The maternally expressed zebrafish T-box gene *eomesodermin* regulates organizer formation

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Summary

Early embryonic development in many organisms relies upon maternal molecules deposited into the egg prior to fertilization. We have cloned and characterized a maternal T-box gene in the zebrafish, *eomesodermin* (*eomes*). During oogenesis, the *eomes* transcript becomes localized to the cortex of the oocyte. After fertilization during early cleavage stages, *eomes* is expressed in a vegetal to animal gradient in the embryo, whereas Eomesodermin protein (Eom) is distributed cytoplasmically throughout the blastoderm. Strikingly, following midblastula transition, nuclear-localized Eomesodermin is detected on the dorsal side of the embryo only. Overexpression of *eomes* results in Nodal-dependent and *nieuwkoid*/*dharma* (*nwk*/*dhm*) independent ectopic expression of the organizer markers *goosecoid* (*gsc*), *chordin* (*chd*) and *floating head* (*flh*) and in the formation of secondary axes. The same phenotypes are observed when a VP16-activator construct is injected into early embryos, indicating that *eomes* acts as a transcriptional activator. In addition, a dominant-negative construct and antisense morpholino oligonucleotides led to a reduction in *gsc* and *flh* expression. Together these data indicate that *eomes* plays a role in specifying the organizer.

Key words: Zebrafish, T-box, *eomesodermin*, Maternal, *VegT*, Nodal, *squint*, *bozozok*, *goosecoid*, *chordin*, *floating head*, *nieuwkoid/dharma*

Introduction

In many organisms, maternal mRNAs and proteins are localized to specific regions of the egg and influence the development of the embryo following fertilization. Localized maternal determinants contribute to the establishment of the embryonic body axes, the specification of the germ layers and the formation of the germ line (reviewed by Bashirullah et al., 1998). The role of maternal molecules in the formation of the anterior/posterior and dorsal/ventral (D/V) axes of the early embryo has been particularly well characterized in *Drosophila*, *Xenopus* and ascidians (reviewed by De Robertis et al., 2000; Jeffery, 2001; St Johnston and Nusslein-Volhard, 1992). In the zebrafish, *Danio rerio*, maternal mRNAs have been shown to localize during oogenesis, however the function of these genes and the significance of their localization patterns are not well understood (Bally-Cuif et al., 1998; Howley and Ho, 2000). Furthermore, although there is indirect evidence that maternal molecules play important roles in axis and germ-layer formation in fish embryos, the identity of the molecules and the mechanisms involved remain elusive.

T-box genes belong to a highly conserved gene family that share a sequence specific DNA-binding domain, called the T-box, that was first identified in the mouse *brachyury* or *T* gene (Herrmann, 1992) and identifies these genes as putative transcription factors. Recently, maternally expressed T-box genes have been identified in ascidians, newts and frogs, and some show localized expression in the egg and early embryo. For example, the maternal T-box gene, *VegT*, plays a critical role in germ layer formation in the *Xenopus* embryo (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang et al., 1998a; Zhang and King, 1996). A number of T-box family members have been identified previously in zebrafish (Ahn et al., 2000; Begemann and Ingham, 2000; Dheen et al., 1999; Griffin et al., 1998; Griffin et al., 2000; Hug et al., 1997; Ruvinsky et al., 2000a; Ruvinsky et al., 1998; Tamura et al., 1999; Yonei-Tamura et al., 1999); however, all of these genes, including the *VegT* zebrafish ortholog (*tbx16/spadetail*), are expressed zygotically and have no maternal expression in the oocyte and early embryo. In view of the presence of maternally expressed T-box genes in other species, and the importance of VegT in Xenopus, we performed a screen for maternal T-box genes in the zebrafish.

Here we describe the isolation and characterization of the first known maternally expressed zebrafish T-box gene, a homolog of Eomesodermin (*Eomes*). *Eomes* was identified originally in *Xenopus*, where it is zygotically expressed immediately following midblastula transition (MBT) in a dorsal to ventral gradient within the marginal zone (Ryan et al., 1996). *Eomes* has since been identified in other vertebrates including mice and humans where it is also zygotically

expressed. However, in newts, *Eomes* is maternally and zygotically expressed (Hancock et al., 1999; Sone et al., 1999; Yi et al., 1999). Functional analyses in *Xenopus* and mouse demonstrate common roles for *Eomes* in mesoderm formation and early gastrulation movements (Russ et al., 2000; Ryan et al., 1996).

Although expression of zebrafish *eomes* in the nervous system of segmentation-stage embryos has been described previously (Mione et al., 2001), we report its maternal and early zygotic expression, as well as its role during early zebrafish development. The zebrafish *eomes* transcript localizes cortically during oogenesis and to the vegetal region of the blastoderm during early embryogenesis in a pattern reminiscent of *VegT* localization in the frog embryo. Eomes protein is observed in nuclei on the dorsal side of the embryo shortly after the MBT. Overexpression of *eomes* results in Nodal-dependent ectopic expression of a subset of organizer specific genes, which can lead to the formation of complete secondary axes. Loss-of-function studies also support a role for *eomes* in induction of organizer-gene expression.

Materials and methods

Zebrafish care and mutant stocks

Zebrafish embryos were obtained from natural matings and staged as described (Kimmel et al., 1995). Wild-type strains used were a local pet-shop strain, *AB and TLF. Mutant strains used were *bozozok*^{m168} and MZ*oep* (rescued *oep*^{m134}/*oep*^{m134}, gifts from M. Halpern and R. Warga). *bozozok*^{m168} embryos were genotyped as described (Koos and Ho, 1999).

Isolation of *eomes*

Degenerate PCR amplification, using standard conditions, was performed on a zebrafish T3/T7-primer-amplified ovary cDNA library (λZapII, gift of H.Takeda) using primers designed to amplify a 150-base pair (bp) fragment within the T-box (Ruvinsky et al., 2000b). A 150 bp product was cloned into pGEM-T easy (Promega, Madison, WI), labeled with [^{32}P] and used as a probe to screen the ovary library at high stringency (1.2×10^6 pfu). Filters were hybridized in Church buffer overnight at 65°C, washed once in 2×SSC/0.1% SDS at room temperature and twice at 65°C in 0.1×SSC/0.1% SDS. Positive plaques (n=28) were purified and cored. Dot-blot analysis revealed 19 of these positives to be the same gene. Half of these were excised using the Rapid Excision Kit (Stratagene, La Jolla, CA) and sequenced, including clone 2.5 (2.8 kb) which contained a complete open-reading frame. Sequencing was carried out at the Princeton University Syn/Seq facility. The GenBank Accession Number for zebrafish *eomes* is AF329830.

Genetic mapping

Mapping was performed according to standard protocols (Hukreide et al., 1999) using the LN54 radiation hybrid panel. Primers used were: 5′-ACAAAGTGGTGCGACCACCAAACTGG-3′ (forward) and 5′-TGGTAGGAACTTCTGCTGCTCCATCC-3′ (reverse).

Northern analysis

Total RNA from 20 dechorionated embryos at various stages was extracted using the APGC RNA extraction method (Chomczynski and Sacchi, 1987). RNA was separated on a 0.8% agarose/RNA borate/formaldehyde gel, blotted overnight onto a nylon membrane, and hybridized in Church buffer with either ^{32}P-labelled *eomes* probe or *β-actin* probe (gift of I. Ruvinsky). Membranes were washed at room temperature in 2×SSC/0.1% SDS and then in 0.1×SSC/0.1% SDS at 50°C. The blot was exposed for 7.5 and 34 hours at –80°C using an intensifying screen.

Whole-mount in situ hybridization

In situ hybridization was performed as previously described (Jowett and Lettice, 1994) except anti-digoxigenin antibodies (Roche, Indianapolis, IN) were used at 1:10,000 to reduce background *for eomes* in situ hybridizations. Antisense riboprobes to *gsc* (Stachel et al., 1993), *chd* (Miller-Bertoglio et al., 1997), *no tail* (Schulte-Merker et al., 1994), *nwk/dhm* (Koos and Ho, 1998; Yamanaka et al., 1998), *squint* (Erter et al., 1998; Feldman et al., 1998), *cyclops* (Rebagliati et al., 1998; Sampath et al., 1998), *wnt8* (Kelly et al., 1995b), *bmp2b* (Martinez-Barbera et al., 1997) and *vega1*/*vox* (Kawahara et al., 2000; Melby et al., 2000) were synthesized as described previously.

The *eomes* riboprobe was transcribed from a 2.1-kb fragment of clone 2.5 that was generated by PCR amplification and ligated into pGEMT-easy. The forward primer was 5′-TGCTCACTGACT-GTTTGAATG-3′ and the reverse primer was 5′-CGGTGGT-CATTTTTCTCT-3′. The plasmid was linearized with *Spe*I and transcribed with T7 polymerase. To distinguish endogenous *eomes* mRNA from injected *eomes-VP* mRNA (see below), a riboprobe to the C terminus of *eomes* (starting at nucleotide 1746) was generated, which produces a probe with a three nucleotide overlap with *eomes-VP*. A fragment was generated by PCR amplification using the forward primer 5′-CGCTACGCAATGCAGCCCTT-3′ and the reverse primer 5′-GTTCTAGATTAAGGGCTGGTGTAGAAGGCG-3′ and cloned into pGEMT-easy. To make riboprobe, the plasmid was digested with *Spe*I and transcribed with T7 polymerase.

Full-length template to synthesize *forkhead7* (*fkd7*) (Odenthal and Nusslein-Volhard, 1998) riboprobe was obtained by PCR amplification of a T3/T7 amplified 15-19 hour cDNA library (gift of B. Appel) using primers based on the published sequence. The *fkd7* riboprobe was generated using T7 polymerase following *Sal*I digestion.

