1:5 in PBS for 30min.
- Add 1µl 0.3%H_2O_2 (1µl 30% H_2O_2 + 99µl D.H_2O) and observe while staining

Embedding in Durcupan

- Wash the stained embryos several times in PBT.
- Wash in 50%, 70% and 90% ethanol 10min each.
- Wash 2x in 100% ethanol for 10min each.
- Wash 2x in 100% Aceton 15min each.
- Incubate in Acetone: Durcupan 1:1 overnight at R.T.
- Embedding in Durcupan (Fluka).
- Incubate overnight at 65°C.
- Sectioning by Microtome.

2.5. *Cyclopamine treatments*

Cyclopamine (Toronto Research Chemicals, North York, Ontario, Canada) treatment was carried out as described in Lawson et al, 2002. Cyclopamine was dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM. Embryos were dechorinated with pronase at 50% epiboly and placed in 6 ml of embryo medium containing 50 µM of cyclopamine. Surviving embryos were fixed and processed for insitu hybridization.

3. Results

3.1. Thyroid differentiation:

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

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). 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. So I analysed the thyroid formation in mutants that show different degrees of compromised endoderm specification.

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. *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 compromised (Fig.9 E and F) and the expression of *nk2.1a* indicates that the thyroid primordium is smaller in $b16^{-/-}$ and $m294^{-/-}$ compared with wild-type (Fig.9B and C).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.

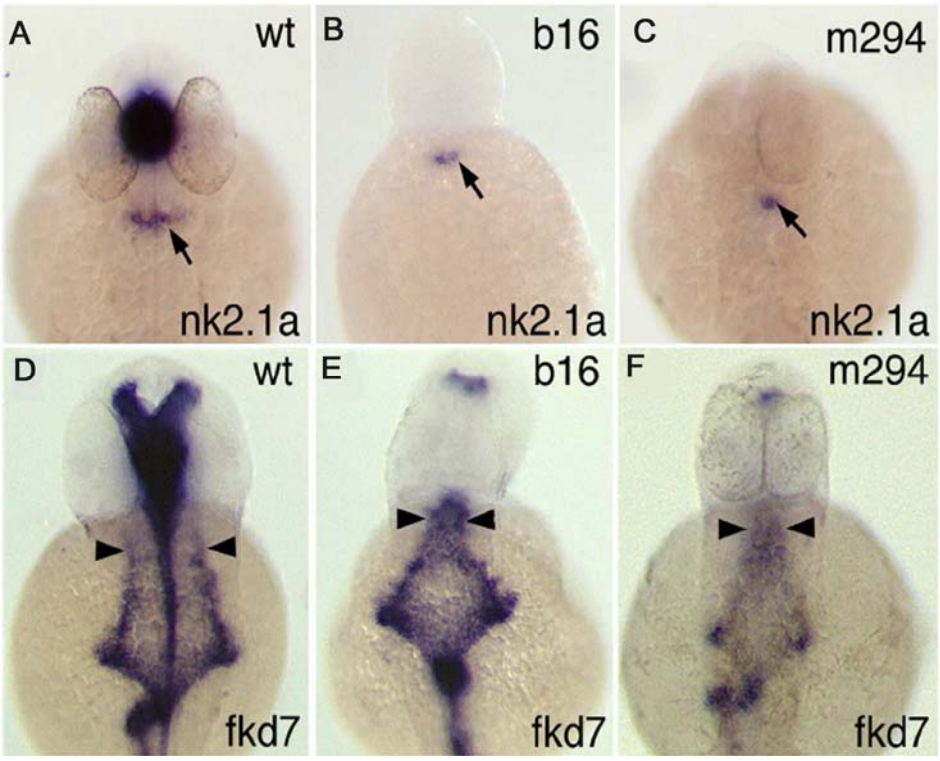

Figure 9: Reduced endoderm coincides with a smaller thyroid primordium in *cyc-/-*. Expression patterns of *nk2.1a* in thyroid precursor cells and *fkd7* in the endoderm (gene expression as indicated bottom right) at 26 hpf in wildtype or mutant embryos (indicated top right). The arrows point to the thyroid primordium in (A-C). Arrowheads indicate the expression of fkd7 in the pharyngeal endoderm on the level of the first to the second branchial arch.

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). I find that the thyroid primordium is completely absent in *bon*$^{-/-}$ mutant. Thus, thyroid development is not only dependent on Nodal signalling, but also on subsequent steps of endoderm specification.

Using an antibody that labels thyroid hormone (T4; Raine et al., 2001; Wendl et al., 2002), I fail to detect functional thyroid follicles at 3–5 dpf in *b16*$^{-/-}$ wholemount embryos (Fig.10B). In contrast, in *m294*$^{-/-}$ embryos, a row of thyroid follicles forms in the midline of the ventral pharynx as in wild-type siblings

(Fig.10C). I 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.10D) and *m294*$^{-/-}$ (Fig.10F) larvae but not *b16*$^{-/-}$ larvae (Fig.10E). In zebrafish larvae treated with drugs that interrupt thyroid hormone synthesis 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*$^{-/-}$.

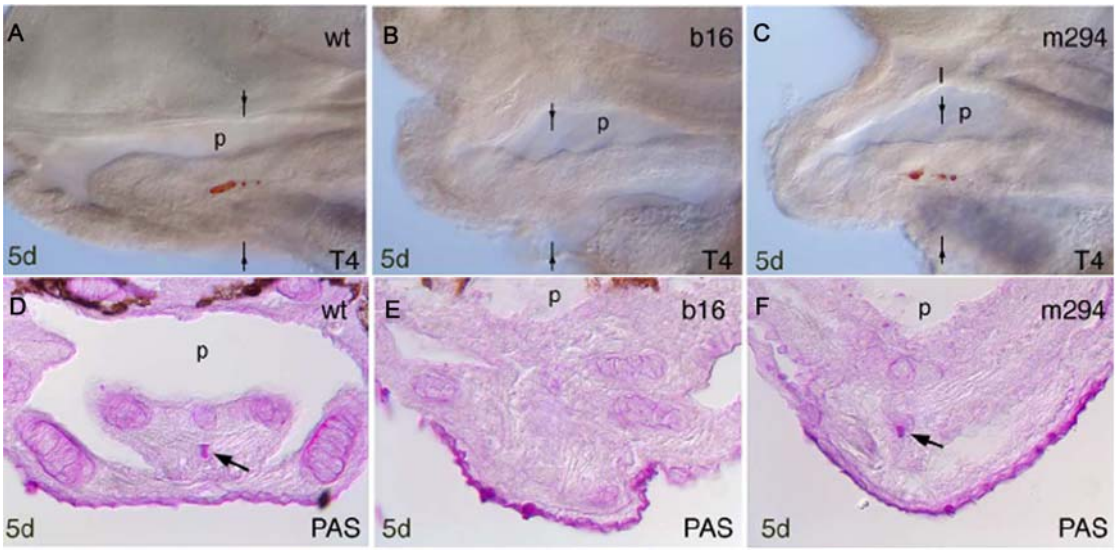

Figure 10: Thyroid follicles are absent in *b16-/-* larvae but present in *cyc m294-/-* larvae. Whole-mount embryos (A–C) or paraffin sections (D–F). Genotype indicated at top right. Antibody staining (T4, brown staining), PAS staining (PAS), Arrows point to thyroid follicles (D, F), Arrow bars in (A–C) indicate level of sectioning as in (D–F).

Both *nk2.1a* and *pax2.1* are expressed from 24 hpf in the thyroid primordium of developing zebrafish, and it has demonstrated previously that *pax2.1* is required for thyroid follicle development (Wendl et al., 2002). Both genes are initially expressed in *b16*$^{-/-}$ embryos (Fig. 11A–F) and so a thyroid primordium initially forms. However at around 60 hpf, both markers disappear in the thyroid primordium of *b16*$^{-/-}$, but not in *m294*$^{-/-}$ (Fig. 11G–L). Altogether, these results show that, although a primordium is initially present, a functional thyroid gland fails to form in *b16*$^{-/-}$ larvae.

