Developmental Cell, Vol. 9, 523–533, October, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.08.010

Eomesodermin Is a Localized Maternal Determinant Required for Endoderm Induction in Zebrafish

Christopher R.R. Bjornson,[1,4,5] Kevin J.P. Griffin,[1,4,6] Gist H. Farr III,[1,3] Akira Terashima,[2] Charis Himeda,[1] Yutaka Kikuchi,[2] and David Kimelman[1,3,*]

[1]Department of Biochemistry
Box 357350
University of Washington
Seattle, Washington 98195-7350

[2]Division of Biological Science
Graduate School of Science
Nagoya University
Furo-cho, Chikusa-ku, Nagoya, 464-8602
Japan

Summary

In zebrafish, endoderm induction occurs in marginal blastomeres and requires Casanova (Cas), the first endoderm-specific factor expressed in the embryo. Whereas the transcription factors Gata5 and Bon are necessary and sufficient for *cas* expression in marginal blastomeres, Bon and Gata5 are unable to induce *cas* in animal pole cells, suggesting that *cas* expression requires an additional, unidentified factor(s). Here, we show that *cas* expression depends upon the T box transcription factor Eomesodermin (Eomes), a maternal determinant that is localized to marginal blastomeres. Eomes synergizes potently with Bon and Gata5 to induce *cas*, even in animal pole blastomeres. We show that Eomes is required for endogenous endoderm induction, acting via an essential binding site in the *cas* promoter. Direct physical interactions between Eomes, Bon, and Gata5 suggest that Eomes promotes endoderm induction in marginal blastomeres by facilitating the assembly of a transcriptional activating complex on the *cas* promoter.

Introduction

A major question in developmental biology is how the early blastoderm becomes segregated into three germ layers: ectoderm, mesoderm, and endoderm. In zebrafish, a mixed population of mesodermal and endodermal precursors is found at the margin of the blastoderm within the four cell diameters closest to the yolk cell (Warga and Nusslein-Volhard, 1999). The first morphological differences that distinguish mesoderm from endoderm become apparent at the onset of gastrulation when endodermal cells involute first, become flattened in appearance, and occupy the deepest layer of the newly formed hypoblast. Newly involuted cells activate the expression of *sox17* and *foxa2/axial* and will eventually form the gut epithelium.

Elegant overexpression studies and mutant analysis have led to a detailed understanding of the molecular cascade leading to endoderm formation in zebrafish (Shivdasani, 2002; Stainier, 2002). An unknown signal from the extraembryonic yolk syncytial layer (YSL) initiates the expression of a number of genes required for endoderm formation including two members of the TGF-β family, the Nodal-related molecules *cyclops* (*cyc*) and *squint* (*sqt*) (Rodaway et al., 1999; Chen and Kimelman, 2000). Nodal-related signaling is absolutely required for endoderm: *cyc;sqt* double mutants and embryos that lack maternal and zygotic One-Eyed-Pinhead (Oep), an essential cofactor for Nodal signaling, are deficient in endoderm (Gritsman et al., 1999; Feldman et al., 2000). Conversely, activation of the Nodal pathway is capable of inducing the expression of endodermal genes (Peyrieras et al., 1998).

Nodal signaling triggers a transcriptional cascade including Gata5/Faust, and the homeodomain containing factors Mezzo (Mez) and Bonnie and Clyde (Bon), which are required to maintain expression of the *sox* factor *casanova* (*cas*), the key endodermal determinant in zebrafish (Reiter et al., 1999; Kikuchi et al., 2000, 2001; Aoki et al., 2002). Despite the obvious importance of this Nodal-regulated transcriptional cascade in endoderm induction, the fundamental issue of how blastomeres adopt the endodermal fate remains to be resolved. Whereas activation of the Nodal signaling cascade or overexpression of Mez or Cas induces robust endodermal gene expression throughout the blastoderm (Peyrieras et al., 1998; Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001; Poulain and Lepage, 2002), overexpression of Bon and Gata5 induces endodermal gene expression only in marginal blastomeres (Kikuchi et al., 2001). These data suggest that an additional factor, localized to marginal blastomeres, is required for Bon and Gata5 to activate endodermal gene expression (Kikuchi et al., 2001).

Here, we describe the unexpected but integral role for the T box transcription factor *eomesodermin* (*eomes*) in zebrafish endoderm induction. Zebrafish *eomes* is a maternally deposited transcript that becomes localized after fertilization to marginal blastomeres. Whereas Eomes alone does not affect endoderm induction, Eomes synergizes potently with Bon and Gata5 to induce endoderm. Conversely, knockdown of Eomes function results in an endoderm deficiency. We find that Eomes binds a site in the *cas* promoter that is essential for high-level *cas* expression. Finally, we show that Eomes physically interacts with both Bon and Gata5, suggesting that Eomes assembles a transcriptional complex on the *cas* promoter. Thus, in addition to its role in mesoderm formation and patterning (Ryan et al., 1996; Russ et al., 2000; Bruce et al., 2003), our results reveal a core role for Eomes in the

*Correspondence: kimelman@u.washington.edu
[3]Lab address: http://depts.washington.edu/kimellab/
[4]These authors contributed equally to this work.
[5]Present address: Department of Neurology, Veteran Affairs Palo Alto Health Care System, 3801 Miranda Avenue, Palo Alto, California, 94304.
[6]Present address: Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, California 90095.

transcriptional event that controls commitment to the endodermal lineage.

