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T-Box Gene *eomesodermin* and the Homeobox-Containing Mix/Bix Gene *mtx2* Regulate Epiboly Movements in the Zebrafish

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Abstract

The T-box gene *eomesodermin* (*eomes*) has been implicated in mesoderm specification and patterning in both zebrafish and frog. Here, we describe an additional function for *eomes* in the control of morphogenesis. Epiboly, the spreading and thinning of an epithelial cell sheet, is a central component of gastrulation in many species; however, despite its importance, little is known about its molecular control. Here, we show that repression of *eomes* function in the zebrafish embryo dramatically inhibits epiboly movements. We also show that *eomes* regulates the expression of a zygotic homeobox transcription factor *mtx2*. Gene knockdown of *mtx2* using antisense morpholino oligonucleotides, likewise, leads to an inhibition of epiboly; moreover, we show that knockdown of *mtx2* function in the extraembryonic yolk syncytial layer only is sufficient to cause epiboly defects. Thus, we have identified two components in a molecular pathway controlling epiboly and show that interactions between deep layer cells of the embryo proper and extraembryonic tissues contribute in a coordinated manner to different aspects of epiboly movements.

Keywords

zebrafish; epiboly; radial intercalation; eomesodermin; mtx2

INTRODUCTION

One of the most significant events during development is gastrulation, when cellular rearrangements produce the embryonic germ layers and set the stage for the emergence of the adult body plan. In zebrafish, several different types of cell movements occur during gastrulation, including epiboly (Warga and Kimmel, 1990). Epiboly is the process by which the blastoderm, which initially sits on top of a large yolk ball, thins and spreads downward to engulf the yolk by the end of gastrulation. Epiboly can be divided into two phases: initiation, which is driven by radial intercalation, and progression, which leads to closure of the blastopore (Strahle and Jesuthasan, 1993). Radial intercalation occurs from the sphere stage (4 hours postfertilization, hpf) to the dome stage (4.3 hpf), when cells move radially toward the surface of the blastoderm to take up more superficial positions, while at the same time the yolk cell domes toward the animal pole (Warga and Kimmel, 1990). The result of these changes is that

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the blastoderm thins and its surface area increases, which allows the vegetal spread of the blastoderm to proceed.

Although epiboly is a common cell movement during gastrulation in several species (reviewed in Kane and Adams, 2002), we know little about its molecular control. We do not know what initiates radial intercalation and the concomitant doming of the yolk and what sort of changes in adhesion molecules and cytoskeletal components occur in cells during this process. How epiboly is coordinated between the different cell layers of the embryo is also not well understood. Only a single epiboly mutant, called *half-baked* (*hab*), was isolated from screens for zygotic mutants in zebrafish (Kane et al., 1996; Kane and Adams, 2002), although recently, several epiboly mutants were isolated in a screen for maternal effect mutants (Wagner et al., 2004).

In previous work, we described the role of the maternal T-box transcription factor *eomesodermin* (*eomes*) in the induction of a subset of dorsal organizer genes (Bruce et al., 2003). Here, we describe an additional function for *eomes* in the control of early cell movements by showing that over-expression of an *eomes* repressor construct inhibits the initiation of epiboly. We also show that *eomes* regulates the expression of the zygotic homeobox transcription factor *mtx2*. Reduction of *mtx2* function using antisense morpholino oligonucleotides leads to disruptions in the progression of epiboly. These findings provide new insights into the molecular control of this critical cell movement and are consistent with work in several species demonstrating the importance of T-box genes in the control of morphogenesis (for recent review, see Showell et al., 2004). In addition, work in the frog and mouse has implicated *Eomes* in the control of early morphogenetic movements, suggesting that this may represent an evolutionarily conserved function of *Eomes* (Ryan et al., 1996; Russ et al., 2000). Despite evidence for a conserved role for *Eomes* in morphogenesis, this work is the first to implicate *Eomes* in epiboly.

RESULTS

Overexpression of the Repressor Construct *eomes-eng* Inhibits Epiboly

We wished to investigate the possible effects on development stemming from a general knockdown of Eomes function, but, as shown previously in Bruce et al., the presence of maternal Eomes protein precluded the successful use of *eomes* morpholinos (Bruce et al., 2003). Instead, we made use of our finding that *eomes* functions as a transcriptional activator in the early embryo, and we injected *eomes-eng* RNA, encoding a repressor construct consisting of the *eomes* DNA binding domain fused to the Engrailed transcriptional repressor domain, into early embryos (Bruce et al., 2003). Although there are caveats to using a repressor construct, this approach has proven quite successful in the study of T-box gene function and such constructs have been shown to recapitulate specific mutant phenotypes (for example, Conlon et al., 1996, 2001; Tada and Smith, 2001; Mullen et al., 2002).

Previously, we described the consequences of injecting *eomes-eng* into just a subset of cells in the early embryo (Bruce et al., 2003). Specifically, we showed that expression of this construct in cells on the dorsal side of the embryo in the organizer leads to an inhibition of expression of some organizer genes, including *goosecoid* (*gsc*) and *floating head* (*flh*). We now describe our finding that when *eomes-eng* was overexpressed more globally in early embryos, defects in epiboly were observed, indicating that *eomes* may have an additional function in early cell movements.

In embryos coinjected with repressor *eomes-eng* RNA and, as a tracer, *gfp* RNA, portions of the blastoderm failed to thin (Fig. 1, compare B,G with A,D). As thinning of the blastoderm at this stage in development is the result of radial intercalation (Warga and Kimmel, 1990), we

infer that the defects we observe in *eomes-eng*–injected embryos are the result of inhibiting radial intercalation. The reduction in intercalation was limited to regions of green fluorescent protein (GFP) expression, suggesting a cell-autonomous defect (Fig. 1, compare I with F). In contrast, cells that did not express GFP appeared to undergo radial intercalation normally (arrow, Fig. 1B,H). In control-injected embryos, GFP-expressing cells became evenly distributed in the blastoderm during epiboly (Fig. 1F), whereas GFP-expressing cells in *eomes-eng*–injected embryos did not (Fig. 1I), which further supports the conclusion that intercalation is impaired. The defect was apparent very early in development, by dome stage, suggesting that injected embryos were unable to initiate radial intercalation. This phenotype was seen in 92% (165 of 180) of *eomes-eng*–injected embryos, was never seen in embryos injected with RNA encoding the engrailed repressor domain alone (0 of 36, Fig. 1D–F), and rarely was seen in uninjected embryos (3%, 4 of 123).