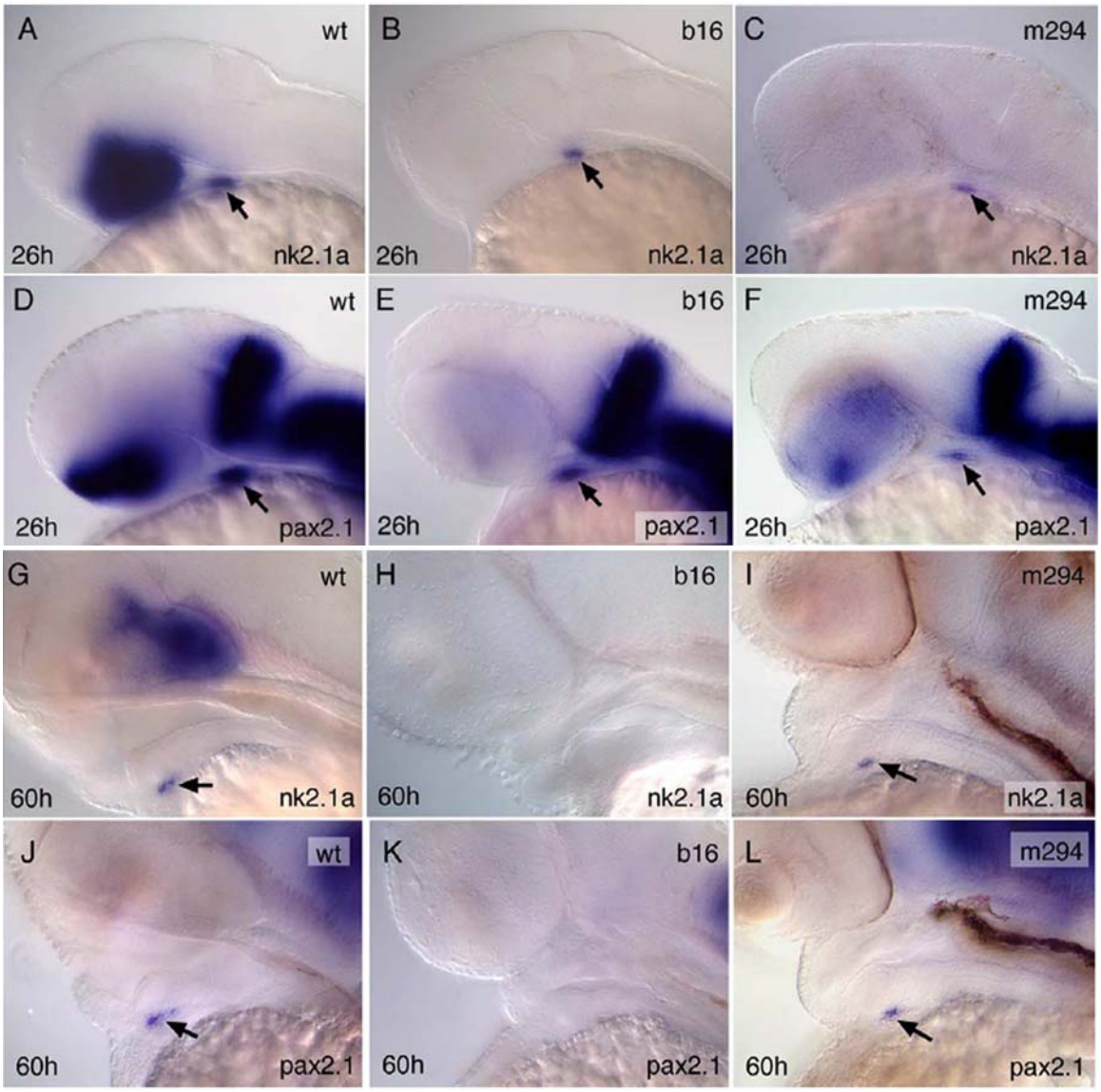

Figure 11: A thyroid primordium is induced in both *b16-/-* and *m294-/-* embryos. Expression patterns of thyroid markers (indicated at bottom right) at different stages of development (indicated bottom left) in mutant embryos (indicated at top right). Arrows point to the thyroid primordium.

3.1.2. *hhex is required for differentiation of thyroid follicles but not for formation or migration of a thyroid primordium*

There are four transcription factors that have been shown to play crucial roles in the differentiation of thyroid follicular cells 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, (Fig. 12), *nk2.1a, hhex,* and *pax8* are also expressed in the developing thyroid (Rohr and Concha, 2000; Wendl et al., 2002). The *hhex* locus maps close to the *cyc* locus in zebrafish and mapping studies have revealed that both loci are deleted in *b16*-/- mutant embryos (Liao et al., 2000). Accordingly, *hhex* mRNA is missing in *b16*$^{-/-}$ embryos (Fig. 12). In *m294*$^{-/-}$ embryos that have a point mutation eliminating exclusively *Cyclops* gene function, *hhex* is expressed (Fig. 12), demonstrating that it is not Cyc activity that is required for *hhex* expression. So 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*$^{-/-}$. I therefore used *hhex* morpholino antisense RNA to test whether *hhex* itself is required for thyroid development.

hhex morpholino oligonucleotides in zebrafish disrupt hepatocyte development in the liver (Wallace et al., 2001), resembling the phenotype of *Hhex*$^{-/-}$ mouse embryos (Martinez Barbera et al., 2000). *hhex* morphants develop heart edema (Fig. 13), but about 50% of injected embryos survive to at least 6 dpf. In contrast to control injected embryos, *hhex* morphants lack T4 immunostaining (Fig. 13A and B), and so with regard to the thyroid follicles, the late phenotype of *hhex* morphants resembles the *b16*$^{-/-}$ phenotype. Injection of lower concentrations of *hhex* morpholino results in a higher percentage of larvae that show some follicle differentiation at 5 dpf (Fig. 13C). This result suggests that reduced *hhex* function results in reduced differentiation or 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. 13D, Table 3). Coinjection of 150 pg mutated *hhex* mRNA results in even higher numbers of

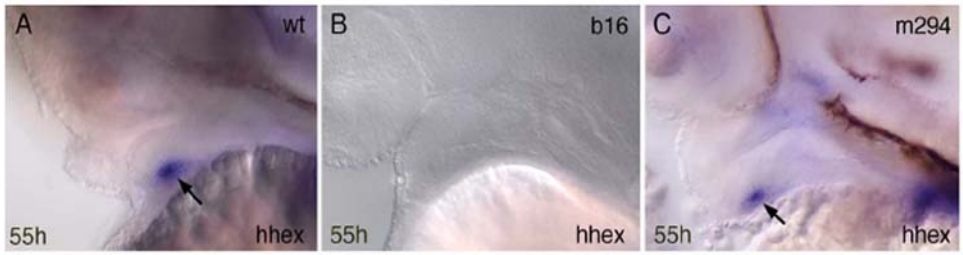

Figure 12: Thyroid follicles are absent in *b16-/-* embryos but present in *cyc m294-/-* embryos. Arrows point to the thyroid primoridum (A, C).

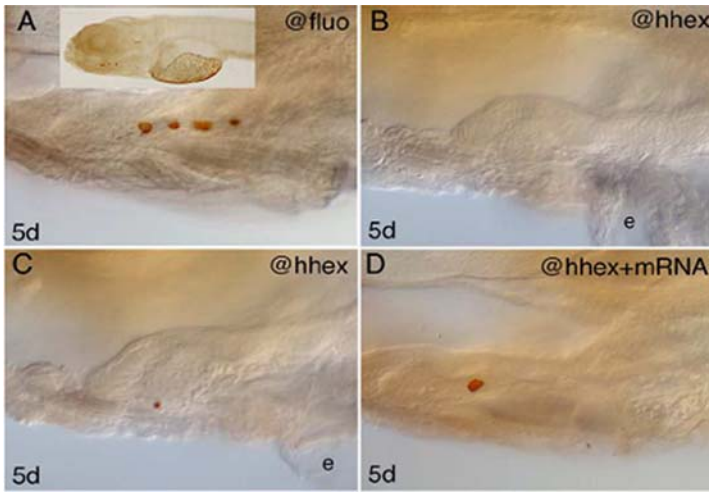

Figure 13: Abrogation of *hhex* function causes absence of thyroid follicles. (A–D) T4 immunostaining in different hhex morpholino-injected (A–C) and morpholino plus *hhex* mRNA coinjected (D) embryos. e,odema; @fluo,control morpholino fluorescein labeled; @hhex, *hhex* morpholino alone; @hhex+mRNA, *hhex* morpholino plus mutated *hhex* mRNA.

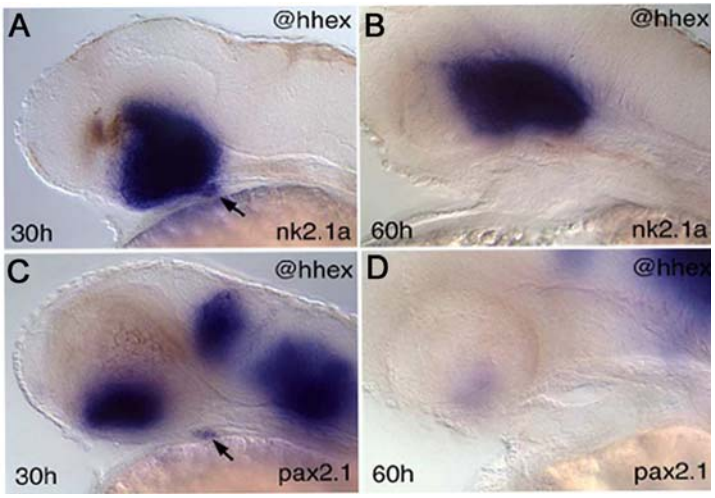

Figure 14: Abrogation of *hhex* function causes absence of thyroid primoridum. Expression patterns of thyroid markers (indicated at bottom right) at different stages (indicated at bottom left) in *hhex* morpholino-injected embryos.

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%)

Table 3: *hhex* morphants fail to develop a functional thyroid gland, but coinjection of *hhex* mRNA restores follicle development in part of the morphants.

larvae with follicles (Table 3). I 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.

Analysis of *nk2.1a* and *pax2.1* expression showed that although follicles are absent, a thyroid primordium is present in *hhex* morphants (Fig. 14A and C). However, expression of these genes disappeared in the thyroid primordium of morphants by around 60 hpf (Fig. 14B and D). These data show that mechanisms such as thyroid differentiation or primordial survival are disrupted in the absence of hhex.

In order to test whether high concentrations of *hhex* morpholino affect the induction or the size of the thyroid primordium, I injected 0.3 mM *hhex* morpholino. Even at these high concentrations that interfere with gastrulation processes, I do not observe a visibly reduced thyroid primordium at early stages. Thus, *hhex* morpholino injection phenocopies *b16*$^{-/-}$ mutant embryos with respect to the loss of the primordium at around 60 hpf. The smaller thyroid primordium in

b16$^{-/-}$ mutants is not caused by *hhex* deficiency and is therefore presumably due to the *cyc* deficiency alone.

As in mammals, the zebrafish thyroid primordium relocalises 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. 15A and B), and is therefore not dependent on *hhex*.