Results

Eomes Is a Maternally Deposited Transcript Localized to the Margin after Fertilization

A highly conserved feature of vertebrate and invertebrate gastrulation is the zygotic expression of T box transcription factors in the involuting/ingressing cells. In the mouse, *Xenopus*, and zebrafish, *eomes* is one component of these zygotic T box genes, and overexpression and loss-of-function studies have demonstrated that *eomes* is important for mesoderm patterning in all of these species (Ryan et al., 1996; Ciruna and Rossant, 1999; Hancock et al., 1999; Tagawa et al., 2001; Fuchikami et al., 2002; Satoh et al., 2002; Bruce et al., 2003), and for proper epiboly movements in zebrafish (Bruce et al., 2005). As in mice (McConnell et al., 2005), zebrafish *eomes* is maternally expressed (Bruce et al., 2003), with an intriguing spatial localization (Figure 1). In mature oocytes, cytoplasm is initially dispersed throughout the yolk, with a small amount of cytoplasm at the animal pole. Upon oocyte activation, cytoplasm streams first toward the center of the yolk, and then to the animal pole. *eomes* mRNA was initially found in the dispersed cytoplasm of the oocyte and the early zygote (data not shown; see also Bruce et al., 2003). Following cytoplasmic streaming, *eomes* transcripts became highly enriched in a ring in the lower, outer domain of the animal pole blastodisc by the time of the first cleavage (Figures 1A–1F). This vegetal ring of *eomes* message was observed in 64-cell stage embryos (Figures 1B and 1E) and through to 3.5 hr postfertilization (hpf; Figures 1C and 1F); after this time, *eomes* mRNA became undetectable by in situ hybridization (data not shown).

To determine whether Eomes protein was also localized to the margin, we generated an anti-peptide antibody to Eomes. In Western blots of immunoprecipitated embryonic extracts, this antibody recognizes a 75 kDa species, the predicted molecular weight of the Eomes translation product (Figure 1G, lane 1). Embryos overexpressing *eomes* and in vitro synthesized Eomes produced products of a similar size (Figure 1G, lanes 3 and 4). Importantly, antibody binding to the 75 kDa immunoreactive species was successfully competed away using the Eomes-specific immunizing peptide, demonstrating that the 75 kDa band represents the endogenous Eomes protein (Figure 1G, lane 2). Although we were not successful in using this Eomes antibody for immunocytochemical analysis, we used dissected embryo fragments to detect a significant enrichment of Eomes in vegetal blastomeres using immunoprecipitation and Western blot analysis. Animal caps were removed from embryos at 3.5 hpf and the levels of Eomes were measured from pools of 50 animal caps versus the remaining vegetal blastomeres and yolk cell. Using the ubiquitously expressed protein β-catenin to normalize for the amount of protein loaded, we found that the levels of Eomes at the margin were on average five times greater than those found in animal caps (Figure 1G, lanes 5 and 6; n = 3). This is likely to be an underestimate of the Eomes distribution due to the technical difficulty of obtaining animal cap samples free of marginal blastomeres. Thus, Eomes protein and mRNA are significantly enriched in marginal blastomeres.

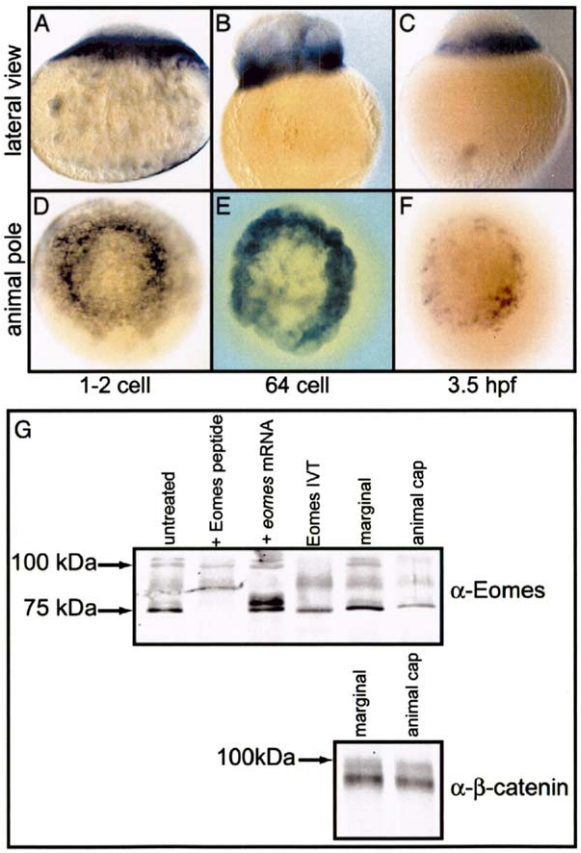

Figure 1. *eomes* Transcripts and Protein Are Localized

eomes mRNA is concentrated to the vegetal margin of the blastodisc in a ring of expression during the first cleavage (A and D), throughout the first cleavages (B and E; shown is a representative embryo 1.5 hpf at the 64-cell stage), and 3.5 hpf (C and F), just after the midblastula transition. Note the exclusion of *eomes* transcripts from the most animal pole region of the blastoderm (lateral view, A–C) and the central domain (animal pole view, D–F).

(G) Western blot analysis of Eomes immunoprecipitated from uninjected embryos (lane 1), uninjected embryos plus the Eomes-specific blocking peptide (lane 2), embryos overexpressing *eomes* (lane 3), and in vitro transcribed/translated Eomes (lane 4). Each lane was loaded with 20 embryo equivalents, except lane 3, which represents a half-reaction. Representative levels of Eomes in marginal (lane 5) and animal cap (lane 6) blastomeres (50 cap equivalents per lane), and β-catenin levels in marginal (lane 7) and animal cap (lane 8) blastomeres (20% of input).

