DOTTORATO DI RICERCA IN

GENETICA E MEDICINA MOLECOLARE

XXII CICLO

In vitro and in vivo analysis of the transcriptional coactivator TAZ

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INTRODUCTION

Gene expression regulation

Gene expression includes all the events that go from the transcription of a gene to the production of the corresponding protein. All these events are finely regulated. Studying gene expression means to understand in which tissue a gene is expressed, how it is expressed and what causes its expression. In fact, phenotypic differences among different cell types are mainly due to differences in gene expression patterns.

Gene expression can be regulated at different levels: activation of DNA structure, start of transcription, maturation of the transcript, transport into the cytoplasm, translation of mRNA and protein stability. Nevertheless, the most important step is the start of the transcription process which determines whether a gene is transcribed or not. RNA polymerase II, as well as RNA polymerase I and III, needs proteins to find the right transcription start site and these proteins necessary for the start of the transcription process are called general initiation factors. Given that RNA polymerases and general initiation factors are the ultimate targets of regulatory events, during the assemblage of the RNA polymerases complex there are several points for regulation to take place. It is important to note that purified RNA polymerases and corresponding general initiation factors have an intrinsic ability to accurately transcribe DNA templates through core promoter elements, thus allowing the fundamental transcription mechanisms to be elucidated, but these activities are generally suppressed in the cell by the packaging of DNA within chromatin and by negative cofactors that directly interfere with the function of the basal transcription factors. As discussed below, this imposes requirements for transcriptional activators and corresponding cofactors that act in a gene-specific manner both to reverse the repression (antirepression) and to effect a net activation above the intrinsic activity of the basal transcription machinery.

It is possible to subdivide transcriptional factors that cooperates directly with RNA polymerase II in three different categories: a) general transcription factors; b) upstream transcription factors; c) inducible transcriptional factors.

a) General transcription factors are necessary for the start of RNA synthesis in all the genes of class II (genes that encodes for proteins). They form a complex with the RNA

polymerase II close to the transcription start site and this complex is called basal complex of transcription.

b) Upstream transcriptional factors are proteins that recognize short consensus sequence situated upstream the transcription start site (an example is the Sp1 factor that recognizes GC boxes). These factors are ubiquitous and they act increasing the transcription efficiency of all the genes containing a GC box in their promoter.

c) Inducible transcriptional factors work like upstream factors, but they have e regulatory role. They are synthesized or activated in a tissue-specific manner and they recognize sequences called response elements.

In general, transcription factors acts directly recognizing specific and well conserved *cis*-sequences situated in promoters or enhancers. Some of these factors and of these sequences are common to more than a promoter and they are used in a constitutive manner; some other are specific and their activity is finely regulated. During the assembly process of these factors onto the regulatory sequences there are a lot of check-points and the speed of transcription can be increased or decreased in response to different regulatory signals. Nevertheless, not all the transcription factors bind these specific sequences on the promoter, they can also recognize other proteins already bound to the promoter, and sometimes they can bind directly to the RNA polymerase.

Often DNA-binding factors that regulate the transcription of protein-coding genes act together with an expanding group of cofactors that act either through modifications of the chromatin structure or, more directly, regulating formation or function (transcription initiation or elongation) of the pre-initiation complex. Requirement for cofactors involved more directly in transcription is somewhat surprising in view of the specificity intrinsic to the various DNA-binding regulatory factors, the structural complexity of their ultimate target (the basal transcription machinery), and documented interactions between regulatory factors and components of the basal transcription machinery. Early studies of activator functions in systems reconstituted with DNA templates and purified RNA polymerase II and corresponding general initiation factors revealed that additional ‘‘coactivator’’ or ‘‘mediator’’ activities were required for the activator function but not for basal (activator-independent) transcription.

Coactivators functions in a variety of ways and often contain the enzymatic activities necessary for an alteration in chromatin structure from a quiescent state to one allowing active gene transcription. Broadly speaking, coactivators can be divided in three

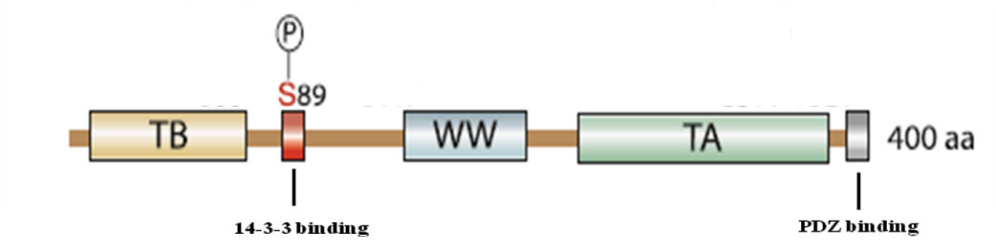
classes. One class of proteins modifies histones in ways that allow greater access of other proteins to the DNA. Examples of these are p300 and CBP, powerful histone acetyltransferases (HATs) that interact with a wide variety of transcription factors and other proteins (Hermanson et al., 2002). These proteins support transcription in vitro from chromatinized templates. A second class of coactivators are members of the TRAP/DRIP/Mediator/ARC complex, proteins that bind to transcription factors, recruit RNA polymerase II and interact with the general transcription apparatus. The Mediator complex supports transcription in vitro from DNA templates but does not support efficient transcription from chromatinized templates. Lastly, protein complexes of the yeast SWI/SNF family (or their mammalian homologs BRG1 or BRM) contain ATP-dependent DNA unwinding activities, necessary for efficient gene transcription in vivo. These groups of proteins will not support transcription from naked DNA, but augment transcription from chromatinized templates in vitro (Lemon et al., 2001).

Co-repressors have the opposite effect on chromatin structure, making it inaccessible to the binding of transcription factors or resistant to their actions. These proteins (such as NcoR) are often associated with histone deacetylase (HDAC) activity, though other mechanisms for gene silencing clearly exist (Hermanson et al., 2002). Although coactivators are defined as proteins that increase transcriptional activity without binding to DNA, it is useful to think of those that bind directly to transcription factors and contain relevant enzymatic activities as primary coactivators. Those that dock on transcription factors and serve as scaffolds for the recruitment of other proteins containing these enzymatic activities can be considered secondary co-activators. This distinction rapidly blurs as proteins that can function as primary coactivators on some transcription factors can also be used as enzymatic tools assembled by secondary coactivators in other contexts.

TAZ

TAZ (Transcriptional co-Activator with PDZ binding motif), also referred to as Wwtr1 (WW-domain containing transcription regulator 1), is a transcriptional co-activator highly expressed in kidney, heart, lung, liver, testis, and placenta. TAZ is characterized by a central WW-domain followed by a highly conserved C-terminal sequence, which

contains a coiled-coil domain that recruits core components of the transcriptional machinery. Both human and mouse TAZ contain a single WW domain that is a very common domain that mediates protein-protein interaction. TAZ also contains a PDZ-binding motif at its C-terminus, that is required for its transcriptional coactivator activity and promotes TAZ nuclear localization particularly to discrete foci, and a 14-3-3 binding motif within the conserved N-terminal portion.



Domain organization of TAZ: Transcriptional enhanced factors binding domain (TB), 14-3-3 binding domain, WW domain (WW), Transcriptional activation domain (TA) and PDZ binding motif.

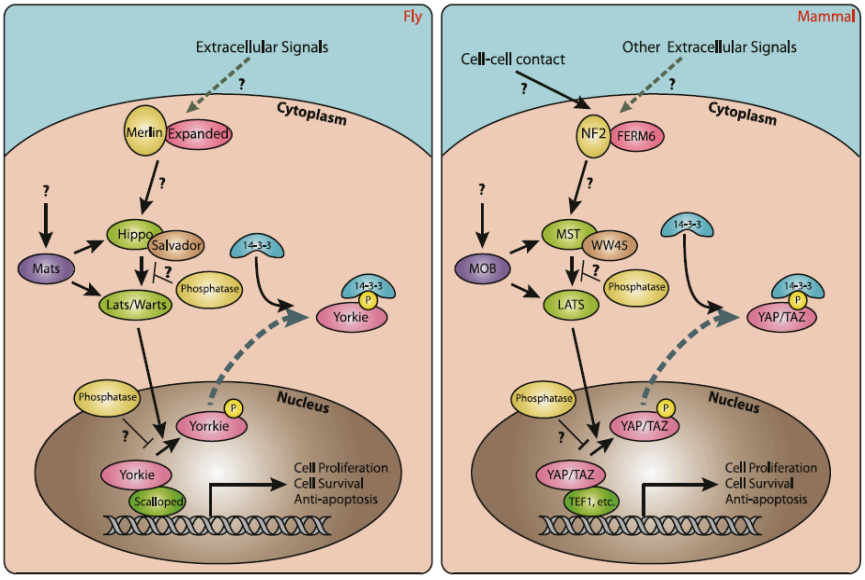
How extracellular cues are transduced to the nucleus is a fundamental issue in biology. In this context, TAZ constitutes a transducer linking cytoplasmic signaling events to transcriptional regulation in the nucleus and the 14-3-3 binding is the key to this regulation.

Interaction of TAZ and YAP (the paralog of TAZ) with the phosphoserine and phosphothreonine-binding proteins 14-3-3 has been known for many years (Kanai et al. 2000; Vassilev et al 2001; Basu et al. 2003). Hence, an important question is which kinases mediate the phosphorylation and link this event to dynamic signaling networks. Different groups have recently demonstrated that the fly Hippo pathway and the mammalian Hippo-like pathway play a key role in stimulating 14-3-3 interaction with the YAP/TAZ family of transcriptional coregulators.

The fly protein Hippo is a Ste20 protein kinase that interacts with and is activated by the WW-domain protein Salvador. Upstream from Hippo are different components, including Expanding and Merlyn and an atypical cadherin Fat, which localizes directly to the plasma membrane. This component might link Hippo to extracellular cues.

Downstream of Hippo are Warts/Lats and its activator Mats. Lats directly interact and phosphorilates Yorkie (the fly ortholog of YAP) and this phosphorylation stimulates 14-3-3 binding to Yorkie and its nuclear export. A series of regulators form a signaling cascade to promote 14-3-3 binding and cytoplasmic localization of Yorkie, thereby controlling its transcriptional coactivator role.

As illustrated in the figure below, these signaling regulators are conserved in mammals. The mammalian counterparts are FERM domain protein (FERM) 6, neurofibromatosis (NF) 2, the Hippo-like kinases MSTs, the regulatory protein WW45, LATS kinases, and Mob1. As shown for Yorkie, LATS kinases phosphorylate YAP and TAZ to promote their 14-3-3 binding and subsequent nuclear export, thereby placing YAP and TAZ downstream from the Hippo-like pathway in mammals. Phosphorylation by LATS is multisite, with five sites on YAP and four sites on TAZ. The four sites on TAZ are analogous to the C-terminal four sites on YAP and three of the analogous sites are conserved in Yorkie but only one is required for 14-3-3 binding, i.e. Ser89, in TAZ.



Comparison between Hippo pathway in fly (Drosophila melanogaster) and mammal.

Apart from the Hippo-like pathway, growth factors such as bone morphogenetic protein 2 (BMP-2) and fibroblast growth factor 2 (FGF-2) regulate TAZ expression and cell

differentiation. In addition, through its WW domain, TAZ interacts with polyomavirus T antigens and this interaction induces nuclear accumulation of TAZ but inhibits its transactivation activity suggesting that nuclear localization of TAZ does not always correlate with its transactivation activity, and may thus have other roles.

TAZ has been defined as a "molecular rheostat" that modulates Mesenchymal stem cells (MSCs) differentiation. Two key transcription factors, Runx2 (also called Cbfa1 or Pebp2aA) and PPARγ, drive MSCs to differentiate into either osteoblasts or adipocytes, respectively, and the differentiation of each lineage appears to be mutually exclusive and transcriptionally controlled. TAZ interacts both in vitro and in vivo with Runx2 and this interaction leads to Runx2-stimulated osteocalcin gene expression. On the other hand TAZ interacts both in vitro and in vivo also with PPARγ but, in this case, it has been demonstrated that TAZ directly inhibit the ability of PPARγ to stimulate gene expression of the endogenous fatty acid-binding protein aP2, both in the presence or absence of the PPARγ-activating ligand Rosiglitazone. All together, these are evidence suggesting that TAZ acts as a transcriptional modifier of mesenchymal stem cell differentiation by promoting osteoblast differentiation while simultaneously impairing adipocyte differentiation.

In the last years, a role of TAZ in the migration, invasion, and tumorigenesis of breast cancer cells has been outlined. In fact, the most highly invasive breast cancer cell lines express TAZ at levels that are approximately four times of those expressed by the majority of the weakly invasive breast cancer cells. The clinical relevance of this observation is supported by the finding that TAZ is overexpressed in a significant fraction of breast cancers (~21.4% of 126 commercially available breast cancer samples examined). Overexpression of TAZ in MCF10A cells (mammary epithelial cells) to a level ~2- to 3-fold of those detected in highly invasive cells caused a morphologic change from an epithelial to a fibroblast-like appearance an dramatically increased the migratory and invasive properties of the cells. Furthermore, shRNA-mediated knockdown of TAZ expression in MCF7 (human breast adenocarcinoma cell line) and Hs578T (non-tumorigenic mammary cells able to form colonies in semisolid medium) cells reduced cell migration and invasion. The epithelial clusters of MCF7 cells became more densely packed with cells when TAZ expression was knocked down. These results indicate that TAZ is a negative regulator of epithelial morphology/architecture, as well as a positive regulator for invasive and migratory behavior. It is conceivable

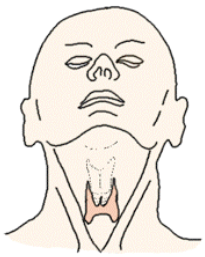
that TAZ overexpression in breast cancer may trigger the loss of epithelial property to promote migration. Moreover, when TAZ expression is knocked down in MCF7 cells, their anchorage-independent growth in soft agar and tumorigenesis in nude mice is retarded, suggesting that TAZ overexpression is an important part of the process involved in breast cancer development and progression.

Varelas et al. (Nature Cell Biology, 2008) described TAZ like a mediator of Smad nucleocytoplasmic shuttling that is essential for TGFβ signaling. In fact, although TGFβ-dependent R-Smad phosphorylation and heteromeric complex formation occur in the absence of TAZ, nuclear accumulation of Smad complexes is lost. This results in loss of TGFβ-dependent signaling, which in hESCs (human Embryonic Stem Cells) results in the failure to maintain self-renewal markers. By modulating TAZ localization they demonstrated that TAZ dominantly controls the subcellular localization of the Smads. They further showed that the Mediator complex component ARC105 binds to and can retain TAZ in the nucleus. As TAZ itself shuttles in and out of the nucleus, these results support the existence of a hierarchical system that regulates the localization of Smad2/3 in the cell.

To date TAZ knock-out mice have been described in four papers (Hossain et al. 2006; Tian et al. 2007; Lei et al. 2008; Mitani et al. 2009). As mentioned before, TAZ was reported as a modulator of mesenchimal stem cell differentiation. Despite this, the staining of embryonic day 17.5 (E17.5) $TAZ^{-/-}$ mice embryos with Alcian blue and Alizarin red to reveal cartilage and bone tissue respectively, showed only minor skeletal abnormalities and knock-out mice present just a slightly smaller stature than wild type. Overall, the most relevant phenotypes described in TAZ null mice are severe polycystic kidney disease and lung emphysema. Signs of histological anomalies in $TAZ^{-/-}$ kidneys were first apparent around E15.5 as dilations of the Bowman's space between visceral podocytes and the parietal cell layer of the Bowman's capsule. Pathological changes in $TAZ^{-/-}$ kidneys included parietal and tubular basement membrane thickening, thinning, and folding, and interstitial fibrosis and inflammation as evidenced by mononuclear leukocyte infiltration. TAZ-deficient mice have also emphysema-like features including enlarged air space and low elastance. These mice exhibit abundant inflammatory cells in bronchoalveolar lavage fluid, evidence of chronic inflammation in lungs, and some $TAZ^{-/-}$ lungs have small focal areas with inflammatory changes. Therefore, it looks like TAZ plays an important role in lung development, especially during alveolar stages.

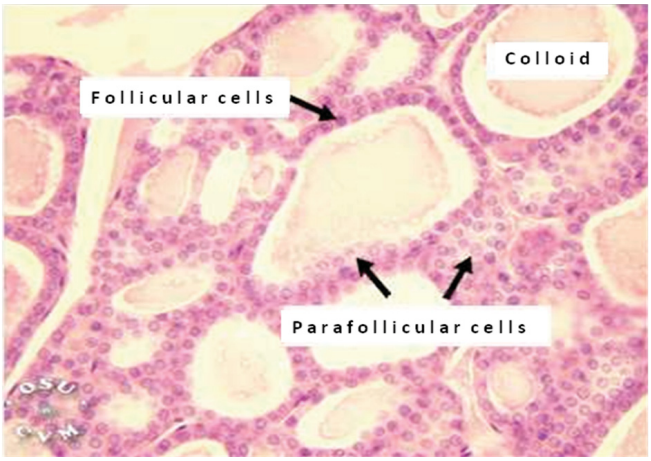
The Thyroid

The thyroid is one of the largest endocrine gland in the body and is composed of two cone-like lobes or wings: *lobus dexter* (right lobe) and *lobus sinister* (left lobe); and it is also connected with the isthmus. The thyroid gland is an organ situated on the anterior side of the neck and its name is due to its shield shape (from the greek *tireòs* = shield).