The phenotypes of *eomes-eng*–injected embryos at 1 day postfertilization (dpf) included head and trunk defects, which presumably were the result of abnormal gastrulation movements, as a consequence of impaired epiboly. The most common defects in embryos were shortened axes and twisted tails (52%, 55 of 106; Fig. 1M). Twisted tails were observed at the level of the anus (the posterior end of the yolk extension), which marks the closure point during epiboly. This finding suggests that closure over the yolk during gastrulation was abnormal. In some cases, cells expressing *eomes-eng* remained at the animal pole during epiboly and, thus, ended up in the head at 1 dpf (24%, 10 of 42; Fig. 1N,O). In addition, axial clefts were sometimes observed (11%, 10/92); these are a hallmark of delayed epiboly (Lunde et al., 2004;Wagner et al., 2004).

We performed several experiments to control for potential nonspecific effects of the repressor construct. We previously performed dose–response experiments and demonstrated that the defects caused by *eomes-eng* can be rescued by coinjection of *eomes* RNA (Bruce et al., 2003). In addition, we made an equivalent construct using the T-box domain of another T-box gene, *no tail*, which does not yield the same phenotypes as *eomes-eng* when injected into embryos (Bruce et al., 2003).

Here, we examined overall cell morphology to ensure that the observed defects were not the result of nonspecific toxicity. To outline cell shapes, we injected a mixture of RNA encoding both cytoplasmic and membrane bound forms of GFP, alone or in combination with *eomes-eng* (Supplementary Figure S1A,B, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat; Ulrich et al., 2003). We saw no obvious differences in cell morphology in *eomes-eng*–injected embryos when compared with control embryos. We also examined the microtubule cytoskeleton of blastoderm cells by immunostaining with anti-tubulin antibody (Supplementary Figure S1C,D) and again saw no dramatic changes in *eomes-eng*–injected embryos that would be indicative of sick or damaged cells.

Eomes Protein Distribution Correlates With a Function in Epiboly

Eomes is expressed at the right time and place to mediate radial intercalation and epiboly. Using a polyclonal antibody that we generated, we previously described the protein expression pattern at the sphere stage (4 hpf), when zygotic genes involved in organizer formation and function are first expressed (Bruce et al., 2003). At this time, Eomes is confined to nuclei on the dorsal side of the embryo. However, Eomes has an earlier pattern of protein expression that is shown in Figure 2. Around the midblastula transition (2.75 hpf), Eomes protein was detected in nuclei throughout the blastoderm (Fig. 2A). This pattern persisted through the high stage at 3.3 hpf (Fig. 2B). Thus, Eomes is present in the nuclei of all embryonic cells, at the time when those cells first become motile (Kane and Kimmel, 1993), and just before the start of radial intercalation.

Regional Patterning Is Normal in *eomes-eng*–Injected Embryos

As we previously showed that regional overexpression of *eomes-eng* in the organizer leads to reduced expression of the organizer genes *gsc* and *flh* (Bruce et al., 2003), we investigated whether the cell movement defects we observed when *eomes-eng* was overexpressed globally resulted from abnormal dorsal–ventral patterning. We examined the expression of the organizer markers *gsc* and *flh* at the shield stage and found that it was generally unaffected. Of embryos injected with *eomes-eng*, 35% (6 of 17) showed a slight reduction in *gsc* expression, whereas uninjected embryos (37 of 37) and *eng*-injected (10 of 10) embryos expressed *gsc* normally. *flh* was expressed normally in 16 of 18 (89%) embryos examined, whereas slightly patchier expression was observed in 2 embryos. Expression was normal in uninjected embryos (22 of 22). Thus, the cell movement defects caused by *eomes-eng* do not seem to be the result of defects in the expression of dorsal–ventral patterning genes.

Eomes protein is also expressed in a subset of cells in the enveloping layer, an extraembryonic tissue, which comprises the outermost layer of the blastoderm and produces a transient epithelium (Kimmel et al., 1995; Bruce et al., 2003). To determine whether the enveloping layer was disrupted in *eomes-eng*–injected embryos, we examined the expression of *keratin4*, which is expressed exclusively in this cell layer (Thisse et al., 2001). We found that *keratin4* was expressed normally in uninjected control embryos (95 of 95) and was normal in 98% (54 of 55) of *eomes-eng*–injected embryos, suggesting that the enveloping layer was unaffected.

eomes Regulates Expression of *mtx2*, a Member of the Mix/Bix Gene Family

As an initial strategy to identify downstream target genes of *eomes*, we took a candidate gene approach. In frogs, the T-box genes *Eomes* and *VegT* have been shown to regulate the expression of members of the Mix/Bix family of homeobox containing transcription factors (Ryan et al., 1996; Lemaire et al., 1998; Tada et al., 1998; Casey et al., 1999). In addition, the mouse *Eomes* gene appears to directly regulate the Mix/Bix gene *Mixl1* (Russ et al., 2000; Sahr et al., 2002). These findings, suggesting a conserved interaction between T-box genes and Mix/Bix genes, lead us to investigate whether Eomes regulated Mix/Bix genes in zebrafish.

We examined expression of three Mix/Bix genes identified in the zebrafish as *bon*, *mtx1*, and *mtx2* (Alexander and Stainier, 1999; Hirata et al., 2000; Kikuchi et al., 2000). Although the functions of *mtx1* and *mtx2* were unknown, their expression patterns partially overlapped with *eomes*; therefore, we examined whether *eomes* could activate the expression of either gene. Overexpression of *eomes* or *eomes-VP* (an activator construct, consisting of the *eomes* DNA binding region fused to the VP16 transcriptional activator domain; Bruce et al., 2003) induced ectopic expression of *mtx2* but not *mtx1* or *bon* (Fig. 3B and data not shown). *mtx2*, a zygotic gene, is expressed in a dynamic pattern that includes expression in marginal cells adjacent to the yolk and in the yolk syncytial layer (YSL; Hirata et al., 2000). The YSL is an extraembryonic structure that forms when marginal cells fuse with the previously anuclear yolk ball around the start of zygotic transcription and, thereby, provides an interface between the embryonic cells and underlying yolk (Kimmel and Law, 1985). Ectopic expression of *mtx2* was observed in cells several cell diameters away from the margin into the animal pole region, particularly in embryos injected with *eomes-VP* (arrowhead, Fig. 3B). Injection of *eomes-VP* resulted in ectopic expression of *mtx2* in 76% of embryos (32 of 42), whereas injection of *eomes* resulted in ectopic expression in 57% of embryos (13/23).