Eomes Synergizes with Gata5 and Bon to Induce Endoderm

The localization of Eomes to marginal blastomeres is highly suggestive of a role in mesoderm and/or endoderm induction because this is the source of mesendodermal lineages. However, overexpression of *eomes* mRNA did not result in widespread induction of mesoderm or endoderm (data not shown), although we did observe ectopic forerunner cells and dorsalization (Fig-

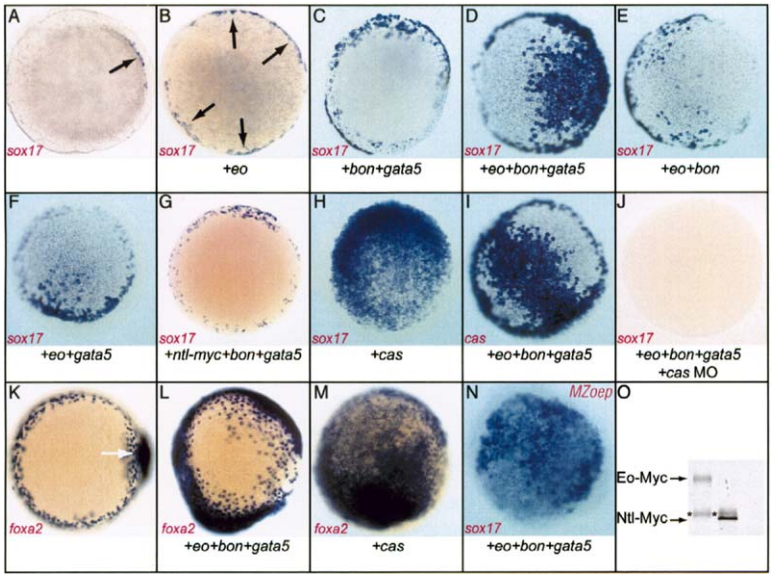

Figure 2. Eomes Acts Synergistically with Bon and Gata5 to Induce Endoderm

Embryos are stained with the following in situ hybridization probes: *sox17*; *cas* (*casanova*); *foxa2/axial*. All embryos are shown from the animal pole. (A)–(J) and (N) are 5 hpf (50% epiboly, late blastula), and (K)–(M) are 7 hpf (60% epiboly, early gastrula).

(A) In uninjected embryos during the late blastula period, *sox17* expression is expressed exclusively in a specialized group of dorsally located cells, the forerunners (arrow).

(B) Overexpression of *eomes* (*eo*) results in the formation of ectopic *sox17*-expressing cells (arrows).

(C) Overexpression of *bon* and *gata5* induces widespread expression of *sox17* in marginal blastomeres, but not in nonmarginal cells.

(D) Overexpression of *bon*, *gata5*, and *eomes* strongly induces expression of *sox17* throughout the embryo, including cells at the animal pole.

(E and F) Overexpression of *eomes* with either *bon* (E) or *gata5* (F) induces *sox17* expression in only relatively small numbers of nonmarginal cells.

(G) Overexpression of *ntl-myc* with *bon* and *gata5* does not induce *sox17* expression at the animal pole.

(H) Overexpression of *cas* causes a more uniform induction of *sox17* compared to the pepper-and-salt induction of *sox17* by *bon*, *gata5*, and *eomes* (compare to [D]).

(I) Overexpression of *bon*, *gata5*, and *eomes* induces expression of the endodermal determinant *cas* in marginal and nonmarginal blastomeres.

(J) *Sox17* expression is completely absent in embryos injected with *bon*, *gata5*, and *eomes* mRNAs and a morpholino to inhibit *cas* mRNA translation.

(K–M) Expression of *foxa2* at 7 hpf (60% epiboly).

(K) In uninjected embryos, *foxa2* is expressed in scattered, involuted endodermal cells around the circumference of the embryo, in addition to notochordal progenitors in the dorsal midline (arrow).

(L) Overexpression of *bon*, *gata5*, and *eomes* greatly increases the number of *foxa2*-expressing cells at the margin, but not in nonmarginal regions.

(M) Overexpression of *cas* induces *foxa2* expression throughout the embryo.

(N) Overexpression of *eomes*, *bon*, and *gata5* induces *sox17* in the absence of Nodal signaling.

(O) Immunoprecipitation and Western analysis was used to confirm that both Myc-Eomes and Myc-Ntl are overexpressed in embryos coinjected with *bon* and *gata5*, though endoderm is induced only when *eomes* was overexpressed. A nonspecific band (asterisk) was detected in all injected samples (20 embryos per lane, n = 3 independent experiments). All embryos shown are wild-type, except (N), which is an *MZoep* mutant.

ures 2A, 2B, and S1; Bruce et al., 2003). This indicated that Eomes does not play an analogous role to *VegT*, a maternally deposited T box transcription factor in *Xenopus* that is a potent inducer of endoderm and mesoderm (Zhang et al., 1998; see also Bruce et al., 2003). We therefore considered alternate possibilities for the function of maternal Eomes in marginal blastomeres.

In zebrafish, endoderm formation is regulated by transcription factors induced by Nodal signaling. Previous studies have indicated that the Nodal effectors Bon and Gata5 may require an unknown cofactor to induce the downstream target *cas*. Whereas activation of Nodal signaling and overexpression of *cas* leads to the expression of the endodermal marker *sox17* throughout the blastoderm, overexpression of *bon* and *gata5* induces *sox17* expression only in marginal blastomeres (Figure 2C; Kikuchi et al., 2001). This suggests that the marginal blastomeres may contain a factor that acts with Bon and Gata5 to induce endoderm. Therefore, we explored the possibility that Eomes might cooperate with Bon and Gata5 in endoderm induction. Coexpression of *eomes* with *bon* and *gata5* had a dramatic and synergistic effect on the expression of the endodermal differentiation marker *sox17*, which was strongly induced in marginal as well as nonmarginal blastomeres (Figure 2D; 77%, n = 75), while coexpression of *eomes* with either *bon* or *gata5* was much less effective at inducing *sox17* expression in nonmarginal blastomeres (Figures 2E and 2F), demonstrating that all three factors are required in combination for the maximal effect. Endoderm induction by *eomes*, *bon*, and *gata5* was significantly mosaic in distribution, in contrast to the relatively uniform expression of *sox17* expression induced by overexpression of *cas* mRNA (Figure 2H; Kikuchi et al., 2001). Furthermore, endoderm induction was specific to *eomes* as another T box factor, *no tail*, did not enhance endoderm induction by Bon and Gata5 (Figures 2G and 2O).