Sectioning and paraffin section in situ hybridization

Following whole-mount in situ hybridization and immunohistochemistry, embryos were dehydrated in an alcohol series and embedded in either JB4 (Polysciences, Warrington, PA), according to the manufacturer's directions, or araldite resin (Polysciences) through a graded series. Sectioning and paraffin in situ hybridizations were performed as previously described (Howley and Ho, 2000), except the *eomes* probe was hybridized for 2 days at 65°C. Oocyte staging was according to Selman et al. (Selman et al., 1993).

eomes expression constructs

For over-expression studies *eomes* was cloned into pCS2+ and pCS2+MT (to produce a Myc epitope-tagged N-terminal fusion protein) (Rupp et al., 1994). The *eomes* ORF was amplified by PCR using the forward primer 5′-CGCCTCGAGACCGCCATGCAGTTA-GAAAGCATCCTC-3′ and the reverse primer 5′-GTTCTAGAT-TAAGGGCTGGTGTAGAAGGCG-3′, and cloned into the *Xho*I/*Xba*I site of pCS2+. To produce *myc-eomes*, the *eomes* ORF was cloned into the *Stu*I/*Xba*I site of pCS2+MT, following PCR amplification with the forward primer 5′-GAGGCCTATGCAGTTAGAAAGCATC-CT-3′ and the reverse primer as above. Plasmids were linearized with *Not*I and transcribed using the SP6 mMessage mMachine kit (Ambion, Austin, TX).

eomes activator and repressor constructs

To test whether *eomes* acts as a transcriptional activator or repressor, N-terminal fusions of the *eomes* T-box to the transcriptional activator domain of VP16 and the transcriptional repressor domain from *engrailed* were made. The *eomes-VP16* construct (*eomes-VP*) was generated by cloning a PCR amplified fragment from *eomes*-pCS2+ corresponding to amino acids 153-431 into the *Cla*I site of pVP16-N (Kessler, 1997). The forward primer was eomesF-enR (5′-CCATC-GATTCCGCCATGGGTTCGGTTCTTCCACCCGCC-3′) and the reverse primer was eomesR-VP16 (5′-CCATCGATGCGGGCGC-CGGGGACAATCTG-3′). As a control, *no tail*-VP16 (*ntl-VP*) was

made by cloning a fragment encoding amino acids 1-232 (Schulte-Merker et al., 1994) into the *Cla*I site of pVP16-N vector. The forward primer was ntlF-enR (5′-CCATCGATTCCGCCATGTCTGCCTCA-AGTCCCGAC-3′) and the reverse primer was ntlR-VP16 (5′-CCA-TCGATAGATTGCTGGTTGTCAGTGCTGTG-3′).

The *engrailed* (*eng*) repressor constructs were made by cloning the same *eomes* fragment as above into the *Cla*I/*Eco*RI site of pENG-N (Kessler, 1997). To construct *eomes-eng* the forward primer was eomesF-enR (described above) and the reverse primer was eomesR-enR (5′-CGGAATTCCGCGGGCGCCGGGGACAATCTG-3′). To generate the control *ntl-eng* the forward primer was ntlF-enR (as above) and the reverse primer was ntlR-enR (5′-CGGAATTCCAGATTGCTG-GTTGTCAGTGCTGTG-3′). Plasmids were linearized with *Sac*II (except *eomes-VP* and *ntl-VP*, which were digested with *Not*I) and transcribed using the SP6 mMessage mMachine kit. Results with the *ntl* constructs differed from those observed using the *eomes* constructs, indicating that the results obtained using the *eomes* constructs were not the result of promiscuous T-box-binding activity**.** In addition, the defects caused by overexpression of *eomes-eng* were rescued by co-injection of *myc-eomes* RNA, indicating that the defects were specific. Injection of *eomes-eng* alone resulted in abnormal phenotypes in 63% (27/43) of injected embryos at 24 hours post fertilization (hpf). In two separate experiments, 65% (34/52) of embryos co-injected with *eomes-eng* and *myc-eomes* had normal phenotypes at 24 hpf. Experiments using the VP16 and *engrailed* constructs lacking any DNA-binding domain gave no phenotype.

Eomesodermin antisense morpholinos

Two antisense morpholinos to *eomes* were designed and synthesized by Gene Tools, LLC (Philomath, OR): Eomes-MO1, 5′-CATTCTTC-ACTGTGCTGATAAAGGG-3′; and Eomes-MO2, 5′-CGCCAGG-GAGGATGCTTTCTAACTG-3′. Morpholinos were injected at 7 ng nl^{-1} as described (Oates and Ho, 2002).

Microinjections

Manually dechorionated embryos were immobilized in 2.5-3% methyl cellulose or in an agarose mold and pressure injected according to Oates et al. (Oates et al., 2000). All embryos injected with RNA were co-injected with 33 ng μl^{-1} of *GFP* RNA to trace the overexpressing cells and to score for proper translation of injected RNAs. Fast green (Sigma, St. Louis, MO) was co-injected (3-5 ng μl^{-1}) as a visual guide of injection volume (approximately 0.5 nl of RNA was injected per embryo). *eomes*, *myc-eomes*, *eomes-VP* and *eomes-eng* RNAs were injected into one or two cells of eight- to 16-cell stage embryos. Two cell injections at the eight- to 16-cell stage were done as described (Koos and Ho, 1998). *antivin* RNA was injected into the yolk of one- to four-cell stage embryos (Thisse et al., 2000). *eomes* and *myc-eomes* RNAs were injected at 200-250 ng μl^{-1}, *eomes-VP* RNA was injected at 50 ng μl^{-1}, *eomes-eng* RNA was injected at 15 ng μl^{-1} and *antivin* RNA was injected at 400 ng μl^{-1}. Initial experiments revealed no differences in activity between the *eomes* and *myc-eomes* constructs. We used the Myc construct preferentially due to our ability to monitor the protein distribution and to distinguish endogenous from exogenous protein. Control embryos were injected with a mixture of *GFP* and *lacZ* RNAs or G*FP* alone and exhibited no specific defects.

Animal pole microinjections

sqt and *myc-eomes* RNAs were injected alone or together into a single cell at the animal pole of 64-256-cell stage embryos and fixed at 50% epiboly (Chen and Schier, 2001). The *sqt* constructs used were either the coding region alone (Rebagliati et al., 1998) or the coding region plus an additional ~600 bp of downstream sequence (gift from and M. Halpern). *sqt* was injected at 4-7 ng μl^{-1} and *myc-eomes* RNA was injected at 250 ng μl^{-1}. Embryos were processed by in situ hybridization for *gsc* and *flh* expression and by immunohistochemistry for either GFP or Myc expression (see below). Embryos were included in the analysis if they were successfully injected (as judged by antibody staining) and displayed normal marginal expression of *gsc* or *flh*. Interestingly, we found that injection of the *sqt* construct containing the *sqt* 3′ UTR sequence was not as potent in this assay as the construct containing the coding region alone. Injection of the construct containing the 3′ UTR induced *gsc* expression less frequently and 10-fold higher concentrations were necessary to induce a ring of *flh* expression as opposed to a solid patch.

Generation of polyclonal antibodies to Eomes

A portion of the *eomes* cDNA, encoding amino acids L3-C165, was PCR amplified as a 5′ *Bam*HI/3′ *Hin*dIII fragment and cloned into the pQE-30 vector containing a C-terminal tag of six histidines (Qiagen, Valencia, CA). This construct was transformed into JM109 cells (Life Technologies, Gaithersburg, MD) and recombinant protein was expressed and Ni/NTA purified under native conditions using the Qiaexpress system according to the manufacturer's instructions (Qiagen). The recombinant protein was used to generate affinity purified polyclonal antibodies in rabbits (Zymed Laboratories, San Francisco, CA).

Immunohistochemistry and western blots

Anti-Myc (9E10), -GFP (Molecular Probes, Eugene, Oregon) and -Eomes antibody staining was performed according to Bruce et al., (Bruce et al., 2001). The 9E10 monoclonal antibody was developed by J. M. Bishop and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biological Sciences. Anti-Myc, -GFP and -Eomes antibodies were used at dilutions of 1:100, 1:500 and 1:500, respectively. Fluorescent antibody staining was performed using anti-rabbit Alexa 488 (Molecular Probes) at 1:500 and embryos were examined on a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY). Western blots were performed as described (Bruce et al., 2001), except that sphere-stage embryos were dissected from the yolk prior to lysis. Approximately two embryo equivalents were loaded per lane, and anti-Eomes and anti-Myc antibodies were used at 1:1000. To test the specificity of Eomes-MO2, transcription and translation of *eomes*-pCS2+ was carried out using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI). The reaction run with plasmid alone or in the presence of 5 ng *cyclops* antisense morpholino as a control (Karlen and Rebagliati, 2001) or 5 ng Eomes-MO2. Western blots were carried out as described above.

Xenopus oocyte and embryo manipulations

VegT depleted embryos were created as previously described by injecting stage VI oocytes with 5 ng of antisense oligonucleotides to maternal *VegT* (Zhang et al., 1998a). The oligo used was an 18-mer: C*A*G*CAGCATGTACTT*G*G*C, where * indicates a phosphorothioate bond. Oocytes were introduced into a female host after maturation and vital dye labeling using the host-transfer technique (Zuck et al., 1998). For rescue experiments, *Xenopus VegT* and zebrafish *eomes* mRNAs were synthesized using the mMessage mMachine kit and were injected into stage VI oocytes 24 hours after antisense injection or, in some cases, into vegetal pole blastomeres of the eight-cell embryo. Embryos were obtained by in vitro fertilization and were maintained in 0.1×MMR.

Imaging

Embryos were photographed on a Zeiss Axioplan microscrope (Zeiss) with a Nikon D1 digital camera (Nikon, Melville, NY) or images were obtained using a Zeiss LSM 510 confocal microscope (Zeiss). Figures were constructed in Adobe Photoshop.

Results

Identification of zebrafish *eomes*

To identify a maternal T-box gene in the zebrafish, we

performed degenerate PCR amplification on an ovary cDNA library (gift of H. Takeda) using primers designed against conserved portions of the T-box (Ruvinsky et al., 2000b) and isolated a 150-bp fragment that was used to probe the ovary library. A number of positive clones were recovered which were identified as the T-box gene *eomes*, based on homology to previously identified *Eomes* genes. Examination of the *eomes* sequence revealed a 1983 bp open-reading frame encoding a predicted protein of 661 amino acids with a high degree of similarity to its vertebrate orthologs in mouse (Ciruna and Rossant, 1999; Hancock et al., 1999), human (Yi et al., 1999), chicken (Bulfone et al., 1999), newt (Sone et al., 1999) and frog (Ryan et al., 1996). Zebrafish *eomes* is most closely related to newt (62% overall and 92% within the T-box) and frog sequences (61% overall and 94% within the T-box). The sequence presented here (GenBank Accession Number AF329830) is nearly identical to the *eomes* gene identified independently (Mione et al., 2001) (GenBank Accession Number AF287007). The chromosomal location of *eomes* was mapped to near the Z13509 marker on linkage group 19 using the LN54 radiation hybrid panel (Hukriede et al., 1999).