The thyroid gland (pink) is situated on the anterior side of the neck in front of trachea.

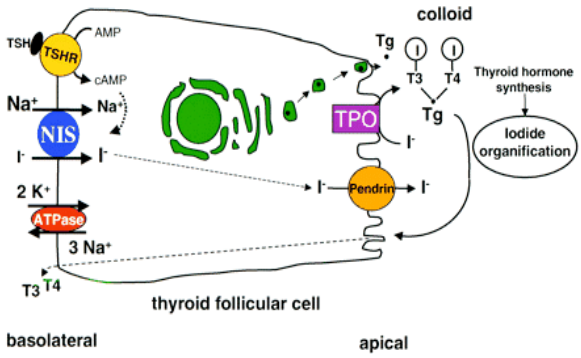
In a fully differentiated thyroid we can find two cellular types: the follicular cells and the parafollicular cells (De Felice and Di Lauro, 2004).



In the thyroid gland are well distinguishable Parafollicular cells and Follicular cells (filled with colloid).

The follicular cells produce thyroid hormones T3 and T4 while the parafollicular cells produce calcitonin. Thyroid hormones are synthesized into colloids by iodination and

condensation of thyroxin molecules bound to thyroglobulin (Tg) by a peptide bond. Tg is a glycoprotein that acts both like a substrate for the synthesis of thyroid hormones and as a storage of them. Via a reaction with the enzyme thyroperoxidase, iodine is covalently bound to tyrosine residues in Tg molecules, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroxine (T4) is produced by combining two molecules of DIT while triiodothyronine (T3) is produced by combining one molecule of MIT and one molecule of DIT.



Mechanism of thyroid gland functioning

Most of the thyroid hormones circulating in the blood is bound to transport proteins. Only a very small fraction of the circulating hormone is free (unbound) and biologically active, hence measuring concentrations of free thyroid hormones is of great diagnostic value. When thyroid hormone is bound, it is not active, so the amount of free T3/T4 is what really matters. For this reason, measuring total thyroxine in the blood can be misleading. T3 bind almost exclusively to Thyroxin Binding Globulin (TBG) while 80% of T4 bind to TBG, 15% to Thyroxin Binding Prealbumin (TBPA) and remaining 5% to seroalbumin.

It is likely that all the cells in the body are targets for thyroid hormones**.** While not strictly necessary for life, thyroid hormones have profound effects on many fundamental physiologic processes, such as development, growth and metabolism. Thyroid hormones stimulate diverse metabolic activities in a lot of tissues, leading to an increase in basal metabolic rate. One consequence of this activity is to increase body heat production, which seems to result, at least in part, from increased oxygen

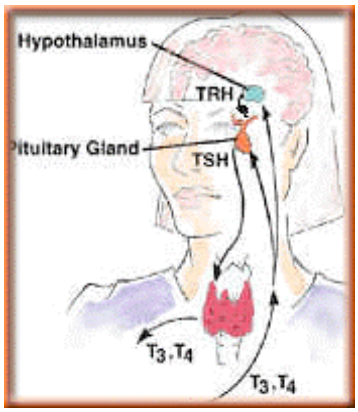
consumption and rates of ATP hydrolysis. Thyroid hormones regulate lipid metabolism; in fact, increased thyroid hormone levels stimulate fat mobilization, leading to increased concentrations of fatty acids in the plasma. They also enhance oxidation of fatty acids in many tissues. Finally, plasma concentrations of cholesterol and triglycerides are inversely correlated with thyroid hormone levels - one diagnostic indication of hypothyroidism is increased blood cholesterol concentration. Thyroid hormone play also a role in the carbohydrate metabolism. They stimulate almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis and glycogenolysis to generate free glucose.

Thyroid hormones are clearly necessary for normal growth in children and young animals, as evidenced by the growth-retardation observed in thyroid deficiency. Not surprisingly, the growth-promoting effect of thyroid hormones is intimately intertwined with that of growth hormone, a clear indication that complex physiologic processes like growth depend upon multiple endocrine controls. Also it is of critical importance in mammals the fact that normal levels of thyroid hormone are essential to the development of the fetal and neonatal brain.

Thyroid hormones also acts on the cardiovascular system where they increases heart rate, cardiac contractility and cardiac output. They also promote vasodilation, which leads to enhanced blood flow to many organs, like for example: a) the central nervous system, in which both decreased and increased concentrations of thyroid hormones lead to alterations in mental state (too little thyroid hormone, and the individual tends to feel mentally sluggish, while too much induces anxiety and nervousness); b) the reproductive system in which normal reproductive behavior and physiology is dependent on having essentially normal levels of thyroid hormone (hypothyroidism in particular is commonly associated with infertility).

The thyroid gland is under the control of the pituitary gland, a small gland the size of a peanut at the base of the brain. When the level of T3 and T4 drops too low, the pituitary gland produces Thyroid Stimulating Hormone (TSH) which stimulates the thyroid gland to produce more hormones. Under the influence of TSH, the thyroid will manufacture and secrete T3 and T4 thereby raising their blood levels. The pituitary senses this and responds by decreasing its TSH production. The pituitary gland itself is regulated by another gland, known as the hypothalamus (shown in light blue in the

picture). The hypothalamus is part of the brain and produces TSH Releasing Hormone (TRH) which tells the pituitary gland to stimulate the thyroid gland by releasing TSH.



A schematic representation of the axis Hypothalamus-Pituitary gland-Thyroid.

Thyroid-enriched transcription factors

TTF-1/Titf1, also named T-EBP or Nkx2.1, is a member of the Nkx-2 subfamily, originally identified as a protein able to bind to a DNA sequence that is present three times on both the Tg and the TPO promoters, and it is involved in the activation of thyroid-specific gene expression. TTF-1 is also expressed in the lung, and its presence is required for the transcriptional activation of surfactant proteins A, B, and C and Clara secretory protein. The critical role of TTF-1 for both thyroid and lung morphogenesis was clarified in TTF-1 null mice that resulted not viable and lacked the thyroid, the pituitary gland and the lung parenchyma. Deep study revealed that in these mice at the begin of the embryogenesis the thyroid primordium forms in its correct position, but subsequently, the thyroid precursor cells undergo apoptosis (Kimura et al., 1999) and disappear at E10.5–11.5. During mouse development TTF-1 expression starts at day E8.5 in endodermal cells that later will become the pharynx and its onset clashes with the proliferation of the cells of the endodermal layer. This event is the first step of the development of the thyroid.

Foxe1 is a member of FOX (Forkhead box) proteins which are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation. FOX proteins are able to bind DNA through the *forkhead* domain that is a domain common among several transcription factors involved in development and differentiation. In the mouse, Foxe1 expression starts at 8 dpc into the endodermal cells of the pharynx and it is more evident in the posterior area of the pharynx, where TTF-1 is not expressed. Hence, this expression pattern suggests that Foxe1 has a less important roles during the first steps of development whereas it's very important for the migration of the thyroid gland during embryogenesis. In fact the analysis of Foxe1 knock-out mice revealed that thyroid starts to develop normally but, at day E10.5, the thyroid primordium fails to migrate towards its correct position in front of the trachea attached to pharyngeal endoderm suggesting that Foxe1 might be important during the migration of the thyroid during its development, probably regulating the expression of genes involved in cellular migration.

Pax8 is a member of the Pax gene family, a family of genes that encode for DNA-binding proteins involved in the regulation of the development of a variety of tissues in different species. The Pax gene family is composed of nine proteins highly conserved in vertebrates and all these proteins are characterized by the presence of the *paired box*. This specific sequence encodes for the *paired domain*, a very important domain for recognizing the target sequences on DNA. The *paired domain* is composed of 128 amino acids and is subdivided in two fundamental subdomains, one at C-terminus the other at N-terminus, called *pai* and *red,* respectively. Each of these domains contain an *helix-turn-helix* motif and both domains are united by a *linker* region. We can explain the subdivision of the *paired domain* into two independent domains with the ability of Pax proteins family to bind DNA in different ways and overall to recognize a lot of sequences. Within the omology rate of the *paired domains* we can subdivide the nine Pax proteins in different groups. One of these groups is composed by Pax2, Pax5 and Pax8. These three proteins are characterized also by the presence of an octapeptide and a partial omeodomain.

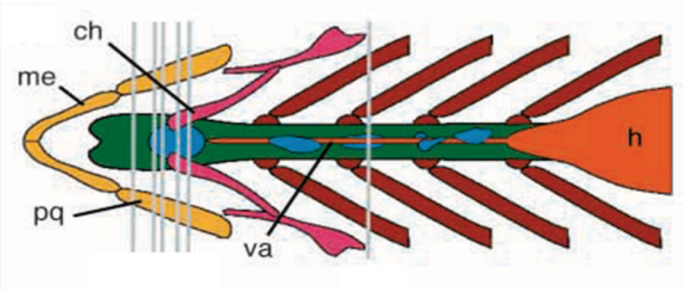
During embryonic development Pax8 is expressed in thyroid, kidney and the nervous tissue. Pax8 has been demonstrated to be required both for the morphogenesis of the thyroid gland and for the maintenance of the thyroid-differentiated phenotype. In Pax8 null mouse embryos initial phases of organogenesis are normal but, at E11.5, the

thyroid primordium appears much smaller than in wild type embryos and at E12 the follicular cells are almost undetectable (Mansouri et al., 1998). Although the relevance of the role of Pax proteins is becoming more and more clear, the molecular mechanisms of their action are still poorly understood.

Thyroid development in Zebrafish

In lower vertebrates such as fish, amphibia, and birds, thyroid follicular cells and C-cells are found in separate organs. Thyroid follicular cells forms 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).

In teleost fish, such as Danio rerio, the thyroid gland differs from that in other organisms. Here, follicle tissue is loosely dispersed along the ventral midline of the pharyngeal mesenchyme, thus, is not encapsulated by connective tissue (Raine and Leatherland 2000; Raine et al. 2001; Wendl et al. 2002).



Schematic drawing of a zebrafish larval head of 4.5 to 6 dpf, ventral view, showing the skeleton, parts of the circulatory system and the thyroid. Blue, thyroid/T4 immunostaining; brown, third to sixth branchial arches; green, basibranchial cartilage; magenta, second branchial arch; orange, heart and ventral aorta; yellow, first branchial arch. ch, ceratohyale; h, heart; me, Meckel's cartilage; pq, palatoquadrate; va, ventral aorta.

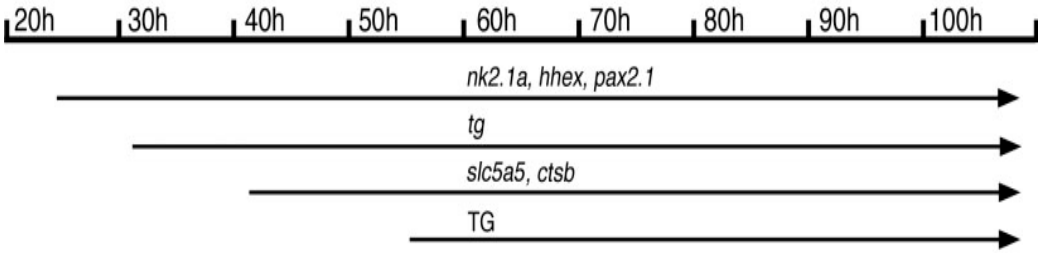
Initial molecular studies suggest that the mechanisms of zebrafish thyroid development are generally comparable to that of human thyroid development (Wendl et al. 2002),

hence zebrafish can be useful as a model organism in further studies that aim at the understanding of the molecular basis of vertebrate thyroid development.

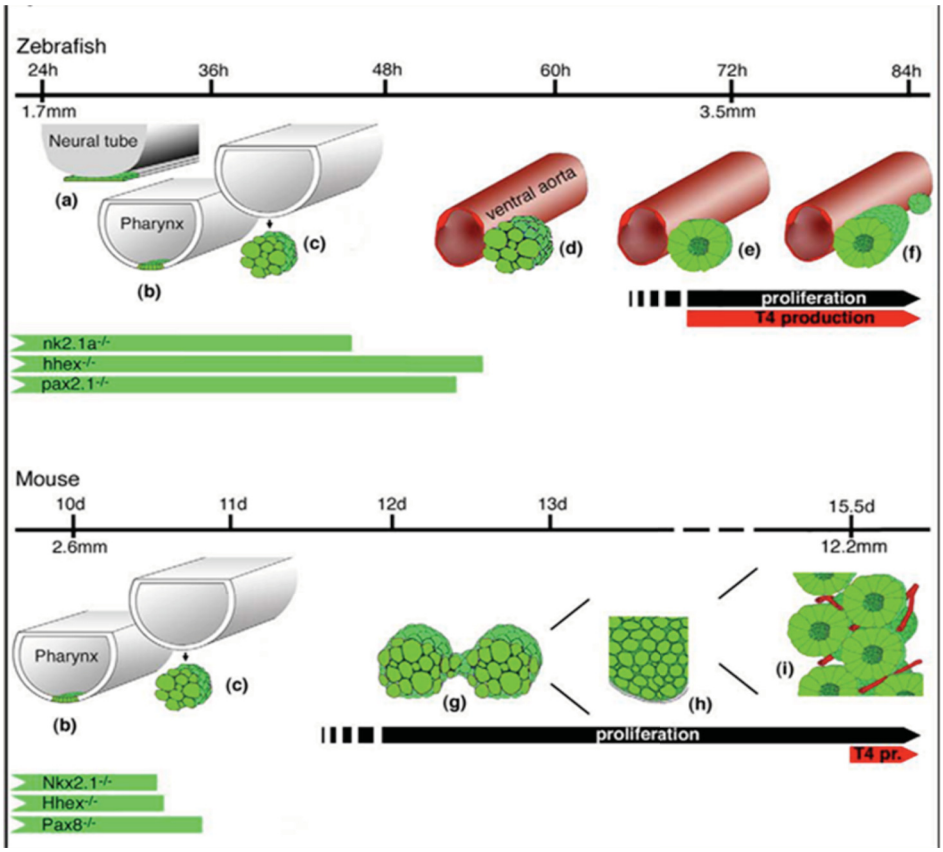
The expression of the thyroglobulin gene in zebrafish is first observed exclusively in the thyroid primordium at approximately 32 hpf whereas, by an antibody against human thyroglobulin (TG), a first signal can be observed as a single small follicle at around 55 hpf. During larval development, more follicles arise, aligning at the pharyngeal midline. This pattern of immunostaining during follicular development is identical to that observed using an antibody against the thyroid hormone T4.

The analysis of zebrafish mutants that doesn't produce TSH reveals that the absence of what is considered, at least in mice and humans, the most important growth factor for the differentiated thyroid, doesn't affect thyroid hormone production or growth of follicles in zebrafish.

The comparison of zebrafish and mouse reveals a different temporal progression of thyroid development in these species. The early appearance of thyroid markers before pharynx formation may be adaptation to fast development of zebrafish embryos. After 4–6 days, when larvae have used up their yolk sac containing maternal thyroid hormone, they rely on a functional thyroid gland. At this stage, the follicles are proliferating as the larva and juvenile fish grow. Mouse embryos, in contrast, are supplied with maternal thyroid hormone up to birth, and after initial specification, 5 days of cell proliferation occur prior to final thyroid differentiation and onset of function. Despite a different time scale of thyroid development in zebrafish and mice, nk2.1a/Nkx2.1, and pax2.1/Pax8 act presumably in the same manner during differentiation of the thyroid gland.



The arrows indicate the onset of developmental gene expression (nk2.1, hhex, pax2.1) and differentiation markers (slc5a5, ctsb and tg).



Comparison between thyroid development in zebrafish and mouse

Three additional aspects of thyroid development in mice imply differences in the genetic networks that direct thyroid development compared with fish. First, the thyroid primordium in mice and humans bifurcates during growth. Second, in all vertebrates apart from most teleosts, the primordium becomes encapsuled by connective tissue. Third, in mammals neural crest-derived C-cells merge with the developing follicular cells, and indeed, these cells can form a thyroid rudiment, even in the absence of thyroid follicular cells (Mansouri et al. 1998). Moreover, it has been described how the correct development of the blood vessels and a right vasculature architecture are essential for the right positioning of thyroid follicles both in zebrafish (Burkhard et al. 2006) and in mouse (Fagman et al. 2004)

In this thesis we demonstrate that, in zebrafish, the correct positioning of thyroid follicles is affected by TAZ, likely because the lack of TAZ alters the development of the vasculature architecture.

