**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* (myc)

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**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 Sellert, 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 (Hisatomi 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*).

**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-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*—ZF Jin) (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).
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 χ² test.

Plasmid constructs
I.M.A.G.E. cDNA clones, from the Lawrence Livermore National Laboratory Consortium, used were: cccd1 (IMAGE IRALp962K2356Q), c-myc (IRBoOp991F125D), meis1 (IRAKp961C08136Q), meis2.1 (IRBoOp991C0733D), meis2.2 (IRBoOp991D0437D), meis3 (IRALp962E1456Q) and meis4 (MPMGp609N1326Q). pCS2-cccd1 was generated by inserting the full-length cDNA into EcoRI and XbaI sites of pCS2+. To generate GFP-meis1, MT-meis1, MT-meis2.2, MT-meis3, MT-meis4, and MT-meis4-MT constructs, we PCR amplified the corresponding Meis coding regions with the primer pairs (5’-3’; EcoRI and XbaI sites underlined): GAATTCATGACAGGACAGT and CTCGAGATGGGTAGGAGTT and CTCGAG for meis1; GAATTCATGACAGGACAGT and CTCGAG for meis2.2; GAATTCATGACAGGACAGT and CTCGAG for meis3; and GAATTCATGACAGGACAGT and CTCGAG for meis4. The PCR fragments were subcloned into pGEMT-Easy (Promega) and sequenced. Meis cDNAs were cloned into pCS2 MT, pCS2p+MTC2 or pCS2+. To generate GFP-meis1, MT-meis1, meis1-MT, MT-meis2.2, meis2.2-MT, MT-meis3, MT-meis4 and MT-meis4-MT constructs, we PCR amplified the corresponding Meis coding regions with the primer pairs (5’-3’; EcoRI and XbaI sites underlined): GAATTCATGACAGGACAGT and CTCGAGATGGGTAGGAGTT and CTCGAG for meis1; GAATTCATGACAGGACAGT and CTCGAG for meis2.2; GAATTCATGACAGGACAGT and CTCGAG for meis3; and GAATTCATGACAGGACAGT and CTCGAG for meis4.

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 DNA content analysis and flow cytometry 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).

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 DISCUSSION
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; Thissee and Thissee, 2005) (this work). meis1, as monitored by in situ hybridization, or by a YFP insertion 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) (this work). 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, 2007). meis1 remained transiently expressed in the ciliary margin zone (CMZ), where the retinal stem cell population resides (Fig. 1D,K,L). At ~24hpf (Fig. 1A), meis1, was expressed in the prospective lens ectoderm, but was turned off as the lens placode started to thicken (Fig. 1J). 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. 1N).
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.
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.

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 redepolyed 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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