Meis regulation and function during eye and nervous system development

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CONTENTS

I Summary

The development of organs is a complex process controlled by gene regulatory networks. In these networks, transcription factors control the expression of target genes which, in turn, determine the specific properties of developing cells. In this thesis, we have focused on two families of transcription factors that have widespread roles in normal development and disease: the *meis* and *teashirt* gene families. For this, we have resorted to the zebrafish, as a vertebrate animal model, with special focus on the formation of the retina.

In Chapter I, we analysed the requirement of *meis* genes during the early retinal development. Previous work had shown that, in *Drosophila,* the *meis* gene homologue *homothorax (hth)* was required for the maintenance of the undifferentiated and proliferative state of retinal progenitors. Due to the partial conservation of the gene network controlling early eye specification between vertebrates and invertebrates, we asked whether *meis* genes were expressed in retinal progenitors and if they did, what their role was. We found that of the four *meis* paralogous genes, *meis1* was expressed in retinal progenitors. In addition, using a combination of strategies, we were able to show that *meis1* was required in retinal progenitors to sustain their proliferative growth. These results indicated that Meis/Hth shared a conserved function.

Since the eye regulatory gene networks in vertebrates and *Drosophila* show significant similarities, we also decided to study other class of genes, which encode the Teashirt zinc-finger transcription factors (Tsh). In *Drosophila,* the two *tsh* paralogues, *tsh* and *tiptop (tio)* were known to collaborate with *hth* and *eyeless* (the *Drosophila pax6* homologue) during fruit fly eye development. However, very little was known, even at the level of expression, about this gene family in vertebrates. In zebrafish only *tshz1* had been described so far. We found new *tshz* genes in zebrafish and placed them in a comprehensive phylogeny. We also described the expression pattern of two of them, *tshz2* and *tshz3b*, and compared it with that of *meis* and *pax6* genes. We concluded that *tshz*, *meis* and *pax6* gene families have coexpression domains in the brain that open the possibility of interaction between their gene products. This work is addressed in Chapter II.

II Resumo

O desenvolvimento dos orgãos é um processo complexo, controlado por redes de regulação genética. Nestas redes, os factores de transcrição controlam a expressão de genes-alvo que, por sua vez, determinam as propriedades específicas das células em desenvolvimento. Nesta tese, centramo-nos em duas famílias de factores de transcrição que têm funções generalizadas no desenvolvimento normal e em doenças: as famílias de genes *meis* e *teashirt*. Para isso, recorremos ao peixe-zebra como um modelo de animal vertebrado, com especial ênfase na formação da retina.

No Capítulo I, analisamos a exigência dos genes *meis* durante o início do desenvolvimento da retina. Trabalhos anteriores mostraram que, em *Drosophila*, o gene homólogo de *meis*, *homothorax* (*hth*), é necessário para a manutenção do estado indiferenciado e proliferativo das células progenitoras da retina. Devido à conservação parcial da rede de genes que controlam o início da especificação do olho, entre animais vertebrados e invertebrados, perguntamo-nos se os genes *meis* eram expressos nas células progenitoras da retina e, se assim fosse, qual o seu papel. Nós descobrimos que, dos quatro genes parálogos *meis*, *meis1* era expresso nas células progenitoras da retina. Além disso, usando uma combinação de estratégias, fomos capazes de mostrar que o *meis1* era necessário para manter o crescimento proliferativo das células progenitoras da retina. Estes resultados indicaram que Meis/Hth compartilham uma função conservada.

Uma vez que as redes de regulação genética do olho em vertebrados e em *Drosophila* apresentam semelhanças significativas, nós decidimos estudar também outra classe de genes, os quais codificam os factores de transcrição Teashirt “zinc-finger” (Tsh). Em *Drosophila*, os dois genes *tsh* parálogos, *tsh* e *tiptop* (*tio*) eram conhecidos por colaborarem com *hth* e *eyeless* (o homólogo *pax6* de *Drosophila*) durante o desenvolvimento do olho da mosca da fruta. No entanto, muito pouco era conhecido, mesmo a nível de expressão, sobre esta família de genes em vertebrados. No peixe-zebra apenas *tshz1* tinha sido descrito até agora. Nós encontramos novos genes *tshz* do peixe-zebra e colocamo-los numa filogenia. Nós também descrevemos os padrões de expressão de dois deles, *tshz2* e *tshz3b*, e comparamo-los com os dos genes *meis* e *pax6*. Nós concluímos que as famílias de genes *meis*, *tshz* e *pax6* têm domínios de coexpressão no cérebro que indicam a possibilidade de interacção entre os seus produtos. Este trabalho é abordado no Capítulo II.

1 General introduction

1.1. *Danio rerio* as an animal model to study vertebrate development

Zebrafish (*Danio rerio*) has become an important animal model to study the mechanism of vertebrate development and disease, since its introduction by George Streisinger. Zebrafish presents several advantages when used as a model system, which have often been cited in literature. For instance, it is easy to maintain in small spaces, it reaches sexual maturity in only 3-4 months and a pair of fish can produce more than 200 eggs per mating. Additionally, eggs and embryos are transparent and, therefore, it is easy to follow the development with just a standard dissection microscope. Development is so quick that merely 24 hours post-fertilization (hpf) all the major organs are formed (Fadool and Dowling, 2008) (Figure 1).

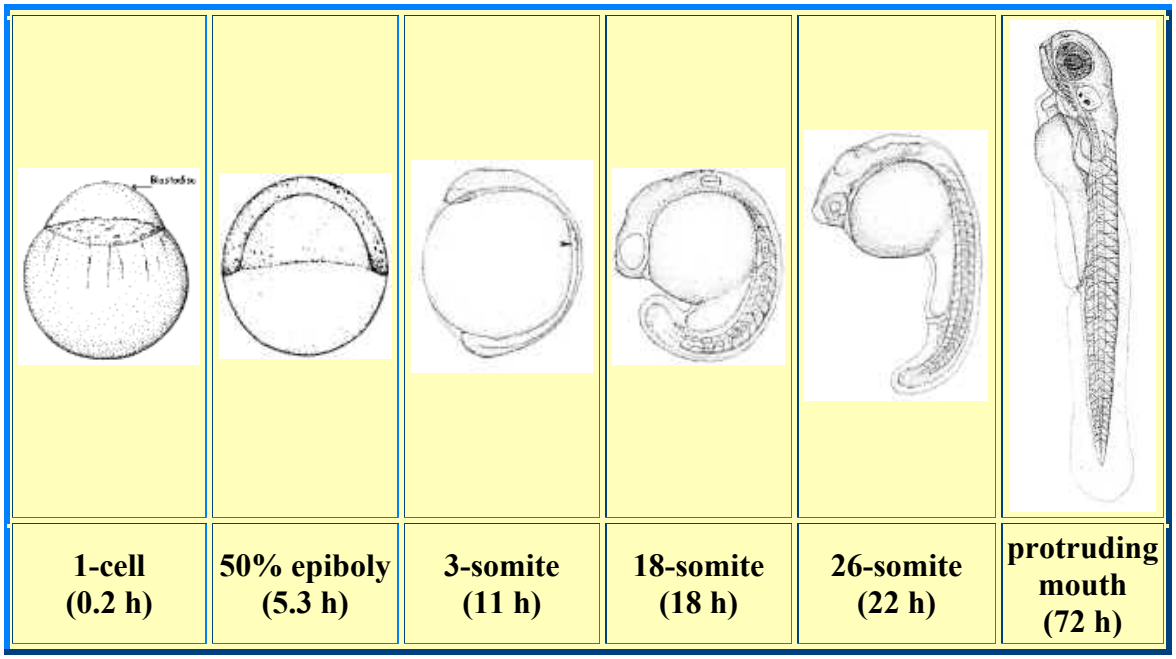

Figure 1. Diagram representing some stages of the zebrafish development. At 1-cell stage, after fertilization, the embryo is divided in two structures, the yolk and on the top of the yolk, the cell. At 11 hours post-fertilization (hpf) stage, the first 5 to 6 somites appear at the rate of about 3 per hour. The brain primordium has now distinctively thickened into the neural keel and one can first distinguish the optic vesicle. At 18 hpf, the otic placode (the precursor of the fish ear) is present beside the hindbrain rudiment that is midway between the optic vesicle and the first somite. At 22 hpf stage of development, the lens and the midbrain-hindbrain boundary are visible. (Adapted from http://www.neuro.uoregon.edu/k12/Development%20Stages.html).

Zebrafish is a particularly good model to study eye development and morphology, since the mechanisms involved are mostly conserved along most classes of the

vertebrate lineage. The eye develops from three distinct embryological tissues called neuroectoderm, skin ectoderm and head mesenchyme. Neural retina arises from the neuroectoderm. The zebrafish neural retina, as happens in many classes of vertebrates, is composed of seven cell types, six neurons (interneurons, amacrine, horizontal, bipolar, ganglion and photoreceptors cells), and a single glial cell, called Muller cell (Fadool and Dowling, 2008).

The retina has a well-characterized laminar organization. The ganglion cells are present innermost in the retina, closest to the lens, and the photoreceptors (rods and cones) lie outermost against the pigment epithelium. Thus, light must pass through all the retina layers before activating the photoreceptors. The light is then translated into an electrical message that stimulates the succeeding neurons of the retina, it is transmitted to the brain through the optic nerve, and it is processed to form an image (http://webvision.med.utah.edu/sretina.html). All vertebrates have a retina with three layers of nerve cell bodies and two layers of synapses. The outer nuclear layer (ONL) contains the cell bodies of the rods and cones, the inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal and amacrine cells, and, lastly, the ganglion cell layer (GCL) is composed of the bodies of ganglion cells and displaced amacrine cells. Dividing these nerve cell layers one finds the outer plexiform layer (OPL) and the inner plexiform layer (IPL), where synaptic contacts occur (http://webvision.med.utah.edu/sretina.html) (Figure 2).

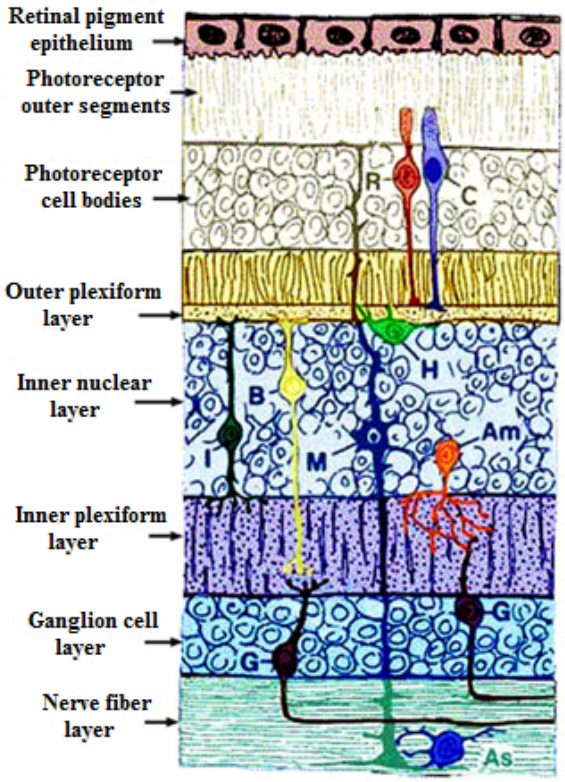

Figure 2. Diagram of a vertebrate differentiated retina´s layers. Retina is up and lens is down. R – rod photoreceptors; C – cone photoreceptors; H – horizontal cells; B – bipolar cells; I – interplexiform cells; M – Muller cells; Am – amacrine cells; G – ganglion cells. (Adapted from: http://webvision.med.utah.edu/anatomy.html)

In zebrafish and in larval amphibians, but not in other vertebrates, retinal neurogenesis also occurs postembryonically, throughout the animal's life, in a stem cell containing zone, a region called cilliary marginal zone (CMZ) or circumferential germinal zone (Raymond *et al.*, 2006; Amato *et al.*, 2004). The CMZ lies between the neural retina and the iris and contains multipotent, self-renewing retinal stem cells, which gene expression recapitulates the expression that occurs in retinoblasts during embryonic development (Raymond *et al.*, 2006; Amato *et al.*, 2004). In fact, in fish and amphibians only the most central region of the retina is formed during embryogenesis and most part of the retina develops from the addition of new cells from the CMZ (Moshiri *et al.*, 2004). CMZ can also regenerate retina after injury (Moshiri *et al.*, 2004) (Figure 3).

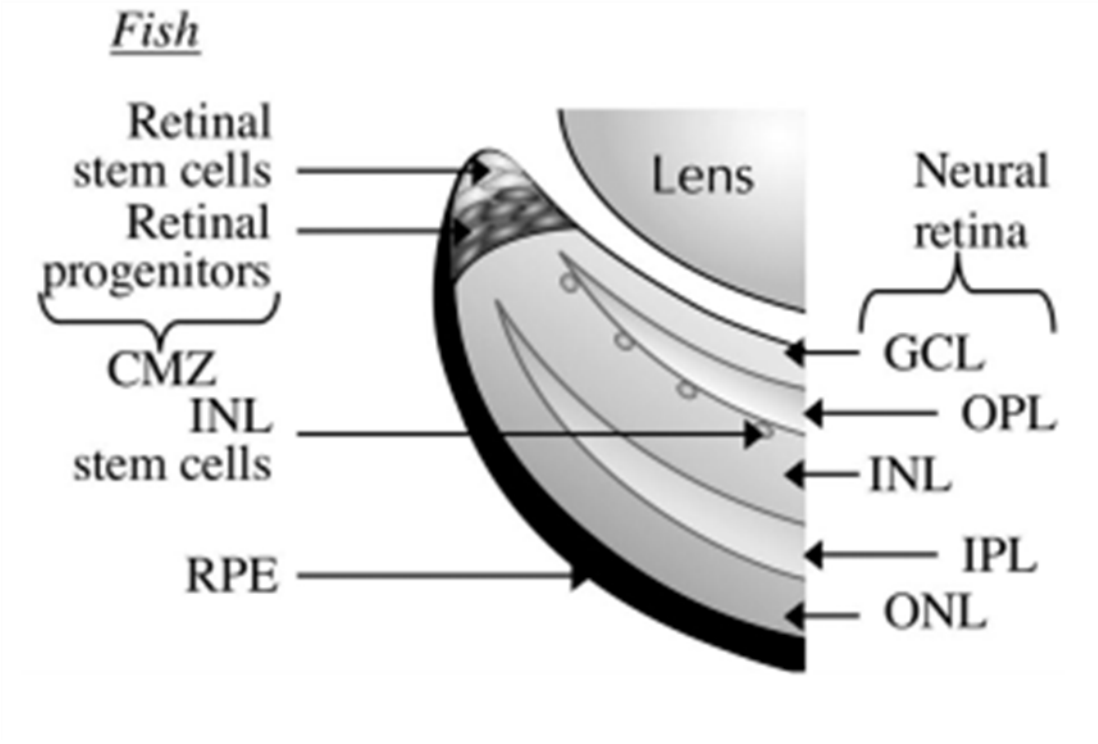

Figure 3. Localisation of the cilliary marginal zone in fish. Multipotent retinal stem cells are found in the most peripheral zone of the cilliary marginal zone of zebrafish. CMZ – cilliary marginal zone; GCL – ganglion cell layer; INL – inner nuclear layer; IPL – inner plexiform layer; ONL – outer nuclear layer; OPL – outer plexiform layer; RPE – retinal pigment epithelium. (Adapted from Amato *et al.*, 2004).

As mentioned before, zebrafish neural retina development is very similar to that of other vertebrates. During neurulation, *six3* and *pax6* expression in the anterior neural plate specify the eye tissues (Kobayashi *et al.*, 2001; Puschel *et al.*, 1992). Through subsequent morphogenetic mechanisms, eyes develop from optic vesicles outpouching

from two sides of the developing neural tube (forebrain) (Fadool and Dowling, 2008). At 24 hpf, the optic cup develops after the invagination of these masses and formation of the optic lumen. The inner layer of the optic lumen gives rise to the neural retina while the outer layer produces the retinal pigment epithelium (RPE) (Fadool and Dowling, 2008) (Figure 4).

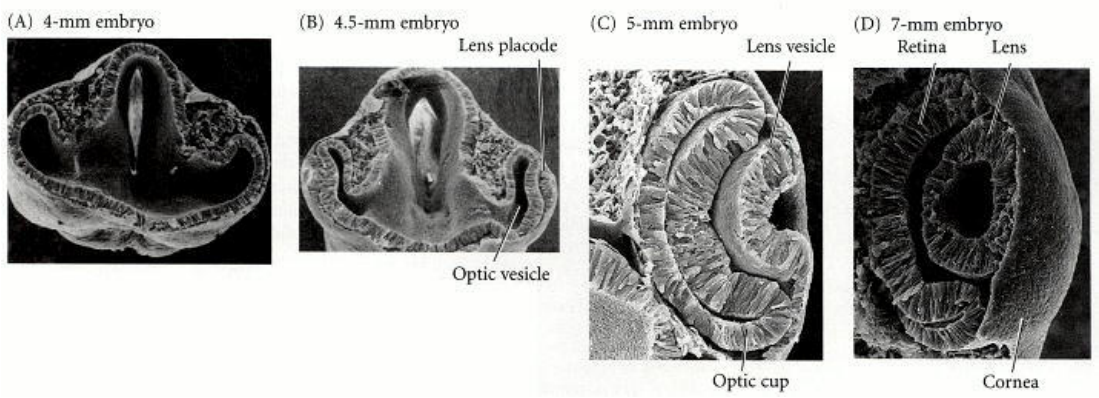

Figure 4. Vertebrate eye development (adapted from Gilbert, S.F., *Part 3. Later embryonic development – Chapter 12. The central nervous system and the epidermis – Development of the Vertebrate Eye* In: Developmental Biology, 6th Ed; Sinauer Associates, Inc: Massachusetts, 2000 (web version))
(A, B) The optic vesicle evaginates from the brain and interacts with the overlying ectoderm, inducing a lens placode. (B, C) The overlying ectoderm differentiates into lens cells as the optic vesicle folds in on itself, and the lens placode becomes the lens vesicle. (C) The optic vesicle becomes the neural and pigmented retina as the lens is internalized. (D) The lens vesicle induces the overlying ectoderm to become the cornea.

Retinal neurogenesis is an orderly process. The first cells to differentiate are ganglion cells. Neural differentiation then follows an inner to outer retinal order. Differentiated ganglion cells appear around 28 and 32 hpf in the ventralnasal retina. Neurogenesis then spreads dorsally around to the ventral temporal retina in a wave-like manner reminiscent of the movement of the morphogenetic furrow in *Drosophila* (Fadool and Dowling, 2008; Hu and Easter, 1999). There are other similarities to *Drosophila*. The wave of differentiation is associated with *sonic hedgehog* expression and the specification of ganglion cells needs *atonal*5 (*ath5*) expression, a homologue of the *Drosophila* gene *atonal* (Fadool and Dowling, 2008) (Figure 5). After the differentiation of the ganglion cells, the differentiation of amacrine cells, interneurons and retinal lamination takes place. At 48 hpf, lamination is across most of the retina. Muller glial cells are among the last to be

formed (Fadool and Dowling, 2008).

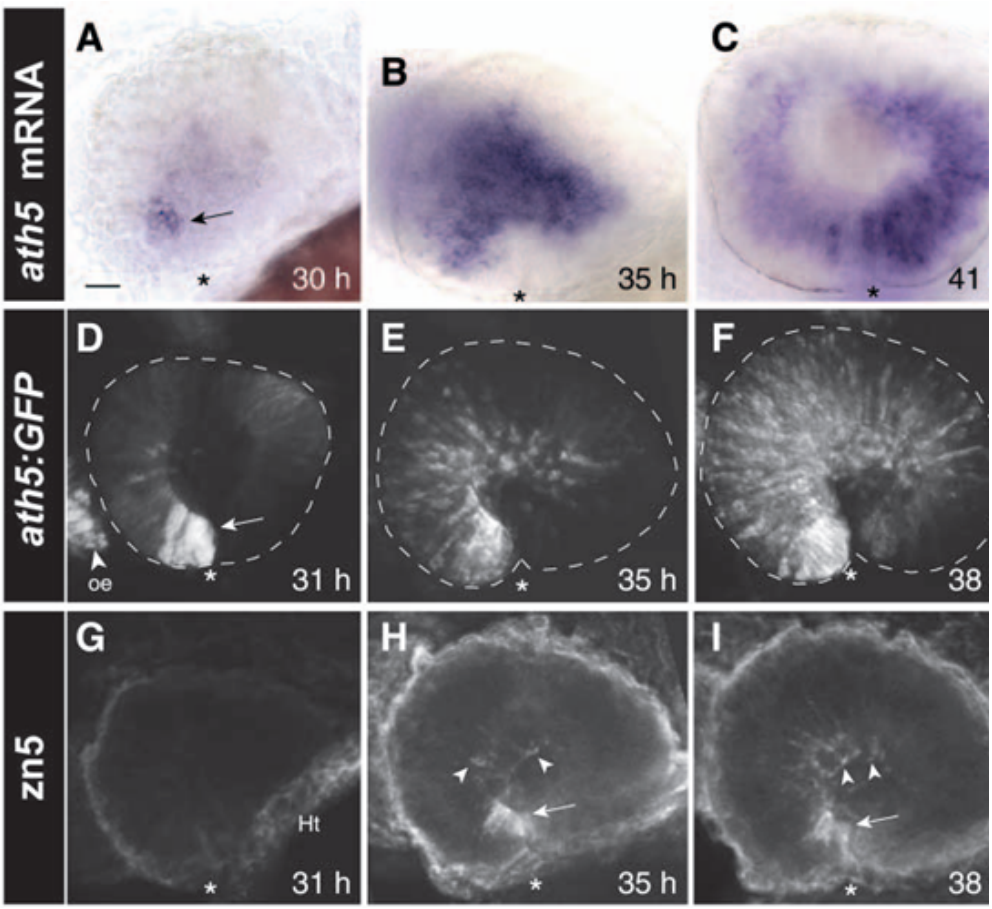

Figure 5. The spatiotemporal pattern of retinal ganglion cells (RGC) neurogenesis in zebrafish. Spread of *aht5* mRNA (A-C), *ath5:GFP* (D-F) and RGC differentiation (G-I) across the retina. (A-C) Whole-mount embryos stained with an antisense *ath5* riboprobe. (D-I) Whole-mount embryos double-stained with anti-GFP (D-F) and zn5 (G-I) antibodies. The *ath5* wave leads the RGC differentiation wave by several hours. Arrows in A, D, H and I indicate the ventralnasal patch. Arrowheads in H and I indicate zn5+ RGCs. Asterisks mark the location of choroid fissure, which delineates the boundary between nasal and temporal retina. Anterior (nasal) is left and dorsal up. oe – olfactory epithelium; Ht – heart. (Adapted from Kay *et al.*, 2005).