Aim of the project

Transcriptional regulation is a fundamental process for cellular differentiation and development. In an adult organism differentiated cells are characterized by the expression of a specific subset of genes and this expression is regulated mainly at the transcriptional level. A good model for studying transcriptional regulation is represented by thyroid follicular cells that express a very specific set of genes, called differentiation markers, that are thyroglobulin (TG), natrium-iodium symporter (NIS), thyroperoxidase (TPO) and TSH receptor (TSHr).

Perhaps, the most important transcription factor for the differentiation and the development of follicular cells is Pax8 (Pasca di Magliano et al. 2000). It has also been described the interaction between Pax8 and another important transcriptional factor in thyroid development, TTF-1. Pax8 interacts with TTF-1 and these two factors are able to synergistically activate the transcription of the thyroglobulin gene (Di Palma et al. 2003). Moreover, Pax8 and TTF-1 are part of an heterocomplex that has an apparent molecular mass greater than the sum of the two factors suggesting the presence of other proteins.

In these last year different laboratories demonstrated that the Transcriptional co-Activator with PDZ-binding motif (TAZ) is involved in several pathways: from cell migration to proliferation, from molecular "rheostat" in Mesenchimal Stem Cell to promoter of invasion in breast cancer cells. Moreover, the recently demonstrated physical and functional interaction between TAZ and TTF-1 in the lung raised the question of whether TAZ could be an important regulatory molecule also in the thyroid.

For this reason, we decided to study whether TAZ is a co-activator of TTF-1 and Pax8 in thyroid as well and, in parallel, we decided also to investigate in vivo the role of TAZ in thyroid development and differentiation.

RESULTS

TAZ functions as a transcriptional coactivator for the transcription factors Pax8 and TTF-1

Protein-protein interaction is a very important event in the regulation of gene expression. Transcription factors and coactivators do not act all alone rather they assemble in multiprotein complexes, and often they are expressed in several tissues while the combination of different factors is unique for a specific cell type.

Previous studies reported that Pax8 and TTF-1 are able to form a heterocomplex corresponding to an apparent molecular mass greater than the sum of the two factors. This observation prompted us to look for the presence of other proteins participating to this heterocomplex and the coactivator TAZ appeared a potential interesting candidate. We analyzed cellular lysates from PC Cl3 thyroid cells by size exclusion chromatography (SEC) followed by Western blot. We detected Pax8, TTF-1 and TAZ in fractions corresponding to an apparent molecular mass ranging between 34–78 kDa thus indicating the presence in these fractions of the three monomers. More interestingly, the three proteins were detected also in fractions corresponding to a higher apparent molecular mass of about 177-440 kDa. This data strengthened our hypothesis of a possible interaction between TAZ and the two transcription factors in thyroid cells (Fig. 1A).

In order to investigate the physical interaction between Pax8 and TAZ, and between TTF-1 and TAZ, recombinant GST-TAZ was purified from bacteria and used in pull down experiments with total protein extract prepared from PC Cl3 thyroid cells. The result of the pull down experiments showed that both Pax8 and TTF-1 proteins are specifically bound by GST-TAZ and not by the GST protein (Figs. 1B, C). To further investigate the possibility of a direct interaction between TAZ and the two transcription factors, pull down experiments were performed using the fusion protein GSTTAZ and bacterial Pax8 and TTF-1 proteins affinity-purified. Results of the binding reactions showed that GST-TAZ is indeed able to coprecipitate bacterial Pax8 and TTF-1,

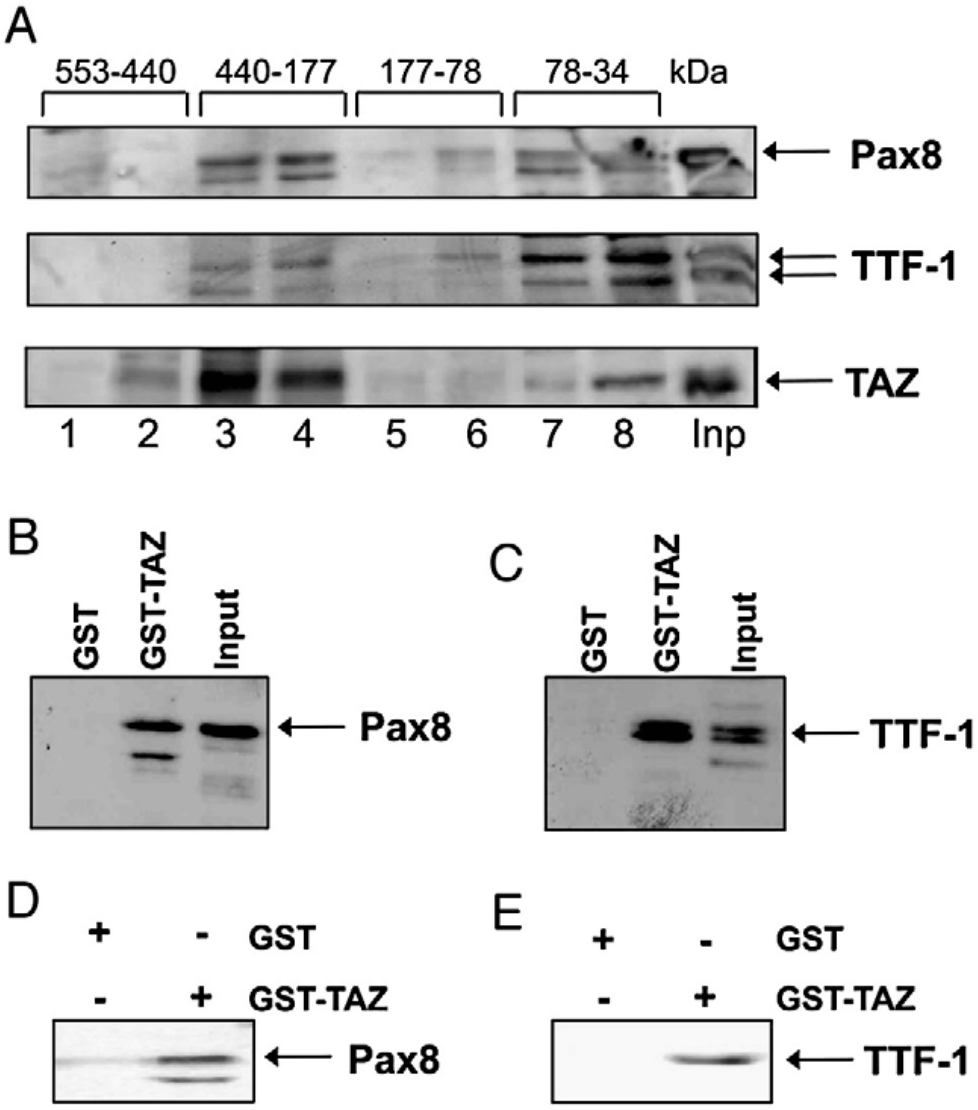


FIG.1: (A) SEC analysis shows TTF1/PAX8/TAZ in the same fractions suggesting interaction between the three proteins that was confirmed by GST pull-down assay with GST-TAZ and protein extracts prepared from PC Cl3 cells (B, C) or full-length purified bacterial Pax8 (D) or TTF-1 (E).

whereas GST alone does not (Figs. 1D, E). These results demonstrate that the interaction between TAZ and Pax8 or TTF-1 already observed in pull down assays with total protein extracts, is indeed a direct protein-protein interaction.

To determine whether the associations observed in vitro could take place also in vivo, HeLa cells were transfected with an expression vector encoding TAZ, alone or in combination with expression vectors encoding either 3xFLAG-Pax8 or 3xFLAG-TTF-1.

Anti-FLAG-agarose affinity gel was used to immunoprecipitate 3xFLAG-Pax8 or 3xFLAG-TTF-1 proteins from total extracts prepared from HeLa cells transiently transfected. Subsequently, the bound proteins were subjected to Western Blot analysis after separation on SDS-PAGE. Western blot developed with a specific anti-TAZ polyclonal antibody showed the presence of TAZ coimmunoprecipitated protein in the presence of either 3xFLAGPax8 or 3xFLAG-TTF-1, thus proving that the association between TAZ and the two transcription factors occurs in vivo (Figs. 2A, B). To further confirm the in vivo association in the thyroid cell environment, we performed the same co-immunoprecipitation experiment using PC Cl3 thyroid cells stably expressing 3xFLAG-Pax8. As shown in Fig. 2C, together with Pax8 also TTF-1 and TAZ proteins are immunoprecipitated.

It was previously reported that in transient transfection assays in HeLa cells, Pax8 is able to activate transcription from a reporter construct named CP5-CAT in which its binding site is present five times in tandem upstream the CAT reporter gene. To investigate whether the physical interaction between Pax8 and TAZ could lead to an increase in Pax8 transcriptional activity, we performed transactivation assays in HeLa cells co-transfecting the reporter construct CP5-CAT together with the expression vectors encoding Pax8 and TAZ, separately or in combination. As expected, the transfection of Pax8 alone leads to a significant activation of the CP5-CAT promoter, while the transfection of TAZ alone has no effect. Interestingly, the co-expression of Pax8 and TAZ leads to a potent enhancement of the transcriptional activity of Pax8 on the CP5-CAT promoter demonstrating that the biochemical interaction between Pax8 and TAZ results in a major functional cooperation between these two proteins (Fig. 3A). In parallel, we also investigated whether the physical interaction between

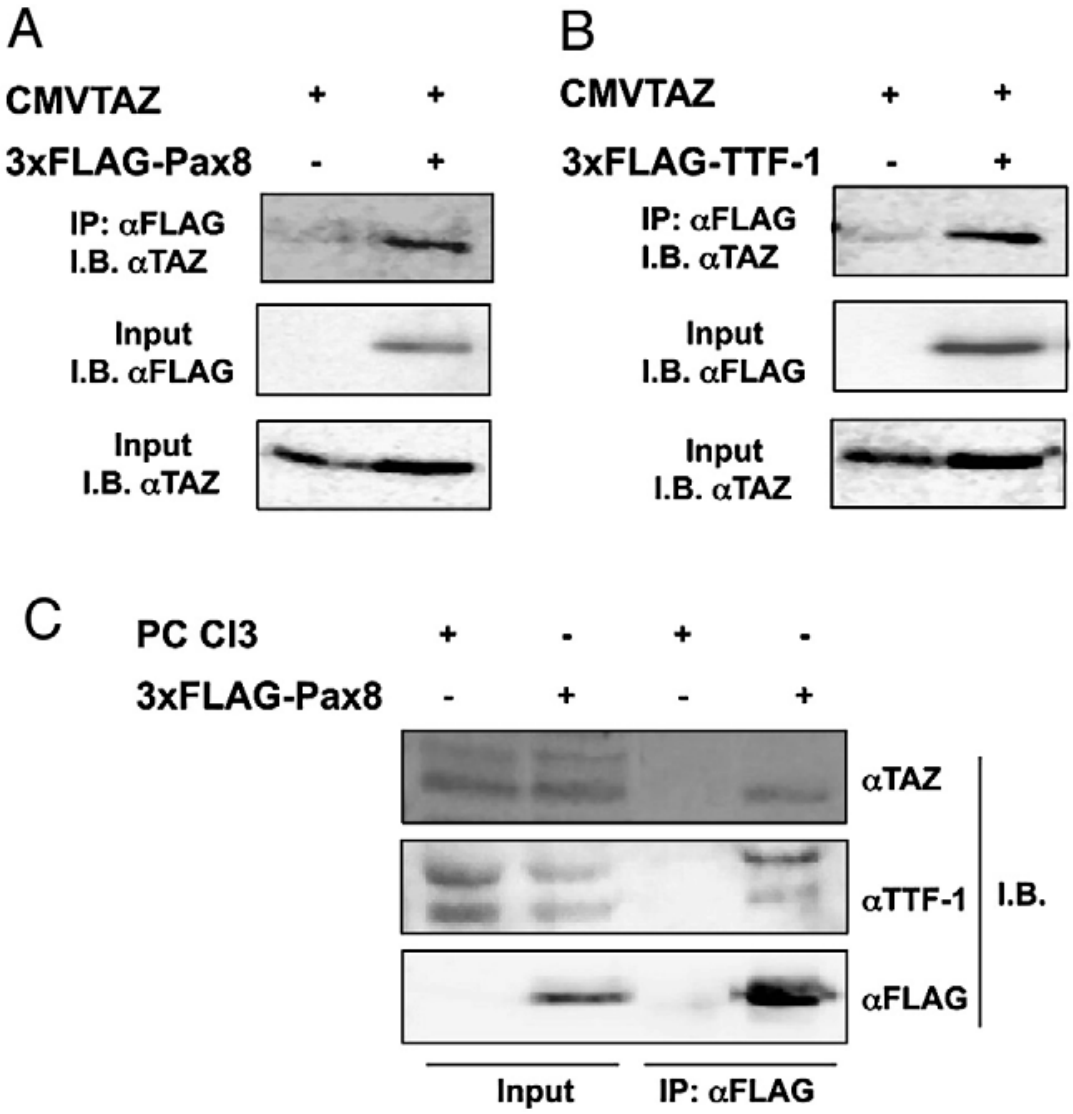


FIG.2: in vivo interaction of TAZ with both PAX8 and TTF1 revealed by immunoprecipitation in HeLa cells transiently transfected with 3xFLAG-PAX8 (A), 3xFLAG-TTF-1 (B) and in PCCl3 cells stably expressing 3xFLAG-PAX8.

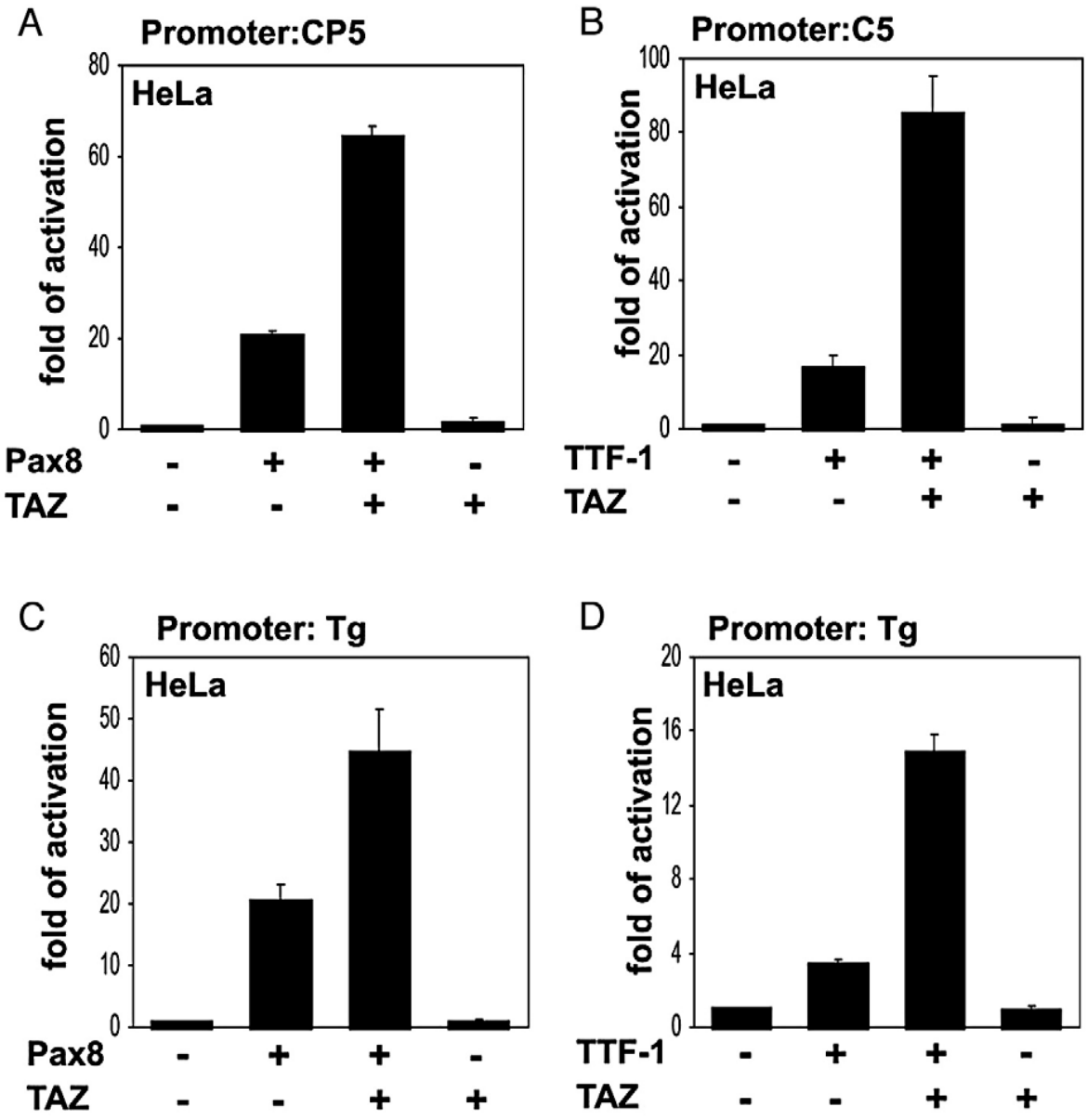


FIG.3: TAZ enhances Pax8 and TTF-1 activity on the CP5, C5 (A-B) and Tg promoters (C-D).