Genetic analysis has shown that Bon and Gata5 act upstream of *cas*, and that Cas function is essential for endoderm formation (Alexander et al., 1999; Alexander and Stainier, 1999; Kikuchi et al., 2001; Stainier, 2002). We therefore analyzed the relationship between Cas and endoderm induction by *bon*, *gata5*, and *eomes*. Similar to *sox17*, we found that *cas* expression was also

strongly induced in nonmarginal blastomeres by *bon*, *gata5*, and *eomes* (Figure 2I; 76%, n = 45). Moreover, inhibition of *cas* mRNA translation by a *cas*-specific morpholino oligonucleotide (MO) completely prevented induction of *sox17* expression by *bon*, *gata5*, and *eomes* (Figure 2J; 100%, n = 36). These data indicate that Eomes acts with Bon and Gata5 early in endoderm induction, upstream of the endodermal determinant *cas*.

In contrast, *bon*, *gata5*, and *eomes* did not induce significant levels of expression of the endodermal marker *foxa2/axial* in nonmarginal blastomeres, although *foxa2* expression was strongly increased at the margin (Figures 2K and 2L). This was intriguing because *bon*, *gata5*, and *eomes* were very effective at inducing *cas* expression in nonmarginal blastomeres (Figure 2I), and overexpression of *cas* mRNA is sufficient to induce *foxa2* (Figure 2M; Aoki et al., 2002). Our results indicate that at endogenous levels, Cas may require an additional factor to activate *foxa2* expression, whereas very high levels of *cas* can override this requirement. Thus, while Cas is essential for endoderm formation, additional inputs are required downstream of Cas for activation of the full complement of endodermal genes.

Regulation of Endoderm Formation by Eomes Does Not Involve Nodal Signaling or Mez

Prior to this study, only Nodal signaling and the Nodal-regulated *mix*-related factor Mez had been shown to induce *cas* expression or endoderm formation in nonmarginal cells (Kikuchi et al., 2001; Poulain and Lepage, 2002). We therefore examined whether the combination of Eomes, Bon, and Gata5 induced endoderm via activation of Nodal signaling or the Nodal effector Mez. Overexpression of *eomes*, *bon*, and *gata5* did not induce ectopic expression of *mez* or the Nodal ligand *cyc*, and only a small change was observed in expression of *sqt* (Figure S2). More importantly, overexpression of *eomes*, *bon*, and *gata5* induced the expression of the endodermal marker *sox17* in *MZoep* embryos, which cannot respond to Nodal ligands (Figure 2N; 70%, n = 64). Together, these data indicate that endoderm induction by *eomes*, *bon*, and *gata5* does not involve the Nodal effector Mez, or activation of Nodal signaling. However, induction of *sox17* expression by Eomes, Bon, and Gata5 in *MZoep* mutant embryos (Figure 2N) was consistently weaker than in wild-type embryos (Figure 2D), suggesting that active Nodal signaling strengthens endoderm induction by these factors.

Eomes Is Required for Endoderm Formation

We next addressed whether *eomes* was required for endoderm formation. First, for the purpose of constructing a dominant-negative Eomes protein, we analyzed the transcriptional activity of Eomes using a synthetic promoter with high basal activity that contains T domain binding sites. Whereas full-length Eomes or a chimeric protein consisting of the Eomes DNA binding domain fused to the Gal4 activation domain (EoG4A) increased promoter activity, the Eomes DNA binding domain fused to the repressor domain of *Drosophila* Engrailed (EoEnR) repressed the promoter (Figure 3A). These results demonstrated that zebrafish Eomes is a transcriptional activator, like its *Xenopus* homolog (Ryan et al., 1996; Conlon et al., 2001), and predicts that EoEnR should act as a dominant negative when expressed in the embryo. Consistent with these data, coexpression of *eoG4A* with *bon* and *gata5* induced *sox17* expression in nonmarginal cells, although less effectively and in fewer embryos than combinations including full-length *eomes* (Figure 3B; compare with Figure 2D; 26%, n = 46). More importantly, coexpression of *eoEnR* with *bon* and *gata5* prevented induction of *sox17* in marginal blastomeres in the majority of injected embryos (Figure 3C; 66%, n = 33). Significantly, expression of *eoEnR* alone prevented the formation of *cas*-expressing endodermal progenitors (Figures 3E and 3F; 83%, n = 42), and this effect was overcome by coinjection of full-length *eomes* mRNA with *eoEnR* (Figure 3F). Consistent with the observation that *cas* expression in the YSL is regulated differently than *cas* expression in blastomeres (Kikuchi et al., 2001), expression of *eoEnR* did not prevent expression of *cas* in the YSL (Figure 3E). These data strongly suggest that Eomes plays a role in endoderm formation in zebrafish.

To more specifically test the involvement of Eomes in endoderm induction, we attempted to inhibit translation of the *eomes* mRNA using antisense morpholino oligonucleotides. The *eomes* mRNA 5′ UTR consists of an approximately 850 nucleotide noncoding exon spliced to exon 2 at the –12 position relative to the putative translation start site (Figure 4A). Sequence analysis indicated, however, that two distinct splice donor sites were used, approximately 150 nucleotides apart, leading to heterogeneity in the proximal 5′ UTR (Figure 4A). We therefore used multiple *eomes* MOs, one targeted to each of the two variants of the 5′ UTR, and an additional MO targeted to the translation start site. However, we found that the *eomes* MOs did not eliminate Eomes protein from the embryo (Figure S3), and did not decrease the numbers of *cas*-expressing progenitors in injected embryos (Figures 4B and 4D).