Expression of *eomes* mRNA

Northern blot and in situ hybridization analyses confirmed that *eomes* mRNA is expressed maternally in both oocytes and early cleavage-stage embryos, as well as zygotically for a short period following MBT (3 hpf) at approximately the 1000-cell stage. Northern analysis revealed that an *eomes* transcript of ~4.1 kb was present through the 1000-cell stage, whereas a slightly smaller transcript was found during the sphere and dome stages (Fig. 1A). Maternal *eomes* transcript levels decreased dramatically just prior to the onset of zygotic transcription at MBT (~512-cell stage) and immediately following MBT, *eomes* mRNA levels transiently increased, presumably due to new zygotic transcription (Fig. 1A, compare 512-cell lane with 1000-cell lane). Thereafter, *eomes* transcript levels gradually decreased, persisting to the shield stage at 6 hpf (Fig. 1A and data not shown).

To examine the spatial distribution of *eomes* transcript, we performed in situ hybridizations on ovaries and embryos. In the ovary, *eomes* mRNA was detected ubiquitously throughout the cytoplasm of stage I oocytes (Fig. 1B, arrowhead) [for staging details see Selman et al. (Selman et al., 1993)]. In stage II oocytes (when vitellogenesis begins) *eomes* transcript began to accumulate cortically (Fig. 1C, arrowhead), a pattern that was maintained in stage III oocytes (Fig. 1D, arrowhead). A similar cortical localization pattern in oocytes has been described for only one other maternal transcript in zebrafish, namely *vasa* (Braat et al., 1999; Howley and Ho, 2000).

In the one-cell stage embryo after fertilization *eomes* mRNA was detected both in the yolk cytoplasmic streams and at the junction between the yolk and the blastoderm (Fig. 1E arrow). During early cleavage stages, the maternal *eomes* transcript was distributed in a vegetal to animal gradient in the cells of the blastoderm, with the highest concentration of mRNA detected vegetally (Fig. 1F-H). This graded pattern of *eomes* expression was maintained until just prior to MBT when transcripts became difficult to detect by in situ hybridization, in agreement with our northern analysis (Fig. 1A).

Immediately following MBT, a burst of zygotic *eomes* expression was observed in a pattern similar to the maternal distribution because staining was most intense in the vegetal cells located closest to the yolk (Fig. 1I-K). Sectioning of embryos confirmed the vegetal to animal gradient of

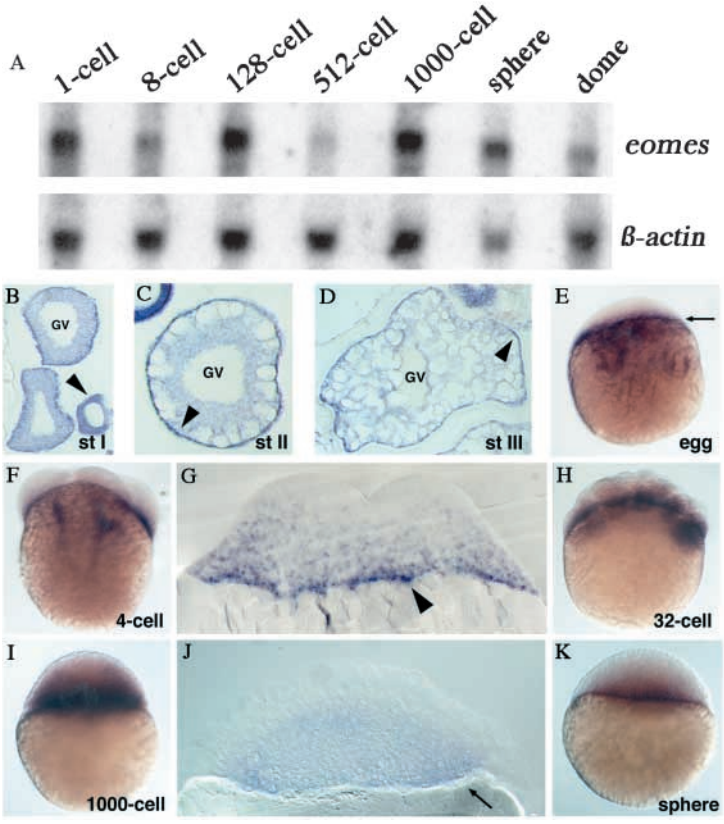

Fig. 1. Localization of *eomes* in the early zebrafish. (A) Northern blot comparing *eomes* transcript levels at one-cell (0.3 hpf), eight-cell (1.25 hpf), 128-cell (2.25 hpf), 512-cell (2.75 hpf), 1000-cell (3 hpf), sphere (4 hpf) and dome (4.3 hpf) stages. Actin was used as a loading control. Exposure time, 7.5 hours. (B-D) Sections of adult ovaries: GV, germinal vesicle. (B) Stage I oocyte (20-140 μm), staining is uniform throughout cytoplasm (arrowhead). (C) Stage II oocyte (0.14-0.34 mm), *eomes* hybridization can be seen along the cortex of the oocyte (arrowhead). (D) Stage III oocyte (0.34-0.69 mm). *eomes* mRNA is detected cortically (arrowhead) and throughout the cytoplasm. (E,F,H,K) Whole-mount embryos, animal pole is toward the top. (G,J) Sections with the animal pole toward the top. (E) Activated egg, *eomes* is detected in the cytoplasmic streams in the yolk and in a gradient along the V/A axis. The arrow marks the region of most intense hybridization at the yolk/blastodisc junction. (F) The expression pattern in a four-cell-stage embryo is similar to that in E. (G) Section of a four-cell stage embryo showing the distribution of *eomes* mRNA. The arrowhead marks the most intense region of *eomes* expression at the yolk-blastomere junction. (H) Expression of *eomes* is maintained in vegetal to animal gradient in a 32-cell-stage embryo. (I) Nearly ubiquitous zygotic *eomes* expression at the 1000-cell stage. (J) Section of an oblong/sphere-stage embryo. *eomes* mRNA is detected in vegetally located cells and is absent from the YSL (arrow). (K) Sphere-stage embryo, *eomes* hybridization is reduced in the animal pole and is most intense in cells closest to the yolk.

expression at the oblong/sphere stage (Fig. 1J). Furthermore, *eomes* mRNA was not detected in the yolk syncytial layer (YSL), an extraembryonic tissue that forms below the blastoderm (Fig. 1J arrow) (Kimmel et al., 1995). Although low levels of transcript were still detected at the dome (Fig. 1A) and shield stages (data not shown) by northern analysis, *eomes* levels were undetectable by in situ hybridization by the dome stage (4.3 hpf, data not shown). As previously described (Mione et al., 2001), zebrafish *eomes* has a later zygotic expression domain in the forebrain beginning at the 4-5 somite stage (11.5 hpf). This is similar to the previously described pattern of *Eomes* expression in the brain of frogs, mice and chicken (Bulfone et al., 1999; Ciruna and Rossant, 1999; Hancock et al., 1999; Ryan et al., 1996).

Eomes protein is localized to nuclei on the dorsal side of the embryo

An affinity purified polyclonal antibody was generated against the N-terminal portion of Eomes, excluding the highly conserved T-box. Eomes is an approximately 94 kDa protein, as shown by western blotting of sphere-stage embryos (4 hpf), which is somewhat larger than the 73 kDa predicted by the *eomes* sequence (Fig. 2A lane 1). Preimmune serum did not detect any proteins (Fig. 2A lane 2). Injection of *myc-eomes* RNA into embryos, which produces a Myc-epitope tagged fusion protein, followed by western blotting, further demonstrated the specificity of the antibody because both anti-Eomes and anti-Myc antibodies recognized the same protein (data not shown). Staining of 24 hpf embryos revealed nuclear expression in the brain, in agreement with the in situ hybridization pattern at this stage (Fig. 2B arrowheads) (Mione et al., 2001). In a further control experiment, no staining was observed in 24 hpf embryos incubated with the pre-immune serum (Fig. 2C).

The anti-Eomes antibody labeled cells of the ovary and embryo in a pattern generally consistent with the distribution of the *eomes* transcript. No protein was detected in stage I oocytes (Fig. 2D, arrowhead), whereas ubiquitous cytoplasmic expression of Eomes was apparent in stage II and older oocytes (Fig. 2D, arrow). This is in contrast to the *eomes* transcript, which was detected from the earliest stage of oogenesis. The distribution of Eomes differs from *Xenopus* VegT protein, which is translationally repressed until oocyte maturation (Stennard et al., 1999). After fertilization, in one-cell-stage embryos, Eomes protein was detected throughout the cytoplasm (Fig. 2E), and remained cytoplasmic throughout early cleavage stages (data not shown).

Eomes, a putative transcription factor, is predicted to function in the nucleus after MBT, when zygotic transcription begins. In accordance with this, reproducible nuclear expression of Eomes protein was first detected in most nuclei of the embryo at 3 hpf, just around the time of MBT (data not shown). A strikingly asymmetric pattern of nuclear staining was observed beginning at the sphere stage (4 hpf). In addition to cytoplasmic expression in most cells of the blastoderm, the protein was detected in nuclei predominantly on one side of the embryo at this stage (Fig. 2F arrow). To determine the region of the embryo that correlated with the nuclear staining, we performed double-labeling studies. Antibody staining for Eomes and in situ hybridization for two different dorsal markers, *gsc* and *flh*, revealed that Eomes localized to nuclei on the dorsal side of the embryo (Fig. 2G,H). Eomes protein appeared to be co-expressed with most *flh*-expressing cells at the sphere (4 hpf) and dome stages (4.3 hpf) (Fig. 2G-I). Eomes was also co-expressed with a subset of *gsc*-expressing cells at the sphere stage (Fig. 2J). The asymmetric expression pattern of nuclear Eomes was detected through the dome stage (4.3 hpf). In addition, we detected Eomes in nuclei of the leading edge of the enveloping layer (Fig. 2K arrowheads). By 50% epiboly (5.3 hpf), Eomes was no longer detected.