TTF-1 and TAZ could lead to a functional cooperation, as seen for Pax8. To this end, we performed transactivation experiments using the reporter construct C5-CAT in which the TTF-1 binding site is present five times in tandem upstream the CAT reporter gene. The results showed that the transfection of TTF-1 alone leads to the activation of the C5-CAT promoter as expected, and the co-expression of TAZ strongly potentiates TTF-1 transcriptional activation as it happened with Pax8 (Fig. 3B). Thus, our data validate that also the biochemical interaction between TTF-1 and TAZ has a major functional outcome. Our laboratory has previously reported that Pax8 and TTF-1 are able to significantly activate transcription from the thyroglobulin (Tg) promoter, and that the two transcription factors display a strong synergism in the transcriptional regulation of this promoter (Di Palma T., et al. 2003). We now asked whether TAZ could potentiate Pax8 and TTF-1 transcriptional activity not only on synthetic promoters such as the CP5-CAT and the C5-CAT ones, but also in a more physiological context represented by the Tg promoter. To this end, we co-transfected in HeLa cells the reporter construct Tg-CAT together with the expression vectors encoding for Pax8, TTF-1 and TAZ, separately or in combination. Also in these experiments, as seen for the CP5-CAT and the C5-CAT promoters, we observed a significant increase in the transcriptional activation of the Tg promoter by Pax8 and TTF-1 in the presence of TAZ (Figs. 3C, D). These results are consistent with our hypothesis that TAZ may act as a critical transcriptional coactivator of thyroid transcription factors and therefore may be involved in thyroid differentiation.

TAZ expression profile during mouse embryonic development

The morphogenesis of the thyroid, like in many endoderm-derived organs, begins with an event that recruits a group of cells, in an otherwise homogeneous cell layer, to the thyroid fate. This event is called, in developmental biology terms, “specification” or “determination” because, as a consequence of it, a group of cells are specified or determined to undertake a definite developmental program.

The first visible change is the thickening of the endodermal epithelium in the foregut, which is referred to as thyroid anlage. The thyroid anlage is first identified in the mouse embryos at E8.5 in the ventral wall of the primitive pharynx, caudal to the region of the

first branchial arch. These small number of cells are univocally characterized by the simultaneous expression of the transcription factors Titf1/Nkx2-1, Foxe1, Pax8, and Hhex. This median thickening deepens, forms first a small pit (thyroid bud, E8.5–E9) and then an outpouching of the endoderm, which is adjacent to the distal part of the outflow tract of the developing heart. An influence of the developing heart on thyroid organogenesis has not been demonstrated, although recent data suggest that there is a correlation between the development of heart and thyroid. Interestingly, cardiac malformations represent the most frequent birth defects associated with TD (thyroid dysgenesis). At E9–E9.5 the endodermal cells of the thyroid anlage form the thyroid bud by proliferation and invasion of the surrounding mesenchyme. By E10, the thyroid primordium appears as a flask-like structure with a narrow neck that rapidly becomes a diverticulum. A small hole at the site of origin in the pharyngeal floor (the foramen cecum) is the remnant of the anlage, connected with the migrating thyroid primordium by a narrow channel (the thyroglossal duct). At E11.5 the thyroglossal duct disappears, and the thyroid primordium loses its connections with the floor of the pharynx and begins to expand laterally. Two days later the thyroid primordium reaches the trachea, which has extended ventrocaudally starting from the primitive laryngotracheal groove. By E15–E16 the thyroid lobes expand considerably, and the gland exhibits its definitive shape: two lobes connected by a narrow isthmus. Genes typical of this stage appear according to a given temporal pattern: Tg, TPO, and TSH receptor (Tshr) genes are expressed by E14.5 (35); sodium/iodide symporter (NIS) is detected by E16 (26). T4 is first detected at E16.5.

By in situ hybridization, we analyzed several embryonic stages between 8.0 and 15.5 days post coitum (dpc), and the adult thyroid. We show that TAZ starts to be expressed at 8.5 dpc in the somites and in the hindbrain (Fig. 4A) and at 9.0 dpc TAZ mRNA is also detectable in the forebrain (Fig. 4B). At both 9.5 (Fig. 4C) and 10.5 dpc (Fig. 4D) TAZ continues to be expressed in the forebrain, hindbrain and somites; however, no expression could be observed in the thyroid anlage (Figs. 4E, F). On the contrary, at the same stages, Pax8 expression is clearly detectable in the thyroid primordium (Figs. 4 G, H). Later on, at 13.5 dpc, when the thyroid completes its migration and reaches its final destination at the base of the neck, TAZ is still not expressed in the thyroid gland (Fig. 4I). Only at 14.5 dpc, hence rather late with respect to Pax8 (Figs. 4J, K) and TTF-1/Titf1, TAZ starts to be expressed in the thyroid (Fig. 4L) and, like for Pax8 and

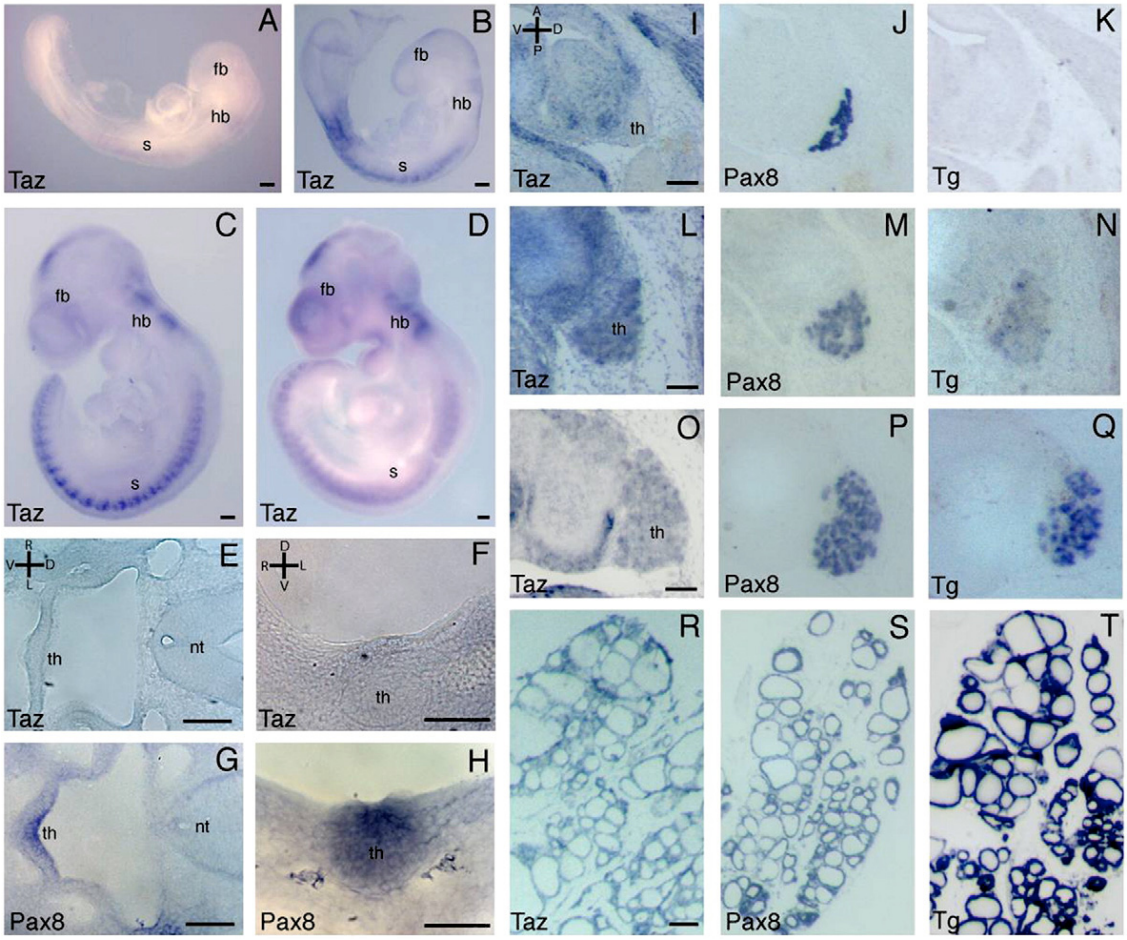


FIG.4: TAZ expression profile during mouse embryo development. (A–D) Whole mount in situ hybridization with TAZ specific probes at 8.5. (E–H) Transverse sections at the level of the thyroid primordium of 9.5. (I–T) In situ hybridization on sagittal sections at the level of the thyroid primordium of 13.5. Abbreviations: A, anterior; D, dorsal; fb, forebrain; hb, hindbrain; L, left; nt, neural tube; P, posterior; R, right; s, somites; th, thyroid; V, ventral. Scale bars represent 100 μm.

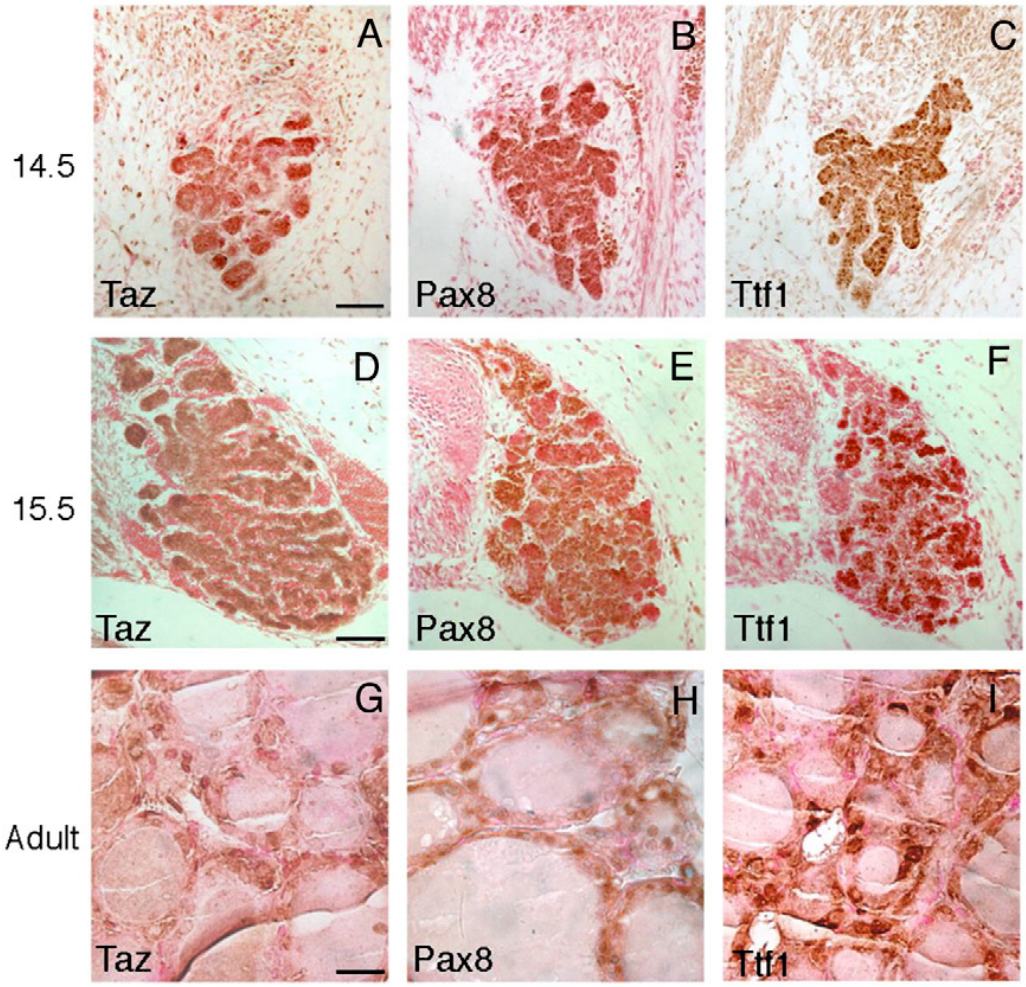


FIG.5: Cellular distribution of TAZ, Pax8 and TTF-1 proteins during thyroid development and in the adult. Immunohistochemistry with anti-TAZ (A, D and G), anti-Pax8 (B, E and H) and anti-TTF-1 (C, F and I) specific antibodies on parasagittal sections of 14.5 days post coitum (dpc) embryos (A–C), on sagittal sections of 15.5 dpc embryos (D–F) and in the adult (G–I).

TTF-1/Titf1, its expression persists at 15.5 dpc (Fig. 4O) and in the adult (Fig. 4R). Immunohistochemistry experiments were also performed to investigate the cellular distribution pattern of the TAZ protein in developing and adult mouse thyroid. As shown in Fig. 5, TAZ immunoreactivity was detected primarily in the nuclei of thyroid follicular cells both in the forming bud and in the adult gland. These results confirm the data observed in the cells (Figs. 5C, D) and indicate that indeed TAZ colocalizes with Pax8 and TTF-1 in vivo in the nucleus of thyroid follicular cells.

Analysis of TAZ knock-out mice

The TAZ locus was targeted in mouse ES cell lines with a β-gal gene (lacZ) knock-in targeting vector, placing the lacZ gene into the second exon immediately downstream of the initiation ATG of the TAZ gene. This strategy results in a TAZ-null mutation with the expression of β-gal under the control of the endogenous transcriptional regulatory elements of TAZ. This mouse was generated in the laboratory of Prof. Walter Hunziker at the Institute of Molecular and Cell Biology (Republic of Singapore) We established a collaboration with him and we obtained four TAZ $^{-/+}$ mice with the aim to spread the line and to analyze the thyroid in TAZ $^{-/-}$ mice. We crossed TAZ $^{-/+}$ mice for several generations but we were not able to obtain viable TAZ $^{-/-}$ mice (Table 1). This result led us to hypothesize a possible embryonic lethality of TAZ $^{-/-}$ mice and for this reason we reasoned that it might be useful to sample embryo at E15.5. Unfortunately, we genotyped 77 embryos and we obtained only 4 TAZ $^{-/-}$ (Table 1). To solve this problem we are actually backcrossing TAZ $^{-/+}$ mice from the sv129 background to the c57bl6 one, hoping that changing the strain could reduce the severity of the phenotype.

	+/+ (25%)	+/- (50%)	-/- (25%)
Adult	137 (53.1%)	121 (46.9%)	0
E15.5	31 (40.2%)	42 (54.5%)	4 (5.2%)

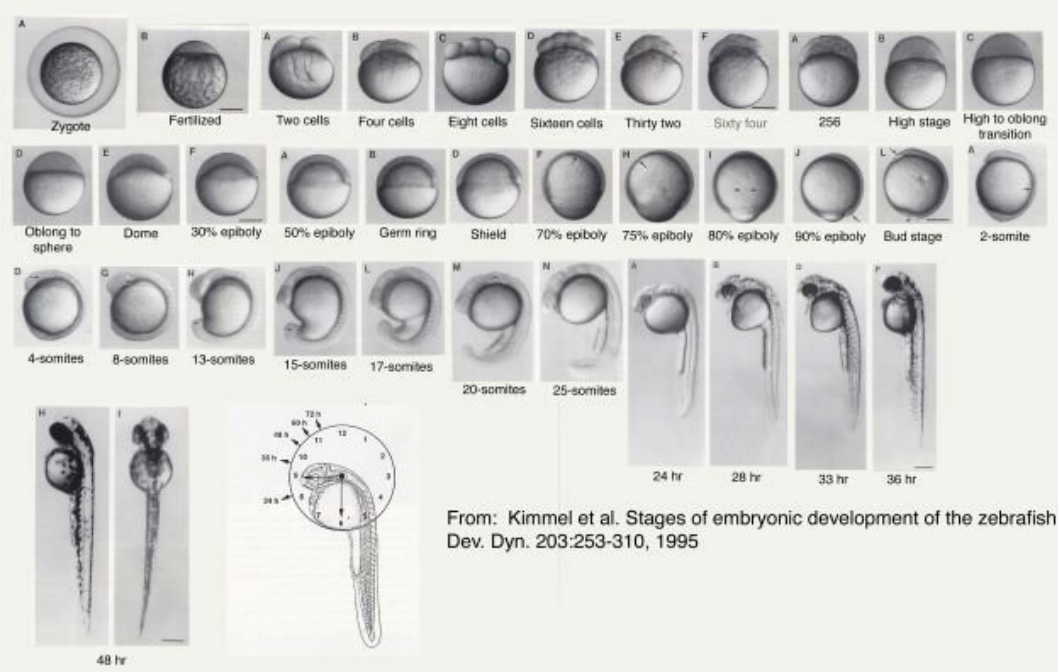
Table 1: numbers (and percentages) of adult and embryos mice genotyped compared with (in white) percentage expected

Zebrafish (Danio rerio) - Introduction to the system -

Zebrafish (Danio rerio) comes close to being the ideal model organism for vertebrate development because it appears to combine the best features of all the other models. Like the frog, zebrafish embryos develop externally and can be viewed and manipulated at all stages. However, zebrafish development is more rapid than in the frog, the organization of the embryo is simpler and (like worms and fruit flies) the embryo is transparent. Like the mouse, zebrafish is amenable to genetic analysis and has a similar generation interval (2-3 months). However, zebrafish are smaller than mice and they produce more offspring in a shorter time. A female zebrafish can lay up to 200 eggs per week, while a mouse may produce a litter of up to 15 embryos in 21 days. Furthermore, it is easy to induce new mutations in zebrafish and large-scale screens have been carried out to identify mutations causing defects in particular biological processes. The technology for gene transfer to zebrafish is highly advanced.