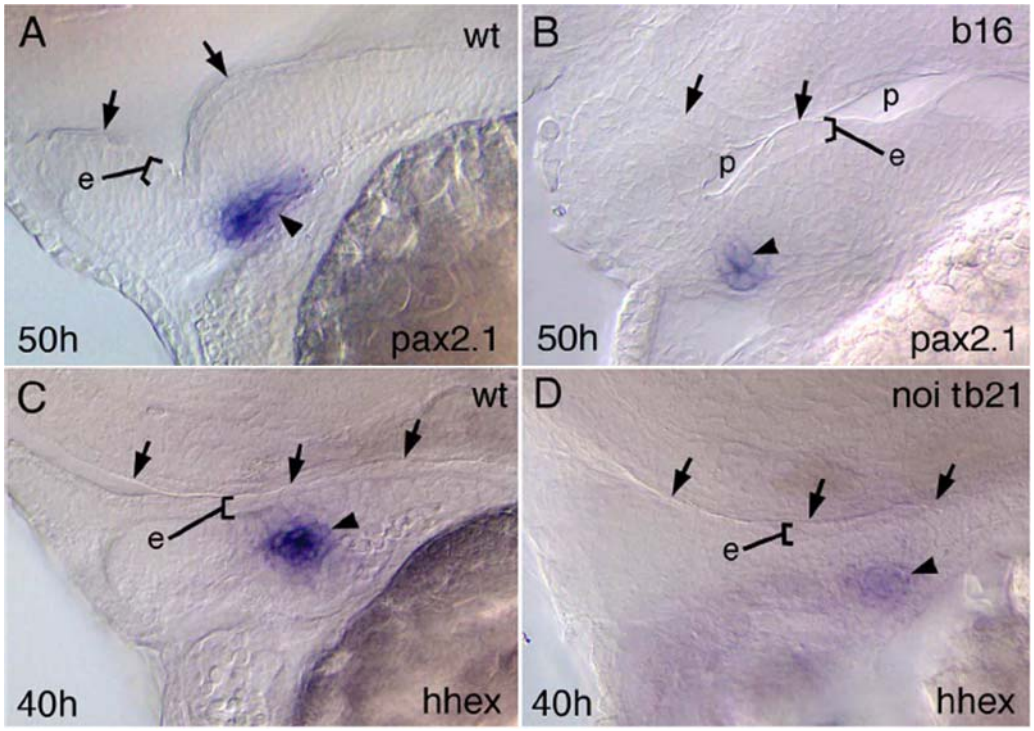

Figure 15: Migration of thyroid primordium occurs in *b16-/-*, and *noi tb21-/-* embryos.
Expression patterns of thyroid markers (indicated at bottom right) in different mutants or wildtype (indicated at top right) at stages when migration of the thyroid is taking place (indicated at bottom left). Note 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.

To see whether Hhex is sufficient for follicle formation, I 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.16 and data not shown). At 28 hpf, I did not observe any visible differences in size or strength of marker gene expression in the thyroid primordium of *hhex* mRNA-injected embryos (Fig. 16 B, E, and F). However, by 40 hpf, about 30% of *hhex* mRNAinjected 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. 16 D, G, and H). This suggests that additional Hhex activity does not affect formation of the thyroid primordium but can increase its later growth.

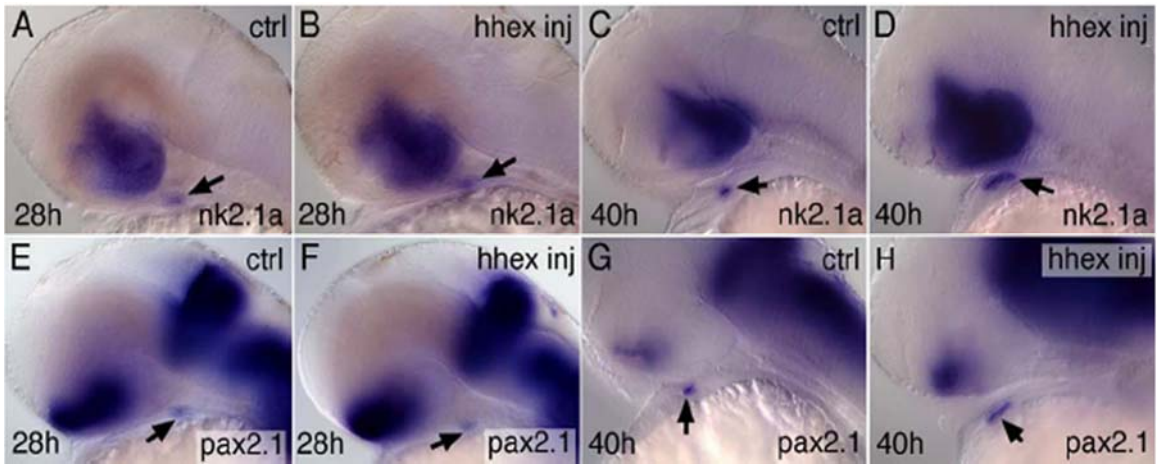

Figure16: Overexpression of *hhex* leads to late expansion of the thyroid primordium. Embryos are injected with *hhex* mRNA or *gfp* mRNA as a control (ctrl; indicated top right). Marker gene expression as indicated bottom right; stages as indicated bottom left.

In the absence of the *hhex* gene, *nk2.1a* is expressed during early development of the thyroid primordium. I wanted to know whether *hhex* in turn is expressed in the absence of *nk2.1a* function. As there is no *nk2.1a* mutant available, I used two different *nk2.1a* morpholinos to knock down Nk2.1a protein. As a control, I also injected *nk2.1b* morpholino. *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. As both genes are expressed in the ventral forebrain, 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.17A, Table 4), whereas they are unaffected in *nk2.1b* morphants (Table 4). Coinjection of *nk2.1a* mRNA-containing mismatches in the morpholino target sequence leads to normal thyroid follicles in about 40% of the embryos (Fig.17B, Table 4), 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.

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%)

Table 4: A functional thyroid is missing in *nk2.1a* morphants.

I find that both *hhex* and *pax2.1* are initially expressed in the thyroid primordium of *nk2.1a* morphants (Fig.17C,D,G,H; 72/72 (100%)), but expression fades around 35 hpf and is absent by 60 hpf (Fig.17 E,F,I.J; 50/57 (88%) for *hhex* and 58/62 (94%) 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*.

In conclusion, the *hhex* gene is not involved in relocalisation of the primordium, but is required for proper differentiation of the functional follicles. *nk2.1a* has a similar role in differentiation.

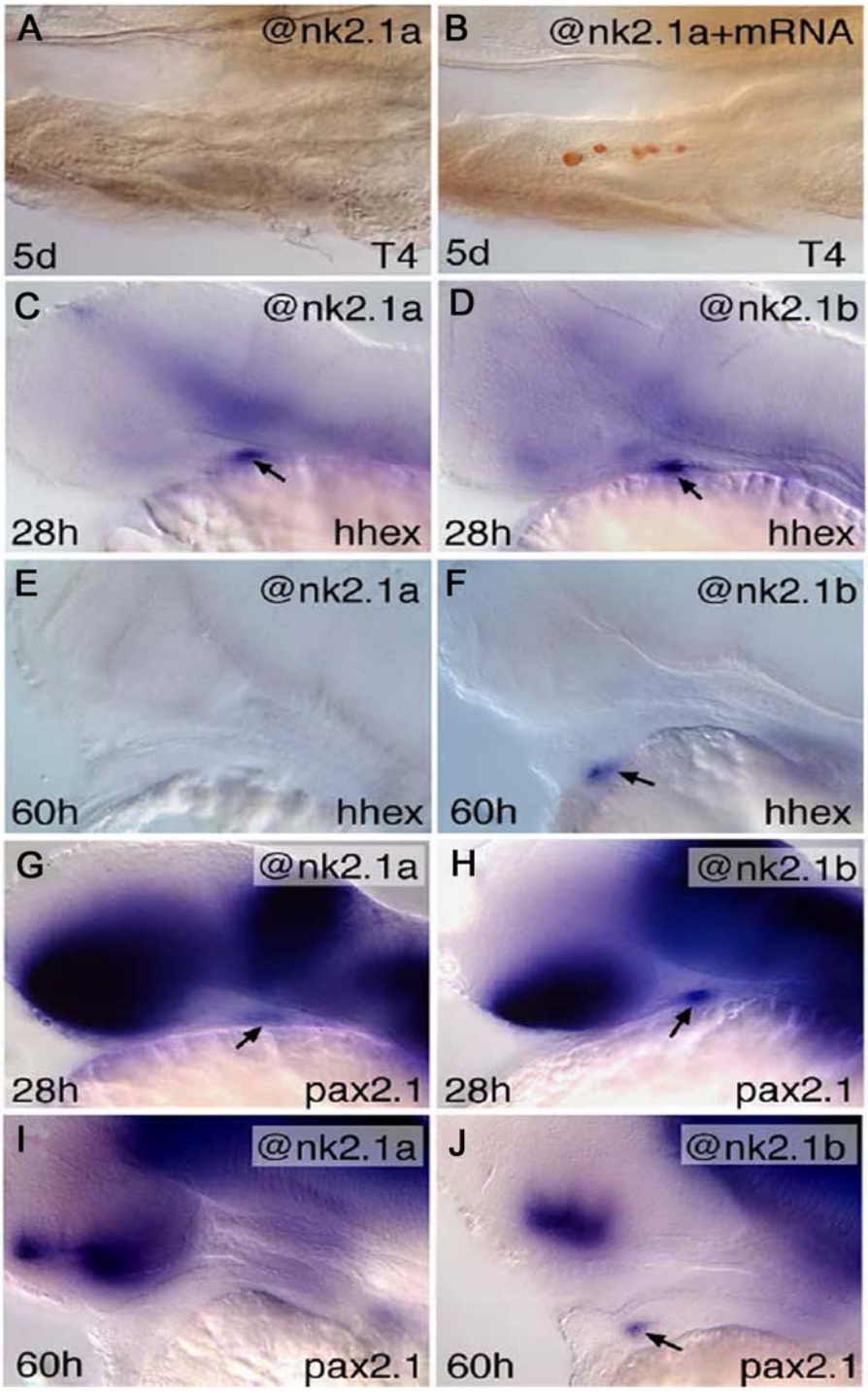

Figure 17: Abrogation of *nk2.1a* function causes failure of thyroid differentiation. (A, B) T4 immunostaining. (C–J) Marker genes expression. Arrows point to the thyroid primordium; stages at bottom left. Marker genes are indicated at bottom right.