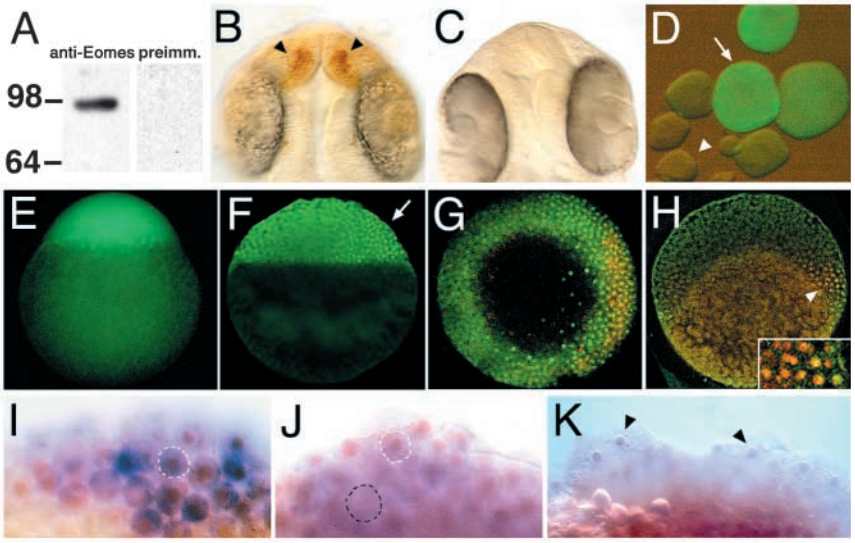

Fig. 2. Eomes protein expression. (A) Western blot of sphere-stage embryos. A 94 kDa band is recognized by the Eomes antibody but not by preimmune serum. (B) Embryo (1 dpf), dorsal side up and anterior to the top, stained with anti-Eomes antibody. Nuclear staining in the brain is light brown and indicated with arrowheads. (C) Embryo (1 dpf) in the same orientation as B incubated with preimmune serum. No staining is visible. (D) Eomes was not detected in early stage oocytes (arrowhead) but cytoplasmic staining (green) was detected in older oocytes (arrow, see text for details). (E) Lateral view of a one-cell stage embryo. Eomes is distributed throughout the blasoderm. (F) Lateral view of a sphere-stage embryo (4 hpf). Eomes is observed in nuclei on one side of the embryo (arrow). (G) Animal-pole view of sphere-stage embryo stained for Eomes (green) and *flh* transcript (red) demonstrates that Eomes is nuclear localized predominantly on the dorsal side of the embryo. (H) Lateral view of a dome-stage embryo (4.3 hpf) stained as in (G). Eomes and *flh* colocalize in some cells (yellow) as can be observed in the enlarged region, bottom right. (I) Animal-pole view of Eomes staining and *flh* in situ staining in a sphere-stage embryo. The white outline indicates a cell that co-expresses Eomes in the nucleus (brown) and *flh* in the cytoplasm (blue). (J) As in I, except *gsc* expression is in blue. The white oultine indicates a co-expressing cell and the black outline indicates a cell that expresses *gsc* but not Eomes. (K) Animal-pole view of sphere-stage embryo with Eomes expression visible in nuclei of the enveloping layer (arrowheads). F-H are images from Z-series taken on a confocal microscope.

Zebrafish *eomes* cannot fully rescue *VegT*-depleted *Xenopus* embryos

We noted that the embryonic expression pattern of maternal *eomes* transcript was strikingly similar to that of maternal *VegT* in *Xenopus*, which is also distributed in a vegetal to animal gradient within the early embryo and appears to be required for the generation of vegetal signals involved in endoderm and mesoderm formation (Casey et al., 1999; Kofron et al., 1999; Zhang et al., 1998b). By contrast, the subcellular distribution of Eomes is more similar to *Xenopus* Eomes, which is expressed zygotically in a D/V gradient (Stennard et al., 1999). Because *eomes* and *VegT* are the only known maternal T-box genes that are expressed in zebrafish and *Xenopus*, respectively, we hypothesized that maternal Eomes function in the fish might be analogous to maternal VegT function in the frog. As one test of this hypothesis, zebrafish *eomes* was assayed for its ability to rescue *Xenopus* oocytes that had been depleted of *VegT* by injection of antisense oligonucleotides. *Xenopus* embryos depleted of maternal *VegT* failed to form endoderm and induce mesoderm, with the result that the morphogenetic movements of gastrulation, including blastopore formation and epiboly, did not occur (Kofron et al., 1999; Zhang et al., 1998a). In three experiments, injection of 300 pg of *VegT* mRNA into *VegT*-depleted embryos rescued 83% (40/48) of embryos (data not shown). By contrast, injection of *eomes* RNA [300 pg (n=13), 150 pg (n=18), 50 pg (n=19), 10 pg (n=38), and 2 pg (n=48)] failed to fully rescue *VegT*-depleted *Xenopus* embryos. Injection of *eomes* at concentrations of 300 pg or higher resulted in exaggerated gastrulation-like movements (invagination) that initiated equatorially, a position that is significantly higher than in normal embryos, and resulted in very large abnormal blastopores that failed to close. Such results indicate that zebrafish *eomes* is much more potent than *VegT* at inducing cellular movements in *Xenopus*. *VegT*-depleted embryos injected with *eomes* failed to form a normal blastopore lip and did not gastrulate, but a limited degree of ectodermal streaming characteristic of epiboly was observed in a small percentage of cases (21%). Our results indicate that zebrafish Eomes is unable to functionally replace maternal VegT in the frog and that these two genes have different activities in early embryos. This raises the possibility that the function of zebrafish *eomes* might be more similar to the zygotic activity of its ortholog, *Xenopus Eomes*.

Overexpression of *eomes* induces secondary axes

Localization of Eomes to nuclei on the dorsal side of the zebrafish embryo indicated that Eomes might play a role in patterning the organizer. To investigate the role of *eomes* during early development, synthetic mRNAs containing either the coding region (*eomes*) or a coding region-Myc-epitope fusion (*myc-eomes*) were used to evaluate the molecular and morphological effects of overexpressing *eomes* in early embryos. Injection of either *eomes* or *myc-eomes* RNA (and *GFP* RNA as a tracer) into early zebrafish embryos led to the formation of secondary axes (Table 1), some of which possessed fully formed heads, including eyes (Fig. 3C). These secondary axes were examined during somitogenesis for expression of *ntl/Brachyury*, a marker of the notochord (Schulte-Merker et al., 1994), and at 24 hpf for expression of *fkd7*, a marker of ventral neural tube and endoderm (Odenthal and Nusslein-Volhard, 1998). Ectopic patches of *ntl/Brachyury*

Table 1. Overexpression of *eomes* induces secondary axes

Stage scored	mRNA injected	Second axis (total)
1-9 somites	*myc-eomes*	18% (49)
	lacZ	0% (24)
	Uninjected	0% (21)
9-16 somites	*myc-eomes*	11% (19)
	lacZ	0% (16)
	*myc-eomes**	33% (43)
	*lacZ**	0% (11)
	Uninjected	0% (41)
24-27 hpf	*myc-eomes*	7% (87)
	eomes	5% (38)
	lacZ	0% (55)
	Uninjected	0% (124)
	*eomes-VP**	41% (17)
	Uninjected	0 (47)

Injections were made into one cell of each embryo at the eight- or 16-cell stages.
Total numbers of embryos are in parentheses.
*Injections were made into two cells at the eight- or 16-cell stages.

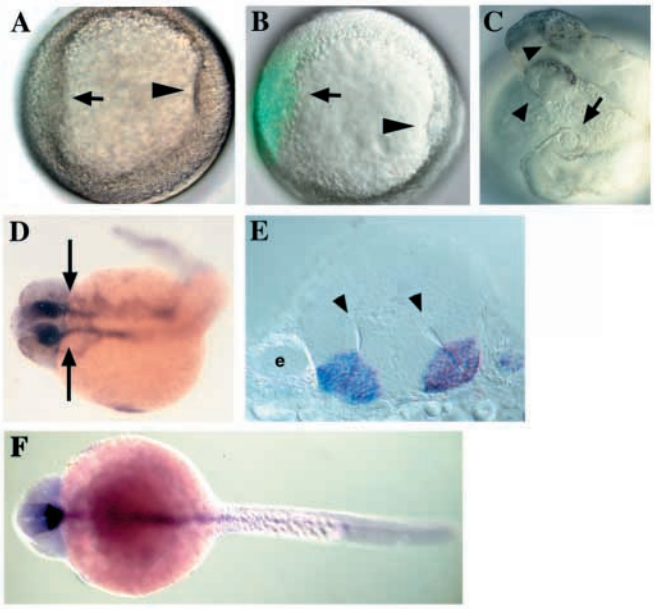

Fig. 3. Overexpression of *eomes* induces secondary axes. (A-C) Animal pole views. (D,F) Dorsal views with anterior to the left. (A) Embryo injected with *myc-eomes*. There is a thickened region (arrow) opposite the native shield (arrowhead). (B) Animal-pole view at shield stage of a live embryo injected with *myc-eomes* and *GFP*. Composite of white light and fluorescent images with GFP-expressing cells in green (arrow) opposite the native shield (arrowhead). (C) Live image of embryo in B at 1 dpf. Two heads are visible: the eyes from one axis are indicated by arrowheads and the eye from the second axis is indicated by arrow. (D) Embryo injected with *myc-eomes* and stained for *fkd7* (black) has two axes side by side at 26 hpf. Arrows indicate the level of the section shown in E. (E) Section of embryo in D, dorsal is to the top. Two neural tubes are visible (arrowheads) and both stain with *fkd7* (blue/purple staining). The eye is marked e. In addition to secondary axes, two other phenotypes were seen in *eomes*-overexpressing embryos. Embryos with bifurcated notochords in the trunk region were observed as well as embryos that resembled the dorsalized mutants previously described (Mullins et al., 1996). (F) Wild-type control embryo at 26 hpf stained for *fkd7*.

expression were observed in 55% of injected embryos (12/22). Although in most cases the two axes were separate and distinct, at least to the level of the tail, in some cases *fkd7* staining revealed double axes that were side by side (Fig. 3D). Analysis of transverse sections of these embryos revealed two axes that were well patterned, containing two neural tubes which both expressed *fkd7* (Fig. 3E) as well as two regions of separate notochords (data not shown). Importantly, secondary axes consisted of cells that expressed either *eomes* or *myc-eomes* (confirmed by GFP expression) and unlabeled cells, indicating that *eomes*-expressing cells could recruit their non-expressing neighbors into the duplicated axis.

The dorsal shield is the fish equivalent of the Spemann organizer in amphibian embryos (Oppenheimer, 1934). Examination of injected embryos at gastrulation stages often revealed a region of the blastoderm that appeared thickened, similar in appearance to the native shield (Fig. 3A,B). This ectopic shield-like region always included GFP-expressing cells. A molecular pathway for shield formation in the zebrafish has been partially described. Maternally deposited β-Catenin activates expression of zygotic genes involved in patterning and specifying the organizer (Kelly et al., 1995a). We sought to determine at what point in this pathway *eomes* acted, and therefore we examined the expression of a number of zygotic genes that are normally expressed in the shield, including *gsc*, *chd* and *znot*/*flh* (Table 2, Fig. 4).

Although ectopic expression of *gsc*, *chd* and *flh* was observed reproducibly in embryos injected with *eomes* or *myc-eomes*, a number of other genes implicated in early patterning were unaffected by overexpression of *eomes*, including the Nodal-related factors *cyclops* and *squint* (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998), *wnt8* (Kelly et al., 1995b), *bmp2b* (Martinez-Barbera et al., 1997), *vega1*/*vox* (Kawahara et al., 2000; Melby et al., 2000) and *nwk/dhm* (Koos and Ho, 1998; Yamanaka et al., 1998). These results indicate that the perturbation caused by *eomes* overexpression is specific and restricted to a small number of genes.