Because we found that the *eomes* MOs could block the translation of *eomes* mRNA in vitro (data not shown), we reasoned that the partial depletion of Eomes due to the *eomes* MOs might cause a hypomorphic effect that would sensitize these embryos to partial and simultaneous depletion of one of the factors that cooperates with Eomes in endoderm induction. Others have shown (Kikuchi et al., 2000; Reiter et al., 2001) that loss of Bon function has a greater effect on endoderm formation than loss of Gata5, so we therefore combined the *eomes* MOs with an MO to deplete Bon. We used a dose of the *bon* MO that did not completely eliminate Bon function (Figure 4; compare Figures 4E and 4C; see also Kikuchi et al., 2000), to better observe any potential synergy. Whereas the *bon* MO alone decreased the number of *cas*-expressing progenitors to approximately 50% of the wild-type number (Figures 4B and 4E), embryos injected with the *bon* and *eomes* MOs were substantially lacking in *cas*-expressing endodermal progenitors, with large areas of these embryos completely devoid of endoderm (Figures 4B and 4F). This effect was rescued by coinjecting the *bon* and *eomes* MOs with either an *eomes* mRNA lacking

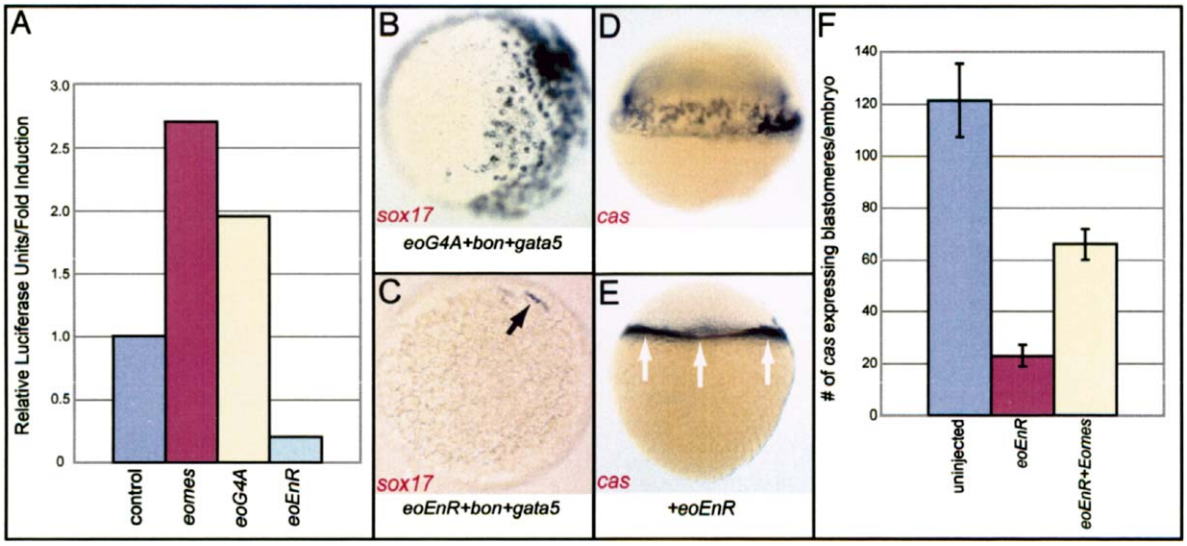

Figure 3. Eomes Is a Transcriptional Activator Involved in Endoderm Induction

(A) Luciferase reporter gene assay to detect transcription from a synthetic promoter containing T domain binding sites (scale: arbitrary units relative to control). EoG4a, Eomes DNA binding domain fused to the activation domain of Gal4; EoEnR, Eomes DNA binding domain fused to the repressor domain of *Drosophila* Engrailed. Eomes and EoG4a both activated the promoter, whereas EoEnR repressed the high basal activity.

(B) At 5 hpf, *Sox17*-expressing nonmarginal cells are observed after expressing *eoG4A* with *bon* and *gata5*.

(C) Embryo injected with *bon* and *gata5* in combination with *eoEnR*; *sox17* expression resembles wild-type (see Figure 2A) with expression just in the forerunners (arrow).

(D) Lateral view of *cas* expression in uninjected embryo at the onset of gastrulation (5.5 hpf). *cas* is expressed in endodermal progenitors scattered throughout the marginal domain, as well as throughout the underlying YSL.

(E) *cas* expression is absent from blastomeres following expression of *eoEnR*, although *cas* expression in the YSL is unperturbed (arrows).

(F) Average numbers of *cas*-expressing blastomeres present in uninjected embryos compared to embryos injected with mRNAs, as indicated (minimum of 10 embryos per condition). Overexpression of full-length *eomes* mRNA significantly reverses the depletion of *cas*-expressing cells due to *eoEnR*.

the MO binding sites or a similarly mutated *bon* mRNA (Figures 4B and 4G; compare to embryos injected with the *bon* MO alone; Figures 4B and 4E). It was not possible to rescue Bon and Eomes function simultaneously because this resulted in ectopic endoderm formation. These data demonstrate that Eomes and Bon act cooperatively in vivo, and establish an in vivo role for endogenously expressed Eomes in the regulation of *cas* expression and endoderm formation.

Eomes Directly Regulates *cas* Transcription

Our findings that Eomes functions in combination with Bon and Gata5 to activate endodermal gene expression, together with the observation that Eomes function is epistatic to Cas (Figure 2J), led us to investigate whether Eomes regulated *cas* transcription directly. We cloned a fragment of the *cas* gene containing the predicted translational start site and approximately 1500 bp of upstream sequence into a luciferase reporter plasmid to examine the effect of Eomes on *cas* transcription. Injection of *p(1476)cas-luc* alone into zebrafish embryos produced a baseline level of luciferase activity due to activation of the *cas* promoter by endogenous factors (Figure 5A). Whereas coinjection of the *p(1476)cas-luc* reporter with *bon* and *gata5* resulted in a 3-fold increase in luciferase activity, coinjection of the reporter with *eomes* resulted in a 16-fold increase (Figure 5A). Because *eomes* alone does not significantly increase endogenous *cas* expression, this result suggests that the *cas* promoter fragment may lack a repressor element. The greatest increase in luciferase activity was seen when *p(1476)cas-luc* was coinjected with *eomes*, *bon*, and *gata5* RNAs (Figure 5A). These data show that the 1500 bp region upstream of the *cas* translational start site is responsive to Eomes, Bon, and Gata5, and that the combination of all three factors produces the most robust effect.