3.2. Thyroid Morphogenesis:

3.2.1. *Hedgehog signalling is required for alignment of thyroid follicles along the midline*

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 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). In mammals the thyroid develops close to aortic sac, aortic arch (Fagman et al., 2006).This close correlation of vascular and thyroid development in both mammalian and teleost systems prompted me to investigate possible molecular interactions between vessels and thyroid in zebrafish.

Recent work has shown that *Shh* deficient mouse embryos develop an abnormal, unilateral single thyroid lobe (Fagman et al. 2004). Furthermore, *Shh* deficiency leads to cardiac malrotation and atrial dilatation (Tsukui et al., 1999), suggesting further defects in cervical vessel development. I first analysed if *shh* deficient zebrafish embryos display similar defects as mice. However, *syu* mutant zebrafish lacking the *shh* gene have an apparently normal ventral aorta and concomitantly normal thyroid morphology (Fig.18B). Zebrafish *syu* embryos show generally weaker defects than the corresponding $Shh^{-/-}$ mouse embryos, what can be explained by different regulation of duplicated *Hedgehog* genes in these

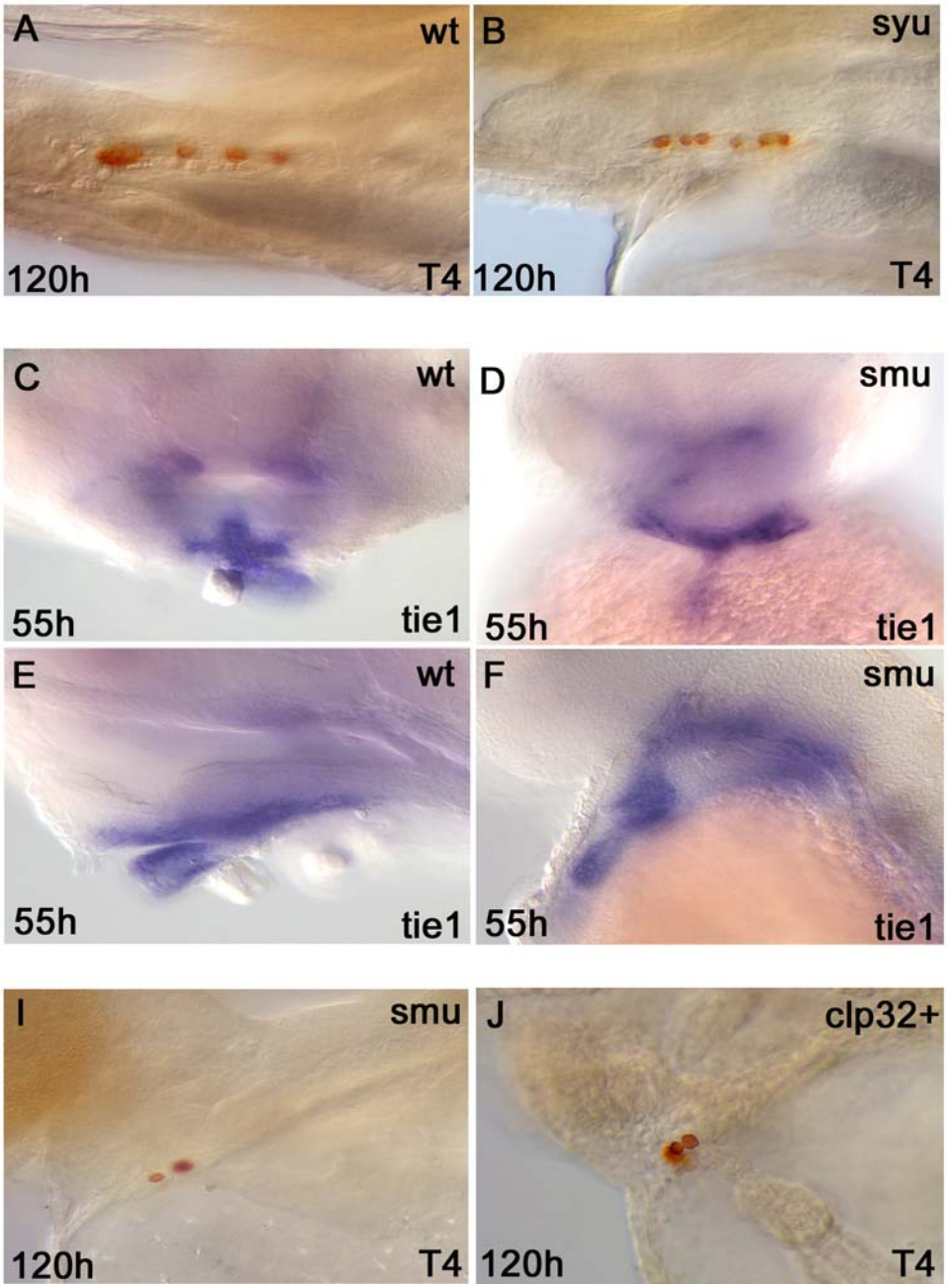

Figure 18: Hedgehog signalling is required for development of the ventral aorta and for dispersal of thyroid follicles along the anterior-posterior axis in developing zebrafish.(B) *syu-/-* mutants lacking *shh* gene and have normal thyroid morphology, (D)ventral view and (F) lateral view in *smu-/-* mutants show severe reorganization of branchial blood vessels (*tie1* expression). (I) thyroid follicles cluster around bulbus areteriosus in *smu-/-*mutant and the same in cyclopamine treated embryos in (J). (A,B,I and J)T4 immunostaining. (C,D,E and F) tie1 in situ staining.

species (Varga et al., 2001). I therefore investigated next a zebrafish mutant with more severe disruption of Hedgehog signalling. In *slow muscle omitted* (*smu*) mutants, where Hedgehog signal transduction is disrupted due to a mutation in the transmembrane signal mediator *smoothened* (Varga et al. 2001), severe reorganisation of branchial blood vessels occurs. Here, the bulbus arteriosus leads directly into abnormally patterned branchial arteries, omitting any blood vessel that resembles a ventral aorta (Fig.18 D and F). Apart from some specimens that lack a thyroid completely, the thyroid primordium starts to develop normally in *smu*. Later in development, however, follicles always cluster around the bulbus arteriosus and do not align along the anterior-posterior axis (Fig.18I).

The teratogenic plant alkaloid cyclopamine can be used to block Hedgehog signalling stage-specifically (Chen et al., 2002). Cyclopamine treatment beginning as late as 32 h is sufficient to prevent thyroid follicles from aligning along the anterior-posterior axis (100% of n=37) (Fig.18J), showing that Hedgehog signalling is required after evagination from the pharyngeal epithelium for guidance of thyroid follicles along the midline.

To further evaluate the role of Hedgehog signalling in thyroid morphogenesis I ectopically expressed *shh* in zebrafish embryos by mRNA injection. Ubiquitous *shh* expression does not alter initial thyroid development (Fig.19A,B) at around 30 h (100% of n=45), however, at 55 h two to three primordia can be found scattered around the midline instead of one single primordium (62% of n=105) (Fig.19C,D). In some *shh* injected embryos that survived to 96h mislocated follicles were found (Fig.19F). Together these results show that Hedgehog signalling is required for proper localisation of thyroid follicles along the anterior-posterior axis in zebrafish at the time of ventral aorta development. Interestingly, ectopic *shh* expression also causes abnormal distribution of endothelial cells in the pharyngeal area, indicating that the thyroid defects might be caused indirectly by altered vessel development (Fig. 20B).