Normally, *gsc* is expressed in cells that give rise to the prechordal plate (Stachel et al., 1993) whereas *chd* is expressed more broadly on the dorsal side of the embryo (Miller-Bertoglio et al., 1997). Ectopic expression of *gsc* and *chd* was detected in *eomes*- and *myc-eomes*-injected embryos along the margin at shield stage (6 hpf, Fig. 4A-F), although anti-Myc antibody staining revealed that nuclear expression of Myc-Eomes was not confined to the margin (Fig. 4C). Immunostaining with the anti-Myc antibody also revealed that the cells that ectopically expressed *gsc* (Fig. 4C, Myc and *gsc* staining are in separate focal planes) were primarily adjacent to cells that expressed Myc-Eomes. Thus, overexpression of *eomes* appeared to induce *gsc* expression non-cell autonomously. Ectopic *chd* was usually adjacent to and partially overlapped with the Myc-Eomes expressing cells. Cells expressing ectopic *chd* and Myc were observed (white Fig. 4F outline) as were cells that expressed *chd* alone (Fig. 4F red outline). Thus, our experiments indicate that *eomes* induced ectopic *chd* expression in *myc-eomes* expressing cells as well in neighboring non-*myc-eomes* expressing cells, indicating both cell autonomous and non-cell autonomous induction.

Early notochord precursor cells express *flh* (Talbot et al., 1995). Injection of *myc-eomes*, induced the ectopic expression of *flh* in scattered marginal cells in a pattern unlike the uniform domains of ectopic expression of *gsc* and *chd* seen under similar experimental conditions (Fig. 4H, arrowhead; Table 2). Immunostaining of *myc-eomes* injected embryos with the anti-Myc antibody revealed that ectopic *flh* expression was confined to cells that expressed Myc-Eomes (Fig. 4I). Thus, ectopic expression of *myc-eomes* induced *flh* expression cell-

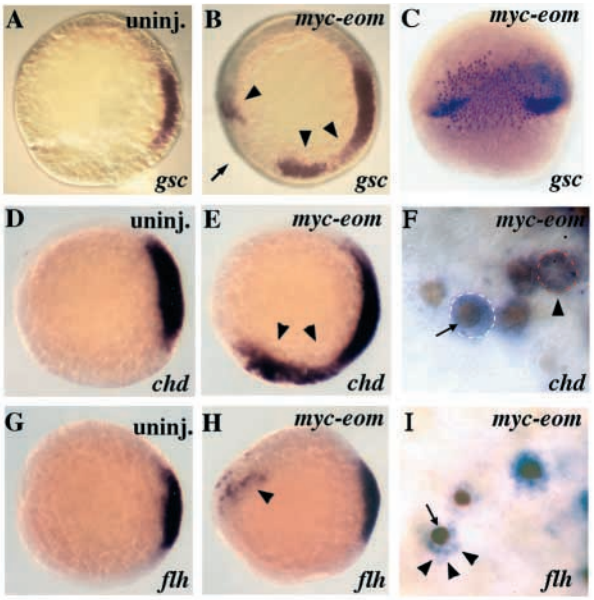

Fig. 4. Analysis of gene expression in embryos injected with *eomes* mRNA. All embryos are at the shield stage (6 hpf) and all views are from the animal pole, except (C,) which is a lateral view. In A,B,D,E,G,H the shield is to the right. Bottom right corner indicates in situ probe used, top right corner indicates the gene construct, if injected. (A) Uninjected embryo stained for *gsc* expression in the shield region. (B) Injection of *myc-eomes* induced ectopic *gsc* expression at the margin (arrowheads). Arrow marks region shown in lateral view in C. (C) Embryo in B after anti-Myc antibody staining (brown). Myc-labeled cells and cells ectopically expressing *gsc* are in different focal planes. (D) Expression of *chd* in an uninjected control. (E) Ectopic *chd* expression in an embryo injected with *myc-eomes* (arrowheads). (F) High-magnification view of margin of an embryo injected with *myc-eomes*. White outline surrounds a cell that expresses *chd* and Myc. (arrow). Red outline demarcates a cell expressing *chd* but not Myc (arrowhead). (G) Expression of *flh* in an uninjected control. (H) Embryo injected with *myc-eomes* with ectopic *flh* expression (arrowhead). (I) High-magnification view of embryo injected with *myc-eomes*. The same cells stain for *flh* (arrowheads) and Myc (arrow).

Table 2. Overexpression of *eomes* and *eomes-VP* induces ectopic expression of *gsc*, *chd* and *flh*

mRNA	*gsc*	*chd*	*flh*
myc-eomes	63% (62)	65% (26)	72% (43)
eomes-VP	71% (38)	70% (33)	81% (32)
VP	0% (5)	0% (37)	12% (34)
lacZ	4% (26)	0% (9)	6% (31)
Uninjected	0% (129)	0% (70)	1% (79)

Expression was analyzed by in situ hybridization at the shield stage. Total numbers of embryos are in parentheses.

autonomously, in contrast to the non-cell-autonomous induction of *gsc* and *chd*.

We next examined the timing of induction of these three genes by comparing the onset of ectopic expression with the normal temporal expression profile of each gene. Endogenous *gsc* expression is first detected just after the MBT at 3.5 hpf (A.E.E.B., unpublished) but the earliest stage at which ectopic *gsc* expression was observed was the dome stage (4.3 hpf, 5/9 embryos). The *chd* transcript is first expressed at the oblong stage (3.7 hpf) (Miller-Bertoglio et al., 1997), whereas ectopic *chd* was first detected at the dome stage (8/14 embryos). Initially, *flh* is expressed at the dome stage (4.3 hpf) (Talbot et al., 1995) and ectopic expression of *flh* was first detected at this stage also (4.3 hpf, 4/8 embryos) in *myc-eomes* injected embryos. Thus, unlike *gsc* and *chd*, the first ectopic expression of *flh* corresponded to the onset of endogenous expression.

To investigate whether *eomes* acted as a transcriptional activator or repressor to induce ectopic organizer markers, we fused the putative DNA-binding region of *eomes* (the T-box) to the VP16 transcriptional-activator domain (*eomes-VP*) and to the transcriptional-repressor domain of *engrailed* (*eomes-eng*). These domains have been shown to impart transcriptional activation and repression when fused to a heterologous DNA-binding domain in several organisms, including flies, frogs and zebrafish (Conlon et al., 1996; Han and Manley, 1993; Kessler, 1997; Koos and Ho, 1999). We then compared the overexpression phenotypes of *eomes-VP* and *eomes-eng* with that of native *eomes*. Overexpression of *eomes-VP* produced phenotypes identical to those seen following *eomes* and *myc-eomes* overexpression (Tables 1, 2), indicating that *eomes* acts as a transcriptional activator to induce ectopic expression of organizer markers and induce the formation of secondary axes. This is consistent with the fact that many characterized T-box genes, including frog *VegT* and *Eomes*, function as transcriptional activators (Tada and Smith, 2001). In contrast, overexpression of *eomes-eng* failed to induce ectopic expression of *gsc*, *chd* and *flh* (data not shown). When *eomes-eng* expressing cells were located dorsally, expression of *gsc* and *flh* was inhibited (Fig. 5B,D), which was opposite to the effect seen after overexpression of *eomes*. Co-injection of *eomes* and *eomes-eng* resulted in normal embryos at 24 hpf, demonstrating the specificity of the *eomes-eng* construct (see Materials and methods for details). These results indicate that *eomes* might be required during normal development to induce the expression of a subset of organizer genes.

Eomes reduction of function

The results with the *eomes-eng* indicateded that *eomes* might be required for normal induction of *gsc* and *flh*. We were interested in investigating the effects of reducing early activity of Eomes in the embryo using antisense morpholino oligonucleotides. However, the presence of maternal protein in both oocytes and early cleavage-stage embryos led us to suspect that *eomes*-targeted antisense morpholino oligonucleotides might have little or no effect. This is because morpholinos inhibit translation of specific transcripts and would have no direct affect upon extant proteins (Nasevicius and Ekker, 2000). We first tested the efficacy of Eomes-MO2 and found that, when injected at the one cell stage, this morpholino abolished zygotic expression of Eomes protein in the brain at 24 hpf (Fig. 6B,C). Furthermore, the reduction in protein was dose dependent (data not shown). In addition, in vitro transcription and translation of *eomes* plasmid was performed. The presence of 5 ng of a morpholino against the gene *cyclops* had little effect on Eomes translation (Fig. 6A, compare lanes 1 and 2) but the presence of 5 ng of Eomes-MO2 dramatically reduced the amount of protein produced (Fig. 6A, compare lanes 2 and 3). Thus, Eomes-MO2 inhibited the production of Eomes.

We next examined Eomes expression in embryos injected with Eomes-MO2 at earlier stages in development. Although protein levels were clearly reduced at the sphere and dome stages, protein localized to nuclei on the dorsal side of the embryo could still be detected (Fig. 6D,E). This indicated that dorsally confined nuclear Eomes protein is comprised of both zygotic and maternal protein, which cannot be entirely eliminated by the morpholino. We next examined the expression of *gsc* and *flh* in morpholino-injected embryos to determine whether the reduction in Eomes was sufficient to disrupt their expression. When in situ hybridizations were developed in blue using NBT/BCIP we were unable to detect convincing changes in the expression of *flh* and *gsc*. We have found that development in red using Fast Red is a more sensitive indicator of differences in transcript level than NBT/BCIP (A.E.E.B. and R.K.H., unpublished). When morpholino injected embryos were developed using Fast Red, a reduction in, but not a complete absence of, *gsc* and *flh* expression was detected (Fig. 6F-I). This is further evidence that *eomes* is required for the normal expression of *gsc* and *flh*. However, this reduction in expression was not sufficient to dramatically disrupt development because the majority of morpholino injected embryos were morphologically normal at 24 hpf (data not shown).