1.2. Analogies between fly and vertebrate eye development

Morphologically, the eyes of insects and vertebrates are completely different. Nevertheless, the mechanism of development of this sensory organ is similar in some core aspects. The most remarkable is the role of Pax6. Mammal's *pax6* and its fly homologue, *eyeless (ey)*, when mutated lead to the abolishment of eye development, and when under targeted expression both are capable of inducing ectopic eye (Gehring, 2002). Fly and vertebrate eye development is similar in other remarkable aspects. *Drosophila* retina neuronal differentiation spreads like a wave across the eye imaginal disc. Neurogenesis in zebrafish retina also spreads through several sequential waves. In both cases, the progression of these waves requires the fly gene *atonal* and its vertebrate homologue *ath5*. This suggests that the molecular mechanisms driving the retina neurogenic wave have been conserved from flies to fish (Kay *et al.*, 2001; Brown *et al.*, 2001; Masai *et al.*, 2000) (Figure 5).

The parallelisms found in these previous works in *Drosophila* and zebrafish eye development led us to study the role of other genes, known to be involved in *Drosophila* eye development, in vertebrates. In the next chapters, we will describe the studies on the role played by *homothorax* (*hth*) and *teashirt* (*tsh*) homologues during zebrafish development.

1.3. The *meis* gene family of transcription factors

Hox proteins are transcriptional regulators essential for cell fate specification in early embryonic development. However, these proteins have poor specificity and affinity with their DNA sequence targets. Consequently, they need cofactors to enhance their DNA specificity and affinity (Mann and Affolter, 1998). There are two vertebrate families of Hox cofactors: Pbx and Meis, which belong to the TALE (Three Amino acid Loop Extension) homeodomain superfamily (Choe *et al.*, 2002). The Meis class comprises fly Hth and vertebrate Prep and Meis homeoproteins. Hth and mouse Meis1 protein are highly homologous in two regions called homeodomain (HD) and MH domain (for Meis and Homothorax). The MH domain is found only in Hth and in mouse, human and *Xenopus* Meis1 homologues (Pai *et al.*, 1998); Prep1 and Meis are able to form complexes with Pbx in the absence of a DNA target and there is a strong correlation between *meis* gene expression and nuclear PBX proteins (Toresson *et al.*, 2000; Ferretti *et al.*, 1999; Chang

et al., 1997; Saleh *et al.*, 2000). In several developmental pathways, Pbx1 binds with Meis family members in the cytoplasm to be imported to the nucleus, but during mammalian female genital tract development the control of Pbx1 intracellular distribution is independent of Meis proteins (Dintilhac *et al.*, 2005). While Meis proteins are present in a specific pattern of expression during development, Prep1 is expressed ubiquitously in the embryo (Ferretti *et al.*, 1999). In *Drosophila*, Hth is required for the nuclear localization of the PBX homologue, Extradenticle (Pai *et al.*, 1998; Rieckhof *et al.*, 1997), suggesting that interactions between Meis and Pbx molecules have been conserved from *Drosophila* to mouse (Toresson *et al.*, 2000). *meis1* (myeloid ecotropic viral integration site 1) was first identified as a new site of viral integration in myeloid leukemic tumours arising in BXH-2 mice (Moskow *et al.*, 1995). It was mapped on mouse chromosome 11 and on human chromosome 2p23-p12 (Moskow *et al.*, 1995; Smith *et al.*, 1997). Meis homeobox proteins have important roles in vertebrate development and disease as one of the Hox cofactors, but they also have Hox-independent functions (Moens and Selleri, 2006; Choe *et al.*, 2002; Shen *et al.*, 1997). Choe and colleagues (2009) proposed that Meis proteins act as Hox cofactors through the modulation of the accessibility of histone deacetylases and CBP histone modification enzymes to Hox-regulated promoters, during zebrafish development. Meis proteins are not just involved in DNA binding and complex stabilization on the target DNA but also recruit TALE partner proteins into the nucleus (Geerts *et al.*, 2005). Meis proteins also form complexes with other homeodomain transcription factors, like Engrailed and Pdx, and also with bHLH proteins, like MyoD (Sagerstrom, 2004; Heidt *et al.*, 2007). Meis genes have been reported to have several different full-length splice variants, which probably recruit different proteins, resulting in varying transcriptional activity of the target promoter sequence with functional and tissue distribution differences (Geerts *et al.*, 2005). Noro and colleagues (2006) reported a homeodomain-less isoform encoded by gene *meis1* and *hth,* suggesting that alternative splicing is an evolutionary conserved mechanism to expand and diversify these transcription factors' functions. Nevertheless, *Drosophila* Meis protein Hth *in vivo* function has been much better studied (Mann and Affolter 1998 and Ryoo *et al.*, 1999). Hth is required early in development for a normal eye formation and is an important suppressor of eye differentiation (Pichaud and Casares, 2000, Pai *et al.*, 1998). Data from *Drosophila* studies suggested that other proteins may function as Hox cofactors, such as Teashirt (Tsh). Vertebrate genomes contain *tsh* homologs that, when expressed in flies, can rescue *tsh* mutant phenotypes (Manfroid *et al.*, 2004). However, it is not yet known if these proteins also function as Hox cofactors in vertebrates.

All vertebrate genomes have three functional *meis* genes (Geerts *et al.*, 2005). As

mentioned above, the *meis1* gene was first identified as a major integration site for leukemogenic virus in a murine leukemia model. Similar oncogenic functions were also proposed for its paralogues *meis2* and *meis3* genes (Geerts *et al.*, 2005; Fujino *et al.*, 2001; Hara *et al.*, 2008). Several studies show that *meis* genes enhance early cell proliferation and suppress differentiation during development (Geerts *et al.*, 2005). *meis* genes also play a role in oncogenesis, being best defined in leukemia (Argiropoulos *et al.*, 2008; Wong *et al.*, 2007; Lawrence *et al.*, 1999; Geerts *et al.*, 2005; Esparza *et al.*, 2008; Kumar *et al.*, 2009; Li *et al.*, 2009, Rozovskaia *et al.*, 2001; Sitwala *et al.*, 2009). Indeed, in human leukemia, high expression of *meis1* was found in bone marrow cells of acute myeloid leukemia (AML) patients (Geerts *et al.*, 2005; Afonja *et al.*, 2000). It is reported that *meis1* cooperates with Hox genes to induce AML in mice and these genes are coexpressed in human AML (Calvo *et al.*, 2001; Kroon *et al.*, 1998; Thorsteinsdottir *et al.*, 2001). Recent work offers some inklings on the molecular mechanisms involved: GSK-3 maintains the MLL (Mixed lineage leukemia) leukemia stem cell transcriptional program by promoting the conditional association of CREB, and its coactivators TORC and CBP (CREB binding protein) with homedomain protein Meis1, a critical component of the MLL-subordinate program, which in turn facilitates Hox-mediated transcription and oncogenesis (Wang *et al.*, 2010). There is some evidence promoting a linkage of the leukemogenic activities of Meis1 to the cyclin cell cycle control pathway (Argiropoulos *et al.*, 2010). The role of *meis* genes in other forms of cancers is still mostly unknown, but upregulation was found in pancreatic endocrine neoplasms, in lung adenocarcinoma tumours (Fernandez *et al.*, 2004), in neuroblastomas (Geerts *et al.*, 2005, 2003; Jones *et al.*, 2000; Spieker *et al.*, 2001), in ovarian carcinomas (Crijns *et al.*, 2007) and also in nephroblastomas (Dekel *et al.*, 2006).

In mice, Meis1 is also required for normal hematopoiesis and angiogenesis (Azcoitia *et al.*, 2005; Hisa *et al.*, 2004; Pineault *et al.*, 2002). Hu and colleagues (2009) reported that HoxA9 indirectly modulates Meis1 and that this process is biologically important during normal hematopoiesis, and Simsek and colleagues (2010) found that Meis1 regulates hematopoietic stem cells metabolism through transcriptional activation of Hif-1a (Hypoxia Inducible Factor 1). In zebrafish, Meis1 and Pbx are involved in erythropoietic cell lineage specification and in myelopoiesis inhibition (Pillay *et al.*, 2010). Meis1 was also recently implicated as a regulator of endothelial cell development in zebrafish (Minehata *et al.*, 2008). *meis1* knock-down has a profound effect on primitive erythropoiesis and erythromyeloid progenitor cells and also severely disrupts the proper development of the vasculature (Cvejic *et al.*, 2011).

Meis proteins are reported to be involved in the development of the central nervous

system (CNS) of vertebrates. Meis2 (and Pax6) is expressed during early fetal forebrain development in humans (Larsen *et al.*, 2010). In mice, Meis1 and Meis2 are present and define distinct territories in the developing telencephalon (Toresson *et al.*, 2000). Previous studies suggest that microRNA-9 regulates neurogenesis in mice telencephalon through regulations of several transcription factors, including Meis2 (and Pax6) (Shibata *et al.*, 2011). Elkouby and colleagues (2010) reported that earliest expression of Wnt3a protein in paraxial mesoderm directly activates Meis3 in the overlying neuroectoderm to induce posterior cell fates, suggesting a new model for neural anteroposterior patterning. In zebrafish, Meis2 is expressed in distinct domains of the central nervous system during development, with the strongest expression in the hindbrain (Biemar *et al.*, 2001; Cecconi *et al.*, 1997). In chicken, Meis2 is necessary and sufficient for tectal development through direct interaction with Otx2, a transcription factor required for the formation of all forebrain- and midbrain-derived structures (Agoston and Schulte, 2009). In zebrafish, all Meis proteins have been suggested to promote hindbrain fates (Choe *et al.*, 2002; Waskiewicz *et al.*, 2001) (Figure 6). It was defined that Meis proteins act in the same pathway as Pbx in zebrafish hindbrain development and may function as a DNA-binding partner of Pbx proteins and as a post-transcriptional regulator of Pbx protein levels (Waskiewicz *et al.*, 2001). Zebrafish Meis3 act synergistically with Pbx4 and Hoxb1b to promote hindbrain fates during development (Vlachakis *et al.*, 2001), and Meis1 interacts functionally with Irx7 to activate anterior hindbrain markers, such as *hoxb1a*, *hoxa2* and *krox20* (Stedman *et al.*, 2009). In rhombomere 3, *krox20* transcription is controlled directly by Meis, probably by Meis2 (Wassef *et al.*, 2008). Through morpholino-mediated knockdown of Meis1 protein in zebrafish, Erickson and colleagues (2010) concluded that Meis1 contributes to retinotectal map formation by specifying positional information in both the retina and tectum.

In *Xenopus*, Meis1 and Pbx1 form a transcriptional activation complex and regulate hindbrain and neural crest development (Maeda *et al.*, 2002, 2001). *Xenopus* Meis3 is required for hindbrain patterning during development (Dibner *et al.*, 2001, 2004) and is also essential for primary neuron and neural crest cells fates (Gutkovich *et al.*, 2010). In *Xenopus*, Meis1 and Pbx1 are important direct or indirect regulators of Zic3 expression, which is involved in the control of left-right asymmetry and neural tube closure in vertebrates (Kelly *et al.*, 2006).

Recent studies revealed linkage of the *meis1* locus to the human neurologic disorder Restless Legs Syndrome (Trenkwalder *et al.*, 2009; Xiong *et al.*, 2009).

In the olfactory epithelium (OE) of mice, slowly dividing self-renewing precursors express Meis1 and Meis2 at high levels and these precursors primarily reside in the lateral

OE (Tucker *et al.*, 2010).

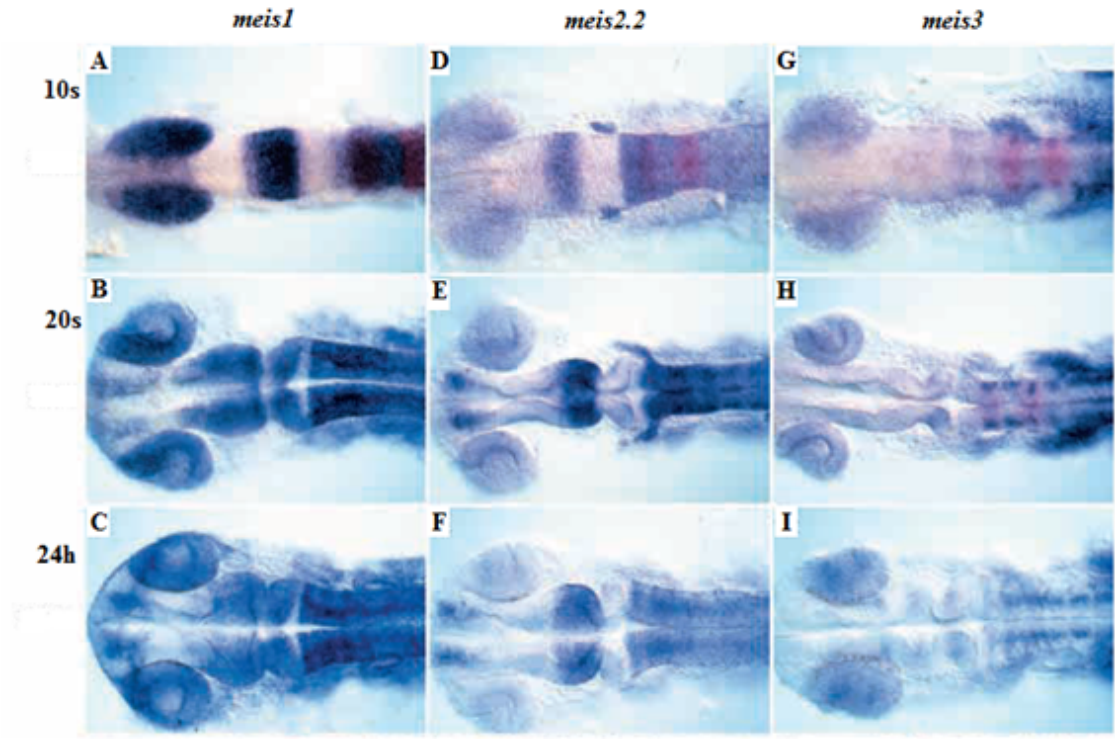

Figure 6. Expression patterns of *meis1*, *meis2.2* and *meis3* during segmentation stages of zebrafish development. RNA *in situ* hybridization of *meis1* (A-C), *meis2.2* (D-F) and *meis3* (G-I) at the stages of 10 somites, 20 somites and 24 hpf. *meis* expression is in blue, *krox20* is in red. In each picture anterior is towards the left. (Adapted from Waskiewicz *et al.*, 2001)

In mice and chicken limbs, *meis1* and *meis2* are expressed in the proximal domain, and the ectopic expression of *meis1* in the distal domain disrupts its development and induces distal-to-proximal transformations in a way similar to *Drosophila*'s homologue *hth* (Mercader *et al.*, 1999, 2000, 2009; Capdevila *et al.*, 1999).

Sánchez-Guardado and colleagues (2011) reported *meis1* and *meis2* expression in the developing chicken inner ear, suggesting a possible role of Meis assigning regional identity in the morphogenesis, patterning, and specification of the developing inner ear.

meis3 is expressed in pancreatic islets and cultured β-cells and is required for β-cell survival, and has PDK1 (3-phosphoinositide-dependent protein kinase 1) as a direct target that mediates Meis3 role in β-cell survival (Liu *et al.*, 2010). Meis1 may also be involved in pancreas organogenesis through its downregulation by the homeobox transcription factor PDX-1 (von Burstin *et al.*, 2010).

As mentioned before, eye development and structure are very different in invertebrates and vertebrates but some components of the regulatory network involved in eye development are conserved (Heine *et al.*, 2008). Postmitotic neurons in the compound eye of *Drosophila melanogaster* are generated in the wake of a single neurogenic wave, called morphogenetic furrow (MF), that goes across the eye imaginal disc (Heine *et al.*, 2008). Proliferating progenitor cells ahead of the MF express the protein Hth, which together with Ey and Tsh promotes rapid, asynchronous proliferation of retinal progenitor cells and prevents their premature differentiation (Bessa *et al.*, 2002). *hth* homologs, *meis1* and *meis2*, are expressed in the eye during development (Hisa *et al.*, 2004; Heine *et al.*, 2008). Heine and colleagues (2008) showed that Meis have an evolutionary conserved role in maintaining the proliferating state of early retinal progenitor cells and their results also place these proteins upstream of CyclinD1 and Pax6 in retinal progenitor cells. *pax6* is an essential gene for eye development in numerous organisms, including humans, but the direct upstream molecular regulators of *pax6* expression in the developing eye are poorly known (Zhang *et al.*, 2002). In mice, *pax6* lens placode enhancer is bound by Meis1 and Meis2 directly, controlling *pax6* expression during early lens ectoderm induction (Zhang *et al.*, 2002). The role of the transcription factors Meis during zebrafish eye development is addressed in Chapter I.

In the medaka fish (*Oryzias latipes*), Meis2 is one of the main targets of the miRNA miR-204 function and together with altered regulation of the Pax6 pathway, the abnormally elevated levels of Meis2 resulting from miR-204 inactivation are largely responsible for microphthalmia, abnormal lens formation, and altered dorsoventral patterning of the retina, which is associated with optic fissure coloboma (Conte *et al.*, 2010).

1.4. *teashirt* gene family, general importance

tsh is the founder member of a family of evolutionary conserved zinc-finger genes, which encode proteins that regulate development, and was first described in *Drosophila melanogaster* (Fasano *et al.*, 1991). *Drosophila* Tsh protein has three distantly spaced zinc-finger motifs (Fasano *et al.*, 1991). In the *Drosophila* embryo, *tsh* expression is present in parasegments 3-13, the central nervous system, first and second midgut constrictions, proximal part of leg imaginal discs and dorsal vessel during hearth morphogenesis (Alexandre, 1996).

During embryogenesis, Tsh has been shown to be involved in the anterioposterior

(AP) determination of the trunk segments and repression of the anterior head development through its function has a Hox cofactor (Fasano *et al.*, 1991; Roder *et al.*, 1992; Bhojwani *et al.*, 1997; Robertson, 2004). In different combinations with Cubitus interruptus (Ci) and Armadillo, Tsh takes part in the specification of naked cuticle along the AP axis and within each trunk segment in the larval epidermis (Angelats, 2002). Tsh mediates trunk-specific Wingless (Wg) signalling activity (naked cell-fate) by binding to Armadillo, and Tsh, in addition to Ci, is required for normal Hedgehog (Hh) maintenance of *wg* expression in the trunk (Gallet *et al.*, 1998). Tsh is also required positively for the expression of the Hh target gene *rhomboid* (*rho*) (Gallet *et al.*, 2000). In trunk parasegments 3-13, Tsh directly interacts with and limits *sex combs reduced* (*scr*) transcription and salivary gland induction (Andrew, 1994, 1998; Taghli-Lamallem, 2007). Tsh is necessary for normal morphogenesis of the anterior and central midgut structures. *tsh* expression in the central midgut mesoderm is regulated by Ultrabithorax, Abdominal-A, Decapentaplegic (Dpp) and Wg (Mathies, 1994). The Wg pathway regulates Tsh post-translationally through its phosphorylation and nuclear accumulation, resulting in the recruitment of Tsh to this pathway (Gallet *et al.*, 1999). Tsh, in cooperation with Brinker, is required for the wingless-mediated repression of *ultrabithorax* and *labial* in the midgut (Waltzer, 2001; Saller, 2002). Embryonic midgut *tsh* expression is dependent not only upon *hox* genes, like *antennapedia* (*antp*), but also upon *extradenticle* (*exd*), and normal morphogenesis of the gut relies on the normal expression of these genes (Rauskolb *et al.*,1994).

In the *Drosophila* wing imaginal disc, *tsh* expression marks the presumptive notum (Ng *et al.*, 1996), and Tsh collaborates with Hth to block wing blade development, probably by repressing some of the activities of the Notch pathway at the dorsoventral compartment boundary (Casares and Mann, 2000). Tsh has also been identified as a positive regulator of *hth* during hinge specification (Azpiazu and Morata, 2000; Soanes *et al.*, 2001). Repression of *tsh* through the combined action of Wg and Dpp, and subsequent repression of *hth* are necessary events for the specification of the wing field (Wu and Cohen, 2002; Zirin and Mann, 2004).