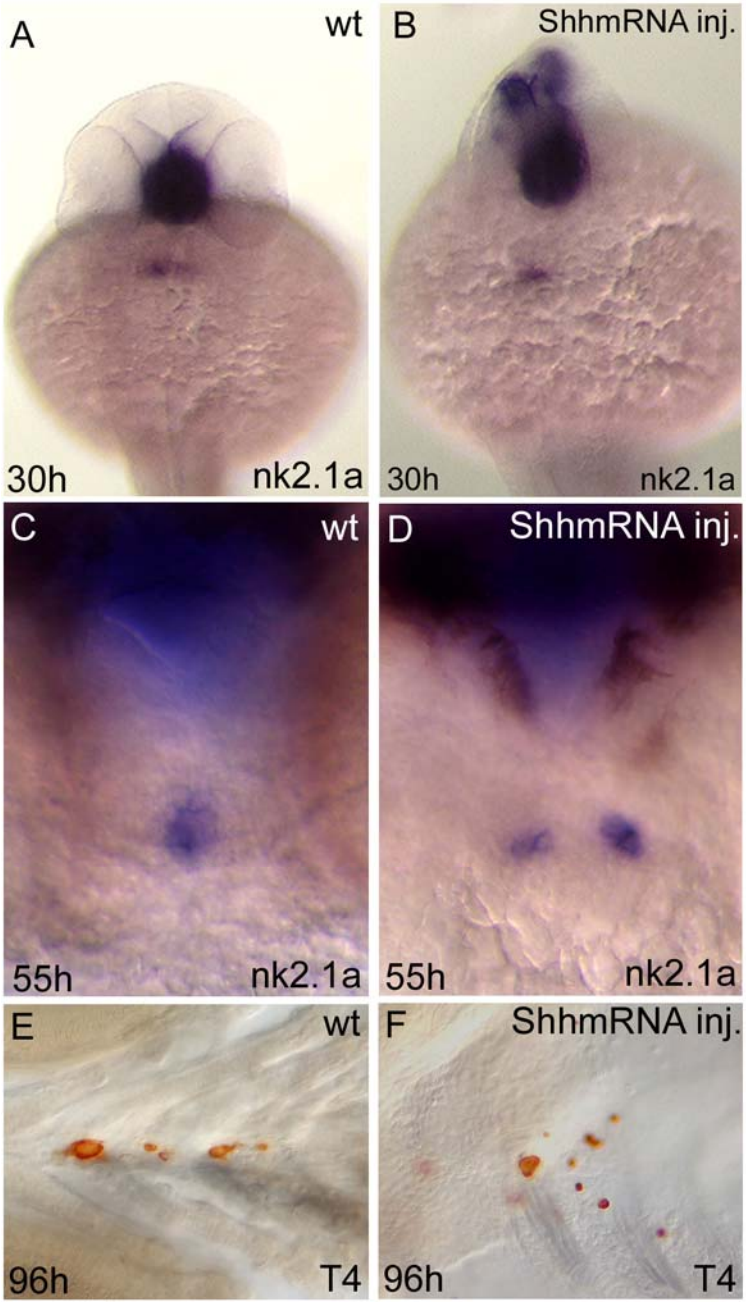

Figure 19: Shh signalling is required for proper localisation of thyroid follicles along the ant.-post. axis in zebrafish. (A-B) Initial thyroid development does not alter at around 30h *shh* mRNA injected embryos and the ectopic *nk2.1a* expression in the brain explain the effect of *shh* mRNA injection.(C-D)Two thyroid primordia scattered around the midline in 55h *shh* mRNA injected embryos. (E-F)Functional mislocated follicles in 96h *shh* mRNA injected larvea,T4 staining.

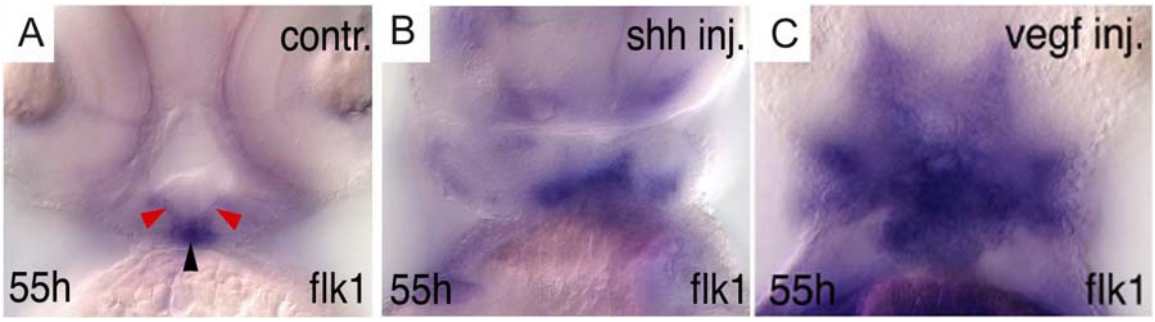

Figure 20: Ectopic *shh* or *vegf* expression causes abnormal distribution of endothelial cells in the pharyngeal area. *flk1 (kdr)* expression labels endothelial cells. In control embryos (*gfp* mRNA injected) (A), a normal ventral aorta (black arrowhead) and branchial arteries (red arrowheads) develop.This pattern is disturbed and laterally expanded in Shh/Vegf injected embryos (B). (C) Ectopic *vegf* expression affects endothelial marker expression more severely than *shh*.

3.2.2. *Disrupted ventral aorta development coincides with abnormal thyroid follicle distribution in vegf deficient zebrafish embryos*

The Hedgehog signalling pathway is known to drive artery development by inducing vascular endothelial growth factor (Vegf) expression in tissue surrounding developing blood vessels (Lawson et al., 2002). To address the possibility that defects in thyroid follicle distribution are mediated through blood vessels, I analysed zebrafish embryos with defects in Vegf signalling. The Vascular Endothelial Growth Factor (VEGF) pathway plays crucial roles in vertebrate hematopoiesis and vasculogenesis (Ferrara, 2004). In zebrafish, the VEGF pathway is required for arterial differentiation of the dorsal aorta (Lawson et al., 2002). Development of ventral aorta and branchial arteries is less well studied; however, VEGF and its receptor KDR are also expressed in this vessel and its surrounding mesenchyme (Fig. 21).

Reduction of Vegf by using *vegf* morpholinos (Lawson et al., 2002; Nasevicius et al., 2000), or absence of Kdr in *kdr*^{y17} mutant embryos (Habeck et al., 2002),

dramatically interferes with pharyngeal vessel formation (Fig. 22A-C). Analysis of the endothelial marker *tie1* in the two deficiency backgrounds indicates that here the ventral aorta fails to form properly. Instead, a misshaped domain of *tie1* expression surrounds the outflow tract of the heart, often expanding laterally, but not along the a-p axis, into one pair of irregular branches. Thus, Vegf pathway is required for normal development and expansion of the ventral aorta along the a-p axis.

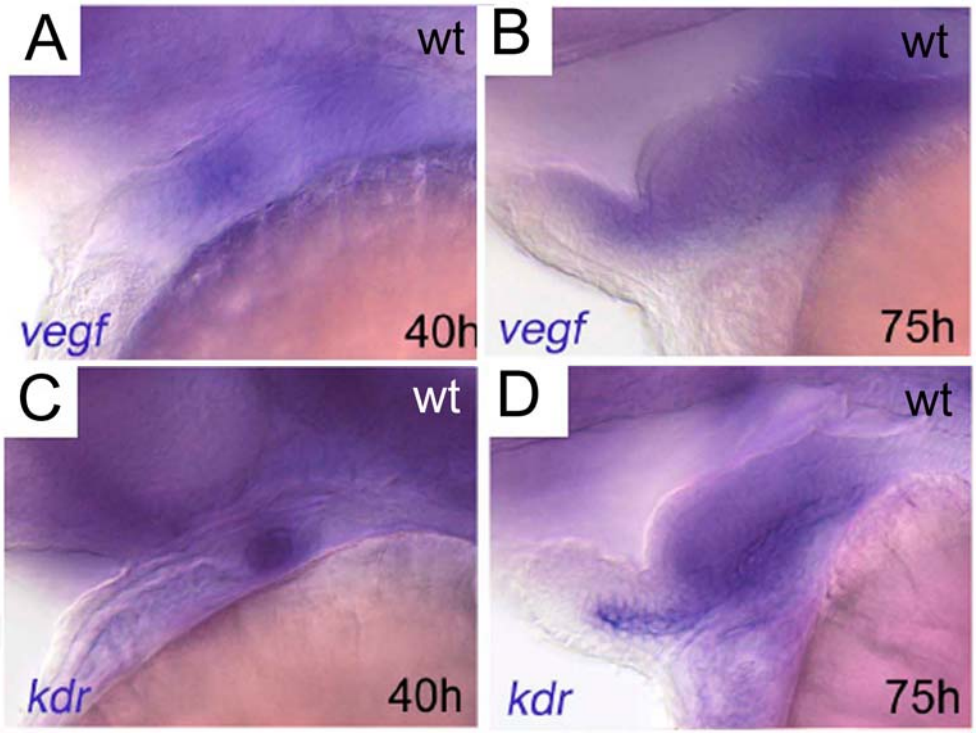

Figure 21: *vegf* and its receptor *kdr* expression in the developmental ventral aorta and its surrounding mesenchym. (A and B) *vegf* is diffusely expressed in the ventral pharyngeal area during ventral aorta development.(C and D) *kdr* is diffusely expressed in ventral aorta and surrounding mesenchyme.Lateral views, anterior to the left.

I then analysed whether it is possible that altered pharyngeal vessel architecture affects thyroid morphology. In the above mentioned two genetic backgrounds with compromised Vegf signalling, the thyroid starts to develop from a normally induced midline primordium at around 26 hpf (normally shaped and sized domain of *nk2.1a* expression data not shown). However, during subsequent growth the

thyroid fails to elongate from its initial position at the cardiac outflow tract along the a-p axis (Fig. 22D-F). Instead, thyroid tissue is not limited to the midline and often expands laterally in irregular fashion, always adjacent to or embedded into ectopic *tie1* expression (Fig. 22G-I). Correspondingly, follicles do not align along the pharyngeal midline during larval growth, and instead form an irregular group around the cardiac outflow tract (Fig. 22J-L). These data strongly suggest that thyroid morphogenesis is linked to pharyngeal vessel development.

In addition I performed Vegf gain of function experiments to further explore a likely interaction of arterial and thyroid development. Ubiquitous *vegf* mRNA expression causes the same effect on thyroid development as *shh* does. Initially a normal thyroid primordium forms at 30 h (100% of n=39) but at 55 h, two to three primordia can be found scattered around the midline (68% of n=94)(Fig.23). Furthermore, pharyngeal endothelial marker gene expression is laterally expanded in *vegf* injected embryos as it is in *shh* injected embryos (Fig.20), confirming that Hedgehog and Vegf act in generating and patterning pharyngeal vasculature, as it has been shown before in similar cases (Habeck et al., 2002; Lawson et al., 2002; Pola et al. 2001).