We next wanted to determine whether Eomes activated *cas* transcription directly. Sequencing of *p(1476)cas-luc* identified a consensus T site 90 bp upstream of the predicted start of translation that is predicted to bind Eomes (Conlon et al., 2001), and Eomes specifically bound this site in our electrophoretic mobility shift assay (Figure 5B). To determine the role of this binding site in *cas* regulation, we introduced point mutations that eliminated Eomes binding (*p(1476mut)cas-luc*; Figure 5B). These mutations led to a 14-fold reduction in promoter activation by endogenous factors, and a 100-fold reduction in activation by exogenous Eomes, Bon, and Gata5 (Figure 5C). Importantly, *bon* and *gata5* were unable to stimulate *cas* transcription when the T site was mutant (Figure 5C). Similar results were obtained using a different combination of point mutations (data not shown), indicating that we had not inadvertently created a novel binding site for a transcriptional repres-

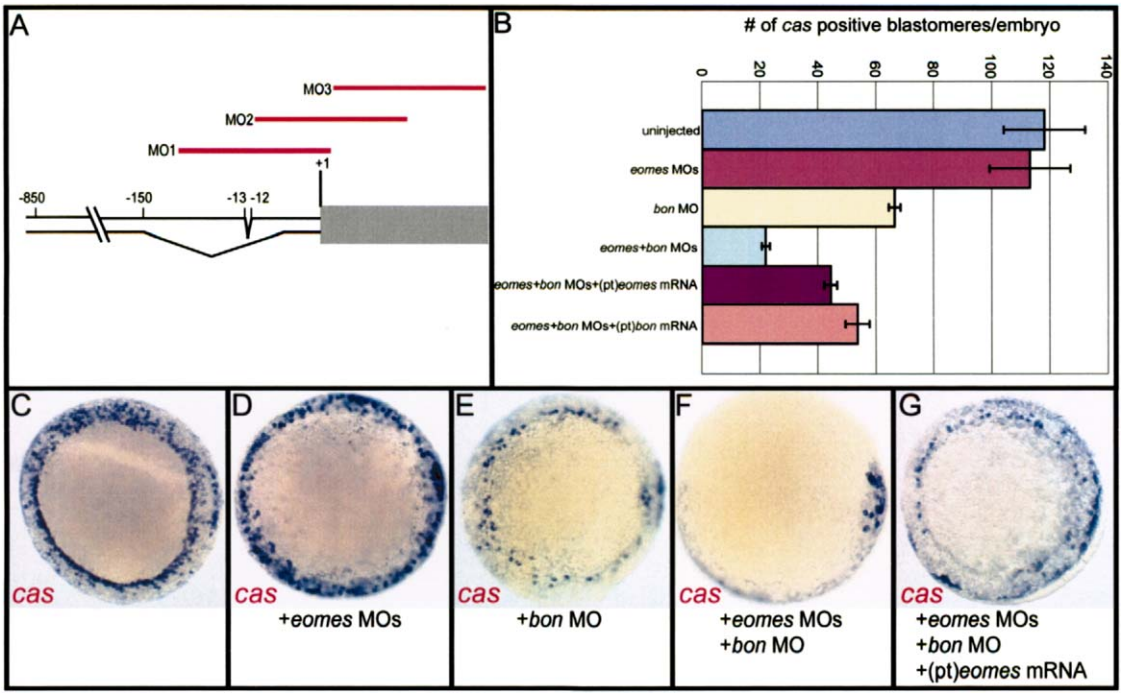

Figure 4. Antisense Morpholino Oligonucleotide Knockdown of Eomes

(A) Cartoon representation of the *eomes* 5′ UTR (not to scale). The *eomes* message contains an 850 bp 5′ UTR, mostly derived from a single upstream exon that is variably spliced to exon 2, containing the putative translation start site. Morpholinos were targeted to +3/–22, +14/–11, and +26/+2 and relative MO binding sites are represented by red bars.

(B) Average numbers of *cas*-positive blastomeres after injection of the MO/mRNA combinations indicated (minimum of 11 embryos per condition).

(C–G) Representative embryos at 5 hpf (late blastula stage) from experiments in (B), animal pole views; *cas* expression. An MO to *mesogenin* (*msg*) was used as a control MO to balance the total dose of MO per embryo. The *msg* MO alone had no effect on endoderm formation (not shown).

(D) Embryo injected with the *eomes* MOs alone had similar numbers of *cas*-positive blastomeres as the uninjected embryo (C).

(E) Embryo injected with the *bon* MO had a significant reduction in the number of *cas*-expressing blastomeres.

(F) Embryo injected with *eomes* and *bon* MOs had a greater overall reduction in *cas*-expressing blastomeres than with the *bon* MO alone (E), and large areas of the embryo are completely devoid of *cas* expression.

(G) Embryo coinjected with the *eomes* and *bon* MOs and a mutant *eomes* mRNA lacking the MO binding sites; *cas*-expressing blastomeres are present around the margin.

sor. These data show that Eomes regulates *cas* transcription by directly binding to the promoter, and demonstrate that Bon and Gata5 are unable to activate this promoter unless Eomes is also bound.

Eomes Binds to Gata5 and Bon

Important physical interactions have been reported between various T box factors and different Gata proteins (Stennard et al., 2003; Garg et al., 2003). We therefore asked whether Eomes can directly interact with Gata5 and Bon. To test this, we fused the T box domain of Eomes to glutathione-S-transferase (pGST-Eomes) for use in GST-pull-down assays. The Eomes T domain bound specifically to in vitro synthesized [^{35}S]Bon as well as [^{35}S]Gata5 (Figure 6A). This interaction was not a general property of T box factors because the T domain from No Tail did not bind either Gata5 or Bon (Figure 6A). Because a recent study demonstrated that a region N-terminal of the Xbra T domain is necessary for certain protein-protein interactions (Messenger et al., 2005), we tested whether sequences N-terminal to the Eomes T domain would enhance binding. However, we found that the interaction of Eomes with Bon and Gata5 was not enhanced when these additional residues were added (Figure 6B), demonstrating that the minimal Eomes T domain is sufficient to bind Bon and Gata5. Finally, we examined the ability of Bon and Gata5 to pull down Eomes, and found that, as expected, the reciprocal interaction occurs (Figure 6C). Intriguingly, we also found that Bon and Gata5 could bind each other (Figure 6C), suggesting that these three proteins may form a trimeric complex. These data demonstrate that Eomes plays an integral role in the regulation of *cas* transcription, possibly by facilitating the assembly of a transcriptional complex on the *cas* promoter.