Zebrafish *eomes* can regulate its own expression

Transcriptional induction of *gsc*, *chd* and *flh* occurs during the zygotic phase of embryonic development. To establish a link between the maternal and zygotic phases of *eomes* expression, we explored the possibility that *eomes* regulates its own

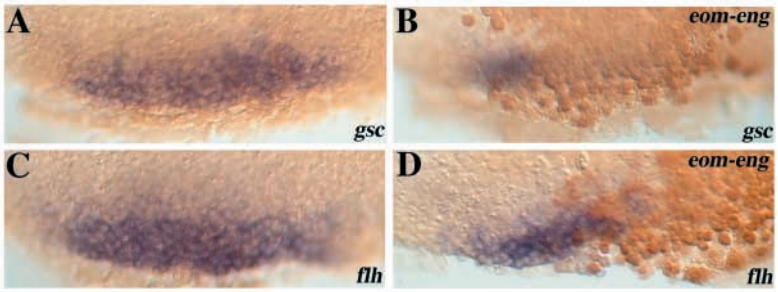

Fig. 5. Dominant-negative *eomes* inhibits dorsal expression of *gsc* and *flh*. (A-D) The dorsal blastoderm margin of embryos at 50% epiboly was dissected and flat-mounted. Bottom right corner indicates in situ probe used, top right corner indicates gene construct, if injected. (A) Expression of *gsc* in an uninjected embryo. (B) Injection of *eomes-eng* inhibits *gsc* expression. Eomes-eng expressing cells shown in brown by anti-GFP antibody staining. (C) Expression of *flh* in an uninjected embryo. (D) Injection of *eomes-eng* inhibits *flh* expression. Eomes-eng expressing cells shown in brown by anti-GFP staining.

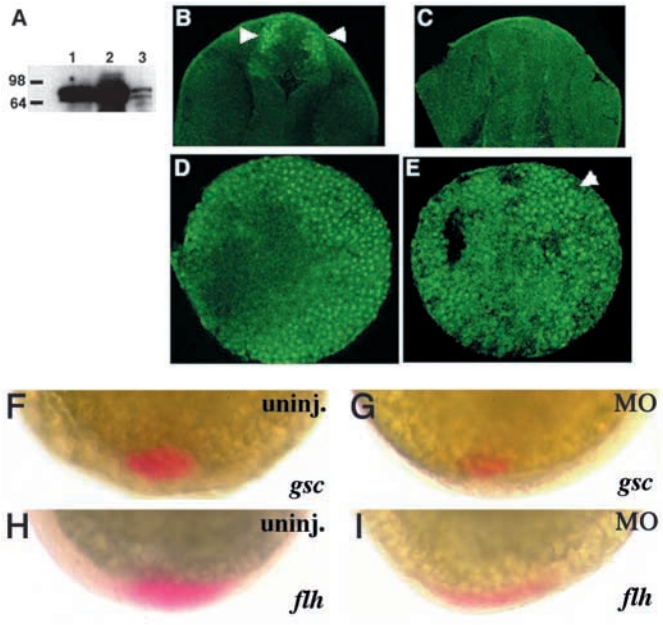

Fig. 6. Eomes-MO2 reduces Eomes protein levels and expression of *gsc* and *flh*. (A) Western blot of in vitro transcription and translation of *eomes*-pCS2+ probed with the anti-Eomes antibody. Lane 1, protein produced in the presence of 5 ng of *cyclops* morpholino. Lane 2, protein produced without morpholino present. Lane 3, protein produced in the presence of Eomes-MO2. The amount of Eomes protein produced is not affected by the presence of *cyclops* morpholino but is dramatically reduced in the presence of Eomes-MO2. (B) Embryo (1 dpf), dorsal side up and anterior to the top, stained with anti-Eomes antibody. Nuclear staining in the brain is in green (arrowheads). (C) Embryo (1 dpf) in the same orientation as B that has been injected with Eomes-MO2 and stained with the anti-Eomes antibody. No staining is visible. (D) Sphere-stage embryo stained with the anti-Eomes antibody. Staining is visible in nuclei on the dorsal side of the embryo. (E) Sphere-stage embryo injected with Eomes-MO2 and stained with the anti-Eomes antibody. Staining is reduced compared to D, but nuclear-localized protein is visible on the dorsal side of the embryo (arrowhead). (F-I) Animal-pole view of shield-stage embryos. Bottom right corner indicates in situ probe used, top right corner indicates gene construct, if injected. (F) Uninjected embryo stained for *gsc*. (G) Eomes-MO2 injected embryo, expression of *gsc* is reduced. (H) Uninjected embryo stained for *flh*. (I) Eomes-MO2 injected embryo expression of *flh* is reduced.

expression. Embryos were injected with *eomes-VP* and fixed at the shield stage, when endogenous *eomes* is undetectable by in situ hybridization. Embryos were processed by in situ hybridization using an *eomes* riboprobe against the C-terminal region, which was not contained in the *eomes-VP* mRNA. Expression of *eomes* was detected in shield-stage embryos injected with *eomes-VP* (83%, 30/36, Fig. 7B), indicating that Eomes-VP and, by inference, Eomes can activate and possibly maintain its own transcription. In addition, we found that injection of *eomes-VP* into MZ*oep* embryos (see below) resulted in induction of *eomes* expression (93%, 50/54), indicating that this autoregulation is Nodal-independent.

Zebrafish *eomes* requires Nodal signaling to induce organizer markers

How does ectopic *eomes* induce the expression of organizer

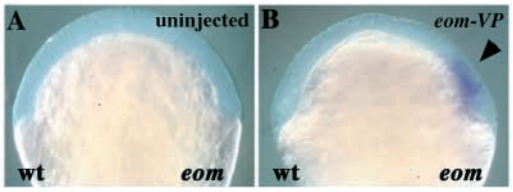

Fig. 7. *eomes* induces its own expression. Lateral views at 50% epiboly. (A) Uninjected embryo with no detectable *eomes* expression at shield stage. (B) Injection of *eomes-VP* leads to ectopic expression of the endogenous *eomes* gene at shield stage (arrowhead, see text for details).

genes? In zebrafish, as in *Xenopus*, maternal β-Catenin is localized to the nuclei on the future dorsal side of the embryo where it activates expression of downstream genes (Kelly et al., 1995a). Zebrafish *β-catenin* is required for dorsal expression of the homeobox-containing gene *nwk/dhm* (*nwk/dhm*) as well as the Nodal-like genes *squint* (*sqt*) and *cyclops* (*cyc*) (Kelly et al., 2000). A variety of experiments including over-expression and mutant analyses indicate that *nwk/dhm* and the Nodals are key regulators of organizer formation and patterning (Schier and Talbot, 2001). Furthermore, *nwk/dhm* and the Nodals appear to act in parallel pathways to establish the organizer (Fekany et al., 1999; Feldman et al., 1998; Gritsman et al., 2000; Koos and Ho, 1998; Koos and Ho, 1999; Yamanaka et al., 1998). We asked whether *eomes* acted through the Nodals and/or *nwk/dhm* to activate expression of *gsc*, *chd* and *flh*, or whether its mode of action was independent of these pathways. To test these possibilities, we took advantage of mutants in each pathway.

Zebrafish *nwk/dhm* is a homeobox gene that is expressed immediately following MBT on the prospective dorsal side of the embryo (Koos and Ho, 1998; Yamanaka et al., 1998). Zebrafish *boz* mutants, which are defective for the *nwk/dhm* gene, lack dorsal structures and exhibit reduced expression of *gsc*, *chd* and *flh* (Fekany et al., 1999; Koos and Ho, 1999; Solnica-Krezel et al., 1996). To determine if zygotic expression of *eomes* is regulated by the *nwk/dhm* pathway, we performed in situ hybridization on *boz*^{m168} embryos and found that *eomes* mRNA expression was normal [data not shown; zebrafish *boz* homozygous mutants were identified either by PCR (Koos and Ho, 1999) or by using embryos obtained from homozygous parents]. To test whether an intact *nwk/dhm* pathway was required for *eomes* function, we injected either *myc-eomes* or *eomes-VP* into *boz* mutants and examined them at the shield stage (6 hpf). Ectopic expression of *gsc* (85%, 17/20), *chd* (100%, 13/13) and *flh* (100%, 15/15) was observed in *boz*-mutant embryos following overexpression (Fig. 8A-F), which showed that *nwk/dhm* gene function does not appear to be required for *eomes* to induce expression of organizer-specific genes. One caveat is that the *boz* phenotype does not appear to be completely penetrant, thus, some partial *nwk/dhm* function may be present in homozygous-mutant embryos (Koos and Ho, 1999).

To determine whether expression of *eomes* was dependent upon Nodal signaling, we examined *eomes* expression in embryos with defective Nodal pathway function. Nodal signaling is reduced in *sqt;cyc* double mutants and in maternal-zygotic *oep* (MZ*oep*) mutants (Feldman et al., 1998; Gritsman

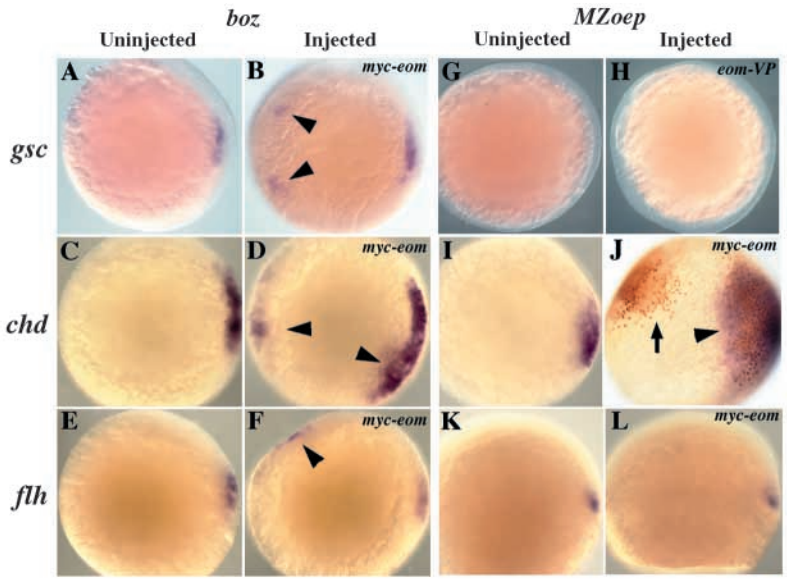

Fig. 8. Overexpression of *eomes* in mutant embryos. All embryos are at 6 hpf (equivalent to the shield stage) with dorsal to the right and are animal-pole views, except K,L which are lateral views. (A-F) *boz*-mutant embryos. (G-L) MZ*oep*-mutant embryos. (A,C,E,G,I,K) Uninjected embryos. (B,D,F,J,L) Embryos injected with *myc-eomes*. (H) An *eomes-VP*-injected embryo. Expression of *gsc* is shown in A,B,G,H, *chd* in C,D,I,J and *flh* in E,F,K,L. Arrowheads indicate regions of ectopic expression. (J) Two cells of an eight-cell-stage embryo were injected and stained with the anti-Myc antibody. Regions of Myc staining are indicated with an arrow and arrowhead. Expanded *chd* expression is only observed on the dorsal side (purple stain, arrowhead).