We investigated whether *eomes* induced *mtx2* expression cell-autonomously or non–cell-autonomously by injecting *myc-eomes* RNA, which produces a myc epitope tagged protein that can be visualized with anti-myc antibody (Bruce et al., 2003). Double labeling with anti-

myc antibody and *mtx2* in situ hybridization demonstrated that *eomes* induced *mtx2* expression cell autonomously in deep cells of the blastoderm (Fig. 3B, inset).

Pursuing this connection further, we found that injection of the repressor construct *eomes-eng* led to a reduction in *mtx2* expression in 78% of embryos (31 of 40), indicating a tight regulation of *mtx2* expression by *eomes* (Fig. 3C). The embryos shown in Figure 3B,C received injections into a single blastomere at the eight-cell stage. We also observed reduced *mtx2* expression when *eomes-eng* was injected globally into one-cell stage embryos (data not shown). In contrast we found that *eomes-eng* had no effect upon the expression of *bon*, a Mix/Bix gene that plays an important role in endoderm specification (Alexander and Stainier, 1999;Kikuchi et al., 2000). Thus, *eomes* is capable of specifically regulating the expression of *mtx2* in the zebrafish, without affecting other Mix/Bix-type genes.

As a control for the specificity of the effect, we examined *mtx2* expression in embryos injected with RNA encoding *ntl-VP*, an activator construct. *ntl* is the zebrafish homolog of the T-box gene *Brachyury* and is expressed in the early embryo (Schulte-Merker et al., 1992). At the sphere stage, expression of *mtx2* was normal in uninjected control embryos (43 of 43), as well as in embryos injected with *gfp* RNA (36 of 36). Expression of *mtx2* was also normal in embryos injected with *ntl*-VP (42/42, Fig. 3D). These results suggest that *eomes* specifically regulates the expression of *mtx2*.

mtx2 Is Also Required for Epiboly

As *mtx2* is a strictly zygotically expressed gene and is regulated by *eomes*, we investigated its potential function in epiboly by gene knockdown using two independent antisense morpholinos (Mtx2-MO1 and Mtx2-MO2). The activity of both *mtx2* morpholinos was tested as described (see Experimental Procedures section; Oates and Ho, 2002).

After injection of either morpholino into one-cell stage embryos, epiboly defects were observed, although the initiation of epiboly appeared to occur normally (Fig. 4, compare A and B). The defects were first observed at and subsequent to the shield stage (6 hpf) when the blastoderm margin failed to continue its vegetal movement and as a result the blastoderm did not thin further. We observed identical phenotypes with Mtx2-MO1 and Mtx2-MO2, thus for simplicity, we focused on Mtx2-MO1. Epiboly defects were observed in 96% (115 of 120) of Mtx2-MO1–injected embryos. The majority of embryos injected with Mtx2-MO1 died by mid-epiboly due to the yolk cell bursting. Of interest, yolk cell lysis is also observed in the zebrafish epiboly arrest mutant *half-baked* (Kane et al., 1996;Kane and Adams, 2002) as well as in the maternal effect epiboly mutant *betty boop* (Wagner et al., 2004). As we did for *eomes-eng*, we examined cell morphology by coinjection of morpholinos and *gpf* RNA and saw no differences from control embryos; similarly, we saw no obvious defects in the microtubule cytoskeleton using an anti-tubulin antibody (data not shown). These data suggest that the morpholino effects are specific and not the consequence of general toxicity.

As *mtx2* is expressed in the YSL as well as in the marginal cells of the blastoderm, we were interested in examining its function there. We injected Mtx2-MO1 into the YSL shortly after its formation and observed defects similar to those seen in embryos injected with Mtx2-MO1 into a single blastomere at the two-cell stage (Fig. 4C). Epiboly defects were observed in 27 of 27 embryos injected with morpholino into the YSL. This finding suggests that *mtx2* expression in the YSL is required for normal epiboly. As *eomes* is not expressed in the YSL, *mtx2* expression may be regulated differently in the YSL than in the blastoderm.

We were also interested in trying to rescue the defects caused by *eomes-eng* by coinjecting *mtx2* RNA. If the defects observed in *eomes-eng*–injected embryos were the result, in part or whole, of repressing *mtx2* expression, then we might expect to see a rescue of the epiboly

defect. However, we were unable to carry out these experiments for technical reasons. We observed a severe overexpression phenotype, even at low doses, when *mtx2* RNA was injected into one-cell stage embryos, thus making it impractical to score for a rescue. In an attempt to overcome this difficulty, we injected *mtx2* RNA into the yolk syncytial layer; however, we found that this injection had no effect on *eomes-eng*–injected embryos (data not shown). This finding suggests that, although expression of *mtx2* in the YSL is required for normal epiboly, it may not be sufficient. In summary, *mtx2* morpholino-injected embryos had defects in the progression of epiboly and *mtx2* morpholinos produce the same defects whether injected into the YSL or the blastoderm.

Regional Patterning Is Normal in *mtx2* Morpholino-Injected Embryos

We examined dorsal–ventral patterning in morpholino-injected embryos by examining *gsc* expression. *gsc* expression was normal in uninjected embryos (90 of 90) and was present in all Mtx2-MO1–injected embryos (99 of 99), although the domains were slightly disorganized in 52% (52 of 99) of the embryos, consistent with defects in morphogenesis. Expression of *flh* was observed in 30 of 30 embryos injected with Mtx2-MO1, although the staining was slightly less intense than in control embryos in 13 of 30 embryos. Normal expression was observed in uninjected controls (22 of 22). We also examined the expression of the ventral marker *evel* (Joly et al., 1993), which was expressed normally in uninjected control embryos (124 of 124) and in 95% (63 of 66) of Mtx2-MO1–injected embryos. Thus, dorsal–ventral patterning was largely unaffected in *mtx2* knockdown embryos.

We also examined the expression of the pan mesodermal marker *no tail* (Schulte-Merker et al., 1992) and found that it was normal in uninjected control embryos (122 of 122) as well as in 91% (91 of 100) of Mtx2-MO1–injected embryos. Similarly, the endodermal marker *bon*, was expressed normally in both uninjected control embryos (50 of 50) and morpholino-injected embryos (78 of 78). These results suggest that the general specification of endoderm and mesoderm was normal in morpholino-injected embryos.