Tsh is also involved in proximal leg morphogenesis, where it is required for proper growth and cell differentiation (Wu and Cohen, 2000; Erkner *et al.*, 1999). Grunge (Gug), an Atrophin-like protein, is a positive regulator of *tsh*, specifically in ventroproximal cells of the proximal leg (Erkner *et al.*, 2002).

tsh is expressed in the eye imaginal disc, anterior to the morphogenetic furrow, and is part of the gene network responsible for the specification of eye identity (Pan and Rubin, 1998). Tsh functions as a complex with Ey and Hth to block the expression of later-

acting transcription factors (Sine oculis (So), Eyes absent (Eya) and Dachshund (Dac)), and to promote cell proliferation, proposing that regulation of *hth*, *ey* and *tsh* is critical for the transition from an uncommitted proliferative state to a mature differentiated state during *Drosophila* eye development (Bessa *et al.*, 2002). Recently, it has been shown that Hth and Tsh require Yorki (Yki), a downstream component of the Hippo tumour suppressor pathway, to induce proliferation and survival of the undifferentiated cells in the eye imaginal disc (Peng *et al.*, 2009). *tsh* has to be first expressed in undifferentiated cells and later has to be turned off to allow retinal cells differentiation, i.e. its expression must be transient. Tsh role as a regulator of eye specification has been proposed to be due to its ability to make eye disc cells responsive to Wg and Dpp signalling in an eye-specific manner (Bessa *et al.*, 2005). Tsh has also functions along the eye disc dorsoventral (DV) axis, as it suppresses eye development close to the ventral margin and promotes eye development near the dorsal margin (Singh *et al.*, 2002). These Tsh functions, require expression of early DV patterning genes; Iroquois-Complex (Iro-C) and Delta (Dl) in the dorsal part and Serrate (Ser) in the ventral (Singh *et al.*, 2004).

tsh has a paralogue gene called *tiptop* (*tio*) (Laugier *et al.*, 2005). *Drosophila*'s Tio and Tsh proteins show the largest similarity in their three shared zinc-finger motifs (Laugier *et al.*, 2005). Curiously, both *Drosophila melanogaster*'s *tio* and the single *Anopheles gambiae*'s *tiotsh* gene have a conserved fourth zinc-finger motif in the C-terminal part, which is also present in vertebrate Tsh proteins (Laugier *et al.*, 2005; Caubit *et al.*, 2000). During *Drosophila*'s early embryogenesis, *tsh* and *tio* expressions are present in distinct domains: *tsh* in the trunk and *tio* in parts of the head and tail. But later in embryo development, they have both common and tissue-specific expression patterns (Laugier *et al.*, 2005). In contrast, *tsh* and *tio* are coexpressed in the imaginal discs (Bessa *et al.*, 2009). In both embryonic and larval development, Tio and Tsh are functionally similar, since Tio can partially rescue Tsh loss. Tio and Tsh can repress each other's expression and Tsh also auto-regulates its own expression through a negative feedback loop (Laugier *et al.*, 2005; Bessa *et al.*, 2009). These authors suggest that these mechanisms could be a way to ensure control of total Tio/Tsh levels.

tsh has also been described in other insects. A single *tiotsh* gene was described in *Tribolium castaneum* (Shippy *et al.*, 2008). In this same work, it is suggested that *Drosphila tsh* and *tio* paralogues resulted from a duplication event after divergence from the mosquito lineage. *Thermobia domestica* has one *tsh* gene that, curiously, is not expressed strongly in the abdomen, unlike *Drosophila*'s *tsh* (Peterson *et al.*, 1999).

tsh genes have also been described in vertebrates but not so extensively. Vertebrate and *Drosophila tsh* genes are similar in their molecular structure, with their

three first and widely spaced zinc-finger motifs. However, vertebrates have additional zinc-fingers and a vertebrate-specific homeodomain (Koebernick *et al.*, 2006; Onai *et al.*, 2007; Caubit *et al.*, 2000) (Figure 7). Despite these differences, the three mice *tshz* genes can rescue Tsh loss-of-function in flies, suggesting that the molecular function of Tshz is phylogenetically conserved (Manfroid *et al.*, 2004). In mammals, three *tshz* genes have been described so far (*tshz1-3*) and the presence of *tshz2* and *tshz3* was recently detected in mice ureters and *tshz3* in human embryonic renal pelvis (Jenkins *et al.*, 2009). These authors and others (Caubit *et al.*, 2008) also suggest that Tshz3 may have a function in ureter morphogenesis in mice and mutations of the gene in humans may be one of the causes for congenital pelvi-ureteric junction obstruction (PUJO) (Jenkins *et al.*, 2009). In mice, Tshz3 is required for proximal ureteric smooth muscle cell differentiation, downstream of Sonic hedgehog (Shh) and Bone morphogenetic protein 4 (Bmp4) (Caubit *et al.*, 2008). Mice Tshz proteins were also suggested to have a role in the establishment of regional identity and specification in the forebrain (Caubit *et al.*, 2005). In mice *tshz3* is expressed in multiple areas of the brainstem involved in respiration and its deficiency leads to breathe failing and death at birth (Caubit *et al.*, 2010). Mice *tshz1* and *tshz2* are detected in neural tube, somites, in developing limbs, branchial arches and gut, suggesting that Tshz and fly Tsh may share conserved functions in axis patterning and limbs specification (Caubit *et al.*, 2000; Long *et al.*, 2001). In humans, reduced expression of *tshz3* may be involved in development of Alzheimer's disease (Kajiwara *et al.*, 2009) and human *tshz2* and *tshz3* are possible candidates for tumour suppressor genes and expression of both is downregulated in breast and prostate cancer (Yamamoto *et al.*, 2011).

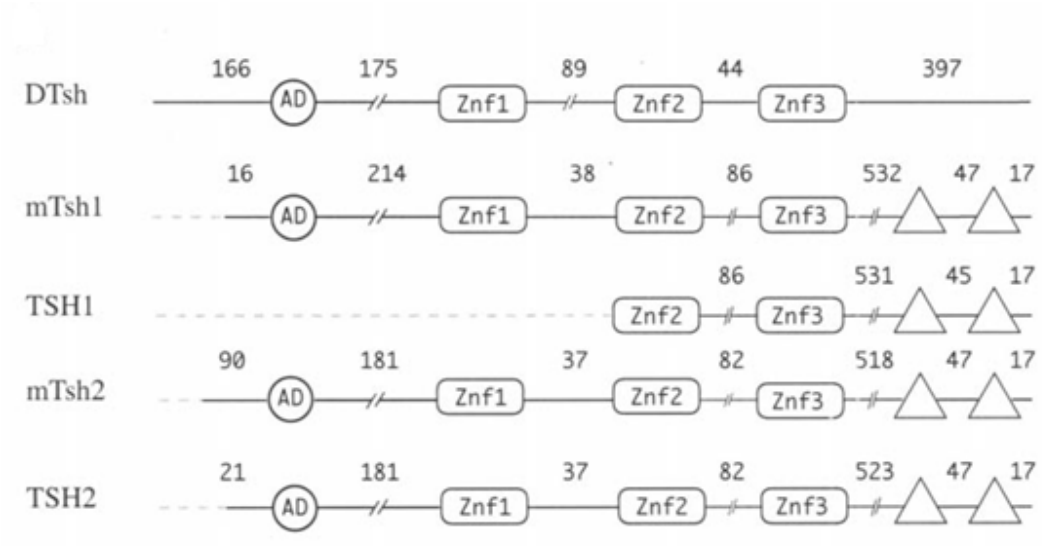

Figure 7. Structural organization of Human (TSH1 and TSH2), Mouse (mTsh1 and mTsh2) and *Drosophila* (DTsh) Tsh proteins. Proteins are aligned according to the position of conserved motifs. Znf1, Znf2 and Znf3 boxes represent typical Teashirt zinc-finger motifs (Cx2Cx12HMx4H). Spacing between these motifs is variable and is indicated by the number above the line. Triangles indicate additional potential zinc-finger motifs (Cx2Cx12Hx3-4H) which are not Tsh-like. AD: indicates a domain rich in glutamic and aspartic residues. Grey dashed lines indicate missing sequence data. (Adapted from Caubit *et al.*, 2000).

Like mammals, the genome of the amphibian *Xenopus tropicalis* also has three *tshz*-related genes (*tshz1-3*) (Onai *et al.*, 2007). In frogs, at tailbud and larval stages of development, *tshz3* is expressed mainly in the caudal central nervous system (hindbrain and spinal cord) and in the caudal branchial arch (Onai *et al.*, 2007). These authors suggest that Xtshz3 is an important promoting factor for the dorsal axis determination in *Xenopus* embryo and this function is exerted by facilitating canonical Wnt signalling. These authors also propose that Xtshz3 play a role in AP axis patterning. In fact, Xtshz1 has also been demonstrated to be essential for the AP patterning of the central nervous system (CNS) (Koebernick *et al.*, 2006). Xtshz1 also controls segmental migration of cranial neural crest cells. Besides expression in the caudal CNS and migrating cranial neural crest cells, Xtshz1 is also detected in diencephalon, pronephros and olfactory placodes (Koebernick *et al.*, 2006).

In chicken, *tshz3* is expressed in the central and in the peripheral nervous system and in myotendinous junctions, muscles and connective tissues. In somites, chicken *tshz3* is activated by FGF8 (Manfroid *et al.*, 2006).

Until now only *tshz1* had been described in zebrafish (Wang *et al.*, 2007). From 2 to 25-somite stages of development, *tshz1* is expressed in the posterior neural tube. From prim-5 stage onwards, its expression in this domain gradually diminishes and shifts to the anterior neural tissues including the telencephalon, the tectum opticum, the midbrain-hindbrain boundary, the hindbrain and the eyes (Figure 8). In Chapter II, we report the finding and description of other *tshz* genes during zebrafish development.

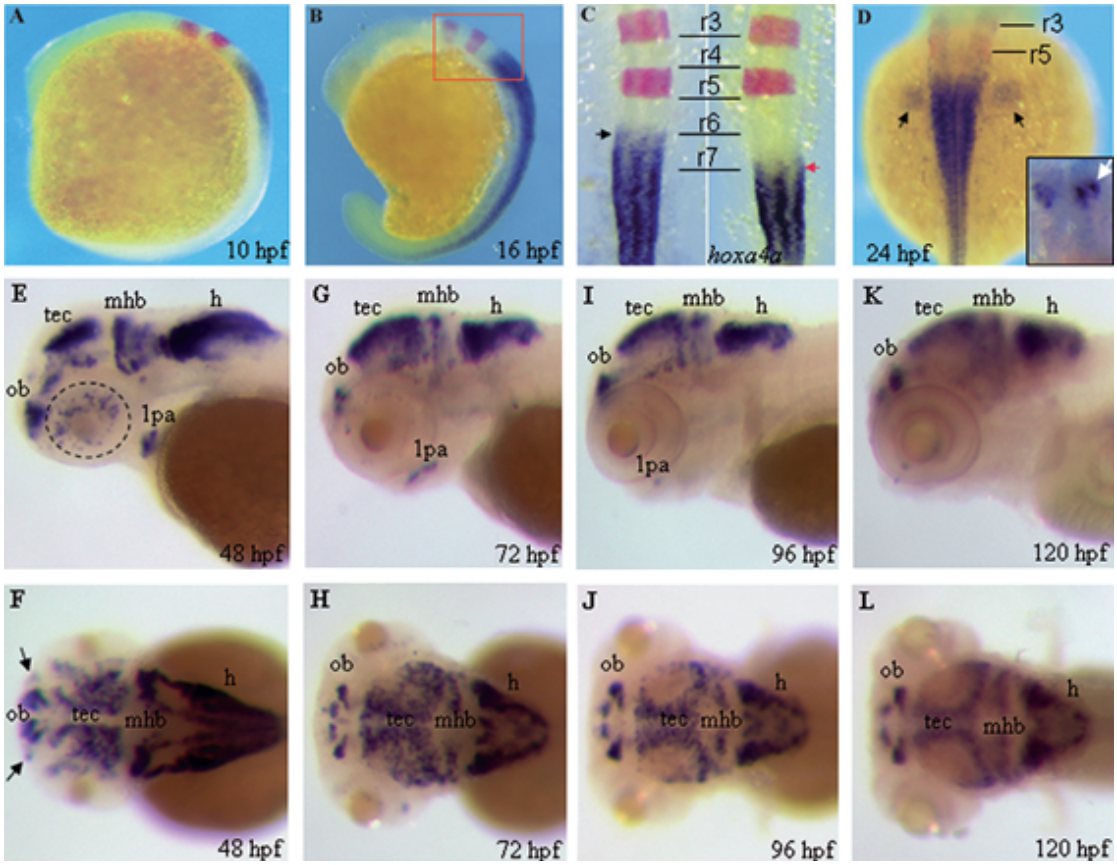

Figure 8. Spatial and temporal expression patterns of *tshz1* revealed by RNA *in situ* hybridization. *tshz1* expression is in blue and *krox-20* is in red. (A, B, E, G, I and K) are lateral views and (C, D, F, H, J and L) are dorsal views. The inset in (D) is an anterior view (dorsal to the top) of the forebrain of the hybridized embryo. (A) At the 2-somite stage (10 hpf), *tshz1* expression is initiated in the hindbrain and anterior spinal cord. (B) At the 14-somite stage (16 hpf), *tshz1* is expressed throughout the spinal cord. (C) Dorsal view of the hindbrain region (red box in B). The expression domain of *tshz1* has a clear anterior boundary at the rostral margin of rhombomere 7 (r7). (D) At the prim-5 stage (24 hpf), *tshz1* is expressed in the spinal cord and pectoral fin buds (black arrows) and in the dorsal forebrain (white arrow in the inset). (E-F) At the long-pec stage (48 hpf), *tshz1* is expressed in the olfactory bulb (ob), tectum opticum (tec), mid-hindbrain boundary (mhb), hindbrain (h), first pharyngeal arch (1pa) and in the eye (dash line-circled), with very weak expression in the olfactory placodes (arrows in F). (G-H) At the protruding mouth stage (72 hpf), lower levels of *tshz1* transcripts are observed in the olfactory bulb, pharyngeal arch and midbrain-hindbrain boundary. (I-L) At the early larvae stages (96-120 hpf), reduced expression of *tshz1* is seen in the tectum and first arch. By 120 hpf, no *tshz1* expression is detectable in the pharyngeal arches, but is still observed in restricted areas of the telecephalon, tectum, mid-hindbrain boundary and hindbrain. (Adapted from Wang *et al.*, 2007).

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2 Chapter I

"*meis1* regulates *cyclin D1* and *c-myc* expression and controls the proliferation of the multipotent cells in the developing zebrafish eye."

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meis1 regulates *cyclin D1* and *c-myc* expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye

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During eye development, retinal progenitors are drawn from a multipotent, proliferative cell population. In *Drosophila* the maintenance of this cell population requires the function of the TALE-homeodomain transcription factor Hth, although its mechanisms of action are still unknown. Here we investigate whether members of the Meis gene family, the vertebrate homologs of *hth*, are also involved in early stages of eye development in the zebrafish. We show that *meis1* is initially expressed throughout the eye primordium. Later, *meis1* becomes repressed as neurogenesis is initiated, and its expression is confined to the ciliary margin, where the retinal stem population resides. Knocking down *meis1* function through morpholino injection causes a delay in the G1-to-S phase transition of the eye cells, and results in severely reduced eyes. This role in cell cycle control is mediated by *meis1* regulating *cyclin D1* and *c-myc* transcription. The forced maintenance of *meis1* expression in cell clones is incompatible with the normal differentiation of the *meis1*-expressing cells, which in turn tend to reside in undifferentiated regions of the retinal neuroepithelium, such as the ciliary margin. Together, these results implicate *meis1* as a positive cell cycle regulator in early retinal cells, and provide evidence of an evolutionary conserved function for Hth/Meis genes in the maintenance of the proliferative, multipotent cell state during early eye development.

KEY WORDS: *meis1*, Zebrafish, Cell cycle, Eye development, *cyclin D1*, *c-myc* (*myca*)

INTRODUCTION

During the development of the eye, in vertebrates and invertebrates, neural progenitors derive from multipotent and proliferative cells (reviewed by Chow and Lang, 2001; Dominguez and Casares, 2005). In the *Drosophila* eye primordium, the TALE-class homeodomain transcription factor Homothorax (Hth) is expressed in this multipotent population, where it is required to maintain these cells in a proliferative state and to prevent their premature differentiation (Pai et al., 1998; Pichaud and Casares, 2000; Bessa et al., 2002). The homologs of *hth* in vertebrates are the Meis and Prep (also known as Pknox) gene families (reviewed by Burglin, 1997; Moens and Selleri, 2006). Whereas the expression of Prep genes is widespread in mice and zebrafish, Meis genes show specific transcription patterns in vertebrates, including expression in the developing eye (Ferretti et al., 1999; Toresson et al., 2000; Waskiewicz et al., 2001; Maeda et al., 2002; Zhang et al., 2002; Hisa et al., 2004).

Recent work points to a role for Meis genes in eye development: Meis1 and Meis2 are upstream regulators of Pax6 in the developing lens in chicken and mouse (Zhang et al., 2002), and mouse embryos homozygous for a homeodomain-less *Meis1* gene show eye malformations (Hisa et al., 2004). Still, the precise role(s) played by Meis genes during eye development remain(s) unknown. If the parallels in early eye development between flies and vertebrates hold true for Hth/Meis, Meis genes might be involved in stimulating proliferation, or preventing premature differentiation in the optic primordium, or both. Here, we investigated these hypotheses in the zebrafish (*Danio rerio*).

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MATERIALS AND METHODS

Probe preparation, in situ hybridization and immunolabeling

Antisense RNA probes were prepared from cDNAs and labeled with digoxigenin. Specimens were fixed, hybridized and stained as described (Tena et al., 2007).

Fluorescent probes and antibodies

Propidium iodide (PI) was used as nuclear stain; FITC-phalloidin to mark filamentous actin; anti-Islet1 mouse monoclonal antibody labels GCL [36 hours post-fertilization (hpf)] and ganglion cell layer (GCL) plus inner nuclear layer (INL) (48-72hpf) (from DSHB, University of Iowa); rabbit anti-GFP (A11122, Molecular Probes), mouse anti-Myc (MMS 150P, Covance), mouse anti-cleaved Caspase 3 (Cell Signaling Technology). Fluorescent secondary antibodies were from Molecular Probes. Dissected eyes from stained embryos were imaged using a Leica-SP2 confocal system, and data processed with Adobe Photoshop.

In vitro RNA synthesis and microinjection of mRNA and morpholinos

cDNAs were linearized and transcribed as described (Tena et al., 2007). One- to two-cell-stage zebrafish embryos were injected in the yolk with mRNA and/or morpholino (MO) diluted in ~5 nl of injection solution (10% Phenol Red in DEPC-treated water).

MOs targeting the ATG region of *meis1*, *meis2.2*, *meis3* and *meis4* mRNAs (see Fig. S1A in the supplementary material) were synthesized by GeneTools. We verified the target specificity of *meis1*- and *meis2.2*-MOs in *Xenopus laevis* assays (see Fig. S1B in the supplementary material), and the biological specificity of the *meis1*-MO by testing its ability to reduce the rhombomere-3 expression of *krox20* (also known as *egr2* – ZFIN) (see Figs S2 and S5 in the supplementary material).

As controls, we injected similar amounts (8-16 ng) of a control MO directed against the *Xenopus tropicalis olig2* gene that shows no match in the zebrafish genome (see Fig. S1 in the supplementary

material). The *meis3*-MO, which has nine and seven mismatches with *meis1* and *meis2.2*, respectively, also served as control MO in some experiments.

Eye phenotype measurements

The polygonal-lasso tool from Adobe Photoshop was used to measure in digital photographs taken with the same magnification, the eye surface area (in pixels) of control and morphant embryos. The volume of each eye was estimated considering it as a hemisphere of radius equal to the radius of a circle with that same area. Measurements from 20 eyes for each condition were compared using a χ^2 test.

Plasmid constructs

I.M.A.G.E. cDNA clones, from the Lawrence Livermore National Laboratory Consortium, used were: ccnd1 (IMAGE IRALp962K2356Q), c-myc (IRBOp991F125D), meis1 (IRAKp961C08136Q), meis2.1 (IRBOp991C0733D), meis2.2 (IRBOp991D0437D), meis3 (IRALp962E1456Q) and meis4 (MPMGp609N1326Q). pCS2-ccnd1 was generated by inserting the full-length cDNA into *Eco*RI and *Xba*I sites of pCS2+. To generate GFP-meis1, MT-meis1, meis1-MT, MT-meis2.2, meis2.2-MT, MT-meis3, meis3-MT, MT-meis4 and meis4-MT constructs, we PCR amplified the corresponding Meis coding regions with the following primer pairs (5′-3′; *Eco*RI and *Xho*I sites underlined): GAATTCGATGGCGCAGAGGT and CTCGAGCATGTAGTGCCAC-TGTCCC for *meis1*; GAATTCGATGGCGCAAAGGTACGA and CTCGAGCATGTAGTGCCACTGGCC for *meis2.2*; GAATTCCATG-GATAAGAGGTATGAGGAGTT and CTCGAGGTGGGCATGTA-TGTCAA for *meis3*; and GAATTCCATGGCGCAACGGTACGA and CTCGAGCATGTAGTGCCACTGACTCTC for *meis4*.

The PCR fragments were subcloned into pGEMT-Easy (Promega) and sequenced. Meis cDNAs were cloned into pCS2 MT, pCS2p+MTC2 or pCS2eGFP (kindly provided by D. Turner, University of Michigan, USA) to generate N-terminal (Myc-meis) and C-terminal (meis-Myc) Myc-tagged meis or N-terminal GFP-tagged *meis1* (GFP-*meis1*), respectively. To generate the Tol2-GFP-*meis1* and Tol2-GFP constructs, we inserted the GFP-*meis1* and GFP fragments, respectively, into *Sal*I and *Ssp*I sites of Tol2 (pT2KXIG).

Acridine Orange staining

Acridine Orange staining was performed as described (Perkins et al., 2005).

DNA content analysis and flow cytometry

Eyes dissected from 19hpf zebrafish embryos were disaggregated, and PI staining carried out as described (Langenau et al., 2003). DNA content was analyzed on a BD FACSAria and results processed with FloJo software (Tree Star). A χ^2 test was used for statistical data analysis.

Induction of ectopic expression mosaics

The Tol2 transposon/transposase method of transgenesis (Kawakami et al., 2004) was used with minor modifications. Four- to 16-cell-stage zebrafish embryos were injected in the yolk with 5-12.5 pg of either Tol2-GFP-*meis1* or Tol2-GFP constructs, plus 125 pg of transposase-encoding mRNA in a final volume of 5 nl of injection solution. Embryos were cultured at 28.5°C, staged and fixed. Anti-GFP antibody was used to detect the GFP- or GFP-*meis1*-expressing clones. A stack of confocal *z*-sections was obtained for each eye analyzed. Three-dimensional reconstruction of the stacks was used to determine the location of the clones.

