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* and MZoeo (rescued oep15/oep15, gifts from M. Halpern and R. Warga). *bozozok* 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 (bZapPII, 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 [32P] and used as a probe to screen the ovary library (reverse primer 5'-GAGGCCTA TGCAGTTAGAAAGCA TC-3' (forward) and 5'-ACAAAGTGGTGCGACCACCAAACTGG-3').

**Genetic mapping**

Mapping was performed according to standard protocols (Hukriede et al., 1999) using the LNS4 radiation hybrid panel. Primers used were: 5'-ACAAAGTGGTGCGACCACCAAACTGG-3' (forward) and 5'-TGGTGAAGACCTCTTGTGCTGCCATCC-3' (reverse).

**Northern analysis**

Total RNA from 20 dechorionated embryos at various stages was extracted using the APCI 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 32P-labelled *eomes* probe or β-actin probe (gift of I. Ruvinsky). Membranes were washed at room temperature in 2xSSC/0.1% SDS and then in 0.1xSSC/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), *cldh* (Miller-Bertoglio et al., 1997), *no tail* (Schulte-Merker et al., 1994), *awkdhn* (Koos and Ho, 1998; Yamanaka et al., 1998), *squin* (Ertet et al., 1998; Feldman et al., 1998), *cyclips* (Rebagliati et al., 1998), *spe* (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'-TGCGCACTGACTGTTGAAATG-3' and the reverse primer was 5'-CGGTTGATTCTTTTCTCT-3'. The plasmid was linearized with *SpeI* 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'-CGTCAGCAGCTACCGCC-3' and the reverse primer 5'- GTTCCTAGTATGAAGGGCTGGTGTGAAGCCG-3' and cloned into pGEMT-easy. To make riboprobe, the plasmid was digested with *SpeI* and transcribed with T7 polymerase.

Full-length template to synthesize forkhead2 (*fd7*) (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 *fd7* riboprobe was generated using T7 polymerase following *SalI* 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'-CGGCTTGAGACGCCATGGCTAATTTTCTCT-3' and the reverse primer 5'-GTTCCTAGTATGAAGGGCTGGTGTGAAGCCG-3' and cloned into the Xhol/XbaI site of pCS2+. To produce *mec-eomes*, the *eomes* ORF was cloned into the StuI/XbaI site of pCS2+MT, following PCR amplification with the forward primer 5'-GAGGCCTATCGTTAGGAGAC-AGCATCC-3' and the reverse primer as above. Plasmids were linearized with NotI and transcribed using the SP6 mRNA message 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 Clal site of pVP16-N (Kessler, 1997). The forward primer was *eomes-ENR* (5'-CCATGATACCTGCTGCTGCTGCTTTCCTCC-3') and the reverse primer was *eomes-VP16* (5'-CCATCAGTCCGGCGGCG- CGGGGACAATCGT-3'). As a control, *no tail-VP16* (nlt-VP) was
made by cloning a fragment encoding amino acids 1-232 (Schulte-Merker et al., 1994) into the Cklai site of pVP16-N vector. The forward primer was ntlR-enF (5'-CCATCGATTCGCCCATGTCGGCTCA-AGTCCCGAC-3') and the reverse primer was ntlR-VP16 (5'-CCA-TGATGCTGTTGTCACTGCTGTG-3').

The engrafted (eng) forward primer was eomesF-enR (described above) and the reverse primer was eomesR-enR (5'-CGGAATTCGCCCGCCTGGGAACATCTG-3'). To generate the control ntl-eng the forward primer was ntlR-enR (as above) and the reverse primer was ntlR-enR (5'-CGGAATTCAGATTGCGTGTTGTCAGTGCTGTG-3'). Plasmids were linearized with SacII (except eomes-VP and ntl-VP, which were digested with NotI) 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 engrafted 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'-CATTTCTC-ACGGTTGCATAAAGGG-3' and Eomes-MO2, 5'-CCGCAAGG-GAGGATCTTCTAATCTG-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 (Thiese 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 GFP 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 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 or 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' BamHI/3' HindIII 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 Qiagen 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*CAGCA TGTACTT*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 RNAs 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 microscope (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 ~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. 1 I-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/blasto disc 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.](image-url)
Zebras**h 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=19), 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 myc-eomes-lacZ 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 and Nusslein-Volhard, 1998). Ectopic patches of 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.

### Table 1. Overexpression of eomes induces secondary axes

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

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.
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 (Erler 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 wnt/dll7a (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-

| 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 indicated 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 (Nasevic 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...

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**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.
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 *Mzoep* 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 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 *squit* (*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* (*Mzoe*; *Mzoe*) mutants (Feldman et al., 1998; Gritsman
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). MZoep 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 MZoep embryos (Gritsman et al., 1999). In addition, overexpression of antivin, a Nodal antagonist, inhibits Nodal signaling, which results in embryos that resemble sqt;cyg and MZoep mutants (Thisse et al., 2000; Thisse and Thisse, 1999).

Both maternal and zygotic expression of eomes mRNA was normal in MZoep 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 MZoep mutants and into antivin-injected wild-type embryos, and examined gene-expression patterns at 6 hpf. In MZoep mutants, neither myc-eomes nor eomes-VP induced gsc (0/90) or flh (0/31) expression (Fig. 8H,L). In addition, coinjection 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 MZoep mutants often expanded the expression of chd. In MZoep 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 MZoep 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 MZoep mutants injected with eomes-VP (0/35). Double axes were never observed in injected MZoep 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. 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) MZoep-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).
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.

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.

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/Thr1/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 tbl16/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 maternally expressed 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 blastomer,es 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 occurred non-cell autonomously and was first detected at the dome stage, ~1 hour after gsc expression is initiated normally. Ectopic chd expression occurred 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 morpholinolo 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 MZoep 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 Nodalts at the dorsal margin to distinguish the notochord and prechordial 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 MZoep 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 MZoep embryos injected with myc-eomes was not observed. This indicates that eomes can interact with a factor that is active in MZoep embryos and confined to the dorsal side of the embryo to induce chd expression. One possible candidate is nk5/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 nk5/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 MZoep 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 Eomes 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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References


Inhibition of Xbra transcription activation causes defects in mesodermal patterning and results in autoregulation of Xbra in dorsal mesoderm. Development 122, 2427-2435.