We also examined the expression of the endodermal marker *sox17*, specifically in dorsal forerunner cells (Alexander and Stainier, 1999). Expression of *sox17* was normal in 99% (68 of 69) of uninjected control embryos and absent in one embryo. No forerunner cell expression of *sox17* was detected in 24% (9 of 37) of morpholino-injected embryos. We suspect that this absence of expression was the result of developmental delay rather than a specific effect on the forerunner cells, because, as a result of the epiboly defect, the morpholino-injected embryos were difficult to stage and most lysed by the shield stage, which is when forerunner expression is first detected (Alexander and Stainier, 1999).

Epiboly Defects Are Nodal-Independent

We next tested whether the effects of *eomes-eng* and *mtx2* morpholinos on cell movements were Nodal-dependent, because induction of organizer genes by *eomes* overexpression requires Nodal signaling (Bruce et al., 2003). The process of epiboly itself does not depend on Nodal signaling, as early epiboly movements are overtly normal in MZ*oep* embryos (Feldman et al., 2000). MZ*oep* embryos lack of Nodal involvement in epiboly, injection of either *eomes-eng* or Mtx2-MO1 into MZ*oep* embryos inhibited epiboly in a manner indistinguishable from their effects on wild-type embryos (13 of 13 for *eomes-eng*–injected embryos and 14 of 14 for Mtx2-MO1–injected embryos; Fig. 5A,B).

Hirata and coworkers demonstrated that *mtx2* expression is not regulated by Nodal signaling (Hirata et al., 2000). This finding suggested that regulation of *mtx2* by *eomes* might also be independent of Nodal signaling. To verify that *mtx2* expression was unaffected by a loss of maternal and zygotic Nodals, we performed in situ hybridizations on MZ*oep* embryos and

found that *mtx2* expression was normal (Fig. 5C). Next, we injected an activator construct, *eomes-VP* (Bruce et al., 2003), into MZ*oep* embryos and found that *eomes-VP* was still capable of inducing ectopic expression of *mtx2* (86%, 37 of 43; Fig. 5D). Therefore, *eomes* regulates *mtx2* through a non-Nodal pathway and the regulation of epiboly by both genes is Nodal-independent.

DISCUSSION

eomes Has a Role in Epiboly

We have shown that *eomes* is involved in two processes in the developing zebrafish embryo. In prior work, we showed that overexpression of *eomes* leads to Nodal-dependent ectopic expression of organizer genes and induction of secondary axes, demonstrating a potential role in organizer function (Bruce et al., 2003). Here, we report that overexpression of a repressor construct revealed an additional, Nodal-independent, role for *eomes* in epiboly. The correct execution of epiboly is essential for gastrulation and, hence, viability of the embryo. Little is known about the genetic control of this process; here, we present evidence that *eomes* may be required for epiboly.

Eomes protein is expressed in two distinct patterns, which correlate with the two Eomes functions that we have described. One pattern correlates with a role in induction of zygotic organizer gene expression (Bruce et al., 2003), whereas an additional pattern is apparent at earlier stages of development around the midblastula transition, when cells in the zebrafish embryo first become motile (Kane and Kimmel, 1993). At this stage, we observed nuclear localized Eomes throughout the blastoderm. Eomes protein is expressed in the cells that will undergo radial intercalation and epiboly at the time when these cell movements are initiating; thus, Eomes is expressed in the right location and at the correct time to influence the behavior of intercalating cells. Consistent with this expression pattern is the observation that the epiboly defects seen in *eomes-eng*–injected embryos were first apparent at doming, which suggests that Eomes is required for the initiation of this cell movement. The mechanism by which *eomes* directs epiboly is unclear but may involve regulation of cell adhesion, which has been proposed to be a common mechanism of T-box function (Ahn et al., 2002). What controls these two different protein expression patterns currently is unknown but, once determined, should provide insight into how the two functions of *eomes* are coordinated. The two *eomes* functions might result from interactions with different partner proteins that are expressed in distinct regions of the embryo. Other groups have demonstrated that T-box genes can act synergistically and interact physically with other transcription factors (Bruneau et al., 2001; Hiroi et al., 2001).

One obvious question is why we did not observe dramatic cell movement defects in our gain-of-function experiments in which *eomes* was overexpressed in embryos. We did observe abnormal thickenings corresponding to regions of overexpressing cells, which we interpreted as ectopic shields, based on marker gene expression (Bruce et al., 2003). However, these thickened regions may also be indicative of changes in the adhesive properties of the *eomes* overexpressing cells. It is also possible that cells cannot epibolize to excess, as might be expected in response to *eomes* overexpression, as they are confined by an outer epithelium called the enveloping layer. Closer examination of the behavior of individual cells in overexpressing embryos may reveal defects that are not apparent at the level of the whole embryo.

In addition, we saw relatively minor effects on the expression of dorsal organizer gene expression in embryos injected globally with *eomes-eng*, in contrast to the near complete repression of *gsc* and *flh* expression that we described in our previous overexpression studies (Bruce et al., 2003). These differences are most easily explained by differences in how the

experiments were performed. Here, *eomes-eng* was injected into the yolk; thus, the RNA was distributed more widely throughout the embryo than in our previous experiments when *eomes-eng* was injected into a single cell at the eight-cell stage. The lower concentration of *eomes-eng* in the injected embryos seems a likely explanation for the observed differences in dorsal marker gene expression.

eomes and *mtx2* Affect Epiboly in Different Ways

T-box genes have been shown to regulate the expression of Mix/Bix class homeobox genes (Ryan et al., 1996; Lemaire et al., 1998; Tada et al., 1998; Casey et al., 1999). We have shown that in zebrafish overexpression of *eomes* induces ectopic expression of the Mix/Bix gene *mtx2*, and, conversely, injection of *eomes-eng* leads to a reduction in *mtx2* expression. Furthermore, injection of *mtx2* antisense morpholino oligonucleotides resulted in defects in epiboly, raising the possibility that the regulation of epiboly by *eomes* occurs in part through *mtx2*.

We have also shown that *eomes* can induce *mtx2* expression cell-autonomously. This finding may seem surprising given that *mtx2* is expressed in only a portion of the early *eomes* expression domain. However, this result is consistent with other work on T-box gene regulation in zebrafish. For example, Goering et al. showed that the T-box gene *ntl* regulates expression of different genes in different portions of its expression domain (Goering et al., 2003). Furthermore, they demonstrated that this differential regulation is accomplished by means of interactions between different T-box genes with overlapping expression domains (Goering et al., 2003). It remains to be seen whether *eomes* interacts with other T-box genes or with other region specific factors to carry out its functions and, further, to be determined is whether the induction of *mtx2* is direct or indirect.