These advantages have allowed the creation of dense genetic maps in zebrafish, which have been useful for the comparative mapping of human genes. There is extensive similarity between the zebrafish and human genomes so many human developmental and disease genes have counterparts in zebrafish. The zebrafish genome is 1700 million base pairs in length, about half the size of the human genome.

A number of zebrafish mutants have been produced that are good models of human diseases, and can therefore be used to test candidate drugs. These include models of Alzheimer's disease, congenital heart disease, polycystic kidney disease and cancer.



TAZ expression in zebrafish

The zebrafish thyroid primordium starts to develop at the outflow tract of the heart, which directly leads into the first primordial branch of pharyngeal arteries. From about 40 hours post fertilization (hpf), the globular primordium expands along the anteroposterior (AP) axis into a strand of follicular tissue, thereby always remaining restricted to the midline.

Of course, our first aim was to demonstrate that TAZ is expressed in the thyroid of zebrafish. To this purpose we performed in situ hybridization with a probe recognizing the entire mRNA of TAZ and we did this experiment at different stages of development.

We observed that TAZ onset in the thyroid is at 48 hpf. At this stage TAZ is expressed also in fins, lens, notochord, otic vescicles, in the pronephric duct, in the pharyngeal arches and into the heart (Fig.6).

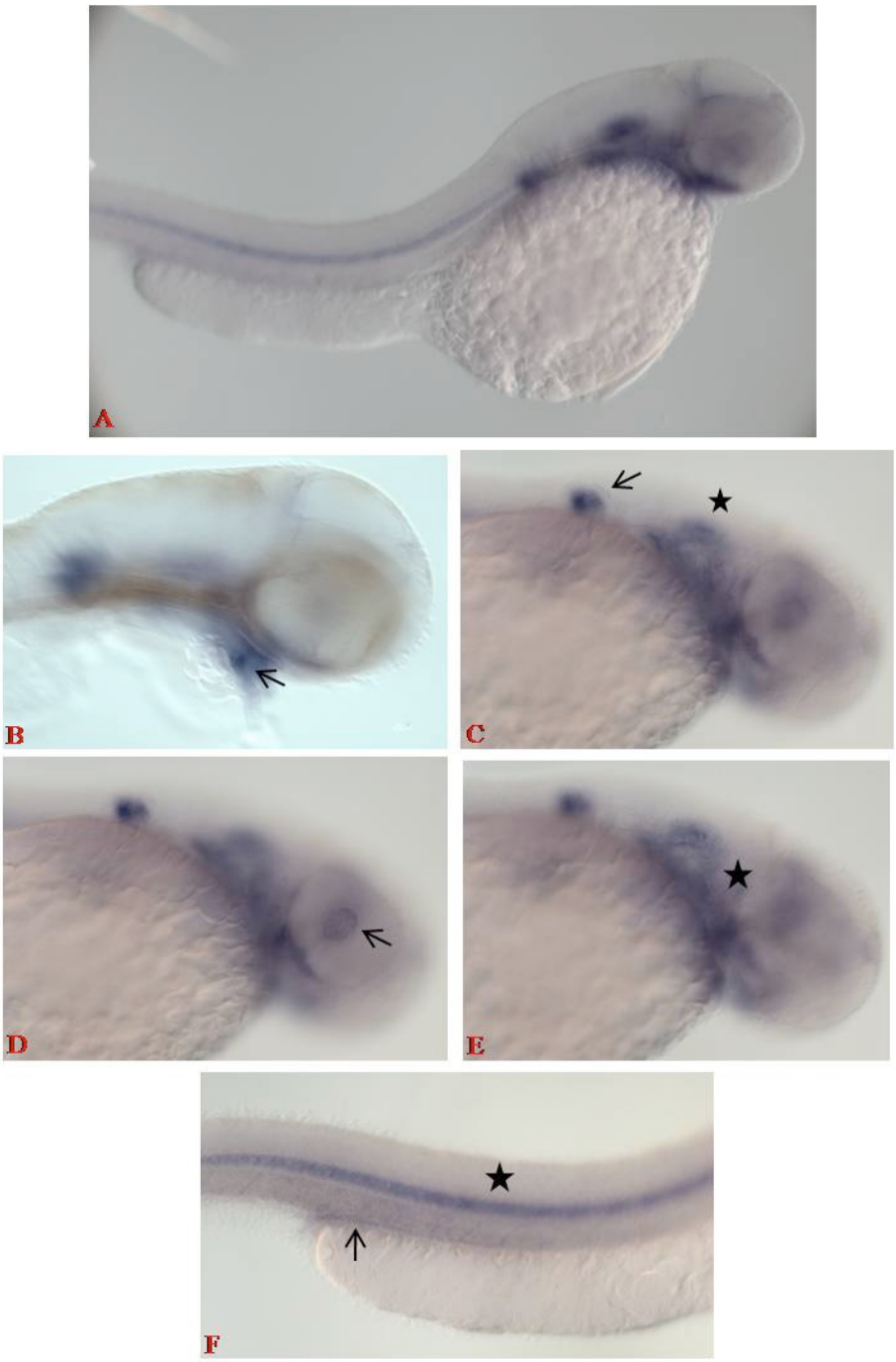


FIG.6: Expression pattern of TAZ at 48 hpf. In particular TAZ is expressed in the thyroid (B), in fins (C -cross-), otic vescicle (C -star-), lens (D), pharyngeal arches (E), notochord (F -star-) and in the pronephric duct (F -cross-).

Use of morpholino oligos to interfere with TAZ expression

The impossibility to obtain TAZ $^{-/-}$ null mice forced us to think at different strategies for studying the TAZ knock-out phenotype. In recent years, morpholino antisense oligonucleotides have been widely used to modify gene expression, sometimes when the knock-out phenotype in mice is lethal and also because the generation of a knock-out by using morpholino is cheaper and takes less time than the production of a knock-out mouse by homologous recombination.

Microinjection or electroporation of morpholino oligos into the embryos of frogs, zebrafish, chicks, sea urchins and other organisms successfully and specifically shuts down the expression of targeted genes, making morpholinos a useful tool for developmental biologists.

Morpholino oligos are short chains of about 25 morpholino subunits. Each subunit is comprised of a nucleic acid base, a morpholine ring and a non-ionic phosphorodiamidate intersubunit linkage. Morpholinos do not degrade their RNA targets, but instead act via an RNAse H-independent steric blocking mechanism. With their requirement for greater complementarity with their target RNAs, morpholinos are free of the widespread off-target expression modulation typical of knockdowns which rely on RISC or RNase-H activity. Depending on the oligo sequence selected, morpholinos either can block translation initiation in the cytosol (by targeting the 5' UTR through the first 25 bases of coding sequence), can modify pre-mRNA splicing in the nucleus (by targeting splice junctions or splice regulatory sites).

For our experiments we designed oligo morpholino against the ATG of the TAZ gene.

We microinjected 1ng of morpholino in zebrafish embryos at 1 to 2 cells stage. Until 40 hpf TAZ morphants appear normal and they are indistinguishable if compared to control embryos not microinjected. From 40 hpf TAZ morphants develop heart edema, ventral curvature and their head is smaller and abnormally shaped. (Fig.7)

The thyroid gland in TAZ morphants is fully differentiated

Four transcription factors have been shown to play crucial roles in the development of thyroid follicular cell precursors in mammals. TTF-1/Nkx2.1, Foxe1, Hhex and Pax8

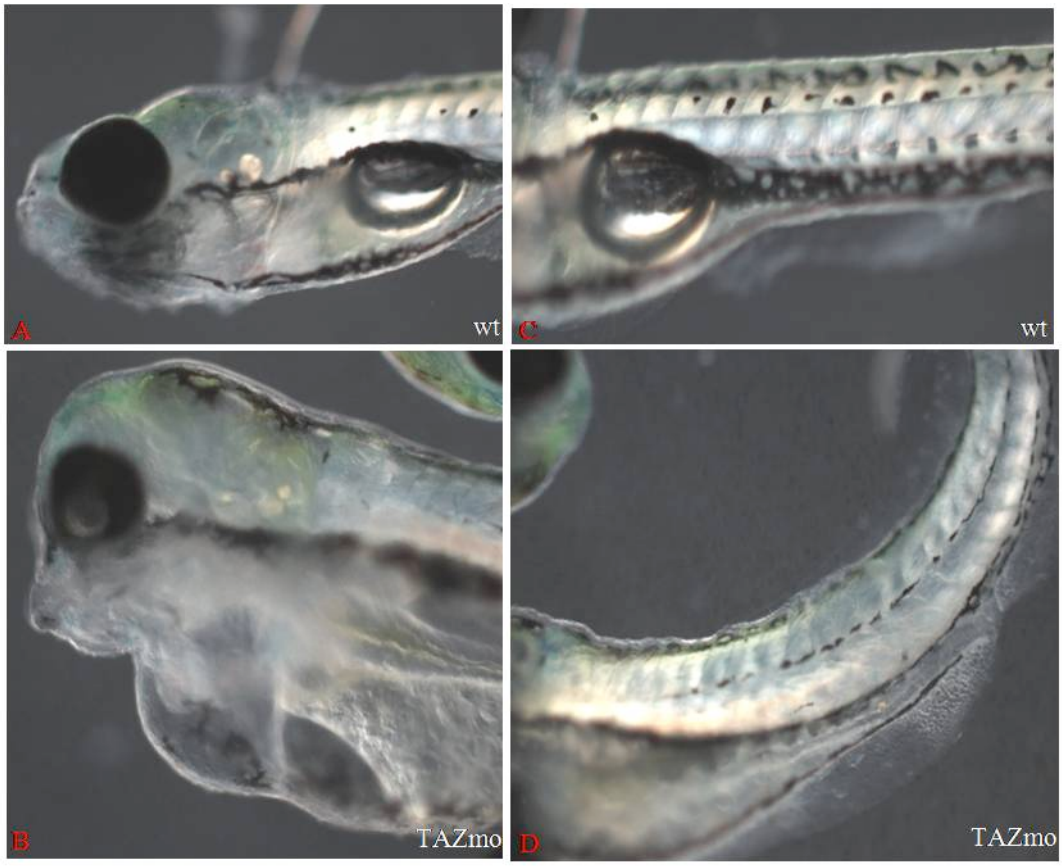


FIG.7: TAZ morphants are characterized by heart edema (B), smaller and abnormally shaped head (B), ventral curvature (D) and defect in pronephric duct (D)

are expressed during thyroid development in mice, and the 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). In zebrafish, nk2.1a, hhex, and pax8 are also expressed in the developing thyroid (Rohr and Concha, 2000; Wendl et al., 2002). Additionally, pax2.1 is expressed in these cells, and its loss-of-function phenotype in noi $^{-/-}$ mutant embryos shows that pax2.1 is required for the development of thyroid follicles (Wendl et al., 2002). pax8 appears to be downstream of pax2.1 in zebrafish thyroid development, and it is rather pax2.1 that has a role comparable to Pax8 in mammalian thyroid development.

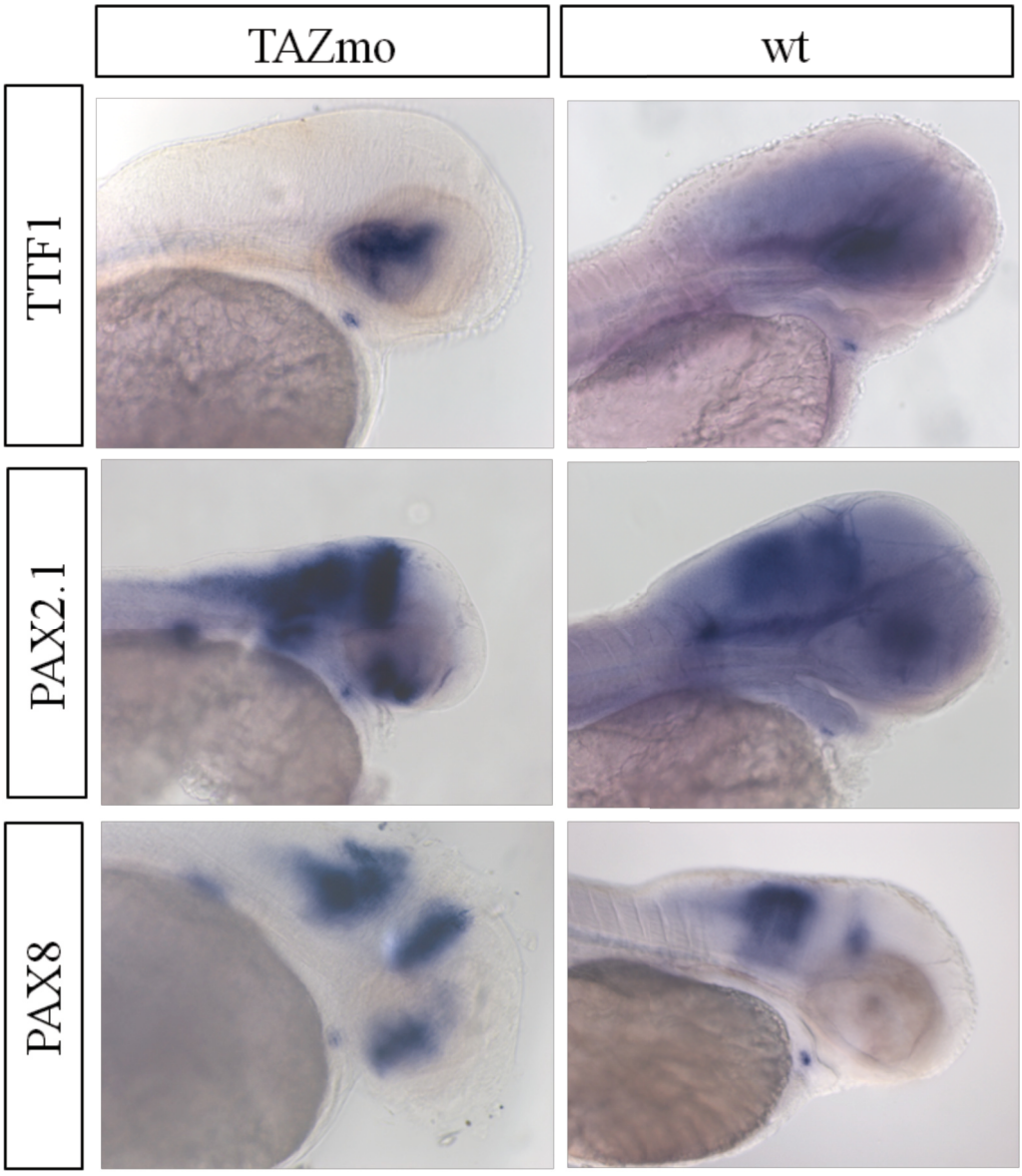


FIG.8: Expression levels of TTF1, PAX2.1 and PAX8 are the same between TAZ morphants (on the left) and wild type (on the right)

Based our data obtained in thyroid cells, we knew that TAZ behaves as a transcriptional coactivator for both Pax8 and TTF-1, hence we wondered whether the lack of TAZ could affect the development of the thyroid gland. To test this hypothesis, we performed in situ hybridization with probes recognizing the mRNAs of TTF-1, Pax8 and Pax2.1 that are the fundamental genes for the correct development of the thyroid gland in zebrafish (Elsalini et al. 2003). At 48 hpf we did not observe any difference in the expression and localization of the three transcription factors, between TAZ morphants and wild type suggesting that, at 48 hpf, thyroid development in TAZ morphants is not affected (Fig.8).

We also know that, in thyroid cells, TAZ is able to enhance Pax8 and TTF-1 synergistic activity on the Tg promoter and that thyroglobulin production is essential for the synthesis of the thyroid hormone T4. Starting from these data we wondered whether the lack of TAZ could lead to a downregulation of Tg expression and, as a consequence, could affect the synthesis of T4. By immunohistochemistry with specific antibodies against Tg and T4 we analyzed the expression of these two proteins in TAZ morphants. At 5 dpf (days post fertilization) the levels of the expression of both Tg and T4 are the same in the wild type and in TAZ morphant embryos (Fig.9).