RESULTS AND DISCUSSSION

meis1 expression is restricted to the undifferentiated and proliferating cells of the early zebrafish eye

Of all five zebrafish Meis genes (*meis1*, *2.1*, *2.2*, *3* and *4.1*), only *meis1* and *meis2.2* are expressed during early stages of eye development (Kudoh et al., 2001; Waskiewicz et al., 2001; Zerucha and Prince, 2001; Thisse and Thisse, 2005) (this work). *meis1*, as monitored by in situ hybridization, or by a YFP insertional reporter inserted close to *meis1*, was seen to be uniformly transcribed in the eye primordium from 15 to ~24hpf (Fig. 1A and see Fig. S3 in the supplementary material), a period in which all cells proliferate (Li et al., 2000). After this time, *meis1* expression progressively retracted in the retina (Fig. 1B-D,K,L) as the neurogenic wave, marked by *ath5* (also known as *atoh7* – ZFIN) expression, expands from antero-nasal to posterior-temporal positions (Fig. 1F-H) (Hu and Easter, 1999; Li et al., 2000; Masai et al., 2000). *meis1* remained transiently expressed in the ciliary margin zone (CMZ), where the retinal stem population resides (Fig. 1D,M). *meis2.2* was also found to be expressed uniformly in early eye primordia, but its expression faded away by 20hpf (see Fig. S3 in the supplementary material). Similar to the situation found in chicken and mouse (Zhang et al., 2002), *meis1* was expressed in the prospective lens ectoderm, but was turned off as the lens placode started to thicken (Fig. 1I,J). Therefore, *meis1* expression is associated with the undifferentiated, proliferative cells during the early development of the zebrafish eye. In addition, a new wave of Meis gene expression starts in postmitotic neurons at around 36-42hpf (Fig. 1M and see

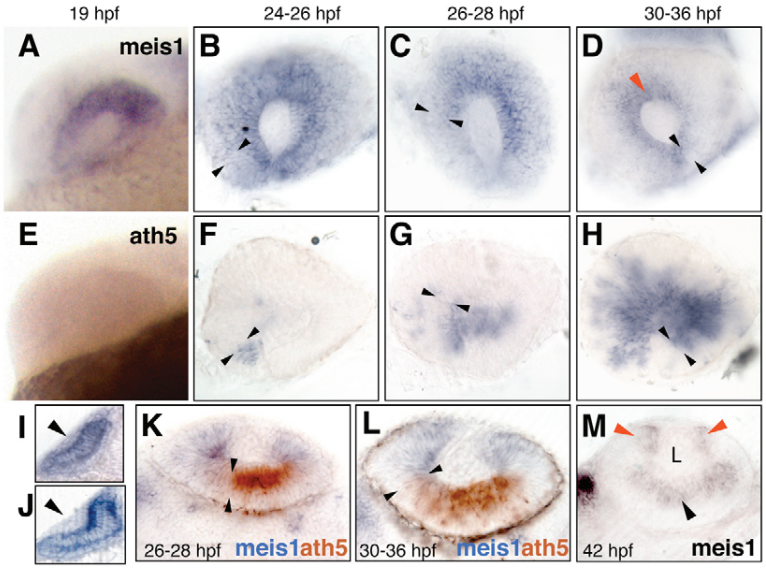


Fig. 1. *meis1* retracts accompanying the *ath5* wave and becomes restricted to the CMZ. (**A-D**) *meis1* and (**E-H**) *ath5* expression analyzed by single in situ hybridization. Developmental stages are indicated as hours post-fertilization (hpf) at 28.5°C. Lateral views of whole-mount (A,E) or dissected (B-D,F-H) eyes, with dorsal up and anterior to the left. The front of the *ath5* domain is marked by black arrowheads. The red arrowhead in D points to *meis1* expression in the prospective ciliary margin. (**I-M**) Transverse 40 μm vibratome sections. (I,J) Dorsal is up. *meis1* is weakly expressed in the lens ectoderm before its thickening (I), but no signal is detected once the lens placode is formed (J). (K-M) Dorsal is to the left. *meis1* and *ath5* expression domains are complementary as shown by double in situ hybridization (K,L). Approximate limits of the *ath5* signal are indicated by the black arrowheads. (M) At 42hpf, *meis1* expression is detected by in situ hybridization in the ciliary margin (red arrowheads) and in the postmitotic ganglion cells (black arrowhead). L, lens.

Fig. S3 in the supplementary material). Interestingly, at 4 days post-fertilization (dpf), *meis2.2* expression had replaced *meis1* at the CMZ.

meis1 is required to promote the G1-to-S transition of the eye primordium cells, and regulates the transcription of *cyclin D1* and *c-myc*

The early expression pattern of *meis1* suggests that it plays a role in the proliferative/multipotent cells of the developing eye. To determine what role that is, we knocked down *meis1* function using *meis1*-specific morpholinos (*meis1*-MO). By the end of embryogenesis, *meis1* morphants were severely microphthalmic (>60% of embryos injected with 8 ng of *meis1*-MO, *n*=163) (Fig. 2A,B,E,F), with eyes containing fewer cells than controls (Fig. 2C,D). Despite this, *meis1*-morphant eyes showed apparently normal retinal lamination (Fig. 2C′,D′). The lens was normal or slightly reduced (Fig. 2D and data not shown). The co-expression of

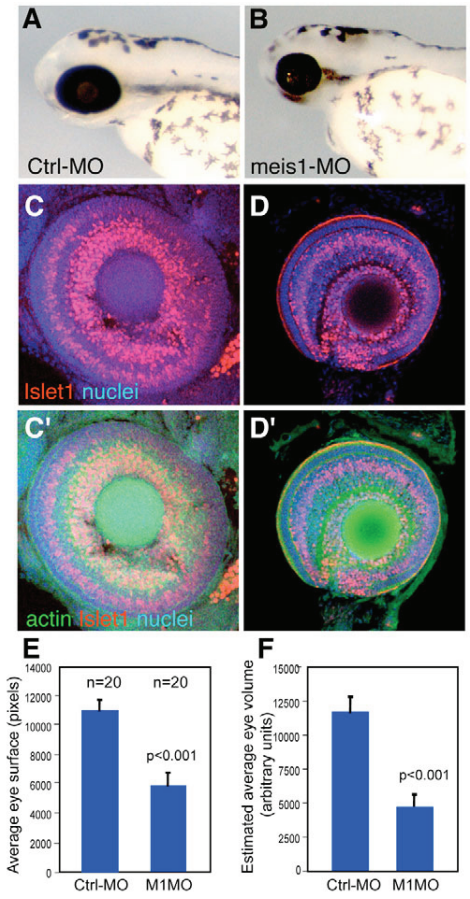


Fig. 2. *meis1* is required for the growth of the eye primordium. (**A**,**B**) Lateral views of representative 72hpf control-MO (A) and *meis1*-MO (B) -injected fish. *meis1* morphants are microphthalmic. (**C**,**D**) Confocal images of dissected eyes stained for propidium iodide (nuclei), rhodamine-phalloidin (filamentous actin) and Islet1, which labels GCL nuclei and some in the INL. The reduced eyes from *meis1* morphants show apparently normal retina lamination (D,D′), but fewer cells than control eyes (C,C′). Area (**E**) and estimated volume (**F**) of control-MO and *meis1*-MO-injected embryos at 72hpf. *meis1*-morphant embryos show a significant ($P<0.001$) reduction in eye area and volume (45% and 60%, respectively). *n*=20 for each condition.

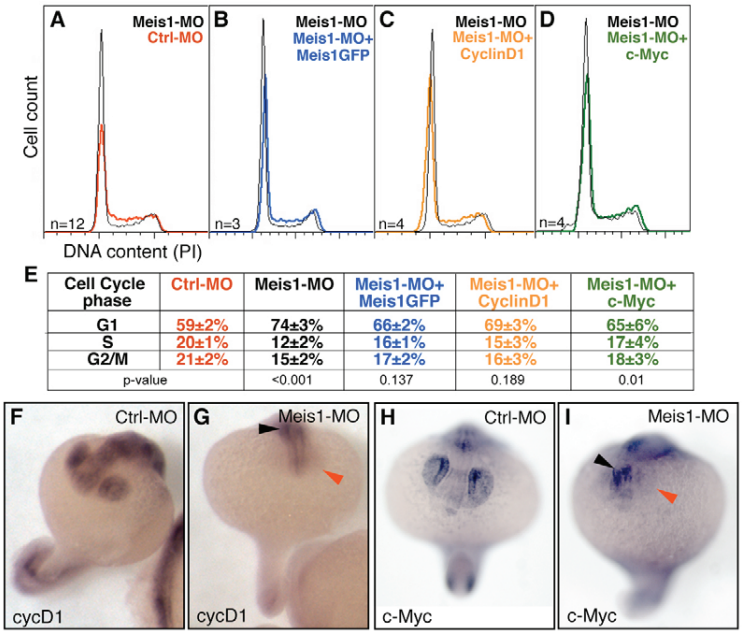
meis2.2 and *meis1* during optic vesicle stages suggested a possible functional redundancy between these two genes. Nevertheless, injection of *meis2.2*-MO (8 ng/embryo) caused only mild eye reductions in 22% of the treated embryos (*n*=299). Furthermore, co-injection of equivalent amounts of *meis1*- and *meis2.2*-MOs (4 ng of each MO/embryo) did not significantly enhance the penetrance or severity of the microphthalmia (29%, *n*=196). These results suggest that *meis2.2* does not have a major role during early stages of eye development in the zebrafish. The phenotype observed in *meis1* morphants does not appear to be due to an abnormal eye primordium specification. Although we found a slight decrease in *pax6b* expression in *meis1*-morphant eyes, as estimated by RT-PCR (see Fig. S4C,D in the supplementary material), the early expression of the eye selector genes *pax6b* and *rx2* (Stigloher et al., 2006) seemed unaffected, when observed by in situ hybridization (see Fig. S4A,B in the supplementary material and data not shown). In agreement with this, we found that the expression of the Pax6 gene *eyeless* is independent of *hth* during the development of the *Drosophila* eye (see Fig. S4E,F in the supplementary material).

To further dissect the mechanisms underlying the observed microphthalmia, we assessed whether *meis1* controls the cell cycle. *meis1*-morphant eyes, at 19hpf, had a significantly higher percentage of cells in G1 phase than control embryos (Fig. 3A,E), indicating a requirement of *meis1* in promoting the G1–S transition. Viability of these cells was not compromised, as *meis1*-morphant eyes did not show a significant increase in the levels of active Caspase 3, or in the vital incorporation of Acridine Orange (not shown).

cyclin D1 (*ccnd1*) and *c-myc* (also known as *myca* – ZFIN) are two major G1 regulators of the cell cycle in vertebrates (Levine and Green, 2004). During the development of the zebrafish eye, *cyclin D1* and *c-myc* are first widely expressed in the optic vesicle, followed by a progressive restriction to the proliferating cells of the neural retina (Thisse and Thisse, 2005; Yamaguchi et al., 2005), a pattern that is reminiscent of that of *meis1*. In addition, recent work shows that *cyclin D1* is required for proliferation in the zebrafish developing retina, as *cyclin D1* morphants are microphthalmic (Duffy et al., 2005). The similarity between the patterns of expression of *meis1*, *cyclin D1* and *c-myc*, and the similar eye phenotypes of *cyclin D1* and *meis1* morphants, prompted us to ask whether *cyclin D1* and *c-myc* were under *meis1* control. Indeed, *meis1* morphants showed a dramatic reduction of *cyclin D1* and *c-myc* expression in the eye when compared with control-injected embryos (Fig. 3F-I and see Fig. S5 in the supplementary material). In addition, the co-injection of either *cyclin D1* or *c-myc* mRNAs partially rescued the cell cycle defects of *meis1* morphants to levels similar to those obtained by co-injection of GFP-*meis1* mRNA (Fig. 3B-E). These results place *cyclin D1* and *c-myc* functionally downstream of *meis1* in the control of cell cycle progression in the developing eye. Whether *meis1* regulates the transcription of *cyclin D1* and *c-myc* directly or indirectly is unknown.

Maintenance of *meis1* expression is incompatible with cell differentiation

In *Drosophila*, *hth* not only promotes proliferation in the eye primordium, but forced maintenance of its expression results in a delay or block of retinal differentiation (Pai et al., 1998; Pichaud and Casares, 2000; Bessa et al., 2002). Similarly, in the early zebrafish eye, *meis1* expression is found in undifferentiated cells but is turned off as neurogenesis advances (Fig. 1). To test whether maintaining *meis1* expression is incompatible with retinal differentiation, we analyzed the distribution of clones of cells expressing either GFP or



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Cell Cycle phase	Ctrl-MO	Meis1-MO	Meis1-MO+ Meis1GFP	Meis1-MO+ CyclinD1	Meis1-MO+ c-Myc
G1	59±2%	74±3%	66±2%	69±3%	65±6%
S	20±1%	12±2%	16±1%	15±3%	17±4%
G2/M	21±2%	15±2%	17±2%	16±3%	18±3%
p-value		<0.001	0.137	0.189	0.01

Fig. 3. *meis1* is required for the G1–S transition and the expression of the G1–S regulators *cyclin D1* and *c-myc*. (**A-D**) Histograms displaying DNA content (cell cycle profile) of cells from dissected 19hpf eyes of *meis1*-MO treated embryos relative to (A) controls (Crtl-MO), (B) *meis1*-MO+GFP-*meis1* mRNA, (C) *meis1*-MO+*cyclin D1* mRNA, and (D) *meis1*-MO+*c-myc* mRNA injected embryos. Number of replicates (*n*) and *P* values are indicated. *meis1* knockdown induces a delay in G1, which is partially rescued by co-injection of GFP-*meis1* (B), *cyclin D1* (C) and *c-myc* (D) mRNAs. (**E**) Average percentages are shown for G1, S and G2/M DNA content. The cell-cycle profiles of control morphants and of uninjected, wild-type embryos are indistinguishable (not shown). (**F-I**) In situ analysis of *cyclin D1* and *c-myc* transcription in control (F,H) and *meis1* morphants (G,I) at 19hpf. *meis1* morphants show a dramatic reduction in *cyclin D1* and *c-myc* in the eye (red arrowheads). *cyclin D1* and *c-myc* are still detected in other body regions (black arrowheads). Hybridization and reaction development were performed strictly in parallel. Representative embryos are shown.

GFP-tagged-Meis1 in developing retinas, prior to and after the initiation of neuronal differentiation (Fig. 4 and see Fig. S6 in the supplementary material). Differentiation was followed using the GCL marker *islet1*. When analyzed between 24 and 30hpf, a stage at which most of the retina is undifferentiated, all GFP- and 80% of GFP-Meis1-expressing clones spanned the whole width of the neuroepithelium (*n*=57 and 46, respectively; Fig. 4A,D). Later in development, when retinal differentiation is ongoing and layering becomes apparent, most GFP clones appeared in the central retina and contained both Islet1-expressing and non-expressing cells (90%) (Fig. 4B,C), whereas only a few (7%) were found in the CMZ (*n*=41). By contrast, of the GFP-Meis1 clones located in the central retina (72%, *n*=39), none contained Islet1-positive cells at this stage (Fig. 4E). In addition, a large portion of these Meis1-expressing clones (28%, *n*=39) was found in the CMZ (Fig. 4F). The fact that Meis1-expressing cells were always found in undifferentiated regions of the neuroepithelium, leads us to conclude that maintenance of *meis1* expression in the first 48 hours of eye development is incompatible with neuronal differentiation.

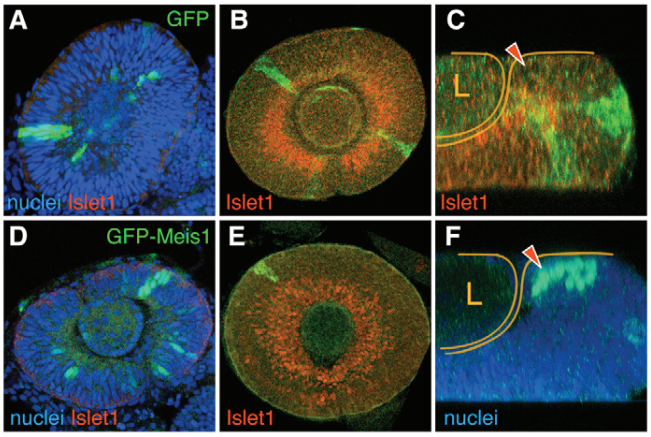

Fig. 4. Clonal overerexpression of *meis1* in the developing eye prevents differentiation and results in cell sorting. Single optical sections from confocal *z*-stacks of GFP (**A-C**) or GFP-Meis1 (**D-F**) expressing clones induced genetically in developing eyes. GFP-*meis1* signal is nuclear. At 24-30hpf, both clone types frequently span the whole width of the neuroepithelium (A,D). Confocal optical sections through the central retina (B,E) and *z*-sections (C,F) of 48hpf eyes. At this stage, GFP clones comprise both Islet1-expressing and non-expressing cells (B,C). By contrast, same-stage GFP-Meis1 clones in the central retina do not contain Islet1-positive cells (E). GFP-Meis1 clones are often located in the CMZ (F). The arrowheads (C,F) point to the CMZ, and the retina and the lens (L) are outlined.

Our results indicate that, during early eye development, *meis1* shares two roles with its fly homolog, *hth*. First, *meis1* is required to maintain proliferation of the multipotent cells of the early eye, by promoting the G1-to-S transition of the cell cycle. Mechanistically, *meis1* regulates the transcription of at least two potent cell-cycle activators: *cyclin D1* and *c-myc*. Second, *ath5* follows receding *meis1* expression in a similar fashion as in *Drosophila*, where *hth* expression retracts as the *atonal*-expressing differentiation wave advances. This finding is in accordance with a model in which the expression of *meis1* has to be downregulated to allow further differentiation of the fish retina, and agrees with our results that the sustained expression of *meis1* is incompatible with neural differentiation. Similar results have been obtained in chicken and mouse by Heine and co-workers (Heine et al., 2008). Although retinal lamination in *meis1* morphants is not grossly affected, we cannot rule out specific effects on the specification and/or differentiation of specific retinal cell types, as *meis1*, together with *meis2.1* and *meis2.2*, is redeployed in postmitotic cells of the ganglion cell and inner nuclear layers (Fig. 1M and see Fig. S3 in the supplementary material).

The expression of *meis1* in the CMZ, and the fact that forcing *meis1* expression results in the localization of the expressing cells to the CMZ, suggest that *meis1* might function in specifying the retinal stem cells of the zebrafish. In this regard, it is interesting to note that *meis1* expression resembles that of *Pax6*, a previously described retinal progenitor transcription factor (Raymond et al., 2006) (reviewed by Amato et al., 2004). In *Drosophila*, previous results showed that *hth* and *eyeless* are co-expressed in the undifferentiated

domain and that their products might directly interact in vivo (Bessa et al., 2002). All these results seem to indicate that a common molecular mechanism to maintain a multipotent stem-like state exists during eye development in vertebrates and invertebrates.

In addition to controlling several developmental processes, Meis genes are overexpressed in an increasing number of cancer types (Lawrence et al., 1999; Segal et al., 2004; Geerts et al., 2005; Dekel et al., 2006). Therefore, the identification of functional targets of the Meis genes involved in the maintenance of the undifferentiated and proliferative state during normal development, such as *cyclin D1* and *c-myc*, is likely to be instrumental in deciphering the mechanisms underlying Meis-associated tumors.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/799/DC1

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A

Meis1	AGGGCGATGGCGCAGAGGTACGAAGATATACCCCACT
Meis2.1	ATGCTGATGGCTCAACGGTACGAAGACCTTGCCCACT
Meis2.2	AGGCTGATGGCGCAAAGGTACGATGAGCTGGCTCACT
Meis3	TATCGCATGGATAAGAGGTATGAGGAGTTGGTGCAGT
Meis4	GTCCTCATGGCGCAACGGTACGAGGATCTGGTCCACT
Ctl MO	TGCTTGAAAGACAGTCTGAATCCAT

B

MO	Target	Mismatches
Meis1 –	Meis2.1	6
	Meis2.2	5
	Meis3	9
	Meis4	6
Meis2.2	Meis1	6
	Meis2.1	5
	Meis3	7
	Meis4	3
Meis3 –	Meis1	9
	Meis2.1	9
	Meis2.2	7
	Meis4	8

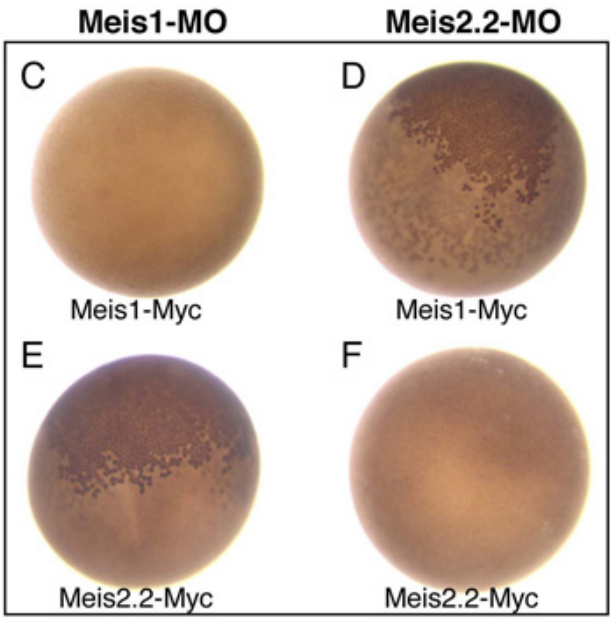


Figure S1. Morpholino (MO) sequences, targets and specificity assay in *Xenopus*. (A) Alignment of Meis sequences around the ATG (underlined). Morpholino (targeted) sequences are shadowed in gray. The control mopholino used in this paper (Ctrl-MO) was originally designed to target the ATG region of *Xenopus tropicalis Olig2*. It does not target any ATG in the zebrafish genome. (B) Mismatches between *meis1*, *meis2.2* and *meis3* morpholinos and the ATG regions of non-target Meis genes. All but one have five or more mismatches, which should prevent cross-targeting. The exception is *meis2.2*-MO, which might cross-target *meis4*. This fact did not interfere in our analysis since *meis4* is not expressed in the developing eye. *meis3*-MO, which has nine and seven mismatches with *meis1* and *meis2.2*, respectively, is included here as it also served as control MO in some experiments. (C-F) Specificity assay in *Xenopus laevis* embryos. *meis1* and *meis2.2* mRNAs, carrying a C-terminal Myc tag (*meis1*-Myc and *meis2.2*-Myc) (10 ng), were co-injected with either *meis1* or *meis2.2* MOs (1 ng) in two-celled *Xenopus* embryos. After injection, embryos (n>50 per experiment) were allowed to develop for 24 hours at 18°C, fixed and immunostained to detect the Myc tag (e.g. Tena et al., 2007). Effective targeting results in the block of mRNA translation and no production of the Myc-tagged protein. Each MO blocks the translation of only its specific mRNA (C,F; absence of nuclear Myc staining). Accordingly, *meis1*-MO is unable to block *meis2.2*-Myc translation, *as meis2.2*-MO is incapable of blocking that of *meis1*-Myc (B,E; presence of nuclear Myc staining, brown).