The epiboly defects observed in *eomes-eng* and *mtx2* morpholino-injected embryos were not identical. One difference was that defects were apparent earlier in *eomes-eng*-injected embryos. In *eomes-eng*–injected embryos, there were regions where the yolk cell did not dome and the blastoderm remained thick, suggesting a failure to initiate radial intercalation. The defects in *mtx2* morpholino-injected embryos were first observed around the shield stage, when the blastoderm margin failed to progress vegetally and the blastoderm did not continue to thin. The earlier onset of defects in *eomes-eng*–injected embryos compared with *mtx2* morpholino-injected ones suggests that *eomes* regulates additional downstream targets, which are responsible for the initiation of radial intercalation.

We also showed that Mtx2-MO1 had the same effect when injected into the YSL as it did when injected into the blastoderm. This finding suggests that *mtx2* is required in the YSL for normal epiboly. However, it may not be sufficient for normal epiboly, as injection of *mtx2* RNA into the YSL cannot rescue the epiboly defects in *eomes-eng*–injected embryos. We hypothesize that *mtx2* is required both in the blastoderm and in the YSL for normal epiboly. As *eomes* is not expressed in the YSL, the regulation of *mtx2* in the YSL may be different from that in the blastoderm. Eomes may regulate *mtx2* expression in the YSL non– cell-autonomously, consistent with our previous work demonstrating that Eomes could induce *gsc* expression by a non– cell-autonomous mechanism (Bruce et al., 2003). Another alternative is that Eomes might only regulate *mtx2* expression in the blastoderm, which could indirectly affect the accumulation of *mtx2* in the YSL when it forms by means of the fusion of marginal blastoderm cells with the yolk.

Another difference was the mid-epiboly death, which was a consequence of the yolk cell bursting. This finding was observed in *mtx2* morpholino-injected embryos but rarely in *eomes-eng*–injected embryos. The effect of *mtx2*-morpholinos may have been more severe due to a more even distribution of morpholinos throughout the embryo than the injected *eomes-eng*

RNA (Nasevicius and Ekker, 2000). In addition, there may be multiple upstream regulators of *mtx2* expression, which act in parallel pathways. This scenario is supported by the phenotype of the recently isolated maternal effect mutant *betty boop*, which is nearly identical to the epiboly defects observed in *mtx2* morpholino-injected embryos (Wagner et al., 2004). Thus, there may be lower levels of Mtx2 protein in morpholino-injected embryos than in *eomes-eng*–injected embryos, leading to a more severe *mtx2* reduction-of-function phenotype.

Mechanism of Epiboly

Little is known about the mechanisms that initiate radial intercalation, although the assumption is that doming of the yolk cell provides the motive force (for review, see Kane and Adams, 2002). This explanation has led to the idea that the cells of the embryo are pushed outward when the yolk cell domes rather than actively initiating movement. More is known about the forces involved in the progression of epiboly, which occurs from the dome stage through the end of gastrulation. Here again, evidence points to the importance of the yolk cell. In a related fish species, *Fundulus*, removal of the blastoderm from the yolk does not prevent epiboly of the YSL, suggesting its independence from the cells of the blastoderm (Trinkaus, 1951; Betchaku and Trinkaus, 1978). However, there is some evidence that the YSL is not the sole force behind epiboly. This evidence comes from analysis of the epiboly mutant *half-baked*, which has defects in the movements of the deep cells but not of the YSL (Kane and Adams, 2002). This finding indicates that epibolic movements of blastoderm cells are genetically separable from the YSL and that blastoderm cells may not be passive participants in epiboly.

Eomes appears to act directly upon the cells of the blastoderm, which would be consistent with the apparent cell autonomy of the defects in *eomes-eng*-injected embryos, and if confirmed would represent the first experimental evidence that cells of the embryo actively participate in epiboly. Although *eomes* can induce *mtx2* expression cell autonomously in the blastoderm it might also be involved in regulating *mtx2* expression indirectly in the YSL. Perhaps the most likely scenario is that Eomes functions by a combination of both cell-autonomous and non–cell-autonomous mechanisms, not only during organizer formation, as we have previously described (Bruce et al., 2003), but also during the earlier process of epiboly.

Conserved Role for Eomesodermin in Morphogenesis

A conserved evolutionary role for *Eomes* in orchestrating gastrulation movements is suggested from work in *Xenopus* and mouse (reviewed in Graham, 2000). Injection of a putative dominant-negative *Eomes* construct into *Xenopus* embryos led to the formation of exogastrulae and gastrulation arrest, indicative of abnormal cell movements (Ryan et al., 1996). Aberrant cell movements were also implicated in the gastrulation failure observed in mice lacking *Eomes*, in which cells fail to migrate into the primitive streak (Russ et al., 2000).

A likely downstream target of murine *Eomes* is the Mix/Bix gene *Mixl1* (Russ et al., 2000). Analysis of the *Mixl1* promoter revealed the presence of putative T-box binding sites (Sahr et al., 2002). In addition, analysis of mice homozygous for a null mutation in the *Mixl1* gene revealed defects in mesoderm and endoderm morphogenesis during gastrulation and the presence of bifurcated axes (Hart et al., 2002), which are also observed in zebrafish embryos injected with *eomes-eng*. In both zebrafish and mouse, this function appears to be mediated, at least in part, by downstream target genes that belong to the Mix/Bix family. Together these results point to a conserved role for *Eomes* in the control of cell movements, although our work is the first to implicate Eomes in epiboly. In mouse and frog, *Eomes* is not expressed maternally; thus, it will be interesting to determine whether the two described functions of Eomes correlate with either the maternal or zygotic expression of the gene.

EXPERIMENTAL PROCEDURES

Zebrafish

Zebrafish embryos were obtained from natural matings and staged as described (Kimmel et al., 1995). Wild-type strains used included a local pet store strain, *AB and TLF. MZ*oep* mutants were produced from crosses of rescued oep^{m134}/oep^{m134}, which were gifts from M. Halpern and R. Warga.

Whole-Mount In Situ Hybridization

In situ hybridizations were performed as previously described (Bruce et al., 2003). Antisense riboprobes to *goosecoid* (Stachel et al., 1993), *bonnie and clyde* (Alexander and Stainier, 1999), *evel* (Joly et al., 1993), *mtx1* and *mtx2* (Hirata et al., 2000), *floating head* (Talbot et al., 1995), *sox17* (Alexander and Stainier, 1999), *no tail* (Schulte-Merker et al., 1994), and *keratin4* (Thisse et al., 2001) were synthesized as described.