et al., 1999). Oep is an essential cofactor of Nodal that is related to EGF-CFC proteins in other vertebrates (Ciccodicola et al., 1989; Gritsman et al., 1999; Kinoshita et al., 1995; Shen et al., 1997; Zhang et al., 1998b). MZ*oep* mutants fail to form a normal shield, and the resulting embryos are cyclopic and lack endoderm and trunk mesoderm. Expression of organizer markers is either absent (e.g. *gsc*) or reduced (e.g. *chd* and *flh*) in MZ*oep* embryos (Gritsman et al., 1999). In addition, overexpression of *antivin*, a Nodal antagonist, inhibits Nodal signaling, which results in embryos that resemble *sqt;cyc* and MZ*oep* mutants (Thisse et al., 2000; Thisse and Thisse, 1999). Both maternal and zygotic expression of *eomes* mRNA was normal in MZ*oep* mutants (data not shown). To test whether *eomes* required Nodal signaling to induce expression of organizer genes, we injected either *myc-eomes* or *eomes-VP* into MZ*oep* mutants and into *antivin*-injected wild-type embryos, and examined gene-expression patterns at 6 hpf. In MZ*oep* mutants, neither *myc-eomes* nor *eomes-VP* induced *gsc* (0/90) or *flh* (0/31) expression (Fig. 8H,L). In addition, co-injection of *antivin* and *myc-eomes* into wild-type embryos did not lead to detectable *gsc* expression in the native shield region (0/10), which was identical to the phenotype observed in embryos injected with *antivin* alone (0/11). Thus, *eomes* did not induce *gsc* or *flh* expression in the absence of Nodal signaling.

By contrast, injection of *myc-eomes* into MZ*oep* mutants often expanded the expression *of chd*. In MZ*oep* mutants, two cells at opposite corners of an eight-cell-stage embryo were injected with *myc-eomes*. After processing for both *chd* and Myc expression at the shield stage, we observed expanded expression of *chd* on the dorsal side (7/22, 32%) (Fig. 8J, arrowhead). However, we never saw ectopic expression of *chd* on the ventral side, despite the presence of ventral Myc-staining cells (Fig. 8J arrow). Thus, overexpression of *myc-eomes* in MZ*oep* embryos led to expanded *chd* expression on the dorsal side of *MZoep* embryos, but failed to induce ectopic expression of *gsc* or *flh*. Interestingly, no ectopic expression of *chd* was detected in MZ*oep* mutants injected with *eomes-VP* (0/35). Double axes were never observed in injected MZ*oep* embryos raised to 24 hpf (0/11). Similarly, no secondary axes were detected in wild-type embryos co-injected with *antivin* and *myc-eomes* (0/13).

These findings indicated that Nodal signaling was required for *eomes* to induce ectopic expression of the organizer genes *gsc* and *flh*, and to induce secondary axes. It also appeared that *eomes* acted through a non-Nodal pathway to induce *chd* expression on the dorsal side of the embryo, but required an intact Nodal pathway to induce ectopic *chd* expression on the ventral side of the embryo.

eomes can modulate *sqt* activity

To further investigate the interaction between *eomes* and Nodals, we performed overexpression experiments at the animal pole. Injection of RNAs into a single cell at the animal pole allows specific interactions to be examined in isolation from marginal signals. Previous work (Chen and Schier, 2001) demonstrated that injection of *sqt* into the animal pole leads to the local induction of *gsc* in *sqt*-expressing and immediately adjacent cells. This work led to the proposal that the prospective shield is patterned by a Sqt morphogen gradient, which normally acts from the margin to activate *gsc* expression at high levels and *flh* expression at lower levels within the marginal region of the embryo (Chen and Schier, 2001). Our experiments revealed that *sqt* induced *gsc* locally but *eomes* induces *gsc* at a distance. Thus, we examined the consequences of injecting both *sqt* and *eomes* into a single cell in the animal pole of embryos at the 64- to 256-cell stage. In agreement with earlier work (Chen and Schier, 2001), we found that injection of *sqt* alone led to induction of *gsc* in a small patch (31/31 embryos, Fig. 9A). By contrast, injection of *myc-eomes* alone did not induce *gsc* expression at the animal pole (0/15 embryos). However, co-injection of *sqt* and *myc-eomes* led to the induction of a ring-like domain of *gsc* expression (30/34 embryos, Fig. 9B) in which *gsc* expression was induced around the injected cells (revealed by Myc antibody staining) but not in the central region where the majority of Myc-Eomes stained cells were located (Fig. 9C). From these results, we concluded that *eomes* appears able to modulate Sqt induction of *gsc*.

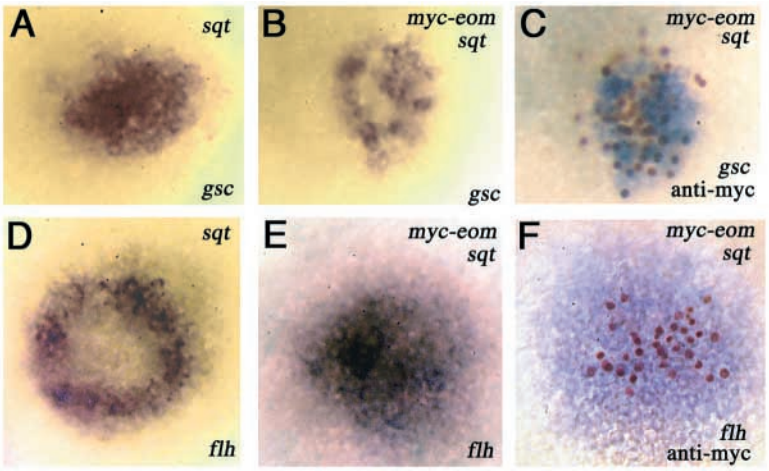

Fig. 9. *eomes* modulates Sqt signaling. Animal pole views at 50% epiboly (5.3 hpf). Bottom right corner indicates probe, top right corner indicates gene construct, if injected. (A) Expression of *gsc* in embryo injected with *sqt* at the animal pole. (B) Expression of *gsc* in embryo injected with *sqt* and *myc-eomes* at the animal pole. *gsc* is induced in a ring-like pattern. (C) Embryo in B after Myc-antibody staining. Note that the region of high Myc staining corresponds to the region of reduced *gsc* expression in B. (D) Expression of a ring of *flh* in embryo injected with *sqt*. (E) Expression of a solid domain of *flh* in an embryo injected with *sqt* and *myc-eomes*. (F) Embryo injected with *sqt* and *myc-eomes*, and stained with the Myc antibody.

We next examined *flh* induction. Chen and Schier (Chen and Schier, 2001) demonstrated that injection of *sqt* RNA into the animal pole caused induction of *flh* at a distance from the *sqt* source. By contrast, *eomes* induces *flh* cell-autonomously. Therefore, we examined the consequences of injecting *sqt* and *eomes* into a cell at the animal pole. Injection of *sqt* alone into the animal pole resulted in induction of a ring of *flh* expression at a distance from the *sqt* source (Chen and Schier, 2001). Injection of *myc-eomes* alone did not induce *flh* expression at the animal pole (0/15 embryos). Co-injection of *sqt* and *myc-eomes* led to induction of *flh* in a solid domain, rather than a ring (17/24 embryos). Thus, the addition of *myc-eomes* resulted in *flh* expression in the central region, which is consistent with *eomes* affecting *flh* expression cell-autonomously.

Thus, it appears that Eomes might modulate Sqt signaling, possibly by dampening it to a level that permits *flh* expression in the central region but is insufficient to induce *gsc*. This intriguing results needs to be confirmed by additional experiments in order to understand the nature of this interaction and how *eomes* and Nodals interact at the dorsal margin.

Discussion

In this paper, we have reported the isolation and characterization of the zebrafish *eomes* gene. We have shown that maternal *eomes* transcript and protein are present in the oocyte and preMBT embryo, that zygotic expression of *eomes* occurs transiently in the pregastrula embryo, and that early Eomes protein can induce zygotic *eomes* expression. Although the *eomes* transcript is distributed uniformly across the D/V axis at the sphere stage, nuclear-localized Eomes protein was detected dorsally, suggesting that nuclear localization of Eomes might play a role in dorsal specification and patterning. Consistent with this, overexpression of *eomes* and an *eomes*-VP16 activator construct induced secondary axes and ectopic expression of a subset of organizer genes, whereas overexpression of a dominant-negative form of *eomes* as well as an antisense morpholino oligonucleotide led to reduced expression of organizer genes. Together, these experiments have demonstrated a potential role for *eomes* in patterning the prospective organizer region. Furthermore, we have shown that induction of organizer markers by *eomes* depends upon an intact Nodal signaling pathway, but not on *nwk/dhm*.

The zebrafish T-box gene *eomes* is maternally expressed

The vertebrate *Eomes* orthologs in humans, mice and chicken (Papaioannou, 2001) are expressed zygotically, but *Eomes* is maternally and zygotically expressed in zebrafish and newts. Genes related to *Eomes* have also been identified in invertebrates. Amphioxus *Eomes/Tbr1/Tbx21*, which is considered an *Eomes*-like precursor gene, is maternally and zygotically expressed (Horton and Gibson-Brown, 2002; Ruvinsky et al., 2000b), whereas the ascidian genes *Ci-VegTR* and *As-mT* are expressed strictly maternally (Erives and Levine, 2000; Takada et al., 1998). Recent work indicates that *As-mT* might be an *Eomes/Tbr1/Tbx21* ortholog but the orthology of *Ci-VegTR* is unclear (Horton and Gibson-Brown, 2002). Therefore, with the exception of *Xenopus VegT*, it appears that most, if not all, maternally expressed T-box genes described to date are closely related to *Eomes*. Thus, it is likely that the last common ancestor of the chordates possessed a maternally expressed *Eomes*-like gene, and that maternal expression was subsequently lost in some vertebrate species. This hypothesis also indicates that maternal expression of *VegT* might be unique to *Xenopus* and, therefore, is unlikely to represent the ancestral condition of *VegT* or a *VegT*-precursor gene. Consistent with this, the zebrafish homologue of the *Xenopus VegT* gene, called *tbx16/spadetail*, is only expressed zygotically (Griffin et al., 1998; Ruvinsky et al., 1998).