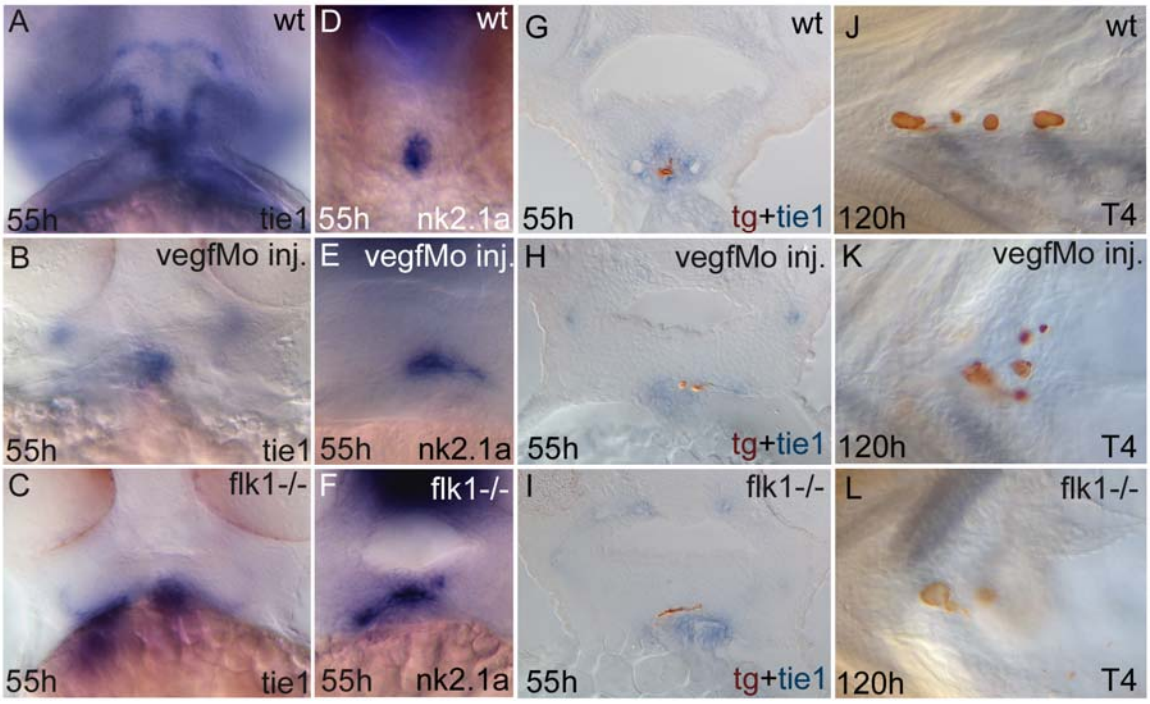

Figure 22: Mutants with defects in ventral aorta development show correlating thyroid abnormalities. (A-F) Frontal views, showing vasculature (*tie1* expression) or thyroid (*nk2.1a* expression). (G-I) Sections showing folliclular lumen (thyroglobulin immunostaining, brown) in relation to endothelial cells (*tie1* expression,blue). (J-L) At 120 hpf, thyroid follicles (T4 immunostaining, brown) fail to align along the ventral aorta. Note that in E,F,H and I the thyroid appears larger due to the lateral expansion. However, in wildtype embryos the thyroid extends along the a-p axis. Concomitant with lateral expansion its a-p extends appears to be reduced, so that the size remains similar. This is also reflected by normal follicle numbers at 120 hpf (J-L).

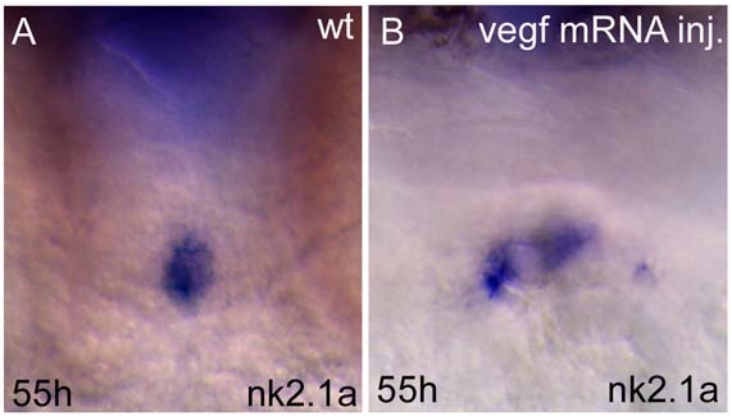

Figure 23: Ubiquitous *vegf* mRNA expression causes the same effect on thyroid development as *shh* does. (B) 55h *vegf*mRNA injected embryo shows two to three thyroid primordia scattered around the midline.

3.2.3. *Endothelial cells are required for directing thyroid growth along the pharyngeal midline in zebrafish*

Since Hedgehog signalling is important in the development of multiple cell types, I tested whether the observed defects in thyroid follicle distribution are mediated through endothelial cells. In the zebrafish *cloche* (*clo*) mutant, differentiation of endothelial cells into blood vessels is arrested at early stages and all blood vessels of head and anterior trunk fail to develop (Liao et al., 1997). Expression of all known blood or endothelial cell–specific genes, including *scl*, *lmo2*, *gata1*, *gata2*, *flt1*, and *flt4*, is almost completely lost in *cloche* mutants (Liao et al.,1998; Sumanas et al., 2005; Stainier et al., 1995). *clo* mutant larvae develop functional but mislocated thyroid follicles clustering around the bulbus arteriosus (Fig. 24A,B). Histological sections confirm that in *clo* mutant larvae blood vessels are missing in the otherwise apparently normal pharyngeal area and reveal that the thyroid follicles are loosely embedded in connective tissue close to the heart (Fig. 24C-F). Thus, endothelial cells are required for directing thyroid growth along the pharyngeal midline in zebrafish. However, as the molecular nature of the *clo* locus is unknown, I started to analyse the role of other, well characterised factors involved in vasculogenesis in thyroid morphogenesis.

Scl (also known as Tal1) is a bHLH transcription factor acting downstream of the *cloche* gene to specify hematopoietic and vascular differentiation (Liao et al., 1998). During normal development Scl is required for hematopoiesis (Gering et al., 1998; Liao et al., 1998), endothelial (Liao et al., 1998), and dorsal aorta development (Patterson et al., 2005) in zebrafish embryos. Correspondingly, the *scl* gene is expressed in haematopoietic and endothelial tissue, as well as in a few cells in the central nervous system (Jin et al., 2006). I found that reduction of Scl by injecting *scl* morpholinos (Patterson et al., 2005; Lawson et al., 2002; Nasevicius et al., 2000) dramatically interferes with pharyngeal vessel formation (Fig. 25 A and B), similar to *smu*$^{-/-}$ mutant embryos. Analysis of the endothelial

marker tie1 in the morphants embryos indicates that the ventral aorta fails to form properly. Instead, a misshaped domain of *tie1* expression surrounds the outflow tract of the heart, often expanding laterally, but not along the a-p axis, into one pair of irregular branches.

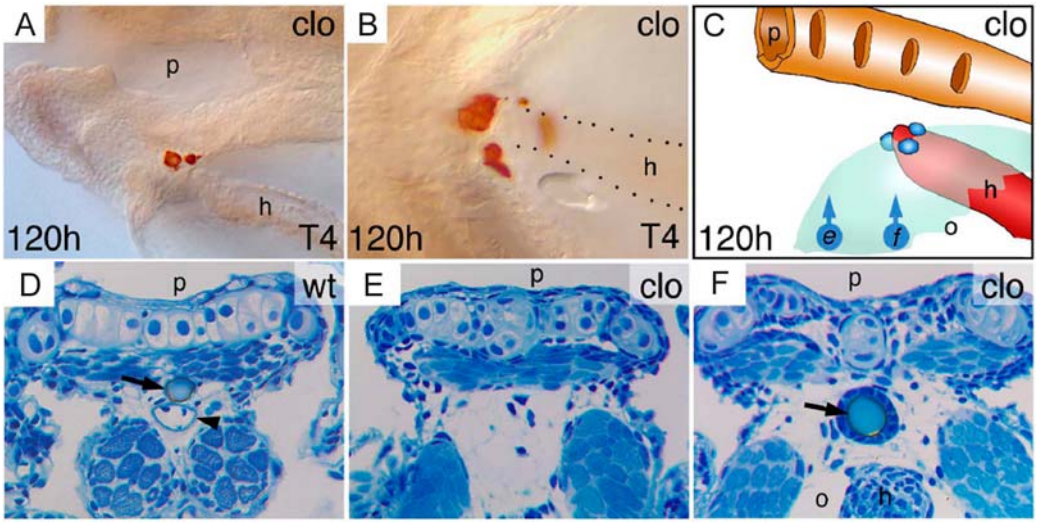

Figure 24: Thyroid follicles cluster around the bulbus arteriosus in *clo* mutant larvae. T4 immunostaining (brown) on 120 h larvae. Lateral view (a), ventral view (b) and sections. Arrows label thyroid follicles. a-c In *clo* mutant larvae, follicles proliferate, but fail to disperse along the anterior-posterior axis of the pharyngeal region. d-f Sections reveal the proximity of the differentiated aorta with its arterial wall (arrowhead in d) to the follicles in wildtype. In *clo* mutants, no blood vessels are detectable (e, f). The level of sectioning is as indicated by the blue arrows in c (the wildtype section corresponds to the position of section e in *clo*). Abbreviations: h, heart; p, pharynx; o, oedema.