Expression Constructs

eomes and *ntl* constructs have been described previously (Bruce et al., 2003).

Antisense Morpholino Oligonucleotides

Two different antisense morpholino oligonucleotides targeted against *mtx2* (accession no. AB034246) were obtained from Gene Tools LLC (Philomath, OR), Mtx2-MO1: 5′-CATTGAGTATTTTGCAGCTCTCTTG-3′, and Mtx2-MO2: 5′-TTGCAGAAAATAAGTAAGTCAAGC-3′. To test the specificity of the morpholino, the coding sequence plus a portion of the 5′ untranslated region (including the morpholino target sequence) was cloned in frame into a plasmid containing *GFP*, to generate *mtx2-GFP* (Oates and Ho, 2002). Injection of *mtx2-GFP* RNA resulted in nuclear-localized GFP expression (20 of 20 embryos), whereas coinjection of *mtx2-GFP* and Mtx2-MO1 led to reduced or undetectable GFP fluorescence in 100% of injected embryos (36 of 36 embryos). Coinjection of Mtx2-MO1 and *GFP* RNA, which did not contain the morpholino target sequence, had no effect on GFP fluorescence (16 of 16 embryos). The results for Mtx2-MO2 were similar. Embryos injected with *mtx2-GFP* RNA alone resulted in nuclear GFP expression (8 of 8 embryos), whereas coinjection of *mtx2-GFP* RNA and Mtx2-MO2 led to reduced or absent GFP expression (22 of 22 embryos). In contrast, coinjection of an unrelated morpholino at the same concentration and *mtx2-GFP* RNA had no effect on GFP fluorescence (21 of 21 embryos). As similar defects were observed with both morpholinos, we conducted most of our studies with MO1.

Microinjections

Microinjections were performed as described previously either through the chorion at the one- to four-cell stage or into dechorionated embryos (Bruce et al., 2003). Morpholino antisense oligonucleotides were injected into the yolk or into a single cell of one- to four-cell stage embryos at a concentration of 2.5 ng/nl. *mtx2-GFP* RNA was injected into the yolk of one- to four-cell stage embryos at a concentration of 100 ng/μl. Morpholino concentrations were determined by testing a range of concentrations and selecting the one that did not cause nonspecific defects. Injections of *eomes-eng*, *eng*, and *eomes-VP*, and *ntl*-VP were performed as described (Bruce et al., 2003).

Antibody Staining and Imaging

Anti-Eomes and anti-myc antibody staining and imaging were performed as described (Bruce et al., 2003). Anti-tubulin antibody staining was performed as described (Topczewski and Solnica-Krezel, 1999).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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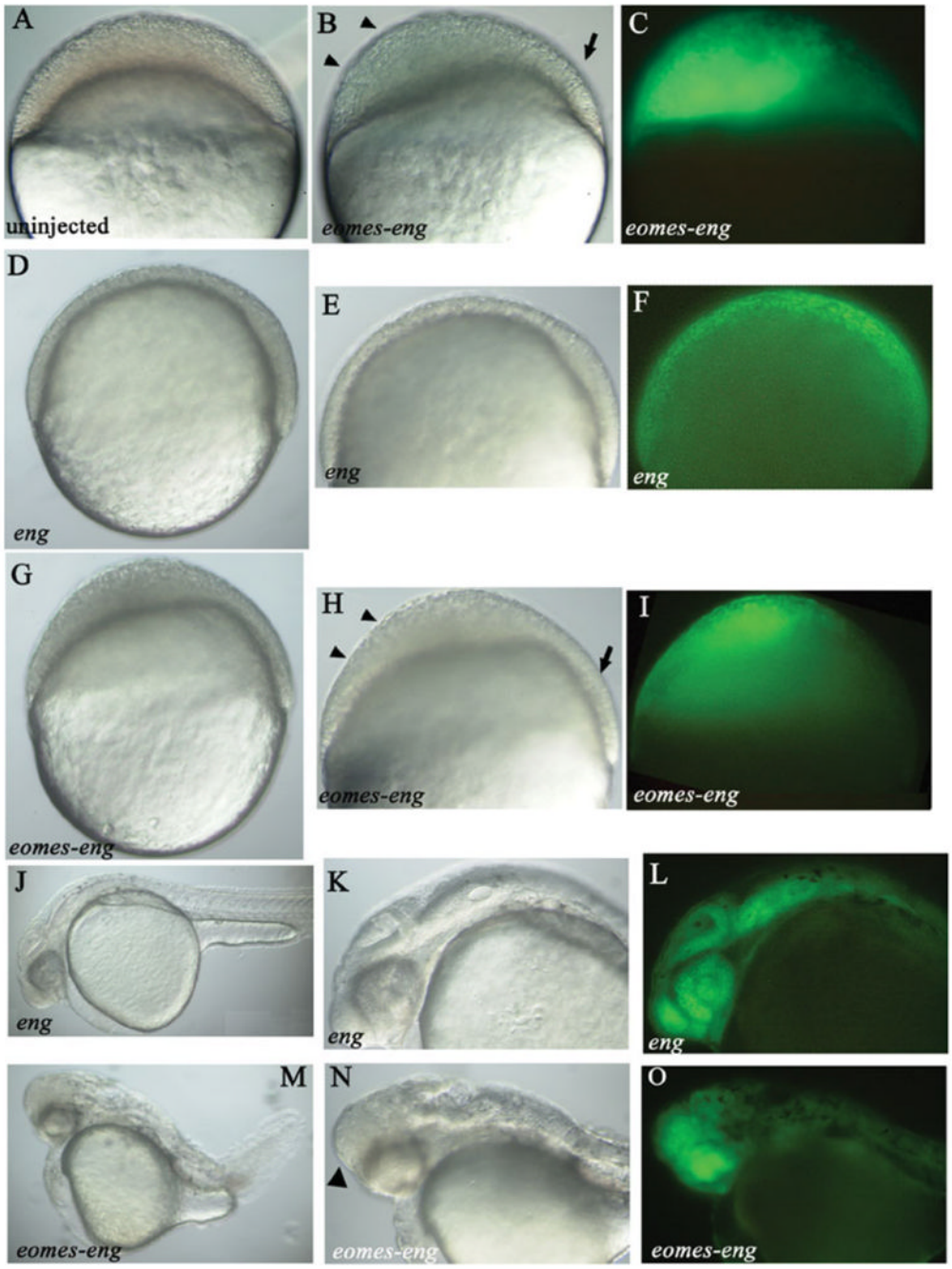