Because of the similarity in expression patterns of *Xenopus VegT* and zebrafish *eomes*, we were wondered if zebrafish Eomes had an analogous functional role to that of *Xenopus* maternal VegT during germ-layer specification. One possible evolutionary scenario is that *VegT* has assumed the functional role previously filled by a maternally expressed *Eomes* gene. Our initial experiments involving injection of the zebrafish *eomes* gene into *VegT*-depleted frog embryos did not completely rescue the depletion phenotype, indicating that zebrafish *eomes* might be involved in different processes to that of frog *VegT*. However, because a library screen is not exhaustive, we cannot rule out the possibility that another maternal T-box gene, which is yet to be identified, functions like *VegT* in zebrafish to establish the primary germ layers. It will be interesting to determine how the presence or absence of maternal *Eomes* influences early developmental programs in

different organisms, and if a maternally expressed *VegT* homologue is identified in organisms other than amphibians.

eomes transcript is localized maternally

As described in this paper, zebrafish *eomes* transcript was expressed in a localized pattern during oogenesis and early embryogenesis. In zebrafish, most maternal transcripts that have been examined are either localized to the future animal pole of the oocyte or remain ubiquitously expressed throughout the cytoplasm. In the early embryo, these transcripts are found evenly distributed throughout the entire blastoderm (Howley and Ho, 2000). To date, only three maternally expressed mRNAs have been identified that exhibit localized patterns of expression in the zebrafish embryo, namely *daz1*, *brul* and *vasa* (Maegawa et al., 1999; Suzuki et al., 2000; Yoon et al., 1997). Although the specific embryonic localization patterns of *eomes* and *vasa* differ, they share some characteristics. Both *eomes* and *vasa* mRNAs localize to the junction between the yolk and the blastomeres, and both are distributed in a vegetal to animal gradient in one-cell-stage embryos (Braat et al., 1999; Howley and Ho, 2000). Furthermore, *eomes* and *vasa* mRNAs are the only transcripts that localize cortically in the oocyte (Howley and Ho, 2000). Thus, the mRNA localization patterns of *vasa* and *eomes* indicates a possible relationship between cortical localization during oogenesis and localization along the yolk/blastomere interface in the early embryo.

Eomes protein is present maternally

Maternal Eomes protein is present in oocytes and preMBT embryos, but does not localize to nuclei until immediately before MBT, which raises the possibility that any function for Eomes as a transcription factor is held latent until the onset of zygotic expression. Overexpression studies revealed that exogenous Eomes localizes to nuclei and induces expression of the endogenous *eomes* gene. Thus, one crucial function of maternal Eomes might be to activate zygotic transcription of *eomes*.

Role of *eomes* in patterning of the organizer

Overexpression of *eomes* resulted in the formation of secondary axes that arise from sites of ectopic expression of the zygotic organizer markers *gsc*, *chd* and *flh* in domains close to the margin of the zebrafish gastrula. These results indicated that *eomes* is sufficient to induce a functional organizer, but only from cells situated in or close to the margin. Overexpression of *eomes* induced the *gsc*, *chd* and *flh* genes at different times and through different mechanisms. Ectopic induction of *gsc* occured non-cell autonomously and was first detected at the dome stage, ~1 hour after *gsc* expression is initiated normally. Ectopic *chd* expression occured both cell-autonomously and non-cell autonomously at the dome stage, which is 30 minutes after endogenous expression begins. These observations indicate that *eomes* induced *chd* and *gsc* by an indirect mechanism that is likely to involve the production of a signaling factor that is diffusible or acts in a cell-cell relay. Furthermore, they indicate that the embryo cannot respond to exogenous Eomes until a distinct time point during the dome stage of development, presumably reflecting a requirement for a competency supplied by some other factor or factors. Importantly, *flh* is induced by *eomes* cell autonomously, and is first detected at the dome stage, when *flh* expression is normally initiated. This timing is consistent with a direct regulation of *flh* expression by *eomes*.

Although several early genes involved in organizer formation, such as *nwk/dhm* and *gsc*, are expressed in spatial domains that closely prefigure the organizer, neither the *eomes* transcript nor the initial distribution of nuclear-localized Eomes protein at MBT were limited to the future organizer region. However, beginning at the sphere stage, Eomes protein was rapidly excluded from most nuclei in the embryo and was retained in the nucleus only on the dorsal side of the embryo. This could be the result of a ventral factor that prevents Eomes entering the nucleus. Alternatively, a dorsal factor might retain Eomes in the nucleus. Combining this expression analysis and the timing of organizer gene induction described above, we propose that the timing of dorsal nuclear localization of endogenous Eomes marks the onset of its activity in organizer patterning.

In *Xenopus*, *Eomes* transcript and protein are expressed in a dorsal to ventral gradient in the mesoderm, and overexpression studies demonstrate that *Eomes* can induce the expression of mesodermal markers in a dose-dependent manner (Ryan et al., 1996). High levels of ectopic *Eomes* induce dorsal mesodermal markers, such as *gsc* and *chd*, and lower levels induce ventral mesodermal markers (Ryan et al., 1996). Thus, the distribution and function of zebrafish Eomes appears broadly similar to that of *Xenopus*, with the exception that the zebrafish gene was not observed to induce ventral mesodermal fates.

Reduction-of-function experiments also support a role for *eomes* in the establishment of organizer-gene expression. Overexpression of a dominant-negative *eomes* construct, as well as injection of an antisense morpholino oligonucleotide, led to either loss or reduction of *gsc* and *flh* expression. These results are consistent with a requirement for *eomes* in the induction of a subset of organizer genes.

Eomes and Nodals

Although the mechanism by which *eomes* regulates organizer gene expression is unclear, Nodal signaling appears to be required and, in the case of at least one gene (*chd*) the requirement for an intact Nodal pathway might be limited to one side of the embryo only. Induction of *gsc*, *chd* (on the ventral side) and *flh* by *eomes* overexpression required Nodal signaling because these genes were not induced when *eomes* was injected into MZ*oep* embryos. Nodals are secreted signaling molecules. Thus, it was surprising to find that the cell-autonomous induction of *flh* by *eomes* depended on Nodal signaling. A likely possibility is that transcription factors activated downstream of the Nodals act in combination with *eomes* to induce the expression of *flh*. In this case, *eomes* and Nodals would act in parallel to induce *flh* expression.

The role of Nodal signaling in the induction of *gsc* and *flh* expression has been examined previously in studies that have demonstrated that induction of *gsc* requires high levels of Nodal signaling whereas low levels are sufficient for *flh* induction (Gritsman et al., 2000). It has been hypothesized that Nodal signaling acts as a classical morphogen in patterning the organizer: cells close to the *sqt*-expressing margin receive high levels of Sqt and express *gsc*, and cells at a distance from the margin are exposed to lower Sqt concentrations and express *flh* (Chen and Schier, 2001). The *gsc*-expressing cells later give rise to the prechordal plate and *flh* expressing cells give rise to

the notochord. Our finding that overexpression of *eomes* resulted in induction of *flh* cell-autonomously and *gsc* at a distance indicates that *eomes* overexpression might have effects on *flh* and *gsc* expression that are the reciprocal of Nodal overexpression.

This intriguing possibility was investigated further by animal pole injections, which indicated that *eomes* could modulate the induction of *gsc* and *flh* by Sqt. Injection of *sqt* alone led to a solid patch of ectopic expression of *gsc*. However, in embryos co-injected with *sqt* and *eomes*, *gsc* was not expressed in a central region in which the density of *sqt* and *eomes* overexpressing cells is highest. One possible explanation for these results is that Eomes dampens Nodal function locally. Additionally, injection of *sqt* alone at the animal pole led to induction of *flh* in a ring, at a distance from the *sqt* source, but co-injection of *sqt* and *eomes* led to expression of *flh* in a solid patch. Thus, we suggest that these results are consistent with *eomes* reducing Nodal function to a level that is sufficient to induce *flh* but insufficient to induce *gsc*.

Additional experiments are required to verify this result and demonstrate such an interaction between *eomes* and *sqt* occurs at the dorsal margin. One possibility that we are currently investigating is that *eomes* may act in concert with Nodals at the dorsal margin to distinguish the notochord and prechordal plate territories, defined by *flh* and *gsc* expression, respectively.

In contrast to *gsc* and *flh*, the ability of *eomes* to induce ectopic expression of *chd* is not entirely Nodal dependent. Overexpression of *myc-eomes* in MZ*oep* embryos resulted in an enlarged domain of *chd* expression on the dorsal side of injected embryos, but ectopic expression of *chd* on the ventral side of MZ*oep* embryos injected with *myc-eomes* was not observed. This indicates that *eomes* can interact with a factor that is active in MZ*oep* embryos and confined to the dorsal side of the embryo to induce *chd* expression. One possible candidate is *nwk/dhm*, which , with the Nodals, has been shown to regulate *chd* expression (Koos and Ho, 1998; Shimizu et al., 2000). Although induction of *chd* by *eomes* occurs in *boz*-mutant embryos, this does not rule out an interaction between *eomes* and *nwk/dhm* that is only apparent in a background in which Nodal signaling is absent. We also observed that injection of *myc-eomes*, but not *eomes-VP*, caused expanded expression of *chd* on the dorsal side of MZ*oep* embryos. This indicates that the VP16 construct lacks a domain necessary for the Nodal-independent interaction, resulting in *chd* induction.

Loss-of-function data in the mouse and frog points to a conserved evolutionary role for *Eomes* in early cell movements (Graham, 2000). Injection of a putative dominant-negative *Eom*es construct into *Xenopus* embryos leads to the formation of exogastrulae, which is indicative of abnormal cell movements (Ryan et al., 1996). Aberrant cell movements are also implicated in gastrulation defects in mice that lack *Eomes*, in which cells fail to migrate into the primitive streak (Russ et al., 2000). The phenotypes we observed when *eomes-eng* was over-expressed globally in early embryos included defects in organizer formation, but also indicated that *eomes* might play a role in the cellular rearrangements of early gastrulation (A.E.E.B., C.H. and R.K.H., unpublished). This indicates a conserved evolutionary role of *Eomes* in regulating cell movements. A detailed analysis of these defects will be presented elsewhere.

eomes in early zebrafish development

In summary, we have shown that the maternal T-box gene *eomes* has both Nodal-dependent and Nodal-independent activities in the early zebrafish embryo. Overexpression of *eomes* resulted in the Nodal-dependent activation of a subset of organizer genes**.** By contrast, overexpression of a dominant-negative construct prevented the expression of these genes. Reducing Eomes protein levels by injection of an antisense oligonucleotide morpholino also reduced the expression of these genes. We also demonstrated that *eomes* can induce its own expression by a Nodal-independent mechanism. Thus, Eomes appears be involved in establishing the expression of a subset of dorsal-organizer genes. It will be interesting to determine how the nuclear localization of Eomes to the dorsal side is controlled and which downstream genes Eomes regulates.

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