At this stage, whereas differentiation seems to be not affected, we found that thyroid follicles in TAZ morphants are not disposed along AP axis, like in wild-type, but they appear larger owing to a lateral expansion. Moreover the number of thyroid follicles is decreased: ~ 4-5 in wild type and ~ 2-3 in TAZ morphants.

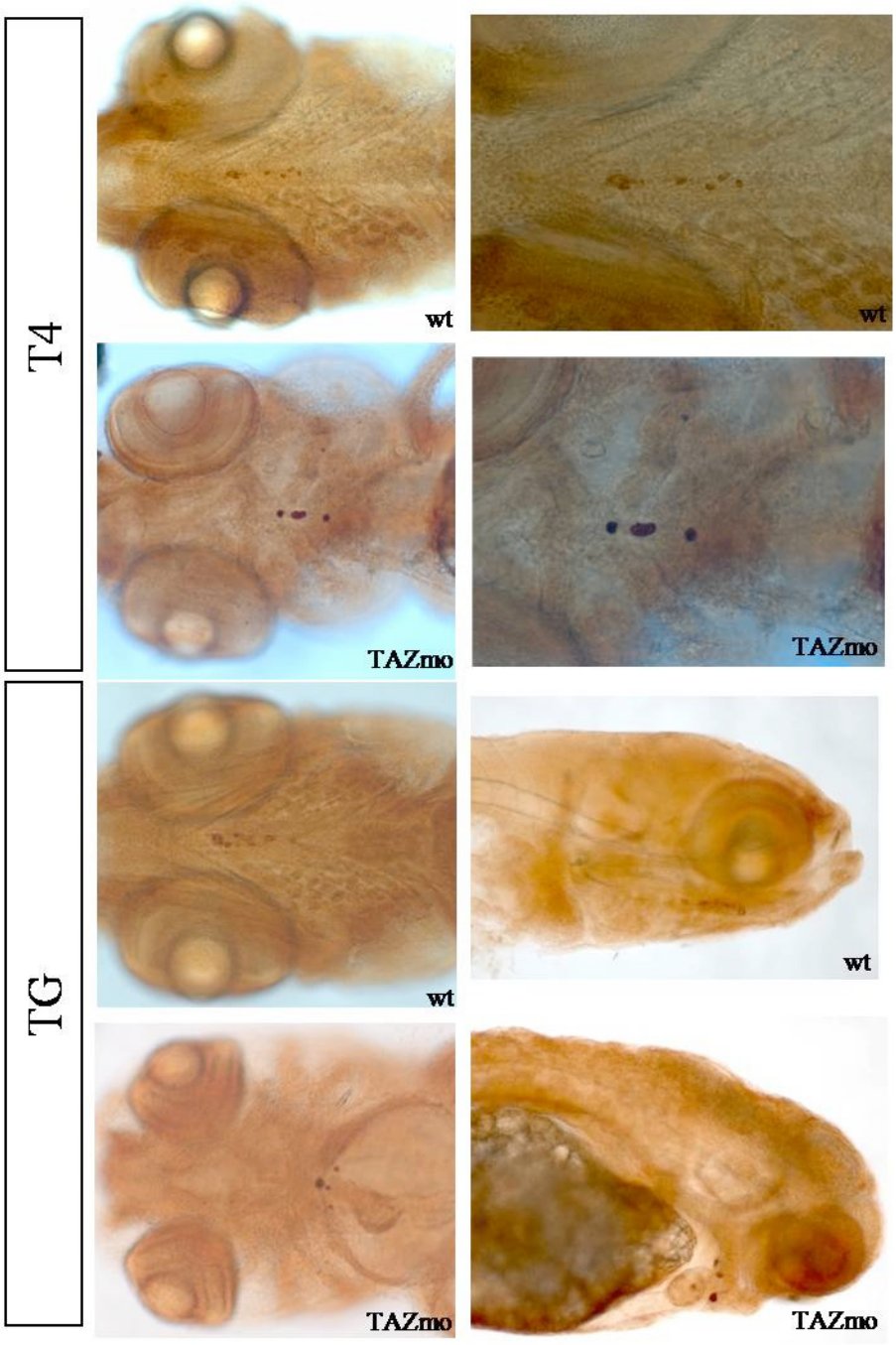


FIG.9: TAZ morphants show thyroid abnormalities in the disposition and in the number of thyroid follicles.

Impaired blood vessel formation in zebrafish lacking TAZ

The thyroid gland in vertebrates develops close to vascular structures. In different studies it has been described that in zebrafish and mouse embryos, with altered vessel architecture, defects in pharyngeal/cervical vessels coincide with abnormal thyroid development in both species (Fagman et al. 2005; Alt et al. 2006).

In zebrafish and mouse 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 and to the aortic sac in mice, respectively. Consequently, follicles cluster at a default position around the cardiac outflow tract in the deficient zebrafish larvae (Vascular Endothelial Growth Factor morphants), and the primordium initially relocalises correctly in the midline of E11.5 Dsh/Dsh mice (Sonic Hedgehog null mice). In a second phase of relocalisation that is dependent on ventral aorta or carotid artery development, the thyroid then adopts its species-specific position further cranially. In the complex and derived circulation of mice, the aortic sac is in part homologous to the cardiac outflow tract and probably also to the ventral aorta in zebrafish. Thus, the first phase of thyroid relocalisation from the pharyngeal epithelium to cardiac structures appears to be evolutionary conserved, whereas the second phase seems to be adapted to the presence of different cranial vessels. These data suggest 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.

The failure of positioning of thyroid follicles along AP axis in TAZ morphants let us to hypothesize that an impairment in blood vessels formation might be the explanation of the TAZ morphants phenotype. To verify our hypothesis we microinjected 1ng of TAZ morpholino in a transgenic line, TG(KDR-like:gfp), in which the Green Fluorescent Protein (GFP) expression is controlled by the kdr-like promoter (Vascular Endothelial Growth Factor receptor) hence by following GFP expression we can monitor the entire vasculature architecture of the embryo.

Immunohistochemistry with the antibody against T4 revealed that, at 4 dpf, vascular architecture in the head of TAZ morphants is completely perturbed if compared with wild type, and that the positioning of the thyroid follicles is affected because the ventral aorta, in the head of the morphants, is absent (Fig.10).

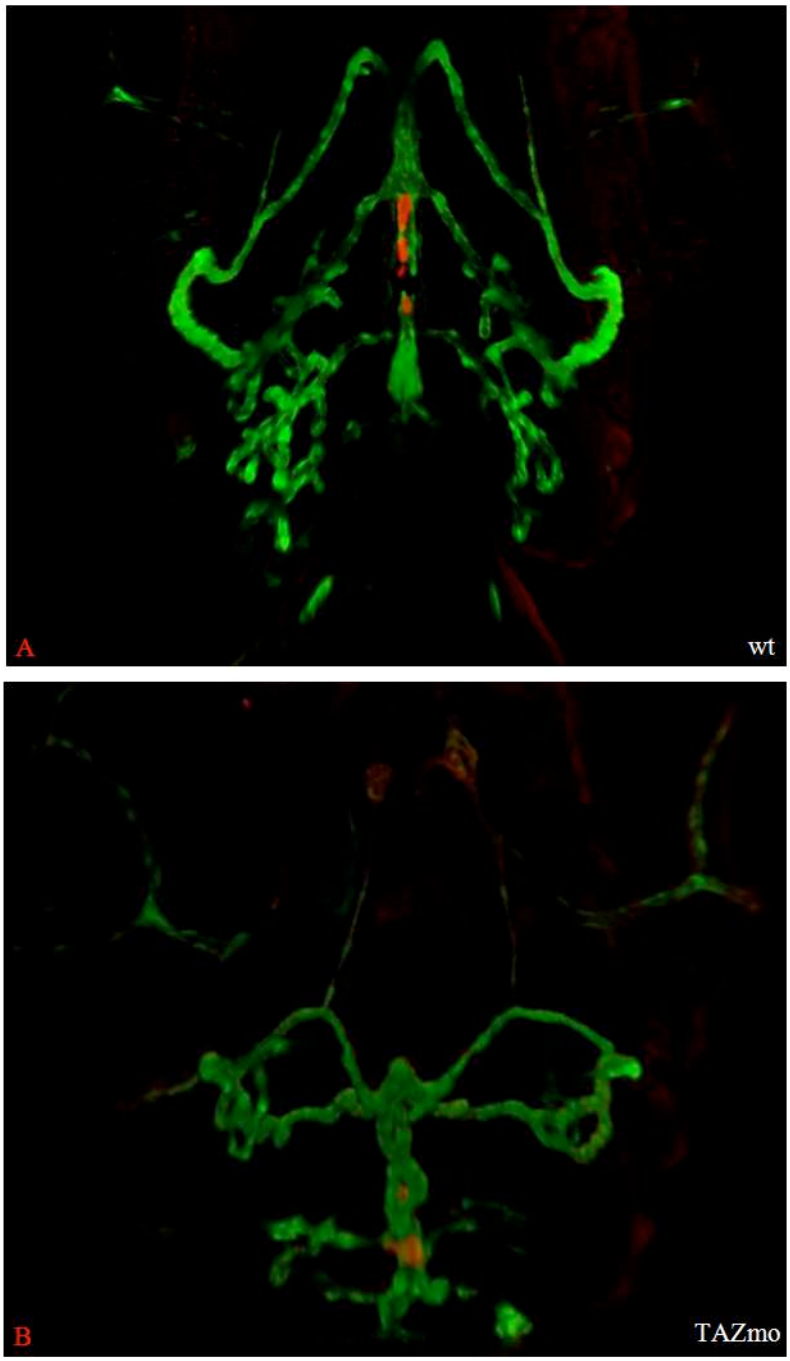


FIG.10: TAZ morphants (B) show completely altered blood vessel architecture in the head if compared with wild type (A); red: thyroid follicles (T4); green: blood vessels (KDR-likebGFP).

The zebrafish thyroid primordium starts to develop at the outflow tract of the heart, subsequently, at about 70 hpf, thyroid follicles start to migrate along the ventral aorta.

Phospholipase C-γ 1 (PLC-γ 1), a well known effector of receptor tyrosine kinase signaling, is involved in the regulation of primitive hematopoiesis in zebrafish embryos (Ma et al. 2007). PLC-γ 1 loss of function mutants (PLC-γ^{y10}) are characterized by a specific defect in the formation of arteries, whereas the development of veins appears unaffected (Lawson et al. 2003).

In the transgenic line TG $(fli1{:}gfp)_{y10}$, GFP expression is controlled by the promoter of the endothelial marker fli1. Hence, by following the expression of fli1 we are able to observe both lymphatic and blood vessels and, into the head, also the precursor of chondrocytes and neural crest cells. Immunostaining with the antibody against T4 revealed that thyroid follicles in PLC-γ^{y10} at 4 dpf are still in close contact with the outflow tract of the heart whereas into wild type thyroid follicles are normally positioned along ventral aorta (Figs. 11A, C).

Mitana et al. (AJRCCM 2009) described a lung phenotype of TAZ $^{-/-}$ mice. These mice showed abnormal alveolarization during lung development, which caused in adult mice airspace enlargement mimicking emphysema. They also measured the expression of various marker genes and they found that expression of vascular endothelial growth factor receptor - 2 (VEGFR-2, KDR), in TAZ null mice, was markedly decreased.

Strikingly we found the PPXY motif in the KDR-like sequence, in zebrafish, hence we wondered if impairment in blood vessel development, in TAZ morphants, might be due to a downregulation of KDR-like expression.

Zebrafish KDR-like loss of function mutant (KDR-like ^{t20257}) develop a normal vascular architecture up to 1,5 dpf. After this stage they show blood vessels malformations, in particular none of the central arteries in the head could be detected in microangiographies up to 4 dpf (Habeck et al. 2002).

We performed immunohistochemistry with the antibody against T4 and we observed that TG $(fli1{:}gfp)_{t20257}$ at 4 dpf show a phenotype very similar to TAZ morphants.

KDR-like ^{t20257} also lacking ventral aorta into the head and the right positioning of thyroid follicles is affected. Moreover the number of thyroid follicles is decreased as well (Figs. 11A, B).

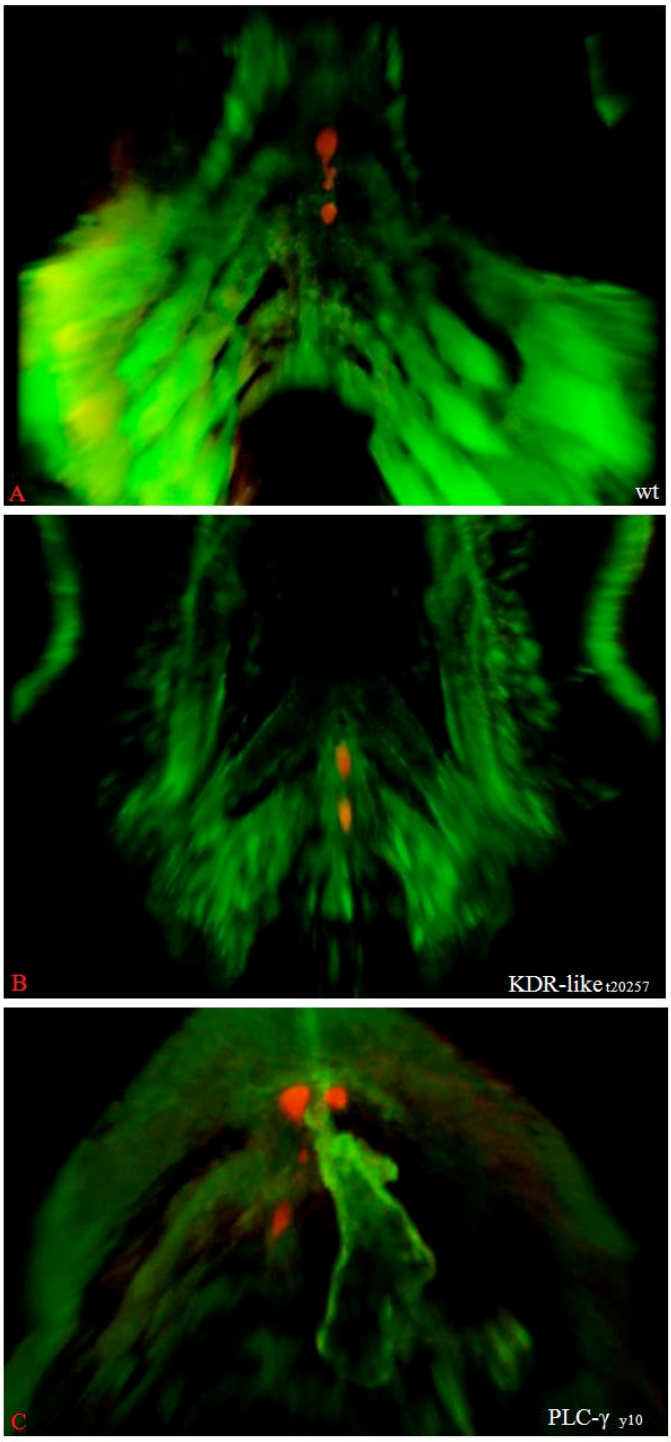


FIG.11: Correlation between defect in blood vessels development and thyroid abnormalities in KDR-like null mutation (B) and in PLC-γ null mutation (C) lines. Red: thyroid follicles (T4)

Lack of skeletal ossification in TAZ-depleted zebrafish

Apart the already mentioned role of TAZ in stimulating differentiation of mesenchimal stem cells (MSCs) in osteoblasts, it has been reported that, in isolated and purified MSCs from bone marrow of multiple myeloma (MM) patients, TAZ expression is correlated with the osteogenic potential of MSCs. In particular, TAZ mRNA expression and protein levels in MSCs were measured and they were both significantly decreased in the MM group compared to the normal group. Takes on the whole these data let us to think that might be interesting to investigate the osteogenic phenotype of TAZ morphants and to observe if they show some abnormalities in bones or cartilages.

Supporting this hypothesis there is the fact that TAZ morphants are smaller than wild type, they develop ventral curvature and their head appear smaller and abnormally shaped.