Fig. 1.

eomes-eng inhibits epiboly. A–O: All views lateral. J–O: anterior toward the left. Construct injected, if any, is indicated in the bottom left corner. A–C: Embryos at 30% epiboly (4.7 hours postfertilization [hpf]), (D–I) embryos at 60% epiboly (6.5 hpf). **A:** Uninjected control. **B:** Embryo injected with *gfp* and *eomes-eng* RNA. Arrowheads indicate the region of the blastoderm that has failed to thin, and the arrow indicates the normal region of the blastoderm. **C:** Same embryo as in B showing green fluorescent protein (GFP) fluorescence. The region indicated by the arrowheads in B is where most of the GFP expression is located. **D:** Control embryo injected with *gfp* and *eng* RNA. **E:** Higher power view of embryo in D. **F:** Same embryo as in E, showing that GFP fluorescence is distributed throughout the blastoderm. GFP-positive

cells are intermingled with unlabeled cells. **G:** Embryo injected with *gfp* and *eomes-eng* RNA. **H:** Higher power view of embryo in G. Arrowheads indicate region of the blastoderm that has failed to thin, and the arrow indicates the normal region of the blastoderm. **I:** Same embryo as in H, showing GFP fluorescence. The region indicated by the arrowheads in H is where most of the GFP expression is located. J–O: Embryos at 1 day postfertilization. **J:** Control embryo injected with *gfp* and *eng* RNA. **K:** Higher magnification of J, showing the head region. **L:** Same embryo as in K, showing evenly distributed GFP fluorescence. **M:** Embryo injected with *gfp* and *eomes-eng* RNA. **N:** Higher magnification of M, showing abnormal head region. **O:** Same embryo as in N showing GFP fluorescence concentrated in the anterior portion of the head.

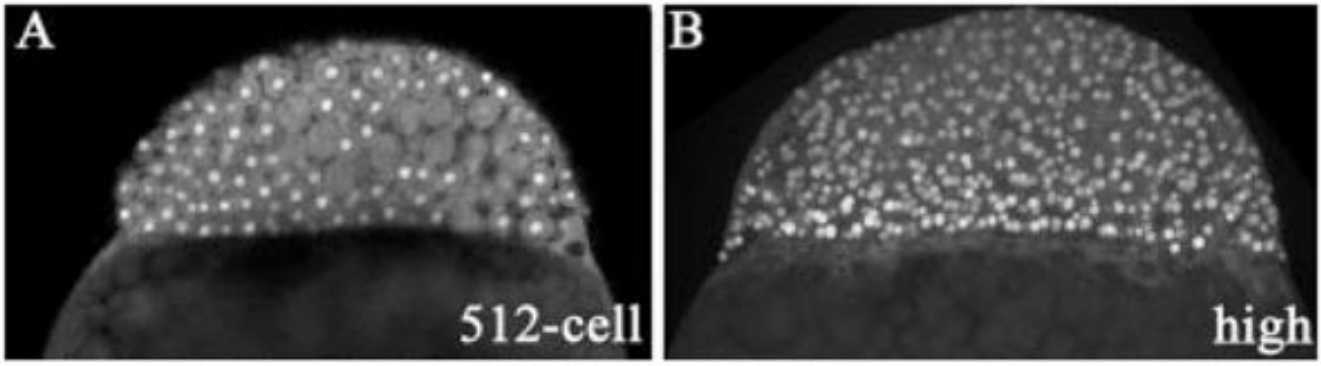

Fig. 2.

Eomes is expressed throughout the blastoderm at early blastula stages. Images are single scans from a confocal z-series of embryos shown in lateral view and stained with the anti-Eomes antibody. **A:** Embryo at 512-cell stage (2.75 hours postfertilization [hpf]). Nuclear staining can be seen throughout the blastoderm, although not all nuclei are in the plane of view. **B:** Embryo at the high stage (3.3 hpf). Protein expression can be seen in nuclei throughout the blastoderm.

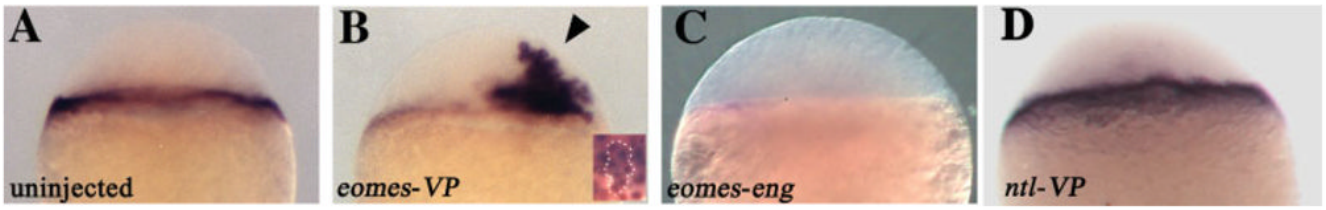

Fig. 3.

eomes regulates *mtx2* expression cell-autonomously. All views are lateral, and all embryos are at sphere stage (4 hours postfertilization [hpf]). Injected construct, if any, is indicated in lower left corner. **A:** In situ hybridization of *mtx2* in an uninjected embryo, showing expression in the marginal cells of the blastoderm and the underlying yolk syncytial layer. **B:** *eomes-VP*–injected embryo with ectopic *mtx2* expression (arrowhead). Inset shows a portion of the blastoderm of a *myc-eomes*–injected embryo with Eomes protein expression in the nucleus in brown and *mtx2* expression in blue. White outline demarcates a group of cells that coexpress Eomes and ectopic *mtx2*, indicating a cell-autonomous induction of *mtx2* by Eomes. **C:** Reduced *mtx2* expression in an embryo injected with *eomes-eng*. **D:** *ntl-VP*–injected embryo with normal *mtx2* expression.