I then analysed whether altered pharyngeal vessel architecture affects thyroid morphology. In the *scl* morphant embryos, the thyroid starts to develop from a normally induced midline primordium at around 30 hpf. However, during subsequent growth the thyroid fails to elongate from its initial position at the cardiac outflow tract along the a-p axis (Fig. 25C and D). Instead, thyroid tissue is not limited to the midline and often expands laterally in irregular fashion, always adjacent to or embedded into ectopic *tie1* expression (Fig. 25E and F). Correspondingly, follicles do not align along the pharyngeal midline during larval growth, and instead form an irregular group around the cardiac outflow tract (Fig. 25G and H). Taken together, disruption of the *scl* gene, which is specific for

vessel development, phenocopies not only the vascular phenotype of smu mutant, but also the thyroid phenotype. This strongly suggests that thyroid morphogenesis is linked to vessel development.

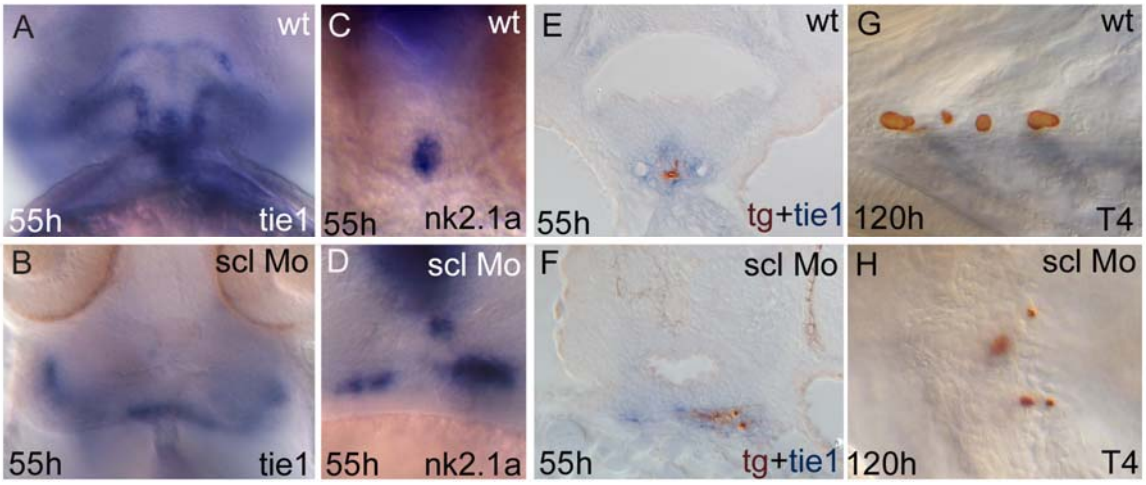

Figure 25: Reduction of Scl causes defects in ventral aorta development and shows correlating thyroid abnormalities. (A-D) Frontal views, showing vasculature (*tie1* expression) or thyroid (*nk2.1a* expression). (E and F) Sections showing folliclular lumen (thyroglobulin immunostaining, brown) in relation to endothelial cells (*tie1*expression, blue).(G and H) At 120 hpf, thyroid follicles (T4 immunostaining, brown) fail to align along the ventral aorta.

To test whether compromised blood circulation evident in all of these deficiency backgrounds causes indirectly thyroid abnormalities, I targeted the *tnnt2* gene by morpholinio knock down. *tnnt2* encodes the thin-filament contractile protein cardiac troponin T, and corresponding morphants lack heart beat and blood circulation (Sehnert et al., 2002). In these morphants, the thyroid is normal at 55 hpf (data not shown), showing that specific disruption of circulation does not account for morphological defects of the thyroid. Thus, it is rather vessel patterning that appears to be correlated to thyroid morphology. As the ventral aorta is the only vessel adjacent to the thyroid in developing wildtype zebrafish embryos, it can be assumed that this is the artery that guides follicular growth along the midline.

4. Discussion

4.1. Thyroid differentiation

4.1.1. *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 morphologically, the early primordium of the thyroid becomes visible as a bud at the ventral floor of the pharynx in different vertebrate species (Macchia, 2000). I show that in zebrafish Nodal signaling 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). I never detected thyroid tissue in *bon*$^{-/-}$ and *gata5/fau*$^{-/-}$ mutant embryos, 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 anterior endoderm, pharynx, or thyroid development rather than due to an indirect consequence of reduced overall endoderm development.

4.1.2. *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, I find that *hhex* deficiency results in the disappearance of 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. 26A; Lazzaro et al., 1991), and then many follicles differentiate simultaneously. In zebrafish, the thyroid primordium does not undergo this extended phase of early growth. 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. 26B; Wendl et al., 2002). Thus, in both species, *hhex/Hhex* is required at a comparable step of thyroid development prior to significant tissue growth.

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, I 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 (Wendl and Rohr, unpublished observations).

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*

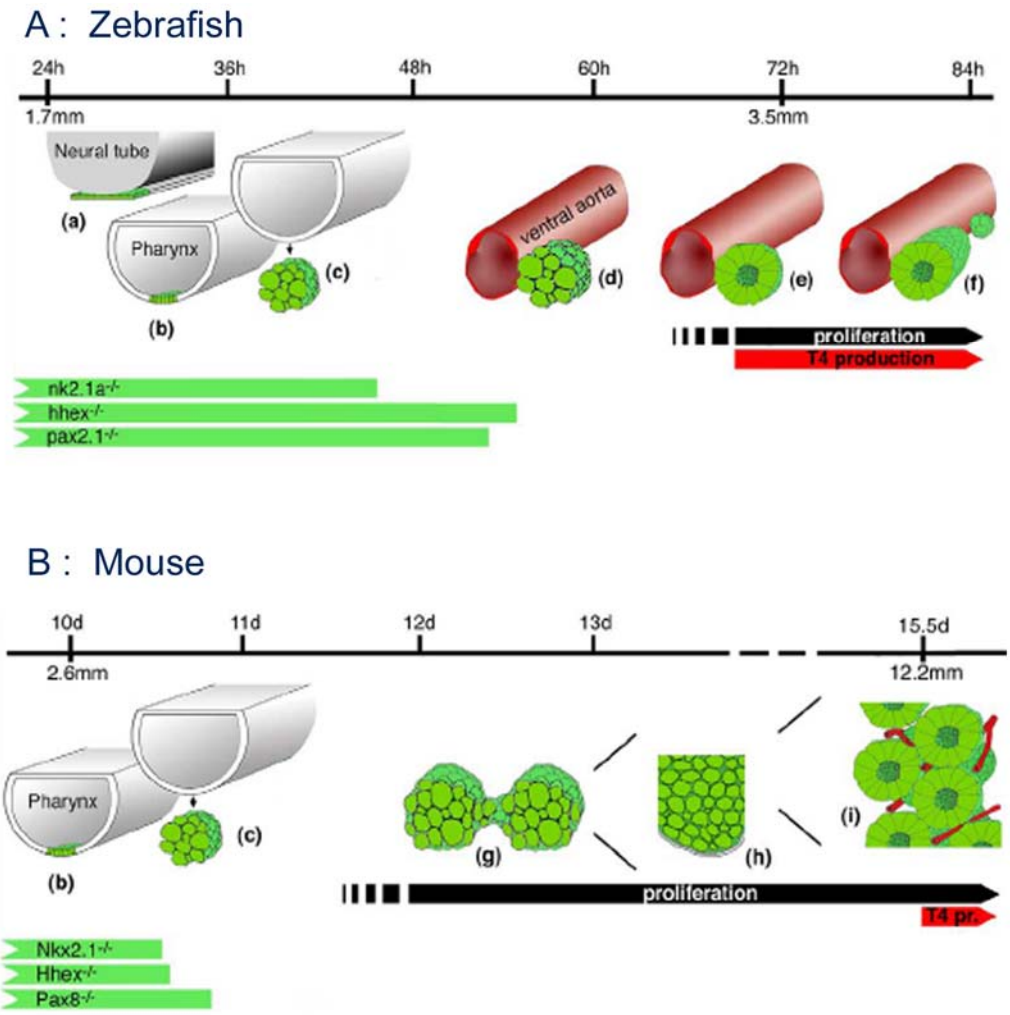

Figure 26: Comparison between thyroid development in zebrafish and mice. Schematic illustrations comparing thyroid development in zebrafish and mice.The scale bar shows time of development (h, hpf for zebrafish; d, dpc for mice). Green colour indicates developing thyroid tissue. (a) Early marker gene 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}.

are initially present (Wendl et al., 2002). However, as not all of the markers have been tested in each of the mouse mutations, I 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, I expect as yet undiscovered genes to be responsible for induction of the thyroid primordium.

4.2. Thyroid Morphogenesis

4.2.1. *Members of both the Hedgehog and the Vegf pathway might be involved in the pathogenesis of localisation defects in thyroid development*

In fish positioning of thyroid tissue and development of the ventral aorta along the anterior-posterior axis depend on Hedgehog signals. Diffuse expression domains of pathway components together with the fast and complex development of the pharyngeal area at present do not allow me to evaluate exactly whether Hedgehog and VEGF signals act directly on thyroid development or indirectly via blood vessels and intermitting additional signalling cascades. Nevertheless, the close correlation between thyroid and vessels in fish strongly suggests that both tissues are directly or indirectly linked by sharing components of these pathways.

It is noteworthy that in the investigated zebrafish, the primordium always undergoes an initially normal first step of relocation from the pharynx to the bulbus arteriosus. This shows that the basic mechanisms of primordial evagination and initial movement are independent from proper development of ventral aorta. A subsequent step of local positioning is responsible for the association of thyroid tissue to adjacent arteries, a relationship that probably evolved early in the vertebrate lineage to ensure efficient hormone release into circulation. It is conceivable that concomitant with the loss of the ventral aorta during vertebrate evolution, the thyroid associated with the aortic arch instead, as seen in a primitive mammal, the echidna (Haynes, 1999; Pischinger, 1937), or with the carotid arteries in higher mammals. My study is the first to identify key components of signalling pathways involved in local positioning of thyroid tissue, also providing candidates for mediating direct interactions between vessels and thyroid tissue. Furthermore, members of both the Hedgehog and the Vegf pathway might be involved in the pathogenesis of localisation defects in human thyroid development.

