

# *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*).

## 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

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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  $\chi^2$  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 *EcoRI* and *XbaI* 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'; *EcoRI* and *XbaI* sites underlined): GAATTCGATGGCGCAGAGGT and CTCGAGCATGTAGTGCCACTGTCCC for *meis1*; GAATTCGATGGCGCAAAGGTACGA and CTCGAGCATGTAGTGCCACTGGCC for *meis2.2*; GAATTCATGATAAGAGGTATGAGGAGTT and CTCGAGGTGGGCATGTATGCAA for *meis3*; and GAATTCATGGCGCAACGGTACGA 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 *SalI* and *SspI* 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  $\chi^2$  test was used for statistical data analysis.

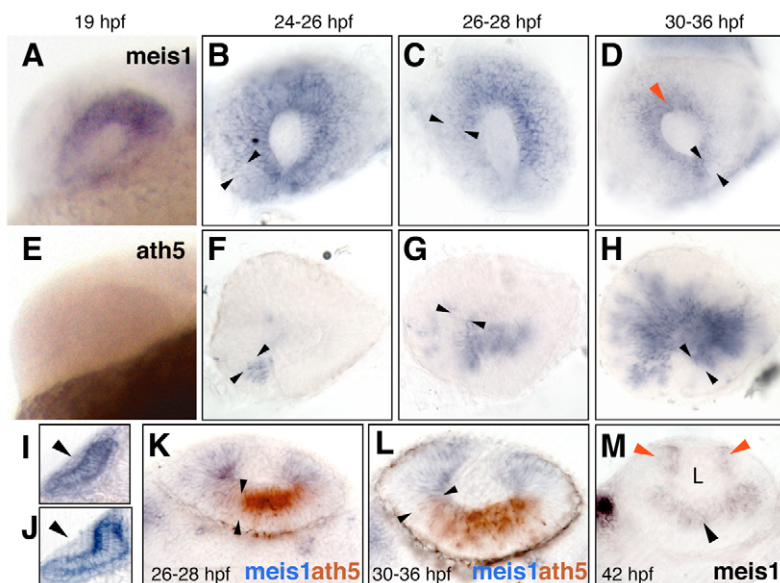
#### Induction of ectopic expression mosaics

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 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; 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

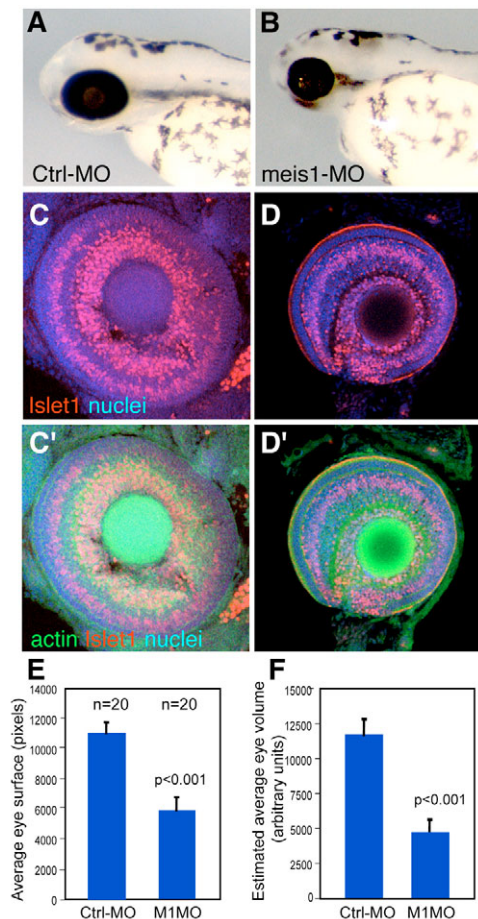


**Fig. 1. *meis1* retracts accompanying the *ath5* wave and becomes restricted to the CMZ. (A-D) *meis1* and (E-H) *ath5* expression analyzed by single in situ hybridization. Developmental stages are indicated as hours post-fertilization (hpf) at 28.5°C. Lateral views of whole-mount (A,E) or dissected (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  $\mu$ m 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 retinal 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.

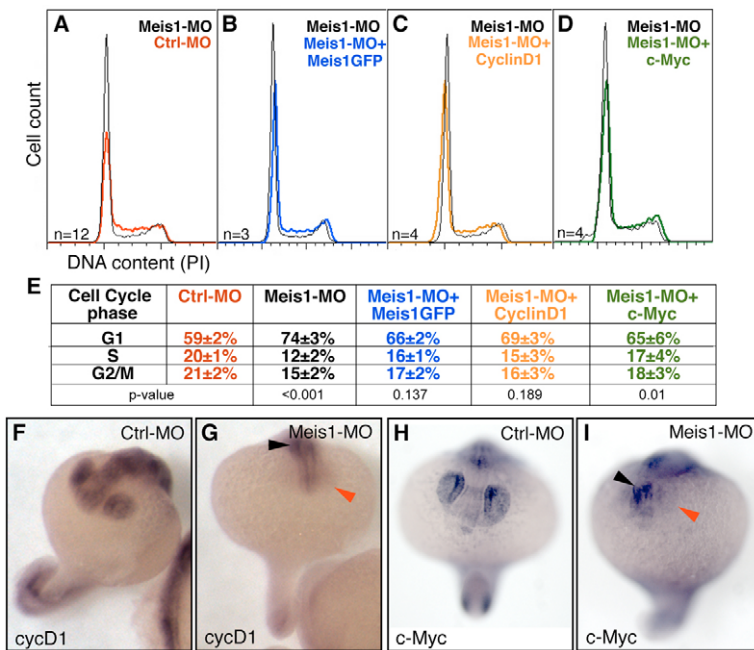
*meis2.2* and *meis1* during optic vesicle stages suggested a possible functional redundancy between these two genes. Nevertheless, injection of *meis2.2*-MO (8 ng/embryo) caused only mild eye reductions in 22% of the treated embryos (*n*=299). Furthermore, co-injection of equivalent amounts of *meis1*- 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



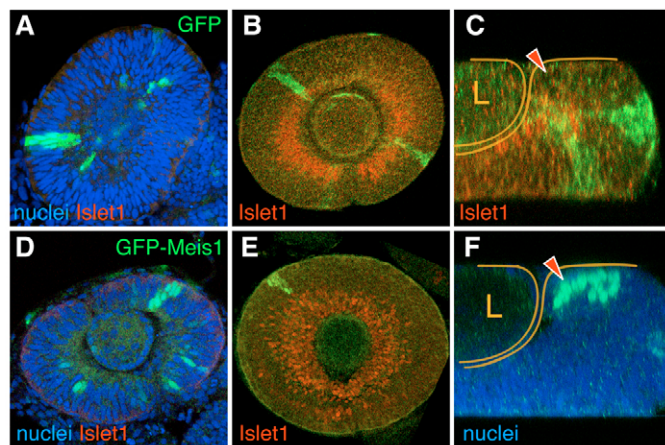
**Fig. 3. *meis1* is required for the G1–S transition and the expression of the G1–S regulators *cyclin D1* and *c-myc*.** (A–D) Histograms displaying DNA content (cell cycle profile) of cells from dissected 19hpf eyes of *meis1*-MO treated embryos relative to (A) controls (Ctrl-MO), (B) *meis1*-MO+GFP-*meis1* 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.

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



**Fig. 4. Clonal overexpression 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.

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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