Discussion

The molecular events underlying endoderm induction in zebrafish have been subject to intense scrutiny over recent years (Stainier, 2002), and because *cas* is the first endoderm-specific gene expressed, a fuller under-

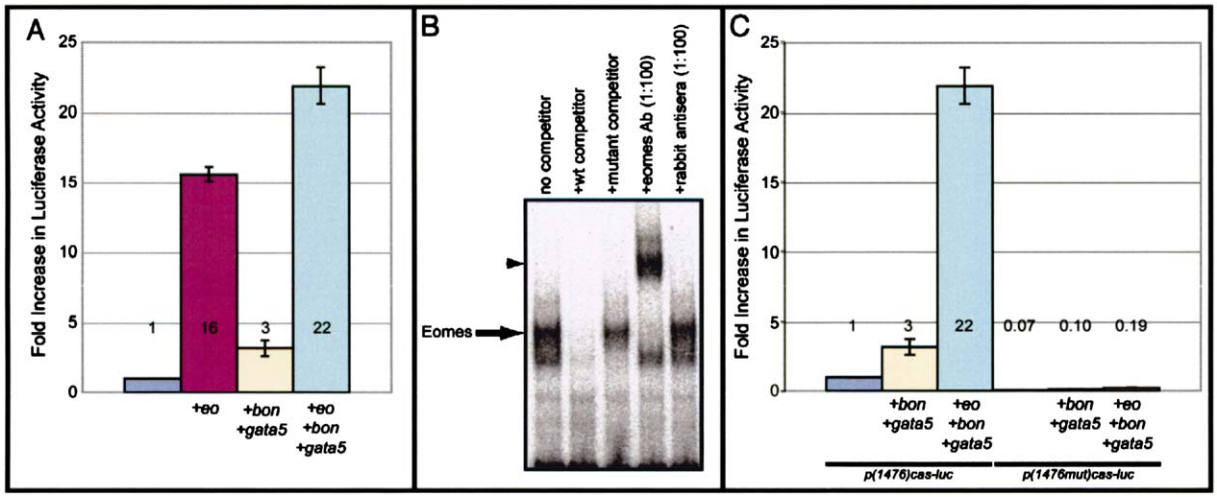

Figure 5. Eomes Binds to the T Site in the *cas* Promoter to Regulate *cas* Transcription

(A) Luciferase activity was measured in embryos injected with *p(1476)cas-luc*, and mRNAs as indicated. Fold increase in luciferase activity was measured compared to the control condition (n = 5–6 in three independent injection experiments), and reported with the standard error. Actual values are indicated.

(B) A radiolabeled probe containing the *cas* T site was incubated with in vitro synthesized Eomes protein for use in electromobility shift analysis (lanes 1–5). Binding of Eomes (arrow) to the probe was eliminated using wild-type competitor (lane 2), whereas a competitor oligonucleotide harboring a mutation in the T site did not inhibit Eomes binding to the probe (lane 3). Preincubation with antiserum to Eomes supershifted the upper of the two shifted bands (arrowhead, lane 4), whereas nonimmune serum did not (lane 5), identifying the upper band as Eomes.

(C) Luciferase activity in embryos injected with either *p(1476)cas-luc* or *p(1476mut)cas-luc*, which contains a mutated T site, and mRNAs as indicated. Fold increase in luciferase activity was measured compared to the control condition (n = 5 over three independent injection experiments), and reported with the standard error. Actual values are indicated.

standing of *cas* regulation is essential to gain insight into endoderm induction in zebrafish. We have identified the T box factor *eomes* as an integral component of the *cas* regulatory machinery. Using a combination of overexpression and loss-of-function studies, we have shown that endoderm induction in zebrafish depends upon the maternally inherited stores of the T box transcription factor Eomes. We demonstrate that Eomes is localized to marginal blastomeres and binds to two other components of endoderm induction, Gata5 and Bon, and show that the transcription of *cas*, the specific indicator of endoderm induction in zebrafish, is directly regulated by Eomes. Thus, we have identified Eomes as a major and previously unrecognized maternal determinant active in zebrafish endoderm induction. We propose that the enrichment of Eomes in marginal blastomeres enables these cells to respond productively to the Nodal-regulated transcription factors, Bon and Gata5, leading to robust *cas* expression.

Eomes Directly Regulates *cas* Expression

cas is essential for endoderm formation and it is the earliest gene expressed in marginal blastomeres that adopt the endodermal fate (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). We show that Eomes plays a direct role in endoderm induction by acting on the transcription of *cas* through the binding of a T site located close to the start of transcription. Mutation of this Eomes binding site in a *cas* reporter construct reduces the level of *cas* promoter activation by endogenous factors in the embryo 14-fold, and renders the *cas* promoter unresponsive to the overexpression of *bon*, *gata5*, and *eomes*. Therefore, our results show that Eomes is a necessary component of endoderm induction, and that Eomes directly regulates the expression of the endodermal determinant *cas*.

Our observation that Eomes binds both Bon and Gata5, in conjunction with the fact that Eomes alone cannot activate endogenous *cas* expression, strongly suggests that an important aspect of Eomes function is assembling a transcriptional complex on the *cas* promoter. Consistent with this, mutations that disrupt the Eomes binding site in the *cas* promoter also prevent Bon and Gata5 from activating *cas* transcription. Although we have not determined whether Bon and Gata5 are able to bind the *cas* promoter independently of Eomes binding, it is clear that Bon and Gata5 cannot activate the *cas* promoter when the Eomes binding site is disrupted. Furthermore, although Eomes, Bon, and Gata5 were capable of inducing *cas* independently of active Nodal signaling, this activity was considerably less robust than in wild-type embryos. Therefore, it is likely that in the endogenous situation, these factors act in parallel with the Nodal signal, most likely through binding of phosphorylated Smad to Bon and Gata5 (Germain et al., 2000; Blokzijl et al., 2002; Randall et al., 2002). Consistent with this, we have observed multiple candidate Smad binding sites in the *cas* regulatory region. In summary, our results suggest that full *cas* activation at the marginal zone requires a combination of many factors including Bon (and/or Mez), Gata5, Nodal signaling, and Eomes, with Eomes acting both to as-