By Alcian Blue/Alizarin Red staining we are able to recognize cartilages and bones respectively. As we hypothesized in TAZ morphants at 6 dpf both ossified than cartilage tissues are absent. (Fig.12)

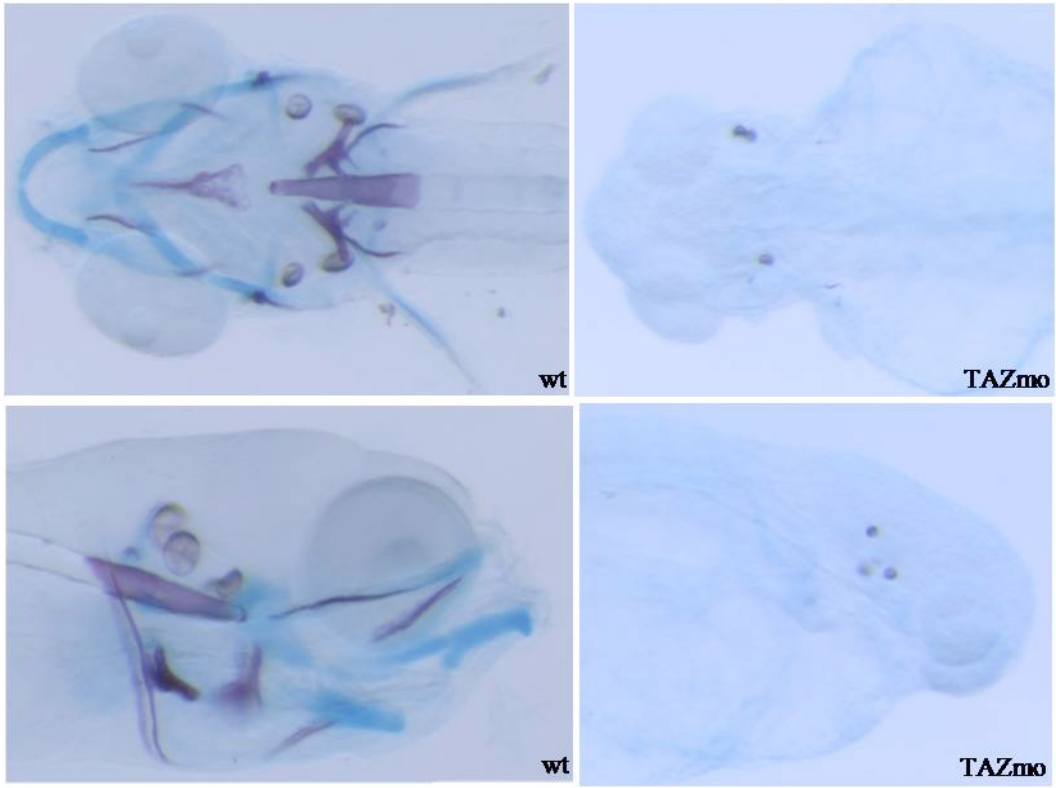


FIG.12: Alizarin Red/Alcian Blue staining show lacking of both ossified and cartilage tissue in TAZ morphant

DISCUSSION

Transcriptional regulation is the most important way of modulating gene expression both in eukaryotes and in bacteria because it contributes to define the proteins expressed by a cell and, as a consequence, the functions characteristic of a cell.

Specifically, this mechanism of regulation is more relevant in pluricellular organism where it allows a gene to be expressed in the right cell and at the right moment during development and differentiation.

A pivotal role in transcriptional regulation is hold by transcription factors. We can define transcription factor any protein other than RNA Polymerase that is required for transcription. Transcription factors however often don't act alone but they are part of protein heterocomplexes in which they interact with different transcription factors, with accessories proteins and with transcriptional co-activators/repressors.

TAZ (Transcriptional co-Activator with PDZ binding motif) is a transcriptional coactivator that, in these last years, has been described to interact with, and to regulate, several transcription factors (Murakami, et al. 2005; Murakami, et al. 2006; Mahoney, et al. 2005; Hong, et al. 2005; Park, et al. 2004). Moreover, it was suggested also a role of TAZ in the migration, invasion, and tumorigenesis of breast cancer cells and it has been demonstrated that TAZ is overexpressed in a significant fraction of breast cancers (Siew Wee Chan, et al. 2008). TAZ is also a component of Hippo pathway in which the activation a TAZ leads to migration and cell proliferation (Zhang, et al. 2009).

During my Ph.D, I have been interested in studying the thyroid gland. In particular, our attention has been focused on thyroid development and differentiation. Functional unit of the thyroid gland is the thyroid follicular cell that is characterized by the expression of specific proteins called, for this reason, differentiation markers; these are thyroglobulin (Tg), thyroperoxidase (TPO) and natrium-iodium symporter (NIS). For the expression of these differentiation markers it is necessary that the thyroid follicular cell expresses three tissue specific transcription factors: TTF-1, Pax8 and Foxe1 (Damante G, et al. 2001).

Previous studies reported that Pax8 and TTF-1 are able to form a heterocomplex, hence we wondered whether TAZ might be part of this complex. By size exclusion chromatography (SEC) on cellular lysates from PC Cl3 thyroid cells we observed the

presence of TAZ, TTF-1 and Pax8 in the same fractions corresponding to a molecular mass of about 177-440 kDa. Starting from this data we performed experiments with the aim to investigate the physical interaction between TAZ and Pax8/TTF-1.

To interact with a transcription factor TAZ needs to find the L/PPXY motif in the activation domain of its partner (Kanai, et al. 2000). We identified the LPGY motif in the Pax8 sequence and LPPY motif in TTF-1. Indeed, we demonstrated the interaction in vitro and in vivo between TAZ and both Pax8 and TTF-1. Since it is well established that TAZ might regulate transcription through the WW domain-PPXY motif interaction (Kanai, et al. 2000), we investigated whether in our case this interaction could regulate the transcriptional activity of either Pax8 and/or TTF-1 and we demonstrated that TAZ is a potent transcriptional coactivator also for these two thyroid specific transcription factors. We demonstrated also that TAZ significant increases the synergistically activation of Pax8 and TTF-1 on the Tg promoter.

Looking at the expression pattern of TAZ in the mouse, we observed that only at 14.5 dpc, hence rather late with respect to Pax8 and TTF-1, TAZ starts to be expressed in the thyroid gland. Intriguingly, Pax8 and TTF-1 onset can be observed at 8.5 dpc while Tg expression, that is regulated by these two factors, starts only at 14.5 dpc. Hence, we always wondered what is missing between 8.5 dpc and 14.5 dpc that could explain the time-lag between the expression of Pax8 and TTF-1, and Tg. For this reason we thought it was really interesting that a gene that is able to increase the synergistic activation of Pax8 and TTF-1 on the Tg promoter starts to be expressed in the nucleus of thyroid follicles at the same time as Tg.

Taken altogether these data suggested us that TAZ might have an important role in thyroid development and differentiation and for this reason we thought that it could be interesting to investigate the role of TAZ in vivo.

We obtained TAZ $^{-/-}$ mice, as a collaboration, with the aim of studying the development and the differentiation of the thyroid gland in mice lacking TAZ.

Unfortunately, we genotyped 258 mice but we were not able to obtain TAZ null mice. We thought at a possible embryonic lethality and for this reason we sampled embryos at E15.5. However, also in this case we obtained only four TAZ knock-out mice suggesting that embryonic lethality happens earlier than E15.5. One way to try to obtain TAZ null mice is to backcross the line from sv129 background to c57bl6 one. At this

time we are doing this crosses and we hope that in a different strain TAZ $^{-/-}$ phenotype could be less severe and we will be able to obtain knock-out mice.

In parallel, while performing the backcross, we decided to study the role of TAZ in the development and differentiation of thyroid cells in zebrafish (Danio rerio). We made this choice for several reasons. Apart from the well known advantages of the zebrafish model, we thought that studying TAZ in zebrafish could allow us to know, in a relative short period of time, if the lack of TAZ in vivo affects in some way thyroid differentiation and development.

Thyroid morphology in zebrafish is peculiar and very different from that in human. Nevertheless, some papers demonstrated that the mechanisms of thyroid development in zebrafish are generally comparable to human thyroid development (Elsalini, et al. 2003).

First of all we demonstrated, by in situ hybridization, that TAZ is expressed in the thyroid of zebrafish and that its expression starts at 48 hpf. Subsequently, we used a specific morpholino oligonucleotide to interfere with TAZ expression.

Despite the data obtained in thyroid cells, we observed that the thyroid in TAZ morphants is fully differentiated and the expressions of Tg and T4 is not affected. Strikingly, immunohistochemistry with antibodies against Tg and T4 revealed that in TAZ morphants the position of the thyroid follicles was completely different if compared to wild type. In fact, in TAZ morphants the follicles fail to align along the AP axis and it seems like the follicles fail to migrate towards their right position.

It has been reported that, both in mouse and in zebrafish models with an abnormal vascular architecture, migration of the thyroid gland strictly depends on the right development of ventral aorta, in zebrafish, or of carotids, in mouse (Alt, et al. 2006; Fagman, et al. 2004). For this reason we wondered whether in TAZ morphants the development of the ventral aorta might be affected and, as a consequence, the thyroid follicles cannot migrate normally.

Microinjection of TAZ morpholino in TG(KDR-like:gfp) revealed that, as we expected, the vascular architecture in the head of TAZ morphants was completely different from that of wild type. Moreover TAZ morphants lack the ventral aorta and the thyroid follicles are still attached to the blood vessel but closer to the heart because they cannot migrate. We further demonstrated that ventral aorta has a role in thyroid follicles migration by looking at thyroid follicles in PLC-γ loss of function mutants in which the

ventral aorta doesn't develop. We observed that, also in this case, the thyroid follicles are still close to the heart attached to the outflow tract.

This is an additional evidence that, with respect to thyroid follicles, migration and differentiation are two independent processes and that, in our case, migration is affected whereas differentiation isn't.

By observing blood vessels phenotype of TAZ morphants our idea is that TAZ could interact with some factor important for blood vessels formation and that, in zebrafish lacking TAZ, this factor is downregulated and for this reason the development of the vessel architecture is affected. The downregulation of KDR in the lung of $TAZ^{-/-}$ mouse and the presence of PPXY motif in KDR-like sequence, let us think that in TAZ morphant KDR might be downregulated. Supporting our hypothesis also a KDR-like loss of function mutant lacks the ventral aorta, like in TAZ morphants, and, moreover, it showed a decreased number of thyroid follicles that don't migrate properly. Of course, we need further data to confirm that the vascular phenotype of TAZ morphant is due to a downregulation of KDR-like, but this is an encouraging data indicating that our hypothesis might be true.

Another well outlined role of TAZ is in the differentiation of mesenchimal stem cell in osteoblasts (Jeong-Ho Hong, et al 2005). Some data demonstrate a downregulation of TAZ in pathologies in which the osteogenic pathway is affected (Bingzong Li, et al. 2007). In fact, TAZ is also able to interact and to activate, under the control of Bmp4, Runx2 that has a pivotal role in osteoblasts differentiation (Jeong-Ho Hong, et al 2005). In our experiments, TAZ morphants are characterized by smaller head and ventral curvature, hence we thought that osteogenesis and chondrogenesis could be affected in zebrafish lacking TAZ. As we hypothesized, TAZ morphants at 6 dpf totally miss both bones and cartilages.

Our data obtained in zebrafish are extremely consistent with those obtained in cells and observed in some pathologies whereas the analysis of $TAZ^{-/-}$ mice shows some inconsistencies. In fact, TAZ null mouse is characterized only by kidney and lung phenotype whereas bones develop normally and there are no data regarding blood vessels and thyroid development. One of the characteristic of zebrafish is that they can live up to the first week of development without a functional vasculature or heart beat, allowing a detailed analysis even in animals with severe cardiovascular defects; by contrast, avian and mammalian embryos die rapidly in the absence of a functional

cardiovascular system. Hence impairment in blood vessels development, that we observe in TAZ morphants, might be the reason of early embryonic lethality that we observed in TAZ null mouse.

MATERIALS AND METHODS

Plasmid constructs

The plasmids used have been previously described and were as follows: Tg-CAT, C5-CAT and CP5-CAT, CMV5-Pax8 , 3xFLAG-Pax8, CMV-TTF-1, Δ14 and Δ3, GST-TAZ and CMV-TAZ. The GST-N-TAZ, GST-W-TAZ and GST-C-TAZ fusion proteins were generated by PCR amplification of the different portions of TAZ coding region and subsequent subcloning in the EcoRI-XhoI sites of the pGEX-4T3 vector. The mutants TTF-1AAPA and Pax8AAGA were constructed using the Quick Change XL Site-Directed Mutagenesis kit (Stratagene) using as template the CMVTTF1 and CMV5-Pax8 vectors, respectively. Mutant Δ349 was generated by PCR amplification of a portion of Pax8 cDNA (corresponding to 349 amino acids) and subsequent subcloning in the HindIII-XbaI sites of pCMV5.

Cell culture, transfections and reporter assays

PC Cl3 and FRTL-5 cells (Berlingieri et al. 1988; Fusco et al. 1987) were grown in Coon's modified F-12 medium (Euroclone) supplemented with 5% calf serum and a six-hormone mixture (6 H) as described by Ambesi-Impiombato and Coon (Ambesi-Impiombato et al. 1979).

HeLa cells were grown in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal calf serum (Hyclone). For transient transfection experiments, cells were plated at 3×105 cells/60-mm tissue culture dish 5 to 8 h prior to transfection. Transfections were carried out with the FuGENE6 reagent (Roche Diagnostics) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:2 in all the experiments. The plasmid CMVLUC was used as internal control and the total amount of transfected DNA was kept constant with an empty expression vector in all the transfection assays. All the data were normalized to the ratio of CAT/LUC activity and the Tg-CAT, C5-CAT and CP5-CAT reporters value was set to 1. Cells extracts were prepared 48 h after transfection to determine either the levels of the CAT

protein with a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics) or the LUC activity by a Luciferase assay as previously described (de Wet 1987). Transfection experiments were done in duplicate and repeated at least three times. CAT activity values on the graph are the means of all experiments (+/–) SD. Statistical analysis has been performed by means of an unpaired two-tailed Student's t test to obtain the P value associated with the observed fold of activation differences.

Protein extracts and immunoblotting

For Western blot and reporter assays, cellswerewashed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 10 mM Hepes pH 7.9, 400 mM NaCl, 0.1 mM EGTA pH 7.8, 5% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmetilsulfonil fluoride (PMSF). The same buffer was used for the lysis of the thyroid tissue. For GST-pull down assays PC Cl3 cells were washed twice with PBS and lysed in WCE buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 0.1% Triton X100, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors (Sigma). For immunoprecipitation experiments cells were washed twice with PBS and lysed in EBC buffer containing 50 mM Tris pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors (Sigma).

Cell fractionation into cytoplasmic and nuclear fraction was prepared by a variation of the standard protocol of Dignam (Dignam et al. 1983), as previously described (D'Andrea et al. 2006)

The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). For Western blot analysis, proteins were separated by SDS-10% PAGE, gels were blotted onto Immobilon P (Millipore, Bredford, MA, USA) for 2 h and the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 2 h or overnight before the addition of the antibody for 1 h. The primary antibodies used were anti-TAZ (kindly provided by T. Benjamin), anti-Pax8 and anti-TTF-1 (kindly provided by R. Di Lauro), anti-Sp1 (Santa Cruz, CA), anti-tubulin (Santa Cruz, CA), anti-FLAG (Sigma), anti-GST (kindly provided by P. Di Fiore). The filters were washed three times in Tris-buffered saline plus 0.5% Tween 20 before the

addition of horseradish peroxidase-conjugated secondary antibodies for 45 min. Horseradish peroxidase was detected with ECL (GE Healthcare)

Pull down assay and coimmunoprecipitation

GST-TAZ protein was purified from BL21 (DE) LysS bacterial cells as previously described [20]. For the synthesis of bacterial TTF-1, E. Coli cells were transformed with pQE12-TTF-1, an expression vector encoding for TTF-1 fused to a His6 tag at the C terminus. The expression and the purification of TTF-1 protein were performed as previously described (Aurisicchio et al 1998). The purification of Pax8 protein was performed using the same protocol described for TTF-1. For pull down assays, GST or GST-TAZ bound to glutathione beads were incubated with 3 mg of total protein extract prepared from PC Cl3 cells or 10 ng of bacterial purified proteins. Binding reactions were carried out for 90 min at 4°C on a rotating wheel. After extensive washing with WCE buffer, bound proteins were detected by Western blot. To analyze the interaction of TAZ with Pax8 and TTF-1, a construct expressing TAZ was transfected into HeLa cells along with vectors containing FLAG-tagged TTF-1 and Pax8. About 48 h after transfection, cells were washed twice with PBS and lysed in EBC buffer. 5 mg of total protein extract was purified on anti-FLAG agarose affinity gel (Sigma) overnight at 4°C on a rotating wheel. Bound proteins were eluted by resuspending the beads directly in 2×SDS-PAGE sample buffer and heating at 95°C for 5 min. before loading on the gel. The same conditions were used when the coimmunoprecipitation experiment was performed using PC Cl3 cells.