4.2.2. *Endothelial cells are able to influence thyroid morphology in zebrafish*

The thyroid gland in vertebrates develops close to vascular structures. In the present study, I analyse zebrafish embryos with altered vessel architecture and find that defects in pharyngeal vessels coincide with abnormal thyroid development. The study of zebrafish embryos with ectopic endothelial cells reveals a functional link between vascular and thyroid development. Additional data show that ectopic endothelial cells influence thyroid morphology in a non-cell autonomous manner (Alt, et al., 2006b). Ectopic induction could probably cause lateral expansion of thyroid tissue in the zebrafish embryos lacking Scl or with compromised Vegf signalling. However in all deficiency backgrounds analysed the thyroid starts to develop from a normally induced midline primordium. Therefore, an inductive role of vascular structures can be excluded to be responsible for the thyroid phenotypes observed in our deficiency backgrounds. Furthermore, in *cloche*, a zebrafish mutant lacking all vessel progenitors in head and anterior trunk region (Liao et al., 1997; Stainier et al., 1995), the thyroid primordium is still induced. Since the myocard forms in *cloche*, it is still possible that the heart or some of its precursor cells are involved in thyroid induction, but an inductive role of vessels can be excluded.

The whole strand of thyroid tissue in zebrafish derives from the small globular primordium that buds off from the pharyngeal epithelium at around 32 hpf (Alt et al., 2006a), and no further cells contribute to the thyroid from outside during its extension along the anterior-posterior axis. This is in contrast to many other vertebrate species where ultimobranchial bodies have been described to fuse with the midline diverticulum, and the fact that in my deficiency backgrounds with vessel defects the thyroid primordium is initially normal, suggests that the lateral expansion of thyroid tissue adjacent to endothelial cells is generated by misdirected growth. Taken together, my zebrafish data show that endothelial

cells are required for proper alignment of thyroid tissue along the anterior-posterior axis during tissue growth.

Under wildtype conditions, the ventral aorta is the only vessel directly adjacent to the thyroid in zebrafish, so that it can be assumed that this is the vessel responsible for interactions with the thyroid. Not only in zebrafish (Wendl et al., 2002), but also in other teleosts such as trouts (Raine and Leatherland, 2000) thyroid tissue remains in close association with the ventral aorta throughout later development and adulthood. It will be interesting to find out how such an interaction is mediated. Extracellular matrix or secreted factors are candidates, but the exact nature of the interaction between vessels and thyroid tissue remains to be elucidated.

In zebrafish development, two morphogenetic phases are distinguishable in thyroid relocalisation. After induction and evagination, the thyroid primordium adopts a position close to the cardiac outflow tract in zebrafish (Fig. 4). In all deficiency backgrounds investigated, this first phase is not disrupted. Consequently, follicles cluster at a default position around the cardiac outflow tract in the deficient zebrafish larvae. In a second phase of relocalisation that is dependent on ventral aorta development, the thyroid then adopts its species-specific position further cranially.

This study suggests that the dependence of thyroid morphogenesis on the development of adjacent arteries is a conserved mechanism that might have evolved to ensure efficient hormone release into circulation.
Thus, the disruption of signalling pathways linking thyroid and blood vessel development represents a novel mechanism that is likely to be relevant for the molecular pathogenesis of congenital thyroid defects in humans.

5. Conclusion

5.1. Thyroid differentiation

- Nodal Signals and their downstream effectors are required for anterior endoderm development including thyroid progenitor cells.
- Despite early onset of its expression in thyroid progenitor cells, the zebrafish *hhex* gene is not required for early steps in thyroid development, but for thyroid growth and differentiation.
- The zebrafish *nk2.1a* gene plays a similar role as *hhex* relatively late in thyroid differentiation.

5.2. Thyroid Morphogenesis

- Under wildtype conditions the ventral aorta is the only vessel close to the thyroid in zebrafish embryos and early larvae.
- Mutant analysis reveals that vascular and thyroid development is linked, and that the ventral aorta guides thyroid growth along the midline in zebrafish embryos. Thus, the ventral aorta defines the position of the thyroid during its relocalisation from the pharyngeal endoderm.
- Main candidate pathways for mediating a link between vessel and thyroid development are Hedgehog and Vegf signaling.

6. Abstract

The thyroid gland is an endocrine organ primarily composed of endoderm-derived cells. During embryonic development, the thyroid primordium evaginates from its site of induction in the pharyngeal endoderm, relocating to a final position deep in the hypopharyngeal mesoderm. In this study, I investigate molecular and genetic mechanisms that control thyroid development. My work can be separated into two parts, the first one dealing with the requirement of thyroid-autonomously acting transcription factors for differentiation, the second one giving first insights into a role of blood vessels in thyroid morphogenesis.

As expected for an endoderm-derived organ, initiation of thyroid development depends on Nodal signaling, which is a key component in endoderm formation. More specifically, I find that this initiation depends on three endoderm-specific downstream effectors of Nodal activity, *casanova (cas), bonnie and clyde (bon),* and *faust (fau)/gata5*. In thyroid progenitor cells initiate expression of the genes encoding thyroid-specific transcription factors such as Hhex and Nk2.1a starts already in the endoderm prior to pharynx formation. However, despite their early Nodal-dependent expression in the endoderm, both *hhex* and *nk2.1a* are required only relatively late during thyroid differentiation.

Although abnormal positioning of the developing thyroid gland is a well known, frequently occurring cause of reduced thyroid function in newborn humans, the molecular mechanisms that are responsible for proper thyroid positioning remain largely unknown. In the second part of my thesis, I investigate zebrafish mutants with defects in blood vessel development. I find that vessel and thyroid development is linked via several molecular pathways such as Hedgehog and Vegf signalling, showing for the first time an influence of vessels on thyroid morphology.

7. Zusammenfassung

Die Schilddrüse ist eine endokrine Drüse, die im Wesentlichen aus endodermalen Zellen zusammengesetzt ist. Während der Embryonalentwicklung löst sich das Schilddrüsenprimordium von dem Ort seiner Induktion im Schlundendoderm ab und relokalisiert zu einer endgültigen Position tief im hypopharyngealen Mesoderm. In dieser Studie untersuche ich die molekularen und genetischen Mechanismen, die die Schilddrüsenentwicklung kontrollieren. Meine Arbeit kann in zwei Teile geteilt werden. Der erste Teil behandelt die Rolle schilddrüsenautonom wirkender Transkriptionsfaktoren in der Differenzierung, der zweite Teil erste Einsichten in die Rolle von Blutgefäßen in der Schilddrüsenmorphogenese.

Wie erwartet für ein endodermales Organ, hängt die Initiation der Schilddrüsenentwicklung von Nodal-Signalen ab, die eine Schlüsselkomponente in der Endoderm-Bildung sind. Im Detail habe ich herausgefunden, dass diese Initiation von drei endodermspezifischen „Downstramfaktoren“ der Nodal-Aktivität abhängt: *casanova* (*cas*), *bonnie and clyde* (*bon*) und *faust* (*fau*)/*gata5*. In Schilddrüsen-Vorläuferzellen beginnt die Expression von schilddrüsenspezifischen Transkriptionsfaktoren wie Hhex und Nk2.1a schon im Endoderm, vor der Pharynx-Bildung. Trotz der frühen Abhängigkeit von Nodal im Endoderm werden jedoch sowohl *hhex* als auch *nk2.1a* erst spät in der Schilddrüsendifferenzierung benötigt.

Obwohl die abnormale Positionierung der sich entwickelnden Schilddrüse eine gut bekannte, häufig auftretende Ursache für reduzierte Schilddrüsenfunktion in neugeborenen Menschen ist, sind die für die korrekte Positionierung verantwortlichen molekularen Mechanismen weitgehend unbekannt. Im zweiten Teil meiner Arbeit untersuche ich Zebrafisch-Mutanten mit Defekten in der Blutgefäßentwicklung. Hier beobachte ich, dass Blutgefäß- und Schilddrüsenentwicklung über verschiedene molekulare Signalwege, wie den

Hedgehog- und Vegf-Signalweg, gekoppelt sind. Dies zeigt zum ersten Mal einen Einfluss der Blutgefäße auf die Schilddrüsenmorphologie.

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9. Abbraviation

°C	Celsius
D.H_2O	distal water
DNA	Deoxyribonucleic acid
dpf	days post fertilisation
g	gram
Gfp	green fluorescent protein
h	hour
Hpf	hours post fertilisation
L	liter
M	Molarity
mg	milligram
Min	minute
ml	milliliter
mM	millimolar
Mo	morpholino oligonucleotide
mRNA	messenger ribonucleic acid
N	Normality
ng	nanogram
NGS	normal goat serum
PBS	phosphate buffered saline
PFA	Paraformaldehyde
pH	(from potential of Hydrogen) the logarithm of the reciprocal of hydrogen-ion concentration in gram atoms per liter.
PTU	Phenythiourea
R.T.	Room temperature
X	times
ZFR	Zebrafish–Ringer
µg	microgram
µl	microliter

10. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Siegfried Roth und Dr. Klaus Rohr betreut worden.

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Teilpublikationen:

Elsalini, O.A. and Rohr, K. (2003). Phenylthiourea disrupts thyroid function in developing zebrafish. Dev Genes Evol **212**,593–598

Elsalini O. A., Gartzen, J., Cramer, M., and Rohr, K. B. (2003). Zebrafish *hhex, nk2.1a,* and *pax2.1* regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors. Developmental Biology **263,** 67–80.

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11. Lebenslauf

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