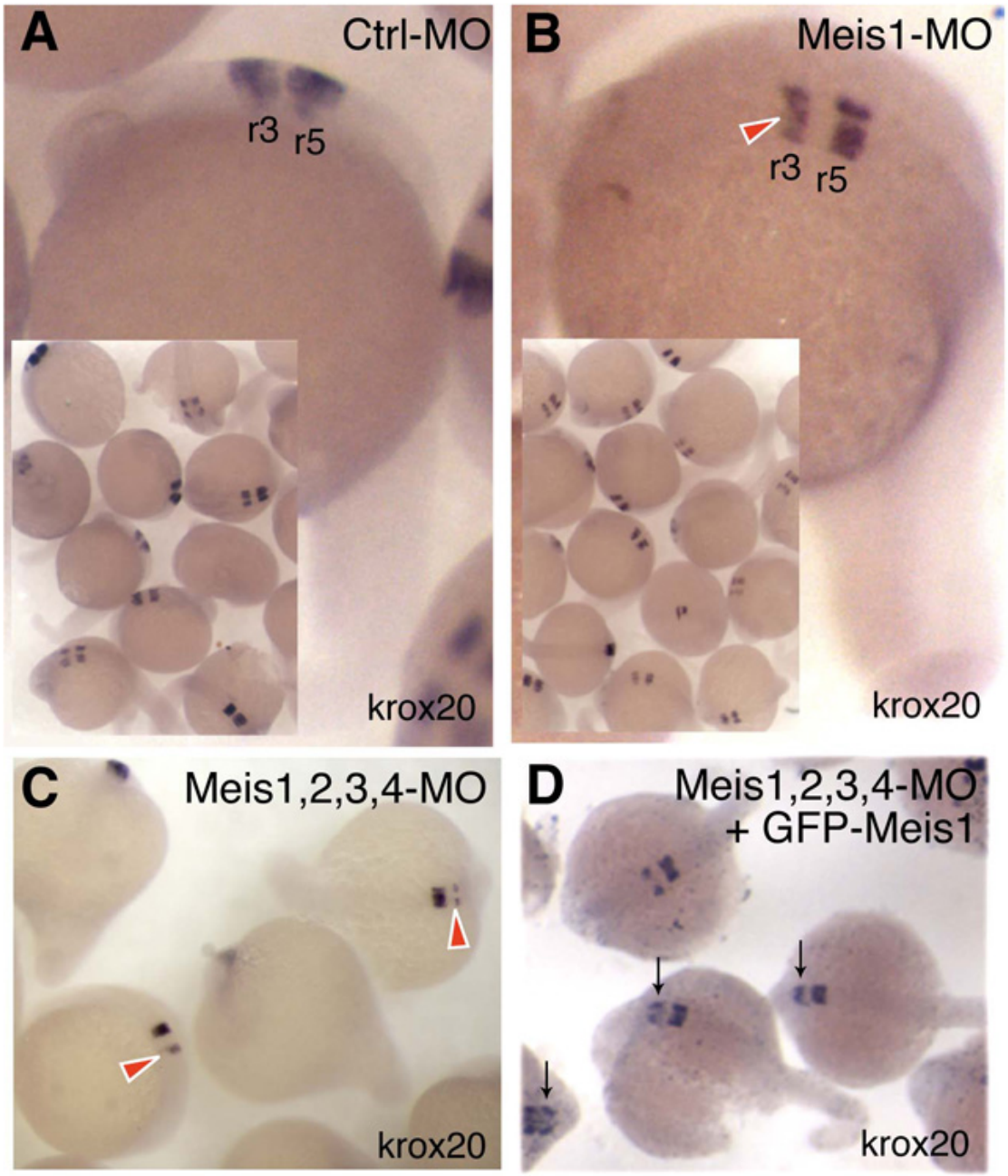


Figure S2. *meis1*-MO injection reduces rhombomeres-3 *krox20* expression specifically. (A) Ctrl-MO has no effect on *krox20* expression in rhombomeres 3 (r3) or 5 (r5). (B) *meis1*-MO embryos show a specific reduction of r3 *krox20* expression (red arrowhead). (C) Injection of a *meis1*, *2.2*, *3* and *4* MO cocktail results in an even stronger inhibition of r3 *krox20* expression (red arrowheads). (D) This effect is partially rescued by injection of GFP-*meis1* mRNA (*meis1*GFP) (black arrows). GFP-*meis1* is insensitive to *meis1*-MO, since the N-terminal tagging of *meis1* with GFP destroys the *meis1*-MO target sequence in the GFP-*meis1* mRNA. These results phenocopy the reported effect on r3 *krox20* expression caused by injection of a dominant-negative Meis form (Waskiewicz et al., 2001) and thus confirm the biological specificity of the Meis mopholinos used in this study.

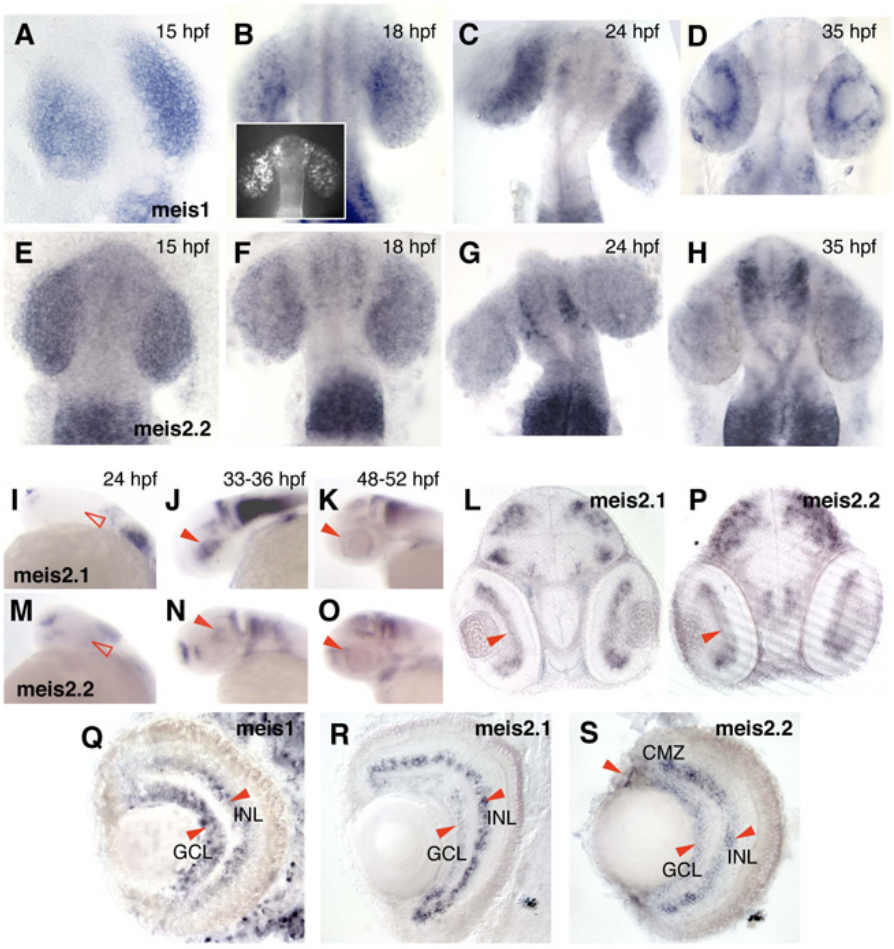
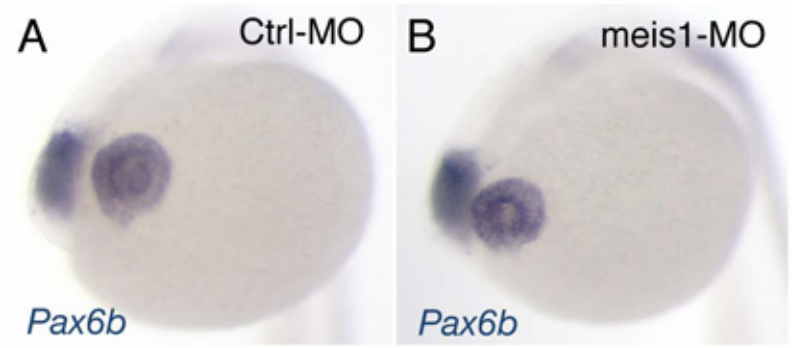
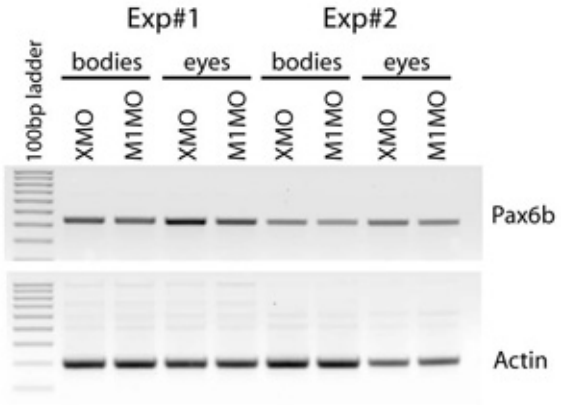


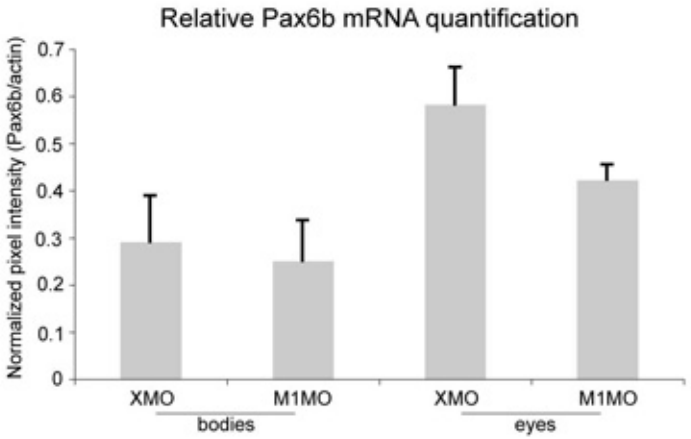
Figure S3. Expression of *meis1*, *meis2.2* and *meis2.1* during eye development. (A-H) Dorsal views of flat-mounted embryos. *meis1* and *meis2.2* are expressed uniformly throughout the optic vesicle (A,E) and early-stage optic cup (B,F). After this developmental point, *meis2.2* expression fades (G,H), while *meis1* becomes progressively restricted to the peripheral retina (C,D). Inset in B is a flat mount of a 16-hpf *meis1*-YFP showing uniform YFP signal in the optic vesicle, which recapitulates *meis1* expression at this stage. (I-K,M-O) Lateral views of whole-mount embryos. *meis2.1* and *meis2.2* are not expressed in the eye (open arrowheads) at 24 hpf (I,M). Both genes begin to be expressed in a central patch of the neural retina at about 33-36 hpf (J,N) that becomes a thin rim of cells by 48-52 hpf (K,O). (L,P) 40-µm vibratome sections of gelatin-embedded embryos show that, at 48-52 hpf, *meis2.1* and *meis2.2* are expressed in postmitotic ganglion cells. (Q-S) 15-µm cryostat sections of eyes from 4-dpf fish, hybridized with probes against (Q) *meis1*, (R) *meis2.1* and (S) *meis2.2*. At this stage, all three genes are expressed in the GCL and in the most central region of the INL − likely amacrine cells − but at different relative levels. Also at this stage, only *meis2.2* is expressed at significant levels in the ciliary margin zone (CMZ). Arrowheads indicate expression detected by in situ hybridization. hpf and dpf, hours and days post-fertilization, respectively, at 28.5°C. The *meis1*-YFP enhancer trap is an insertion of a YFP-retroviral vector (Laplante et al., 2006) located 1,707 bp downstream of the last exon of *meis1* (chr13) and inserted in the same transcriptional direction.


A
Ctrl-MO
Pax6b
B
meis1-MO
Pax6b

C


Exp#1
Exp#2
bodies
eyes
bodies
eyes
100bp ladder
XMO
M1MO
XMO
M1MO
XMO
M1MO
XMO
M1MO
Pax6b
Actin

D


Relative Pax6b mRNA quantification
Normalized pixel intensity (Pax6b/actin)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
XMO
M1MO
XMO
M1MO
bodies
eyes

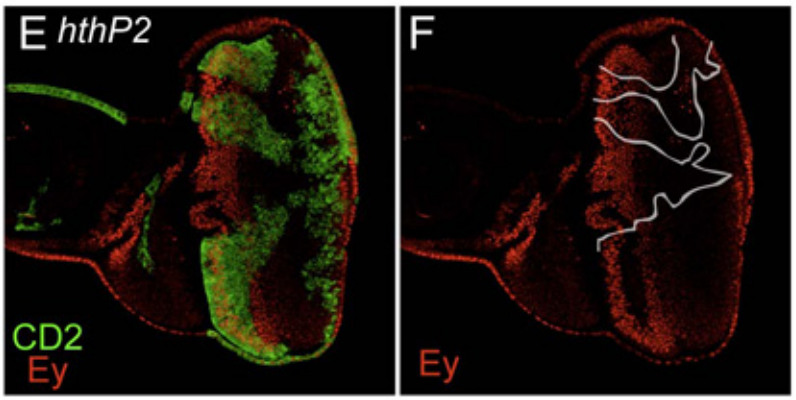

E hthP2
CD2
Ey
F
Ey

Figure S4. Meis/Hth genes are not essential for *pax6/ey* expression. (A,B*) pax6b*, detected at 20 hpf by in situ hybridization, is expressed at similar levels in both control-MO (A) and *meis1*-MO (B) injected embryos, despite the already noticeable reduction of the eye primordium in *meis1*-morphants. We have noticed, though, a consistent reduction of *pax6b* expression in the lens of *meis1* morphants. (C) RT-PCR of two independent experiments (#1 and #2) for *pax6b* and *actin*, as internal control. Total RNA, from dissected eyes or remaining bodies of 19-hpf injected embryos (control-MO and *meis1*-MO), was isolated using an RNeasy Kit (Qiagen) and cDNA prepared with standard methods. PCR was performed with specific primers for *pax6b*, forward 5′-GATTTTGCAGGTGTCGAATG-3′ and reverse 5′-TTCACCACCGTTGTCCTGT-3′; *β-actin* primers as in (Duffy et al., 2005). PCR protocol used: denaturation of 95°C for 2 minutes, followed by 28 cycles of 95°C for 1 minutes, 60°C for 30 seconds, and 72°C for 1 minute, and a final elongation of 72°C for 5 minutes. (D) The image processing and pixel intensity values of ethidium bromide-stained agarose gels were obtained with Typhoon 9410 Variable Mode Imager. Results from two independent experiments (each performed in duplicate), are expressed as mean±STDEVP (standard deviation using the entire data population as argument). (E,F) *Drosophila* late third larval stage eye disc containing clones of cells mutant for a strong *hth* hypomorphic allele, *hthP2*. The clones are marked by the absence of CD2 (clones are outlined in F). *ey* expression remains unaltered in *hth* mutant cells. *Hth* clones were induced between 48 and 72 hours after egg laying by a 20-minute 37°C heat shock on larvae resulting from the cross of *FRT82B hthP2/TM2* males to *yw hsFLP 122; FTR82B hsCD2 y+ M/TM2* females (e.g. Pichaud and Casares, 2000). Expression of CD2 was induced by a 30-minute heat shock at 37°C, 30 minutes prior to dissecting wandering third instar larvae. Mouse anti-CD2 is from Serotec, and rabbit anti-Ey is a gift from Patrick Callaerts. Secondary antibodies were from Molecular Probes. Images were acquired on a Leica SP2 confocal system and processed with Adobe Photoshop.

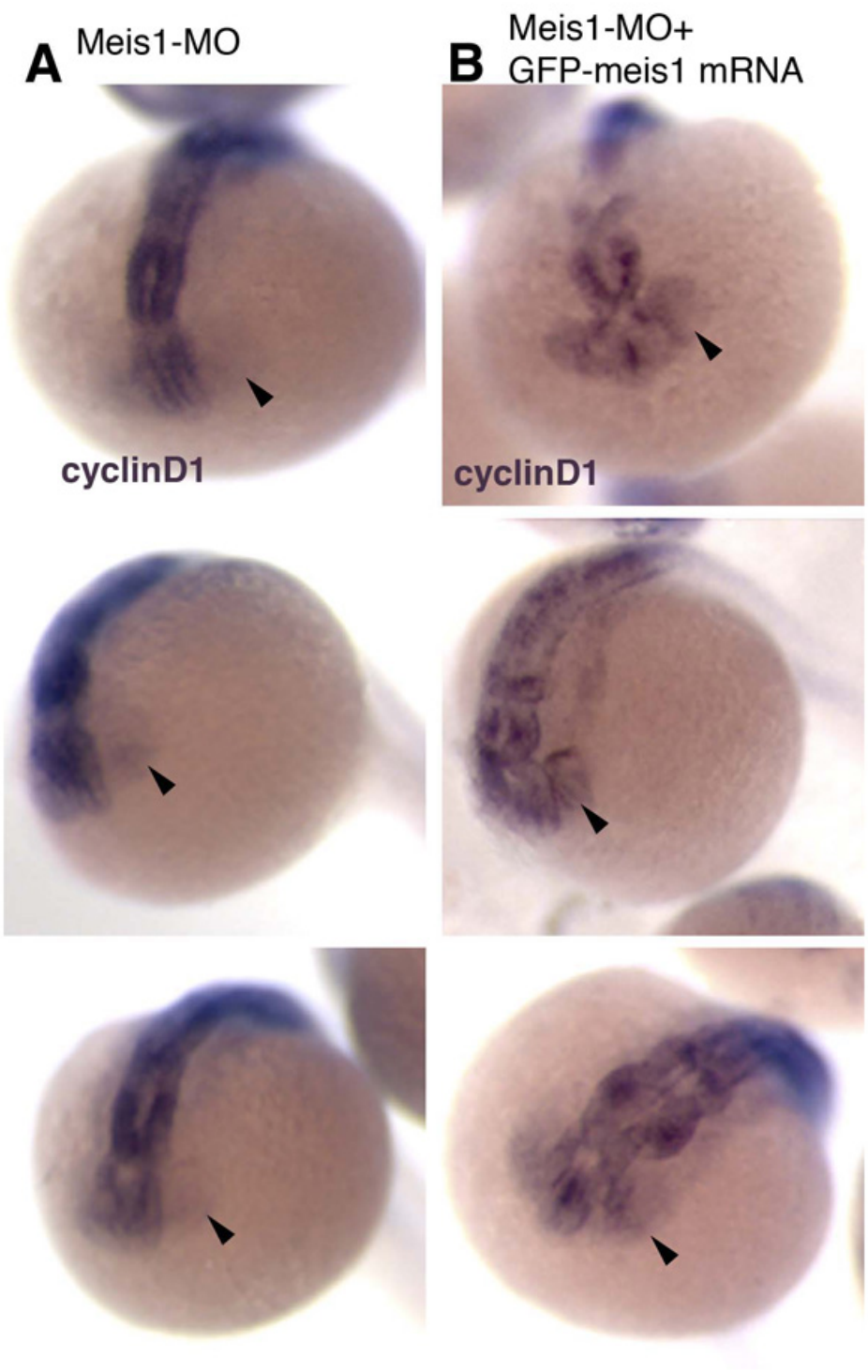


Figure S5. Co-injection of meis1 mRNA rescues the loss of *cyclin D1* expression in *meis1*-morphant embryos. *cyclin D1* in situ hybridization of 20-hpf embryos injected with 12 ng of *meis1*-MO (A), or 12 ng of *meis1*-MO plus 360 pg of GFP-*meis1* mRNA (B). GFP-*meis1* mRNA is insensitive to the MO. Three representative embryos are shown for each condition. Arrowheads point to the eyes.

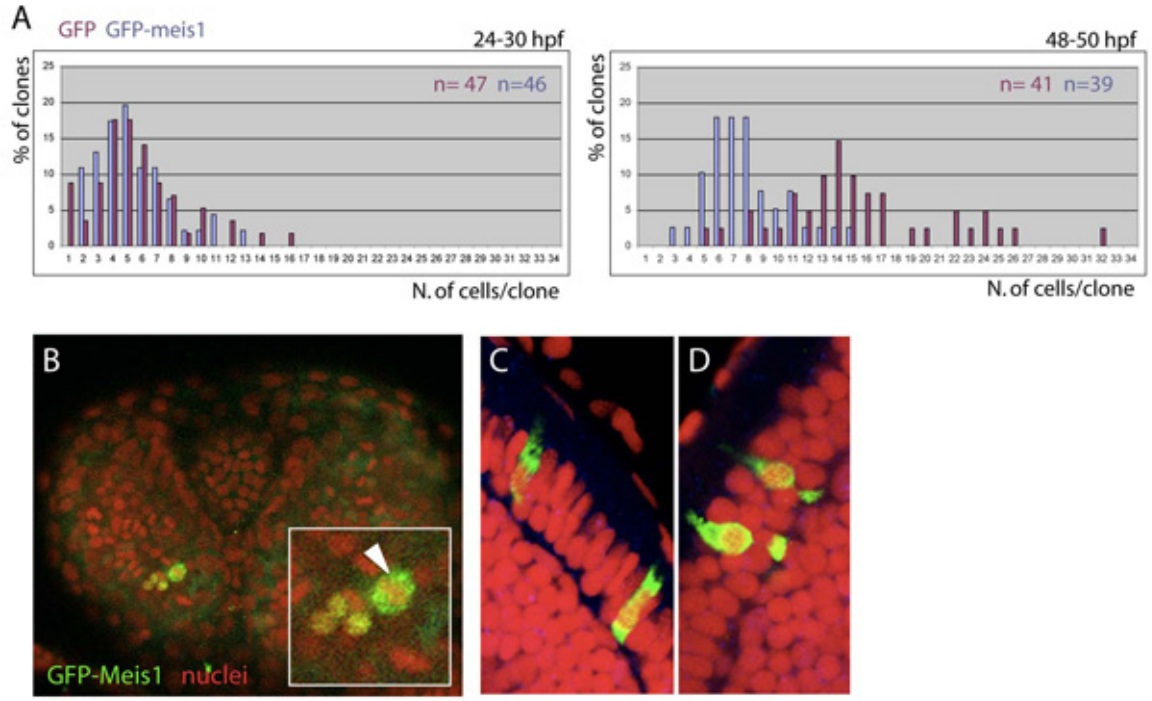


Figure S6. Size of GFP-meis clones at two developmental stages and subcellular localization of the GFP-Meis product. (A) Size of GFP and GFP-*meis1* clones at two developmental stages. The number of clones (*n*) for each clone type is indicated. (B) 30-hpf eye containing GFP-*meis1*-expressing cells counterstained with DAPI to label DNA. GFP-*meis1* cells can be seen undergoing mitosis (arrowhead in the inset). (C,D) 3-dpf GFP-*meis1*-expressing clones can only be recovered in the photoreceptor layer. Here, the GFP-tagged Meis1 transcription factor accumulates mostly in the cytoplasm of the photoreceptors. This contrasts with its nuclear accumulation at earlier stages (see Fig. 4). The nuclear localization of Meis proteins have been shown to depend on their interaction with Pbx products (Vlachakis et al., 2001; Choe et al., 2002). Therefore, one possible cause for the cytoplasmic accumulation of GFP-Meis1 in photoreceptors might be reduced levels, or absence, of Pbx partners in these cells. In addition, Pbx proteins can be retained in the cytoplasm by direct binding to non-muscle myosin II heavy chain B (Huang et al., 2003). If this were the case in photoreceptor cells, the Meis1-Pbx complex would not be able to travel to the nucleus. Alternatively, the overexpressed GFP-Meis1 might be saturating the nuclear import machinery, causing a large fraction of this protein to remain in the cytoplasm.