Size Exclusion Chromatography (SEC)

For SEC analysis 2 mg of total extract was loaded on a Superose 6 10/300 GL (GE Healthcare) gel filtration column equilibrated in sodium phosphate buffer, pH 7.2, containing 120 mM NaCl. For Western blot analysis, the indicated fractions were subjected to trichloroacetic acid precipitation before loading on SDS-PAGE

In situ hybridization on mice

For embryo collection, CD1 mice were crossed. Noon of the day on which the vaginal plug was detected was considered as 0.5 dpc in the timing of the embryo collection. Timed pregnant females were killed by cervical dislocation and embryos were dissected from decidual tissue in cold PBS and fixed overnight in 4% paraformaldehyde in PBS at 4°C. For RNA whole mount in situ hybridization (WISH), embryos have beenwashed in PBT (0.1% Tween 20 in PBS), dehydrated through ascending methanol and stored at-20°C. WISH experiments were performed as previously described (Liguori et al. 2003). The embryos were then photographed using a Leica DFC300 F digital camera. Subsequently, some embryos were embedded in 7.5% gelatin, frozen, sectioned using a MICROM HM 560 cryostat, and then examined and photographed using a Leica DM6000 B microscope and a Leica DFC480 digital camera. For RNA in situ hybridization on sections, embryos and adult thyroids have been washed in PBT (0.1% Tween 20 in PBS), dehydrated through ascending ethanol, washed in Toluene and Toluene-Paraffin 1:1, included in paraffin and then sectioned using a 2030 Reichert-Jung microtome. RNA in situ hybridization on sections was performed as previously described (Rugarli et al. 1993). Embryo sections were then photographed using a Leica DC300 digital camera. The following probes were used: full-length mPax8, full-length mTAZ, TAZ-Pr1, part of TAZ cDNA amplified with the mouse TAZ-specific primers 5′-AGGATCCAGATGGAGAGAGAGAG-3′ and 5′-AGTAGTGATTACAGCCAGG-3′ and a Tg fragment (Lazzaro et al. 1991)

Immunohistochemistry on mice

Embryos and adult thyroids were dissected in cold PBS and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Samples were washed in PBT (0.1% Tween 20 in PBS), dehydrated through ascending ethanol, washed in Toluen and Toluen-Paraffin 1:1, included in paraffin and then sectioned using a 2030 Reichert-Jung microtome.
Sections were dewaxed by standard techniques and heat treated (microwave oven) in citrate buffer 0.01 M ph 6.0 to retrieve the antigen sites. To quench endogenous peroxidases, the sections were treated in 0.3 % hydrogen peroxide in methanol for 30

minutes at room temperature. The sections were incubated in blocking solution (1% BSA, 10% Normal Goat Serum, in PBS1X) for 1 hour at room temperature and then with the primary antibodies in the same solution overnight at 4°C. Sections were washed twice in PBT for 5 minutes and then incubated with the goat anti-rabbit secondary antibody (Dako, 1:400 dilution) for 1 hour at room temperature. Staining procedures and chromogenic reactions were carried out according to the Vectastain ABC kit protocol (Vector Laboratories). After diaminobenzidine (Sigma) reaction, the sections were counterstained with eosin solution (Sigma) for 30 seconds. The following primary antibodies were used: anti-Taz (Santa Cruz Biotechnology; sc-48805, 1:25 dilution), anti-Pax8 (kindly provided by R. Di Lauro, 1:1000 dilution) and anti-TTF-1 (kindly provided by R. Di Lauro, 1:1500 dilution).

Zebrafish

Zebrafish work was carried out according to standard procedures (Westerfield, 2000). Staging in hours post fertilisation (hpf) or days post fertilisation (dpf) refers to development at 28.5°C. The following mutant fish lines were used: plcg ^{y10} (Lawson et al. 2003), kdr-like ^{t20257} (Habeck et al. 2002).

Embryonic manipulation

Antisense morpholino oligonucleotides directed against the zebrafish TAZ translation initiation codon (zTAZ MO: 5'-CTGGAGAGGATTACCGCTCATGGTC-3') and a standard control oligomer (Con MO) were purchased from GeneTools LLC (Philomath, OR). Zebrafish embryos in the 1- to 2-cell stage were injected with 1 ng of oligonucleotides in 1 × Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes pH 7.6). We always injected morpholinos into one-cell-stage embryos. All phenotypes described were consistently found in over 70% of morphants, with the rest showing milder defects or wild-type appearance.

Immunohistochemistry on zebrafish embryos

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.

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) O/N at 4°C, then washed in PBT for 3 h. Incubation with a biotinylated secondary antibody was for 2 h, washing in PBT for 3 h. Incubation with the ABC kit (Vectastain) was for 2 h according to the instructions of the manufacturers. Larvae were washed again in PBT for 3 h, then once in PBS, and incubated in DAB (0.2 mg/ml PBS) for 30 min. To stain, 1 µl of a 0.3% aqueous H_2O_2 solution was added under observation by using a dissection scope. All procedures were carried out at room temperature, washing steps can be extended at 4°C.

For detailed analysis, larvae were postfixed in PFA for 15 min at room temperature, washed in PBT, and gradually transferred to 70% glycerol.

For analysis at confocal microscope whole-mount embryos were embedded in 0,5% agarose and examined and photographed using Leica SPE microscope. Images were processed using Leica LASAF lite software.

Labelling of the probe

0.5-1 µg of the linear plasmidic DNA was used in the in vitro transcription reaction for the synthesis of antisense RNA labelled with DIG. For synthesis of in situ probe, a 20 µl reaction was set up containing 2 µl 10x transcription buffer, 2 µl DIG-labelling mix, 0.5 µl RNasin, 1 µl RNA polymerase and RNAse free H_2O. Transcription mix was incubated at 37°C for 2-4 h. The RNA was then washed in distilled H_2O by using Millipore filter tubes.

In situ hybridization on zebrafish embryos

Embryos up to 48 hpf were fixed in 4% PFA ON and stored in 100% methanol at -20°C. After 12 h, they were washed twice with PBT, digested with 10 µg/ml proteinase-K in PBT for several minutes depending on the stage, washed further and fixed again in 4% PFA for 20 min. After additional washes, the embryos were prehybridized for 2-4 h at 62°C and then digoxigenin (DIG)-labelled RNA probe was added to the final concentration of approximately 50 ng/ml and hybridized ON at 62°C. The embryos were then submitted to several washing treatments such as: at 62°C in Hyb-/2XSSC (75:25) for 15 min; at 62°C in Hyb-/2XSSC (50:50) for 15 min and at 62°C in Hyb-/2XSSC (25:75) for 15 min. To prevent the nonspecific staining, they were then kept at least for 2 h at room temperature (RT) in blocking reagent (Roche) and then incubated for 2 h with anti-DIG-AP antibody (Roche) (1:6000 dilution in blocking reagent), followed by washing in PBT ON. The following day, the embryos were rinsed with staining buffer (X-pho buffer) (0.1 M Tris pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, 0.1% Tween 20) and stained with BM purple AP substrate, precipitating (Roche). The staining reaction was stopped by the addition of PBT, followed by the fixation in 4% PFA. The stained embryos were then washed in PBT, gradually transferred to 90% glycerol and studied for detailed analysis. Whole-mount embryos were examined with an AXIOPLAN 2 microscope equipped with Axiocam digital camera (Zeiss). Images were processed using Axion Vision software.

REFERENCES

- Tina Di Palma, Barbara D'Andrea, Giovanna Lucia Liguori, Annamaria Liguoro, Tiziana de Cristofaro, Dolores Del Prete, Andrea Pappalardo, Anna Mascia, Mariastella Zannini: *TAZ is a coactivator for Pax8 and TTF-1, two transcription factors involved in thyroid differentiation* **Experimental Cell Research** 2009
- Mario de Felice and Roberto di Lauro: *Thyroid Development and Its Disorders: Genetics and Molecular Mechanisms* **Endocrine Reviews** 2004
- Zakir Hossain, Safiah Mohamed Ali, Hui Ling Ko, Jianliang Xu, Chee Peng Ng, Ke Guo, Zeng Qi,Sathivel Ponniah, Wanjin Hong, and Walter Hunziker: *Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1* **PNAS** 2007
- Yu Tian, Robert Kolb, Jeong-Ho Hong, John Carroll, Dawei Li, John You, Roderick Bronson, Michael B. Yaffe, Jing Zhou, and Thomas Benjamin **Molecular and Cellular Biology** 2007
- Ryosuke Makita, Yasunobu Uchijima, Koichi Nishiyama, Tomokazu Amano, Qin Chen, Takumi Takeuchi, Akihisa Mitani, Takahide Nagase, Yutaka Yatomi, Hiroyuki Aburatani, Osamu Nakagawa, Erin V. Small, Patricia Cobo-Stark,Peter Igarashi, Masao Murakami, Junji Tominaga, Takahiro Sato, Tomoichiro Asano, Yukiko Kurihara, and Hiroki Kurihara: *Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ* **Am J Physiol Renal Physiol** 2008
- Akihisa Mitani, Takahide Nagase, Kazunori Fukuchi, Hiroyuki Aburatani, Ryosuke Makita and Hiroki Kurihara: *Transcriptional Coactivator with PDZ-Binding Motif is Essential for Normal Alveolarization in Mice* **AJRCCM** 2009
- Kainan Wang, Cindy Degerny, Minghong Xu, and Xiang-Jiao Yang: *YAP, TAZ and Yorkie: a conserved family of signal-responsive transcriptional coregulators in animal development and human disease* **Biochem. Cell. Biol.** 2009

- William M. Mahoney, JR, Jeong-Ho Hong, Michael B. Yaffe and Iain K. G. Farrance: *The transcriptional co-activator TAZ interacts differentially with transcriptional enhancer factor-1 (TEF-1) family members* **Biochem. J.** 2005
- Siew Wee Chan, Chun Jye Lim, Ke Guo, Chee Peng Ng, Ian Lee, Walter Hunziker, Qi Zeng, and Wanjin Hong: *A Role for TAZ in Migration, Invasion, and Tumorigenesis of Breast Cancer Cells* **Cancer Research** 2008
- Bingzong Li, Mingxia Shi, Jing Li, Hongbing Zhang, Bin Chen, Lei Chen,Weibo Gao, Nicola Giuliani, and Robert Chunhua Zhao: *Elevated Tumor Necrosis Factor-α Suppresses TAZ Expression and Impairs Osteogenic Potential of Flk-1^{+} Mesenchymal Stem Cells in Patients with Multiple Myeloma* **Stem Cells and Development** 2007
- Fumihiko Kanai, Paola A. Marignani, Dilara Sarbassova, Ryohei Yagi, Randy A. Hall, Mark Donowitz, Akihiko Hisaminato, Tsutomu Fujiwara, Yoshiaki Ito, Lewis C. Cantley, and Michael B. Yaffe: *TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins* **The EMBO Journal** 2000
- Xaralabos Varelas, Rui Sakuma, Payman Samavarchi-Tehrani, Raheem Peerani, Balaji M. Rao, Joanna Dembowy, Michael B. Yaffe, Peter W. Zandstra and Jeffrey L. Wrana: *TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal* **Nature Cell Biology** 2008
- Jeong-Ho Hong, Eun Sook Hwang, Michael T. McManus, Adam Amsterdam, Yu Tian, Ralitsa Kalmukova, Elisabetta Mueller, Thomas Benjamin, Bruce M. Spiegelman, Phillip A. Sharp, Nancy Hopkins, Michael B. Yaffe: *TAZ, a Transcriptional Modulator of Mesenchymal Stem Cell Differentiation* **Science** 2005
- Kwon-Sik Park, Jeffrey A. Whitsett, Tina Di Palma, Jeong-Ho Hong, Michael B. Yaffe, and Mariastella Zannini: *TAZ Interacts with TTF-1 and Regulates Expression of Surfactant Protein-C* **The Journal of Biological Chemistry** 2004
- Burkhard Alt, Osama A. Elsalini, Pamela Schrumpf, Nele Haufs, Nathan D. Lawson, Georg C. Schwabe, Stefan Mundlos, Annette Grüters, Heiko Krude, and Klaus B. Rohr: *Arteries define the position of the thyroid gland during its developmental relocalisation* **Development** 2006

- Osama A. Elsalini, Julia von Gartzen, Matthias Cramer, and Klaus B. Rohr: *Zebrafish hhex, nk2.1a, and pax2.1 regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors* **Developmental Biology** 2003
- Burkhard Alt, Saskia Reibe, Natalia M. Feitosa, Osama A. Elsalini, Thomas Wendl, and Klaus B. Rohr: *Analysis of Origin and Growth of the Thyroid Gland in Zebrafish* **Developmental Dynamics** 2006
- Hinrich Habeck, Jörg Odenthal, Brigitte Walderich, Hans-Martin Maischein, Tübingen 2000 screen consortium, and Stefan Schulte-Merker: *Analysis of a Zebrafish VEGF Receptor Mutant Reveals Specific Disruption of Angiogenesis* **Current Biology** 2002
- Nathan D. Lawson, Joshua W. Mugford, Brigid A. Diamond, and Brant M. Weinstein: *phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development* **Genes & Development** 2003
- Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML: *TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm* **Genes & Development** 2001
- Basu S, Totty NF, Irwin MS, Sudol M, Downward J: *Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis* **Molecular Cell** 2003
- M.T. Berlingieri, G. Portella, M. Grieco, M. Santoro, A. Fusco: *Cooperation between the polyomavirus middle-T-antigen gene and the human c-myc oncogene in a rat thyroid epithelial differentiated cell line: model of in vitro progression* **Molecular Cell** 1988
- A. Fusco, M.T. Berlingieri, P.P. Di Fiore, G. Portella, M. Grieco, G. Vecchio: *One-and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes* **Molecular Cell** 1987
- F.S. Ambesi-Impiombato, H.G. Coon: *Thyroid cells in culture* **Int. Rev. Cytol.** 1979
- A.J. Sinclair, R. Lonigro, D. Civitareale, L. Ghibelli, R. Di Lauro: *The tissue-specific expression of the thyroglobulin gene requires interaction between thyroid-specific and ubiquitous factors* **Eur. J. Biochem** 1990

- C. Missero, G. Cobellis, M. De Felice, R. Di Lauro: *Molecular events involved in differentiation of thyroid follicular cells* **Molecular Cell** 1998
- M. Zannini, H. Francis-Lang, D. Plachov, R. Di Lauro: *Pax-8, a paired domain-containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters* **Molecular Cell** 1992
- M. De Felice, G. Damante, M. Zannini, H. Francis-Lang, R. Di Lauro: *Redundant domains contribute to the transcriptional activity of the thyroid transcription factor 1* **The Journal of Biological Chemistry** 1995
- R.J. Bohinski, R. Di Lauro, J.A. Whitsett: *The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis* **Molecular Cell** 1994
- T. Di Palma, R. Nitsch, A. Mascia, L. Nitsch, R. Di Lauro, M. Zannini: *The paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly interact and synergistically activate transcription* **The Journal of Biological Chemistry** 2003
- J.D. Dignam, R.M. Lebovitz, R.G. Roeder: *Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei* **Nucleic Acids Research** 1983
- B. D'Andrea, R. Iacone, T. Di Palma, R. Nitsch, M.G. Baratta, L. Nitsch, R. Di Lauro, M. Zannini: *Functional inactivation of the transcription factor Pax8 through oligomerization chain reaction* **Molecular Endocrinology** 2006
- L. Aurisicchio, R. Di Lauro, M. Zannini: *Identification of the thyroid transcription factor-1 as a target for rat MST2 kinase* **The Journal of Biological Chemistry** 1998
- G.L. Liguori, D. Echevarria, R. Improta, M. Signore, E. Adamson, S. Martinez, M.G. Persico: *Anterior neural plate regionalization in cripto null mutant mouse embryos in the absence of node and primitive streak* **Developmental Biology** 2003
- Heng Zhang, Chen-Ying Liu, Zheng-Yu Zha, Bin Zhao, Jun Yao, Shimin Zhao, Yue Xiong, Qun-Ying Lei, and Kun-Liang Guan: *TEAD transcription factors*

mediate the function of TAZ in cell growth and epithelial-mesenchymal transition **The Journal of Biological Chemistry** 2009

- Marina Pasca di Magliano, Roberto Di Lauro, and Mariastella Zannini: *Pax8 has a key role in thyroid cell differentiation*: **PNAS** 2000
- Westerfield, M.: *The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio)* **Univ. of Oregon Press** 2000
- Fagman, H., Grande, M., Gritli-Linde, A. and Nilsson, M.: *Genetic deletion of sonic hedgehog causes hemiagenesis and ectopic development of the thyroid in mouse* **American Journal of Pathology** 2004
- Murakami M, Nakagawa M, Olson EN, Nakagawa O.: *A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome* **PNAS** 2005
- Murakami M, Tominaga J, Makita R, Uchijima Y, Kurihara Y, Nakagawa O, Asano T, Kurihara H. *Transcriptional activity of Pax3 is co-activated by TAZ* **Biochemical and Biophysical Research Communications** 2006
- Damante G, Tell G, Di Lauro R.: *A unique combination of transcription factors controls differentiation of thyroid cells* **PNAS** 2001