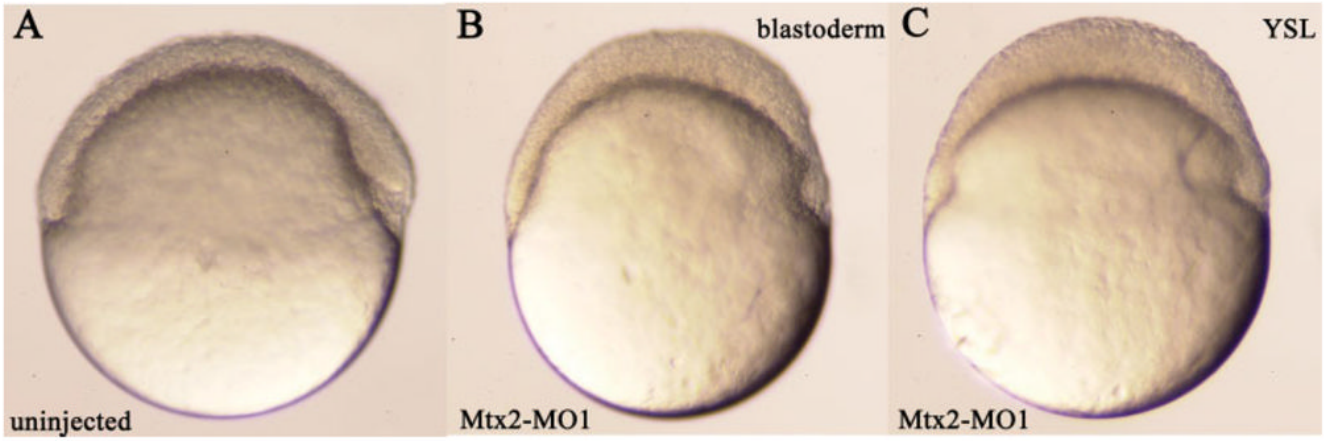

Fig. 4.

mtx2 morpholinos inhibit epiboly. All views are lateral with dorsal to the right; all embryos are at shield stage (6 hours postfertilization). Injected construct, if any, is indicated in lower left corner. **A:** Uninjected embryo. **B:** Embryo injected with Mtx2-MO1 into one cell at the two-cell stage; note that the blastoderm is thickened compared with control. **C:** Embryo injected with Mtx2-MO1 into the yolk syncytial layer (YSL); note that the blastoderm is thickened compared with control.

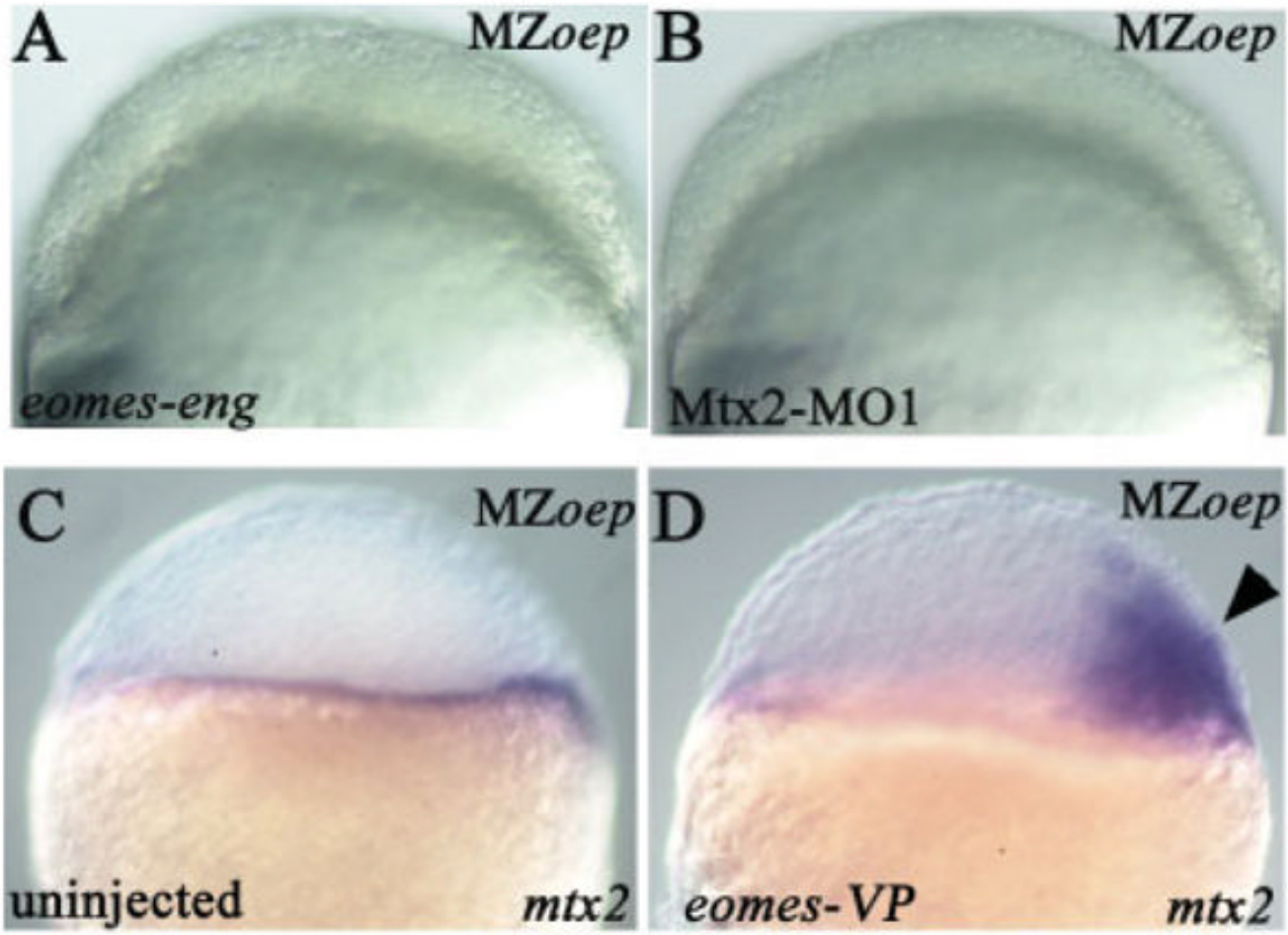

Fig. 5.

Epiboly defect is Nodal-independent. All views are lateral; the mutant phenotype is indicated in upper right corner; the injected construct is indicated in lower left corner. A,B: At 50% epiboly (5.25 hours postfertilization [hpf]). C,D: At sphere stage (4 hpf). **A:** Embryo injected with *eomes-eng*; the blastoderm has failed to thin. **B:** Embryo injected with Mtx2-MO1; the blastoderm has failed to thin. **C:** *mtx2* expression in an uninjected embryo. **D:** Ectopic *mtx2* expression (arrowhead) in an *eomes-VP*–injected (into a single cell at the eight-cell stage) embryo.