3 Chapter II

“Phylogeny of the Teashirt-related zinc finger (tshz) gene family and analysis of the developmental expression of *tshz2* and *tshz3b* in the zebrafish”

DEVELOPMENTAL DYNAMICS 239:1010–1018, 2010

Phylogeny of the Teashirt-related Zinc Finger (tshz) Gene Family and Analysis of the Developmental Expression of *tshz2* and *tshz3b* in the Zebrafish

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The tshz genes comprise a family of evolutionarily conserved transcription factors. However, despite the major role played by *Drosophila tsh* during the development of the fruit fly, the expression and function of other tshz genes have been analyzed in a very limited set of organisms and, therefore, our current knowledge of these genes is still fragmentary. In this study, we perform detailed phylogenetic analyses of the tshz genes, identify the members of this gene family in zebrafish and describe the developmental expressions of two of them, *tshz2* and *tshz3b*, and compare them with *meis1*, *meis2.1*, *meis2.2*, *pax6a*, and *pax6b* expression patterns. The expression patterns of these genes define a complex set of coexpression domains in the developing zebrafish brain where their gene products have the potential to interact. *Developmental Dynamics 239:1010–1018, 2010.* © 2010 Wiley-Liss, Inc.

Key words: tshz gene family; phylogeny; expression; zebrafish; meis; pax6

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INTRODUCTION

The specification and development of organs depend on the localized expression of specific classes of transcription factors. One such class is that of the family of tsh-related Zn-finger (tshz) transcription factors. The *Drosophila teashirt* (*tsh*) gene (Fasano et al., 1991), the founding member of the family, has been shown to play critical roles during the development of the fly embryo and adult: *tsh* is required to pattern the midgut (Mathies et al., 1994) and to specify the identity of the trunk ectoderm in the embryo (Roder et al., 1992). *tsh* is also required for the establishment of the proximodistal axis of adult appendages, such as wings and legs (Abu-Shaar and Mann, 1998; Erkner et al., 1999; Azpiazu and Morata, 2000; Casares and Mann, 2000; Wu and Cohen, 2000, 2002) and the specification of the eye (Pan and Rubin, 1998; Singh et al., 2004; Bessa and Casares, 2005). More recently, it has been shown that the *tsh* paralogue, *tiptop* (*tio*), albeit dispensable for *Drosophila* development, is functionally equivalent to *tsh* and can partially compensate its loss in *tsh* mutants (Laugier et al., 2005; Bessa et al., 2009; Datta et al., 2009). *tshz* genes have been also identified in vertebrates. At least, three *tshz* have been reported in mouse, chicken, and *Xenopus* (*tshz1-3*; Caubit et al., 2000; Long et al., 2001; Manfroid et al., 2004, 2006; Koebernick et al., 2006; Onai et al., 2007) and one in zebrafish (*tshz1*; Wang et al., 2007). Molecularly, invertebrate and vertebrate *tshz* genes

Additional Supporting Information may be found in the online version of this article.

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show similarity in their three first and widely spaced Zn-finger domains. Vertebrate *tshz*'s show additional Zn-fingers and a vertebrate-specific homeodomain (Koebernick et al., 2006; Onai et al., 2007). Despite these molecular differences, the three murine *tshz* genes can function as the endogenous *tsh* when expressed in *Drosophila* (Manfroid et al., 2004). These various studies show that vertebrate *tshz* genes are expressed also with dynamic and complex patterns in many developing organs, including the central nervous system, mesodermal derivatives (somites, pronephros), limbs, and branchial arches. However, the functional characterization of this gene family in vertebrates is still fragmentary. During early *Xenopus* development, *tshz3* is required for dorso–ventral axis formation (Onai et al., 2007), while *tshz3* knockout mice show ureteral defects (Caubit et al., 2008).

In this study, we have further investigated the evolution of the tshz gene family and made use of the zebrafish to explore the developmental expression of two of its members, zebrafish *tshz2* and *tshz3b*.

RESULTS AND DISCUSSION

Phylogenetic Analyses of *tshz* Genes

To establish a comprehensive phylogeny of the *tshz* gene family, a total of 119 nucleotide sequences from insects, cephalochordates, urochordates, and vertebrates were used for phylogenetic analyses (see the Experimental Procedures section). Because there is no multiple sequence alignment scheme that outperforms the rest in producing reliable phylogenetic trees (Essoussi et al., 2008), the possible effect on sequence relationship inferences of the use of a given alignment scheme must be taken into consideration. Therefore, Supp. Fig. S1A shows the consensus tree of the four consensus Bayesian trees obtained with four different alignment procedures (Supp. Fig. S1, which is available online). Supplementary Fig. S1B shows the consensus Bayesian tree obtained when using the alignment that produced the largest number of filtered informative positions (the alignment generated with M-Coffee), here taken as a measure of the quality of the alignment. It should be pointed out that, as suggested by Notredame and coworkers (2000), only aligned amino acid positions with a high degree of confidence were used in the phylogenetic analyses (see the Experimental Procedures section). Zinc-finger domain positions represent approximately 45% of the positions used in the phylogenetic analyses. An abridged phylogeny of the *tshz* genes is shown in Figure 1.

All Dipteran species (*Anopheles*, *Aedes*, and *Culex*), but *Drosophila*, show one *tshz* gene (Fig. 1). Coleoptera (*Tribolium*), Hymenoptera (*Nasonia*), and Hemiptera (*Acyrthosiphon*) species also show a single *tshz* gene (Fig. 1). The evolutionary scenario put forward by Shippy and colleagues (Shippy et al., 2008) of a relatively recent gene duplication in the lineage leading to *Drosophila* seems the most logical explanation for such a pattern. Nevertheless, when three out of four alignment schemes are used, the retrieved phylogenies suggest one old gene duplication before the divergence of the Diptera, Hymenoptera, Coleoptera, and Neoptera, followed by multiple independent losses (Fig. 1 and Supp. Fig. S1). Taking into consideration the difficulties in getting a credible alignment when using highly divergent sequences, at present, the scenario put forward by (Shippy et al., 2008) should still be viewed as the most likely hypothesis.

In all mammals and reptiles, there are always three *tshz* genes. Furthermore, we found a single *tshz* gene in Urochordata and Cephalocordata species (Fig. 1) as well as in lamprey (this sequence was not used in the phylogenetic analyses because it is very incomplete; data not shown). Two rounds of genome duplication, leading to four *tshz* genes in a common ancestor of jawed vertebrates, one of which was lost before the diversification of the group, could thus explain the data. The hypothesis of two rounds of genome duplication (2R theory) was first advanced to explain the presence of four Hox clusters in mammals (Sidow, 1996; Furlong and Holland, 2002; Larhammar et al., 2002). Recent analyses based on a large number of genes have provided further support to the 2R theory (Sundstrom et al., 2008). The analyses presented in Figure 1 suggest that the first genome duplication generated a *tshz1-2* and a *tshz3–4* genes. The second round of duplication led to *tshz1* and *tshz2*, as well as *tshz3* and *tshz4* (that was lost). However, in the absence of additional data (like the presence of tshz4 in some species but not in others) partial genome duplications, rather than whole genome duplications, could as well explain the tshz gene number in the different species. It should be noted that the phylogenetic position of urochordata and cephalocordata *tshz* sequences is unexpected but is not well supported (Fig. 1 and Supp. Fig. S1). Two different *tshz1* genes are reported in GenBank for *Xenopus laevis*. Taking into consideration this information, there are four *tshz* genes in amphibians. It is unclear whether both *tshz1* genes are functional.

In all fish species analyzed but *Danio rerio*, there are five *tshz* genes. This observation is in agreement with an extra genome duplication event in the fish lineage and subsequent loss of one gene (3R hypothesis; see Sundstrom et al., 2008, for results based on a large number of genes that support this hypothesis).

Identification of the *tshz1a*, *1b*, *2*, *3a*, and *3b* Zebrafish Genes

As a step toward identifying the full complement of zebrafish (*Danio rerio*) *tshz* genes and their evolutionary relationships, we used the phylogenetic information given in Figure 1 plus synteny information. Although five *tshz* genes are always observed in fish, we found only four genes in zebrafish (*tshz1a*, *tshz2*, and two *tshz3* genes). In the phylogenetic analyses shown in Figure 1, the two zebrafish *tshz3* genes cluster with the *tshz3a* gene from other fishes. Nevertheless, in *Oryzias latipes*, *Gasterosteus aculeatus*, and *Takifugu rubripes* the *tshz3b* gene is flanked by the ZNF536 and ZNF507 genes. One of the two zebrafish's *tshz3* genes is

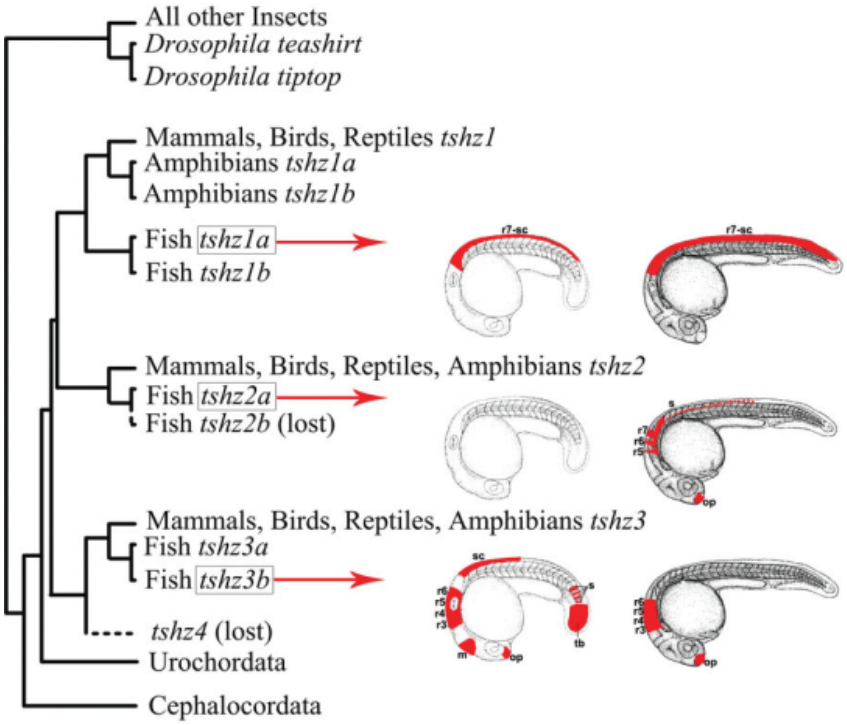


Fig. 1. Evolutionary relationship of *tshz* genes. Schemes show expressions of *tshz1a* (Wang et al., 2007), *tshz2a*, and *tshz3b* in embryos at 19 (left) and 24 (right) hours postfertilization (hpf) as red domains. op, olfactory placode; m, midbrain; r3–7, rhombomeres 3 to 7; s, somites; tb, tail bud; sc, spinal cord.

flanked by gene ZNF536 and LOC560119 (there is no similarity between LOC560119 and ZNF507). Due to its proximity to ZNF536, we labeled that sequence as *tshz3b* (Fig. 1). In *O. latipes*, *G. aculeatus*, and *T. rubripes tshz3a* is flanked by the gene coding for the RPB5-mediating protein and a novel gene of unknown function. The novel gene shows approximately 80% nucleotide similarity in the three fish species thus it is very likely the same gene. In ENSEMBL there is no gene annotated as orthologous of this novel gene in zebrafish, as there is no *tshz3* gene annotated in the vicinity of the gene coding for the RPB5-mediating protein (zgc:110109). However, there are many *tshz* expressed sequence tags (ESTs) from other species that map to the region where the *tshz3a* gene should be. Thus, we named that novel gene *tshz3a*. Finally, no *tshz1b* gene was found in the zebrafish. In *O. latipes*, *G. aculeatus*, and *T. rubripes* the following genes can be found in the vicinity of *tshz1b*: Gal1-R, MBP, ZNF236, ZNF516, (*tshz1b*), ZADH2,

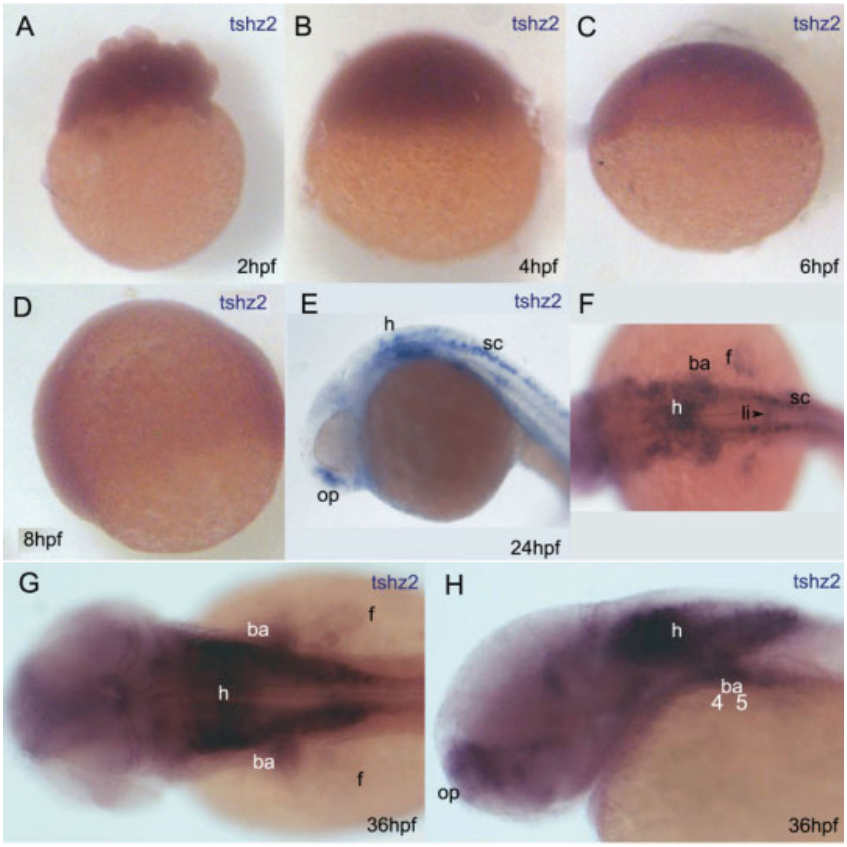


Fig. 2. Spatial and temporal expression pattern of *tshz2* detected by RNA in situ hybridization. **A–D**: Embryos at 2 (A), 4 (B), 6 (C), and 8 (D) hours postfertilization (hpf). *tshz2* mRNA is detected in early stages but signal fades by 8–10 hpf. Re-expression of *tshz2* occurs around the prim-5 stage (24 hpf) (**E,F**). **G,H**: *tshz2* expression pattern in a 36 hpf embryo. At 36 hpf, *tshz2* is also expressed in the liver primordium (not shown). F,G: Dorsal views with anterior to the left. (E and H) Lateral views with dorsal up and anterior to the left. ba, branchial arches; f, pectoral fin; h, hindbrain; l, liver; op, olfactory placode; sc, spinal cord.

HAS2, ZHX2, and DERL1. However, there is a gap in the zebrafish genome sequence (estimated to be approximately 400 kb) between genes MBP and HAS2. Therefore, this gap is the likely reason why *tshz1b* could not be found in zebrafish, but we predict that due to its syntenic conservation in other teleosts, the zebrafish *tshz1b* should be found in the same genomic region.

Zebrafish *tshz2* and *tshz3b* RNA Expression

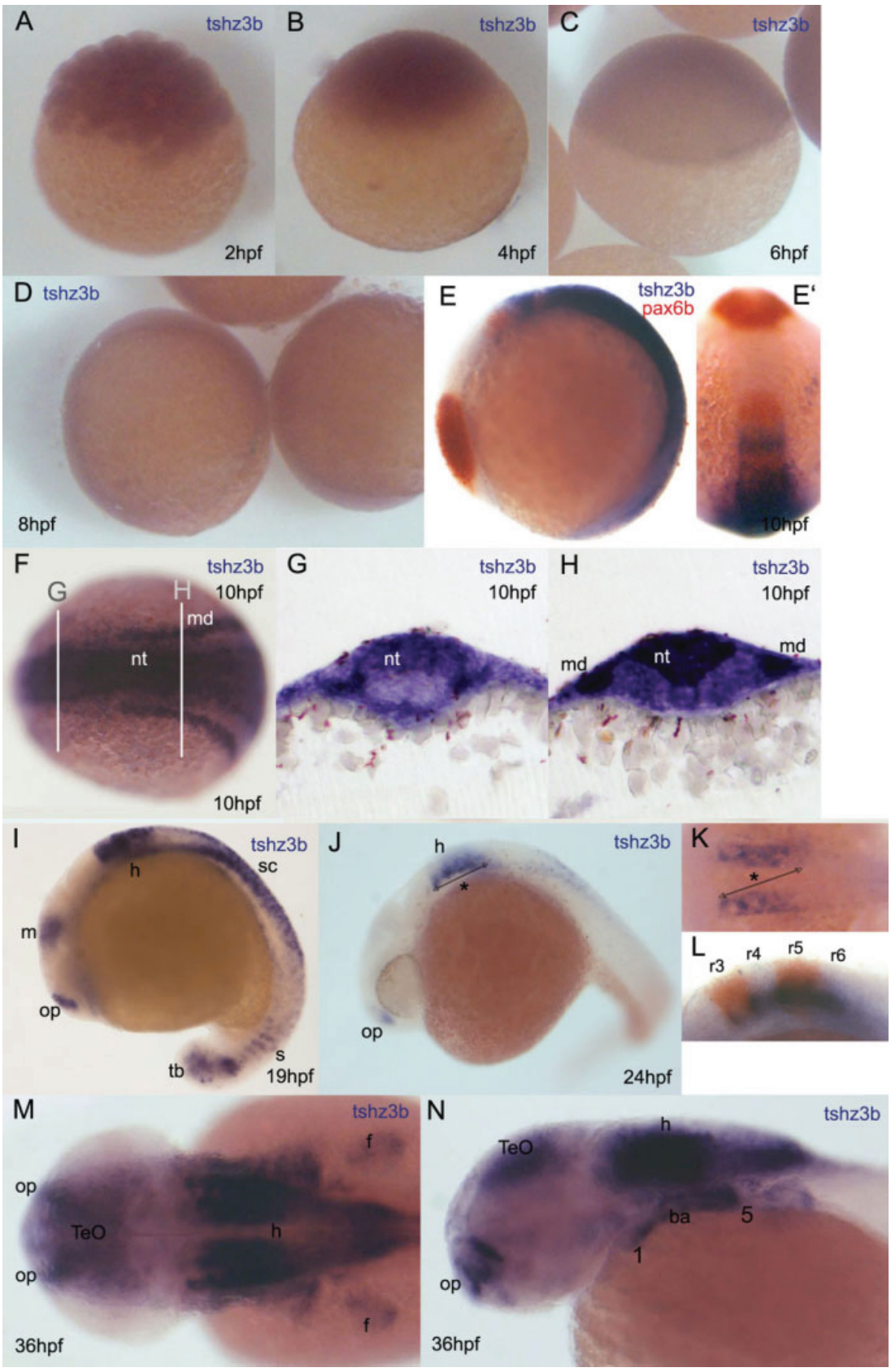
In the zebrafish, the expression of only one *tshz* gene has been described to date, *tshz1* (renamed here *tshz1a*; Wang et al., 2007; Fig. 1). Here, we have studied in detail the expression of the *tshz2* and *tshz3* genes. While we could not detect any in situ hybridization signal using *tshz3a* probes at any time between 10 and 72 hours postfertilization (hpf; not shown), *tshz2* and *tshz3b* are expressed dynamically in several tissues during development (Figs. 2–5). We detect maternal *tshz2* and *tshz3b* mRNA in early embryos (2–8 hpf; Figs. 2, 3). *tshz2* expression disappears around 10 hpf and reappears in 24 hpf embryos (Fig. 2E,F and Supp. Fig. S2A). At 24 hpf, *tshz2* is expressed in pectoral fin buds, liver, olfactory placodes, hindbrain, and spinal cord (Fig. 2E,F and Supp. Fig. S2A). At this stage, the rostral limit in the hindbrain is difficult to define, as expression is weak (Fig. 2E,F and Supp. Fig. S2A), while expression increases in more posterior rhombomeres (Fig. 2E and sections in Supp. Fig. S2A). At spinal chord level, *tshz2* expression is restricted to a lateral cell cluster (Fig. 2E and Supp. Fig. S2A). At 36 hpf, expression is conspicuous in the olfactory placodes and hindbrain, and fades in the spinal cord (Fig. 2G,H and Supp. Fig. S3A,B). *tshz2* is also expressed in branchial arches (ba) 4 and 5 at 24 and 36 hpf, although faint and transient expression in more anterior branchial arches can be detected at 24 hpf (Fig. 2F–H; Supp. Figs. S2B, S3B).

At 10 hpf stage, *tshz3b* is strongly expressed in the hindbrain and posterior neural tube and also in the lateral mesoderm (Fig. 3E–H). In embryos doubly stained for *pax6b* and *tshz3b*, the rostral limit of *tshz3b* in the hindbrain lies posterior to *pax6b* (Fig. 3E,E′). Because *pax6b* rostral limit is rhombomere 1 (Puschel et al., 1992), *tshz3b* expression does not extent up to the anterior limit of the hindbrain. In the neural tube, *tshz3b* is expressed dorsally in the anterior part of its domain but extends along the whole dorsoventral extent more posteriorly (Fig. 3F–H). At 19 hpf, neural expression is detected in the olfactory placodes, midbrain, hindbrain, and in the spinal cord (Fig. 3I). In addition, *tshz3b* expression is detected in the tail bud and in the nascent somites (Fig. 3I). We do not detect variations among similar stage individuals in their tail bud mesoderm expression, suggesting this expression pattern is not related to the segmentation clock in any obvious way. By 24 hpf, *tshz3b* expression fades from all domains except for the olfactory placodes and hindbrain (Fig. 3J,K). Colabeling with *egr2/krox20* at this stage confirms that *tshz3b* expression extends from r3 to r6 (Fig. 3L). Of interest, at these early stages, the expression of *tshz3b* and *tshz1a* abut each other in the hindbrain (which we have confirmed in double in situ experiments; not shown), with *tshz3b* spanning r3–6 and *tshz1a* starting at r7 (Wang et al., 2007). At 36 hpf, *tshz3b* is expressed in the forebrain, midbrain (tectum opticum), and whole extent of the hindbrain, and in the pectoral fin buds (Fig. 3M,N and Supp. Fig. S4). Of interest, the neural tube expression of *tshz3b* changes dramatically from 24 to 48 hpf. Strong expression is gained in the tectum opticum at 48 hpf (compare Figs. 4I and 5I). In contrast, *tshz3b* expression is strong in ventral–lateral regions of the hindbrain at 24 hpf (Fig. 4P) and 36 hpf (Supp. Fig. S4A), but this expression becomes much fainter at 48 hpf (Fig. 5P). Expression of *tshz3b* in branchial arches is first detected at 36 hpf in branchial arches 4 and 5 (Fig. 3M,N and Supp. Fig. S4B).

tshz, *meis*, and *pax6a* Are Transiently Coexpressed in the Hindbrain and in the Retina

In *Drosophila*, *tshz*, *meis*, and *pax6* homologues (*teashirt*, *homothorax*, and *eyeless*, respectively), have been shown to be functionally related during eye development (Bessa et al., 2002; Peng et al., 2009). The vertebrate homologues, the *meis* and *pax6* genes, have been extensively studied in vertebrates but a functional relation between them and *tshz* has not been described so far. In the zebrafish *meis* and *pax6* genes are known to be expressed in, and required for the development of specific regions of the neural tube (Waskiewicz et al., 2001; Choe et al., 2002; Kleinjan et al., 2008; Stedman et al., 2009). To determine whether the domains of expression of *tshz2* and *tshz3b* overlapped the expression patterns of any of the meis or pax6 genes, we compared their expression patterns in sections of 24 hpf and 48 hpf zebrafish embryos by in situ hybridization using specific RNA probes.

At 24 hpf, *tshz2* and *tshz3b* share expression with *meis1*, *2.1*, and *2.2* in the olfactory placodes (Fig. 4A–G). In addition, *tshz2* and *tshz3b*, *meis1*, 2.1, and *2.2*, and *pax6a* and *6b* are expressed in the hindbrain (Fig. 4A–G). However, the precise patterns of expression differ, and overlap in distinct domains. *tshz2* and *3b* are strongly expressed in ventral/lateral regions of the hindbrain where *meis2.1* and *2.2* are also expressed (Fig. 4O–U). In addition, *tshz2* is expressed along the dorso–ventral axis of the neural tube, where it overlaps the expression of *pax6a*, which labels more strongly the medial zone (Fig. 4). At 48 hpf, *tshz2* is expressed in the ganglion cell and inner nuclear layers of the neural retina (Fig. 5H). *meis2.1* and *meis2.2* are expressed in the inner nuclear layer, while *pax6b* (and more weakly *pax6a*) is expressed in the ganglion cell and inner nuclear layers (Fig. 5K–N). However, this *tshz2* expression pattern is transient, as we do not detect any neural expression in 72 hpf embryos (not shown). *tshz3b* expression in the tectum opticum is reminiscent of that of *meis1* (Fig. 5I,J). In this region, it is likely that its expression overlaps with *meis2.1* and *2.2* as well (Fig. 5K,L). Of interest, in more posterior regions of the neural tube (medulla oblongata) *tshz2* and *tshz3b* expressions are more reminiscent of the expression of *meis2.1* than that of *meis1* (Fig. 5O–


A
tshz3b
2hpf
B
tshz3b
4hpf
C
tshz3b
6hpf
D
tshz3b
8hpf
E
tshz3b
pax6b
E'
10hpf
F
tshz3b
10hpf
G
H
md
nt
10hpf
G
tshz3b
10hpf
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H
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h
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s
19hpf
J
tshz3b
h
*
op
24hpf
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r3
r4
r5
r6
M
tshz3b
f
op
TeO
h
op
f
36hpf
N
tshz3b
h
TeO
ba
5
1
op
36hpf

Fig. 3.

R). Also, we have noted that at 36 hpf, *meis2.1* and *meis2.2* show expression in branchial arches 1–5 similar to that of *tshz2* and *tshz3b* (not shown). This gene expression data indicates that *tshz2* and *tshz3b* are likely coexpressed with *meis* and *pax6* genes in different subdomains of the cephalic region of the nervous system, which might allow functional interactions among these genes.

CONCLUSIONS

A comprehensive phylogenetic analysis of the family, including invertebrate and vertebrate sequences, uncovered four *tshz* genes in the zebrafish genome: *tshz1a*, *tshz2*, *tshz3a*, and *tshz3b*, and predicts the existence of a fifth, *tshz1b*. Of these, we investigated in detail the developmental expression of *tshz2* and *tshz3b* compared their patterns with those of meis and pax6 genes. At 10 hpf, *tshz3b* is expressed in the prospective hindbrain and spinal cord and in the mesoderm, while *tshz2* is not expressed. By 24 hpf, *tshz3b* is predominantly expressed in the hindbrain (rhombomeres 3 to 6) and in the olfactory placode and *tshz2* is expressed in the hindbrain and olfactory placode and also in the spinal cord, branchial arches, pectoral fin buds, and liver. At later stages, new domains of *tshz3b* expression are

Fig. 3. Spatial and temporal expression pattern of *tshz3b* detected by RNA in situ hybridization. **A–D**: Embryos at 2 (A), 4 (B), 6 (C), and 8 (D) hours postfertilization (hpf). *tshz3b* mRNA is detected in early stages but signal fades by 8 hpf. **E,F**: At 10 hpf, *tshz3b* expression resumes and is present from intermediate until the caudal region of the neural tube: lateral (E) and dorsal (F) views. The embryo in E is costained with a pax6b antisense RNA probe. (brown). (E′ is a dorsal view of the same embryo; rostral is up). **G,H**: Transversal Vibratome sections of a 10 hpf embryo at the anteroposterior levels marked in F. In G, *tshz3b* is expressed in the dorsal neural tube (nt), but in its most posterior regions (H), *tshz3b* expression is present both dorsally and ventrally. (md) in (H) marks *tshz3b* expression in the mesoderm (md). **I**: At the 20-somite stage (19 hpf), *tshz3b* is expressed in the olfactory placodes, midbrain, hindbrain, spinal cord, somites, and tail bud. **J**: At prim-5 stage (24 hpf), *tshz3b* expression is present in the olfactory placodes and in the hindbrain. **K**: Dorsal view at the hindbrain level (*) of the embryo shown in J. **L**: Lateral view of the hindbrain of a 24 hpf embryo marked for *tshz3b* (blue) and *krox20* (brown); *tshz3b* is expressed from r3 to r6. **M,N**: *tshz3b* expression in the anterior region of a 36 hpf embryo. Dorsal (M) and lateral (N) views are shown. ba, branchial arches; f, pectoral fin; h, hindbrain; m, midbrain; md, mesoderm; nt, neural tube; op, olfactory placode; r3–6, rhombomeres 3 to 6; s, somites; sc, spinal cord; tb, tail bud; TeO, tectum opticum.

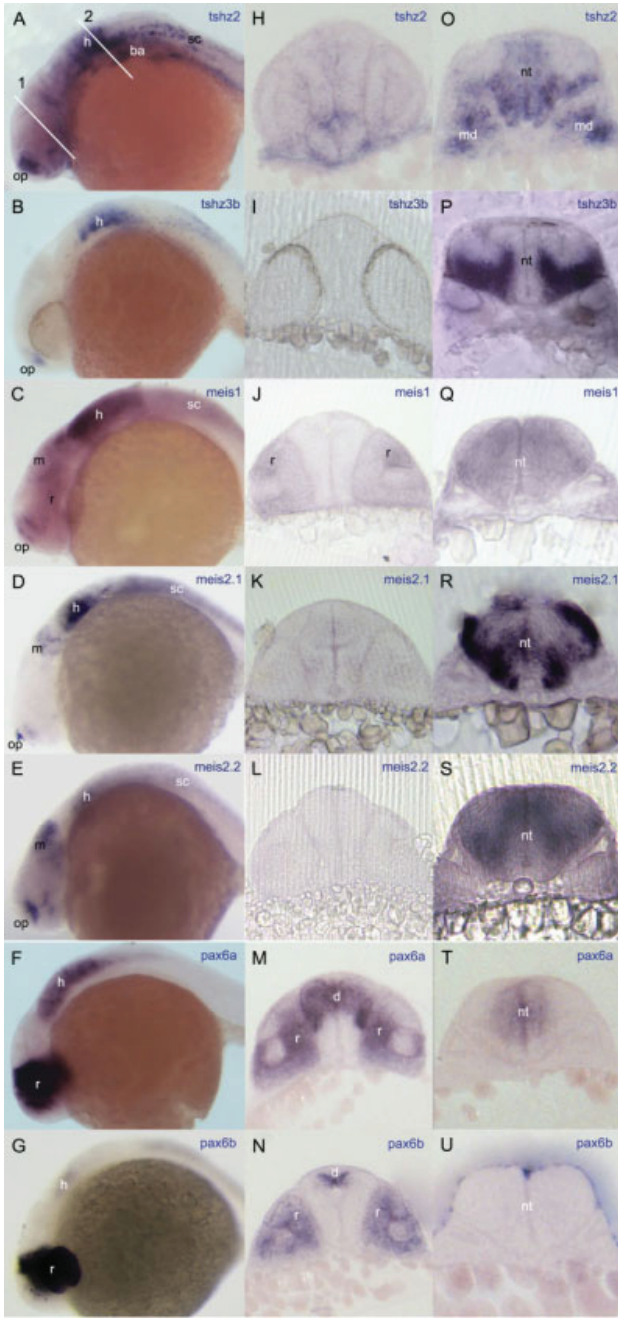

Fig. 4. Spatial expression pattern of *tshz2*, *tshz3b*, *meis1*, *meis2.1*, *meis2.2*, *pax6a*, and *pax6b* in 24 hours postfertilization (hpf) embryos revealed by RNA in situ hybridization. **A–G**: Lateral views with dorsal up and anterior to the left. **H–U**: Transversal vibratome sections at the eyes (left pictures) and rhombomere 5 (right pictures) at the level marked by the lines (1 and 2, respectively) in A. A–G: All genes are expressed in the hindbrain. In Vibratome sections (O–U), it is possible to observe that *tshz2* coexpresses with *tshz3b*, *meis*, and *pax6a*, although at different dorsoventral levels. *tshz* and *meis* genes also coexpress in the olfactory placodes (A–E) and *tshz2* and all the *meis* genes coexpress in the spinal cord (A,C,D,E). Lines (1) and (2) in A mark the approximate section planes shown in the middle and right columns. ba, branchial arches; d, diencephalon; h, hindbrain; m, midbrain; md, mesoderm; nt, neural tube; op, olfactory placode; r, retina; sc, spinal cord.

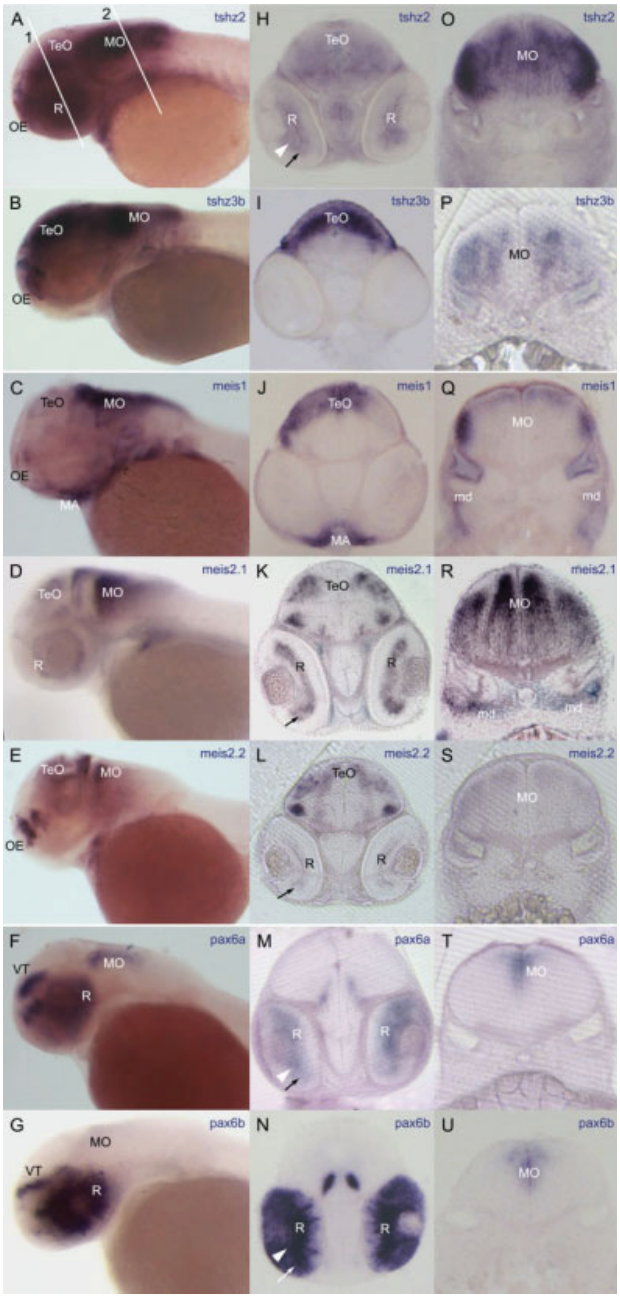

Fig. 5. Spatial expression pattern of *tshz2*, *tshz3b*, *meis1*, *meis2.1*, *meis2.2*, *pax6a*, and *pax6b* in 48 hours postfertilization (hpf) embryos revealed by RNA in situ hybridization. **A–G**: Lateral views with dorsal up and anterior to the left. **H–U**: Transversal vibratome sections at the eyes (left pictures) and rhombomere5 (right pictures) level. A–G,O–U: All genes are expressed in medulla oblongata. H–L: *tshz2* is expressed in the midbrain (H) such as *tshz3b* and *meis* genes, although coexpression takes place at different dorsoventral levels (H–L). A,B,C,E: *tshz2* coexpresses with *tshz3b*, *meis1*, and *meis2.2* in the olfactory epithelium. In H,K,L,M,N, it is possible to observe that *tshz2*, *meis2.1*, *meis2.2*, *pax6a*, and *pax6b*, respectively, are expressed in the neural retina. Lines (1) and (2) in (A) mark the approximate section planes shown in the middle and right columns. MA, mandibular arch; md, mesoderm; MO, medulla oblongata; OE, olfactory epithelium; R, retina; TeO, tectum opticum; Th, thalamus; PTv, ventral posterior tuberculum; VT, ventral tectum. Arrowhead: ganglion cell layer. Arrow: Inner nuclear layer.

added in the tectum opticum, the branchial arches and the fin buds, while *tshz2* expression disappears from the spinal cord but appears in the tectum opticum and in the neural retina. When the expressions of *tshz1a* (Wang et al., 2007), *tshz2*, and *tshz3b* (this report) are globally compared, they show a significant degree of overlap. After 36–48 hpf, these genes are expressed in the olfactory placodes, in the hindbrain and in the tectum opticum (this latter domain only shared by *tshz1a* and *tshz3b*). However, the developmental time at which each of these genes starts being expressed in these domains differ. In addition, the rostral limit of expression within the hindbrain also differs: That of *tshz3b* lies in rhombomere 3 while *tshz1* starts in rhombomere 7. Also, these three paralogues are expressed in the paryngeal arches, but in different ones. While *tshz1a* is detected in the first pharyngeal arch, *tshz2* and *tshz3b* are expressed in the posterior branchial arches. Therefore, *tshz1a*, *tshz2*, and *tshz3b* share some domains of expression, reflecting their paralogy. However, they also show significant spatial and temporal subfunctionalization, the true extent of which will only be uncovered by detailed comparative expression and functional analyses. In addition, we show that *tshz2* and *pax6a* are coexpressed in the hindbrain at 24 hpf stage and in the neural retina at 48 hpf stage of development. When analyzed jointly, the expression patterns of *tshz*, *meis*, and *pax6* gene families define a complex set of coexpression domains in the developing zebrafish brain where their gene products have the potential to interact.

EXPERIMENTAL PROCEDURES

Sequence Analysis

In this work, we used 119 nucleotide *tshz* sequences from insects, cephalochordates, urochordates, and chordates, that are annotated as such in public databases or have significant similarity by BLAST (E-value <0.05) with known *tshz* sequences. This set of sequences represents a compromise between sequence inclusion and loss of information, because the inclusion of very incomplete sequences, or sequences showing large deletions produce many sites showing alignment gaps that are not considered when using Bayesian methods of phylogenetic reconstruction (Ronquist and Huelsenbeck, 2003), as here

performed. Accession numbers are listed in Supp. Table S1.

Establishing the relationship of divergent sequences, such as the *tshz* sequences here considered, can be difficult because different multiple sequence alignment (MSA) algorithms can produce different alignments. Recently Essoussi and colleagues (Essoussi et al., 2008) have shown that there is no single MSA tool that consistently outperforms the rest in producing reliable phylogenetic trees. Furthermore, Golubchik et al. (2007) showed that the absence of amino acid residues often leads to an incorrect placement of gaps in the alignments, even when the sequences were otherwise identical. Therefore, MSA algorithms must perform worst when sequences differ in size, as it is often the case, when considering divergent sequences. Moreover, for a given alignment, not all amino acid positions will be aligned with equal confidence. Therefore, when using divergent sequences, it is advisable to use more than one alignment algorithm, as well as only the amino acid positions that are well supported, and compare the results, as here performed and next described.

Nucleotide sequences were translated and aligned at the amino acid level. The resulting alignment was used as a guide to produce the corresponding nucleotide alignment. The multiple alignment algorithms implemented in the following software were used: ClustalW (Thompson et al., 2002), M-Coffee (Thompson et al., 2002), T-coffee (Notredame et al., 2000), and Muscle (Edgar, 2004). Furthermore, as suggested by Notredame et al. (2000), we used only aligned amino acid positions with a score greater than 3. The number of aligned informative positions with a score greater than 3 obtained using ClustalW, M-coffee, T-coffee, and Muscle was, respectively, 107, 143, 121, and 87. It should be noted that the smaller sets of informative positions are not necessarily a subset of a larger set.

The synteny information was obtained using Ensyntex (Fonseca et al., 2009), a Web-based application that allows the exploration of microsynteny in the regions surrounding a set of gene identifiers provided by the user. The dimension of the region and number of genes considered was, respectively, 500 kb and 5 genes. Note that Ensyntex relies on Ensembl gene annotation (release 53) but it does not use the synteny regions defined by Ensembl (available only for some organisms).

Zebrafish Stocks

AB wild-type embryos were used. Embryonic stages are given as hours postfertilization (hpf) at 28.5°C and, for early stages, referred to number of somites.

Probe Preparation, In Situ Hybridization, and Immunolabeling

tshz2 (IMAGp998k1511982Q), *tshz3a* (IMAGp998E1214819Q), and *tshz3b* (LLKMp964D0651Q) cDNAs were obtained from ImaGenes GmbH. Antisense RNA probes were prepared from cDNAs using digoxigenin as label. The embryos were fixed, hybridized, sectioned, and stained as described in (Tena et al., 2007).

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Santos et al., Supplementary table 1

Gene	Species	Accession number
tiptop	*Acyrthosiphon pisum*	gi|193700069
tiptop	*Aedes aegypti*	gi|157116604
tiptop	*Anopheles gambiae*	gi|158299733
tiptop	*Culex quinquefasciatus*	gi|170056034
tiptop	*Drosophila erecta*	gi|194877844
tiptop	*Drosophila grimshawi*	gi|195033308
tiptop	*Drosophila melanogaster*	gi|6671378
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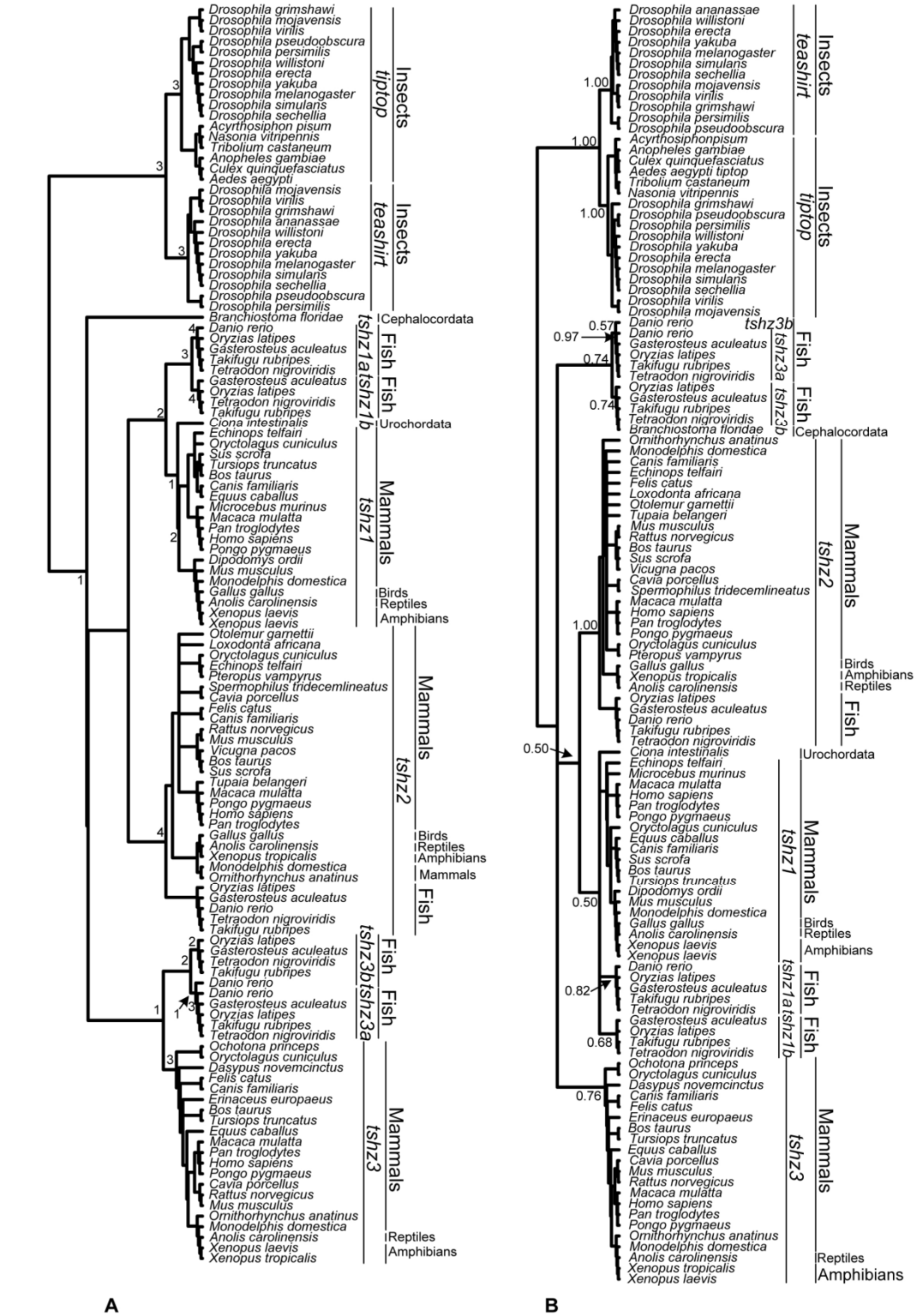
Santos et al., SUP_FIG1



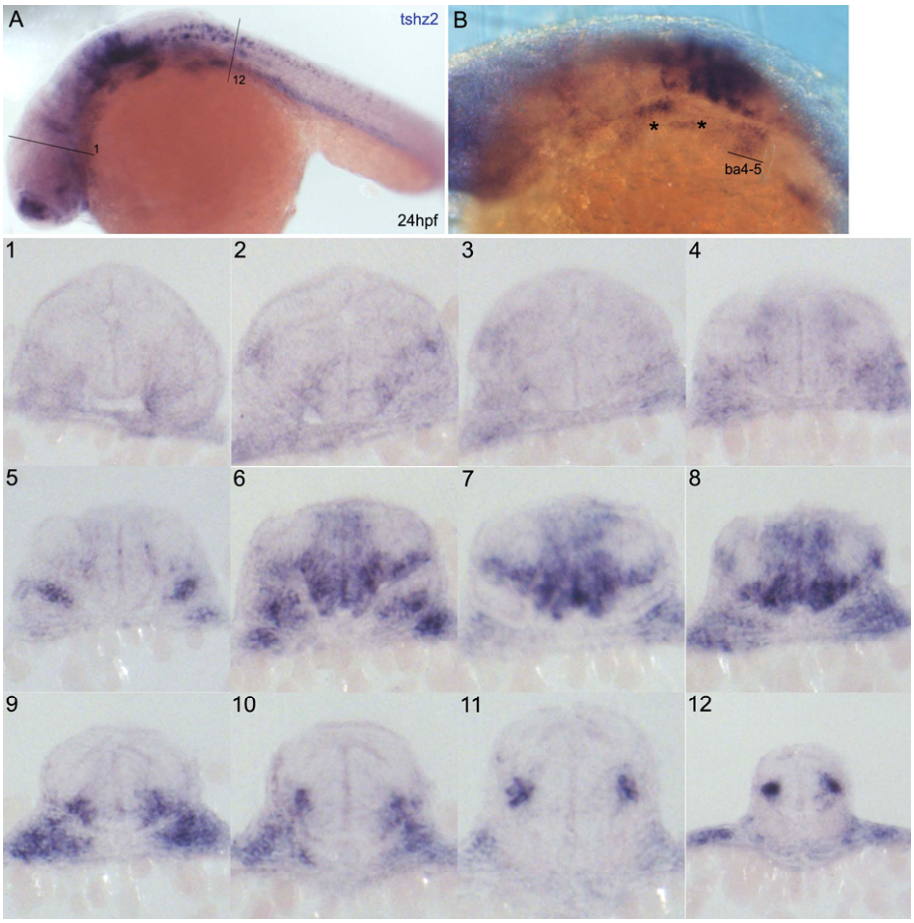


Figure S2. *tshz2* is expressed along the hindbrain and spinal cord and in branchial arches 4-5 in 24hpf embryos.

In situ hybridization of 24hpf embryos using a *tshz2* RNA probe. (A) Lateral view with dorsal up and anterior to the left. (B) Dorso-lateral view with dorsal up and anterior to the left focused at the branchial arches (ba) level. (1-12) 40µm transversal vibratome sections of the embryo in (A) at different anteriorposterior (AP) levels. First and last sections are represented in as 1 and 12, respectively. *tshz2* expression in the hindbrain varies along the AP axis: its expression is stronger in rhombomeres 4 to 6 (sections no. 6-8). Along the dorsal-ventral extent, *tshz2* is expressed more strongly in ventro-medial and ventro-lateral portions of the hindbrain. At spinal cord level, *tshz2* expression is restricted to a lateral cell cluster (pictures 10-12). (B) *tshz2* is expressed in branchial arches 4 and 5. Some signal is detected in more anterior branchial arches (asterisks).

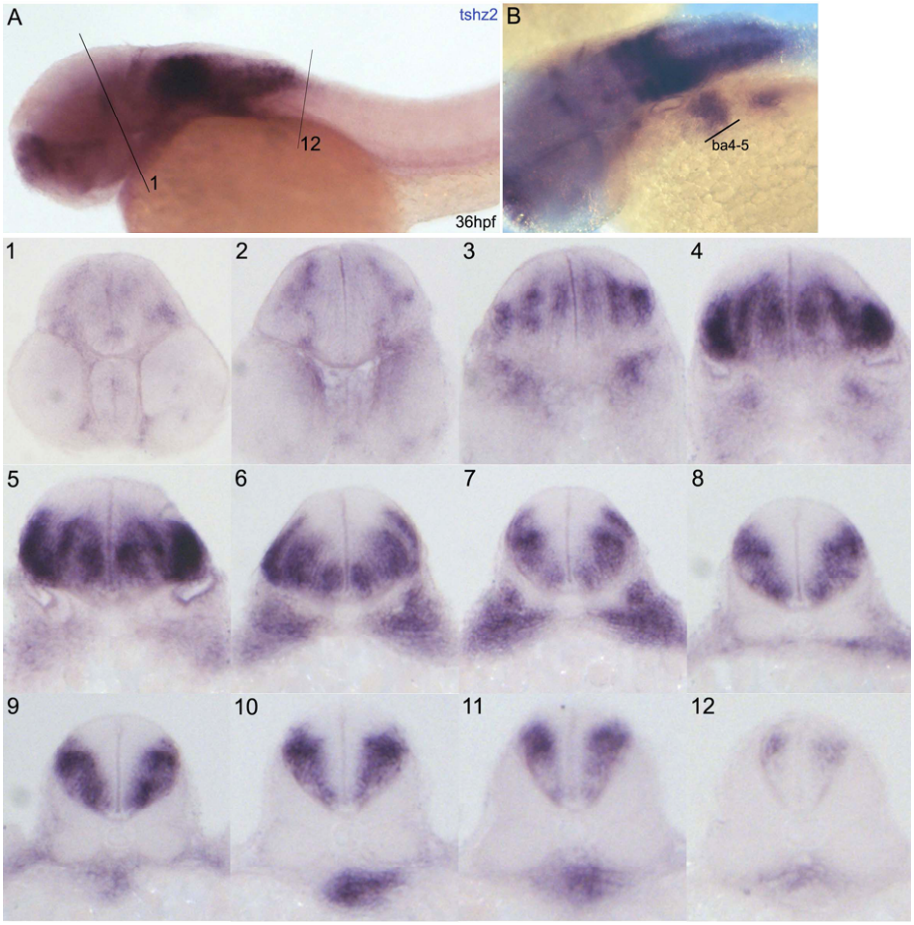


Figure S3. *tshz2* is expressed along the hindbrain and spinal cord and in branchial arches 4-5 of 36hpf embryos.

In situ hybridization of 36hpf embryos using a *tshz2* RNA probe. (A) Lateral view with dorsal up and anterior to the left. (1-12) 40µm transversal vibratome sections of the embryo in (A) at different AP levels. First and last sections are represented in (A) as 1 and 12, respectively. (1, 2) Faint expression is detected in the midbrain and becomes stronger in the hindbrain (3-12). Here, around the otic vesicle level (4,5), *tshz2* is expressed in medial and lateral regions of the medulla oblongata in the ventral neural tube. More posteriorly (7-12), the expression becomes restricted to the lateral portion of the medulla oblongata. The expression in the lateral cell cluster along the spinal cord that was detected at 24hpf is no longer seen. (B) Dorso-lateral view with dorsal up and anterior to the left at the branchial arches (ba) level. *tshz2* is expressed in pharyngeal arches 4 and 5.

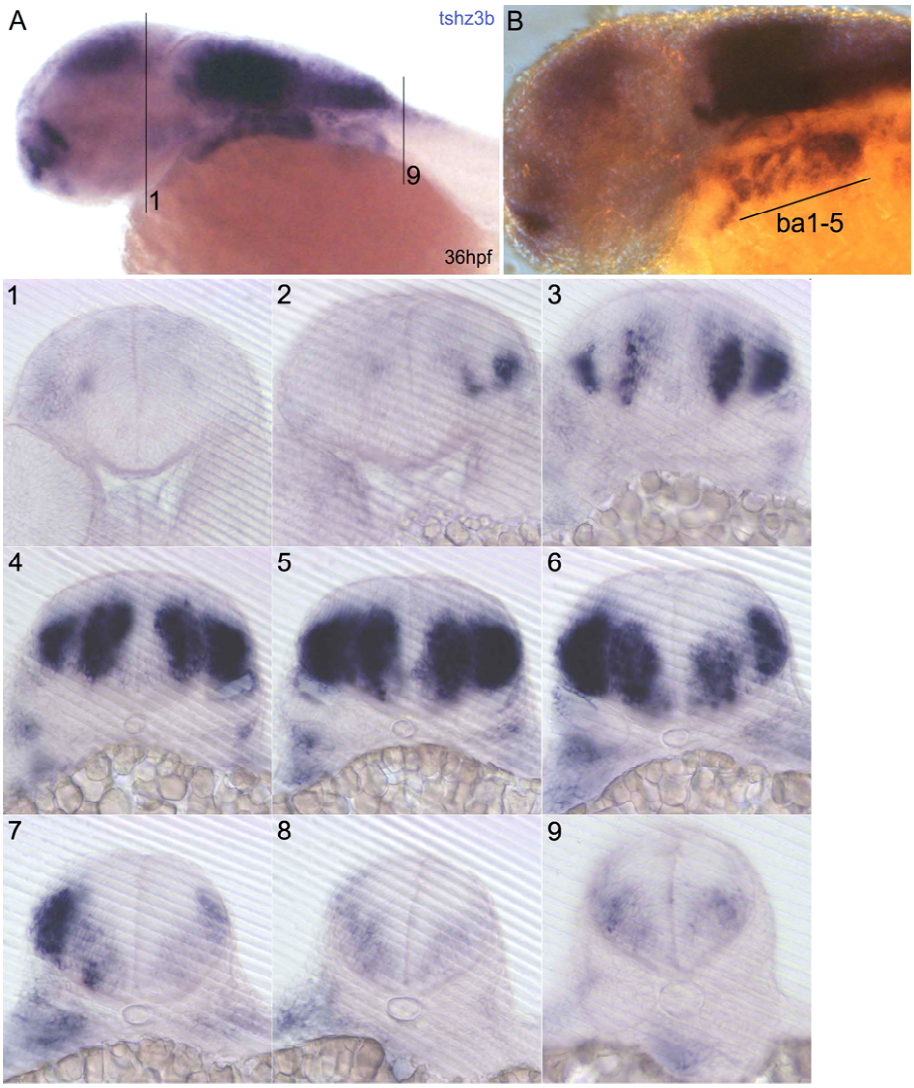


Figure S4. *tshz3b* is expressed along the hindbrain, in the anterior spinal cord and in branchial arches 1-5 of 36hpf embryos.

In situ hybridization of 36hpf embryos using a *tshz3b* RNA probe. (A) Lateral view with dorsal up and anterior to the left. (1-9) 40µm transversal vibratome sections of the embryo in (A) at different AP levels of the hindbrain. First and last sections are represented in (A) as 1 and 9, respectively. *tshz3b* is expressed in medial and lateral regions of the medulla oblongata (4-6) in the ventral neural tube, and this expression fades towards posterior (7-9). (B) Dorso-lateral view with dorsal up and anterior to the left at the branchial arches (ba) level. *tshz3b* is expressed in branchial arches 1-5.

4 Supplementary Information

Author's contribution

Chapter I – Original article "*meis1* regulates *cyclin D1* and *c-myc* expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye".

For the article "*meis1* regulates *cyclin D1* and *c-myc* expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye" the author contributed with: probe preparation, *in situ* hybridization and immunolabeling, *in vitro* RNA synthesis and microinjection of mRNA and morpholinos and to eye phenotype measurements. In addition, the author contributed to the discussion of results and to the writing of the manuscript.

Chapter II – Original article "Phylogeny of the Teashirt-related zinc finger (tshz) gene family and analysis of the developmental expression of *tshz2* and *tshz3b* in the zebrafish".

For the article "Phylogeny of the Teashirt-related zinc finger (tshz) gene family and analysis of the developmental expression of *tshz2* and *tshz3b* in the zebrafish" the author contributed with: probe preparation, *in situ* hybridization and immunolabeling. In addition, the author contributed to the discussion of results and to the writing of the manuscript.

Additional Materials and Methods

On this section of the thesis, we will detail the experimental procedures used, in those cases where they were only succinctly described in the original articles.

1. Probe preparation

For probe preparation, I.M.A.G.E. cDNA clones, obtained from the Lawrence Livermore National Laboratory Consortium were: ccnd1 (IMAGE IRALp962K2356Q), cmyc (IRBOp991F125D), meis1 (IRAKp961C08136Q), meis2.1 (IRBOp991C0733D), meis2.2 (IRBOp991D0437D), meis3 (IRALp962E1456Q) and meis4 (MPMGp609N1326Q). The cDNA clones tshz2 (IMAGp998k1511982Q), tshz3a (IMAGp998E1214819Q) and tshz3b (LLKMp964D0651Q) cDNAs were obtained from ImaGenes GmbH. The clones were then expanded in ampicillin or kanamycin enriched LB medium, linearized by restriction enzymes and purified. Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein as labels.

2. RNA *in situ* hybridization

Zebrafish embryos at specific stages of development were fixated overnight at 4 ºC with 4% paraformaldehyde/PBS (PFA) and then decorionated. Embryos older than 24 hpf were permeabilized with protein kinase and refixed in 4% PFA. Embryos were then prehybridized in hybridization mixture for at least one hour at 65 ºC. Prehybridization mixture was removed and added hybridization mixture plus RNA probe, overnight at 65 ºC. On the next day, post-hybridization washes were proceeded.

3. Immunolabeling

The hybridized embryos were incubated with the antibody (alkaline-phosphatase conjugated anti-digoxigenin or fluorescein), for 2 hours at room temperature. After washing, NBT (nitro-blue tetrazolium) / BCIP (bromo-4-chloro-3-indolyl phosphate) mixture was added and incubated in the dark at room temperature until a blue reaction product is visible.

4. Two-color *in situ* hybridization

Hybridization is carried out as explained above, except that both digoxigenin and fluorescein-labelled probes were added together to the hybridization mix. After hybridization, the first probe was detected as described above. Directly after stopping the first coloration reaction with sterile water washes, we incubated embryos with EDTA for 30 minutes at 65 ºC and then incubated in antibody (alkaline-phosphatase conjugated anti-fluorescein) for 2 hours at room temperature. After washing, we performed the coloration with INT/BCIP mixture in the dark at room temperature, until a red reaction product was visible.

5. Vibratome sections of *in situ* hybridizations

Embryos of several stages of development were sectioned after *in situ* hybridization. For a better result, these embryos were overstained. After *in situ* hybridization, we fixed embryos in 4% PFA overnight at 4 ºC. We then washed embryos with sterile water and transferred them to gelatin at room temperature until polymerization occured. The gelatin blocks were then fixed in 4% PFA for several hours. Using a vibratome, 5 µm sections were cut and cover slip in glycerol.

5 Conclusions

Conclusions

The development of the different body components in all animals is a tightly controlled process and one of the mechanisms involved is the coordination between cell proliferation and specification. One step of control is at the level of transcription. The progenitors of the organs are expanded and specified by a particular network of transcription factors. The role of these transcription factors is essential to achieve a final organ with the right size and morphology. When proliferation and specification become uncoordinated, development abnormalities and diseases such as cancer and neurodegeneration occur. In this thesis we aimed to study some transcription factors that are essential for the coordination between organ specification and progenitor cells expansion. To achieve this, we used zebrafish as a vertebrate model and, in some aspects, compared its development with that of *Drosophila*.

In Chapter I, we analysed the role of the transcription factors Meis, homologues of fly Hth, during zebrafish eye development. In vertebrates, *meis* genes are expressed and have a role in eye development (Ferreti *et al.*, 1999; Toresson *et al.*, 2000; Waskiewicz *et al.*, 2001; Maeda *et al.*, 2002; Zhang *et al.*, 2002; Hisa *et al.*, 2004). Meis1 and Meis2 are upstream regulators of Pax6 in the developing lens of chicken and mice (Zhang *et al.*, 2002). Eye malformations are found in mouse embryos homozygous for a homeodomain-less *meis1* gene (Hisa *et al.*, 2004). In *Drosophila*, *hth* is expressed in the multipotent population of the eye primordium, where it is required to maintain these cells in a proliferative state and to prevent their premature differentiation (Pai *et al.*, 1998; Pichaud and Casares, 2000; Bessa *et al.*, 2002). Early eye development in flies and vertebrates shows some parallels and if this extends also to Hth/Meis, *meis* genes may be involved in stimulating proliferation and preventing differentiation in the eye primordium. We concluded that *meis1* is sufficient and necessary for the proliferation of the undifferentiated cells of the retina. Our results also suggest that *meis1* expression is incompatible with cells' differentiation, since *meis1* ectopic expressing cells tend to segregate to undifferentiated regions of the differentiating retina. These results are reminiscent of those found in *Drosophila* for *hth*.

Another class of transcription factors also involved in specification and development of organs is that of the family of Teashirt related zinc-finger (Tshz) transcription factors. The *Drosophila tsh* gene has critical roles during the development of the fly to pattern the midgut (Mathies *et al.*, 1994), to specify the trunk ectoderm (Roder *et al.*, 1992), to establish the proximodistal axis of adult wings and legs (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2000; Casares and Mann, 2000; Erkner *et al.*, 1999; Wu and Cohen,

2000; Wu and Cohen, 2002) and to specify the eye (Bessa and Casares, 2005; Pan and Rubin, 1998; Singh *et al.*, 2004). In vertebrates, little is known about the role of Tshz during development. Mice, chicken and *Xenopus* have at least three *tshz* genes (*tshz1-3*) (Caubit *et al.*, 2000; Koebernick *et al.*, 2006; Long *et al.*, 2001; Manfroid *et al.*, 2004; Manfroid *et al.*, 2006; Onai *et al.*, 2007). So far, only *tshz1* expression pattern has been described in zebrafish (Wang *et al.*, 2007). Various studies show that vertebrate *tshz* genes are expressed with dynamic and complex patterns during development of the central nervous system, mesodermal derivatives (somites and pronephros), limbs and branchial arches (Caubit *et al.*, 2000; Koebernick *et al.*, 2006; Long *et al.*, 2001; Manfroid *et al.*, 2004; Manfroid *et al.*, 2006; Onai *et al.*, 2007). Nevertheless, the function of this gene family in vertebrates is still poorly known. During early *Xenopus* development, *tshz3* is required for dorsoventral axis formation (Onai *et al.*, 2007) and *tshz3* knock-out mice have ureteral defects (Caubit *et al.*, 2008). In chapter II, we aimed to find and describe other *tshz* genes during zebrafish development. We investigated the evolution of the Tshz family and described zebrafish *tshz* phylogeny and the expression pattern of two of its members, zebrafish *tshz2* and *tshz3b*. In *Drosophila*, Tsh, Hth and Ey are functionally related during eye development (Bessa *et al.*, 2002; Peng *et al.*, 2009). A functional relation between the vertebrate homologues encoding Meis, Pax6 and Tshz transcription factors has not been described so far. To determine whether the domains of expression of *tshz2* and *tshz3b* overlap those of the *meis* and *pax6* genes, we compared their expression patterns. We concluded that the expression patterns of *tshz*, *meis* and *pax6* genes show a complex domain of coexpression in the developing brain, proposing a potential interaction between their gene products. In future work, it would be interesting to do functional studies on Tshz in zebrafish. This work will be made easier when the full and final annotation of zebrafish *tshz* genes is complete.

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6 Abbreviations

Abbreviations

AML: acute myeloid leukemia
AP: anterioposterior
Ath: atonal homologue
bHLH: basic helix-loop-helix
CBP: CREB (cAMP response element-binding) binding protein
CMZ: ciliary marginal zone
CNS: central nervous system
DNA: deoxyribonucleic acid
Dpp: decapentaplegic
DV: dorsoventral
Ey: eyeless
FGF: fibroblast growth factor
GCL: ganglion cell layer
GFP: green fluorescent protein
GSK-3: glycogen synthase kinase 3
H: hour
HD: homeodomain
Hif-1: hypoxia inducible factor 1
Hpf: hours post-fertilization
Hth: homothorax
INL: inner nuclear layer
IPL: inner plexiform layer
Irx: iroquois-class homeodomain
Meis: myeloid ecotropic viral integration site
MF: morphogenetic furrow
MH: Meis and Homothorax domain
miRNA: micro Ribonucleic Acid
MLL: mixed lineage leukemia
MyoD: myogenic differentiation
OE: olfactory epithelium
ONL: outer nuclear layer
OPL: outer plexiform layer
Otx: orthodenticle homeobox
Pax: paired box

Pbx: pre-B cell leukemia homeobox

PDK: 3-phosphoinositide–dependent protein kinase

Pdx: Pancreas Duodenum Homeobox-1

Prep: prolyl endopeptidase

RNA: ribonucleic Acid

RPE: retinal pigment epithelium

TALE: three amino acid loop extension

Tio: tiptop

TORC: trimethylamine oxide reductase

Tsh: teashirt

Wg: wingless

Znf: zinc finger motifs

7 Acknowledgments

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