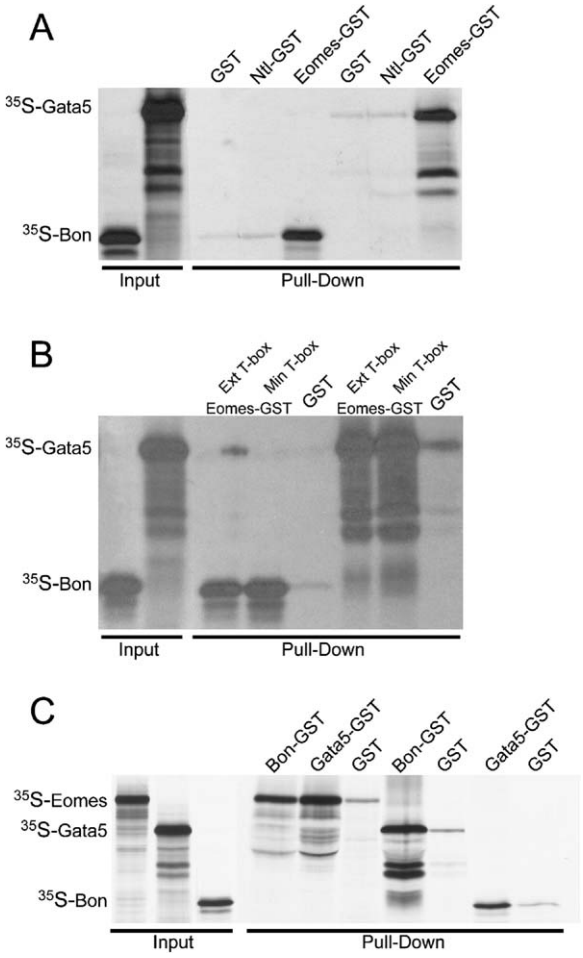

Figure 6. Eomes Interacts with Bon and Gata5

GST-pull-down analysis using bacterially expressed GST-tagged proteins and ^{35}S-labeled in vitro translated Eomes, Bon, and Gata5. (A) The minimal Eomes T domain fused to GST interacts with ^{35}S-labeled Bon and Gata5 (lanes 5 and 8), but GST (lanes 3 and 6) and the T domain from No Tail (lanes 4 and 7) do not bind Bon or Gata5. (B) The minimal Eomes T domain (Min T box; lanes 4 and 7) fused to GST binds to ^{35}S-labeled Bon and Gata5 as effectively as the extended Eomes T domain (Ext T box; lanes 3 and 6), which contains 50 additional residues upstream of the T box.

(C) GST-tagged Bon and Gata5 each bind ^{35}S-labeled full-length Eomes (lanes 4 and 5). Additionally, GST-Bon binds ^{35}S-labeled Gata5 (lane 7) and GST-Gata5 binds ^{35}S-labeled Bon (lane 9).

semble a transcriptional complex and providing an additional transcriptional activating domain.

A Model for Endoderm Induction

Combining previous studies (Poulain and Lepage, 2002; Stainier, 2002) and our data, we provide an expanded model for endoderm induction (Figure 7). Nodal signaling, activated initially by a signal from the YSL, activates the zygotic expression of *bon*, *gata5*, and *mez*. Bon and Gata5 bind to Eomes and form a complex that interacts with the *cas* promoter. Together with Nodal-activated Smads, which bind Bon and Gata5, *cas* expression is initiated at a high level within the marginal blastomeres during the late blastula stage. At the onset

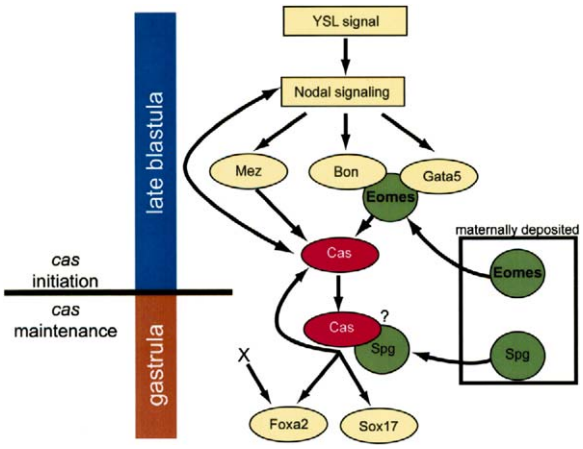

Figure 7. Model of the Endoderm Specification Pathway in Zebrafish

Eomes physically interacts with Bon and Gata5 to initiate *cas* transcription at the late blastula stage. Nodal signaling not only activates the expression of *bon*, *gata5*, and *mez*, but it enhances the activity of these Smad binding factors. At the onset of gastrulation, *cas* transcription is maintained through an autoregulatory loop that is dependent on the activity of Spg (also called Oct4/Pou5f1; a potential physical interaction between Cas and Spg is indicated with a question mark). The combination of Cas and Spg activates the transcription of *sox17* and *foxa2*/*axial*. Our results indicate that an unidentified factor (X) is also required for the activation of *foxa2*/*axial*. Zygotically expressed proteins are indicated in yellow, maternally deposited transcripts are shown in green, and Cas is shown in red.

of gastrulation, *cas* expression is maintained in marginal blastomeres through an autoregulatory loop that also includes the maternally deposited factor Spg/Oct4/Pou5f1 (Lunde et al., 2004; Reim et al., 2004). Cas and Spg/Oct4/Pou5f1 are necessary for the activation of two additional components of endoderm formation, *sox17* and *foxa2*. Interestingly, we have found evidence for an additional factor (Factor X in Figure 7) required for *foxa2* expression because the combination of Bon, Gata5, and Eomes activates *cas* expression but did not activate expression of *foxa2*, whereas overexpression of *cas* mRNA does induce *foxa2* (Aoki et al., 2002). Thus, the fact that Eomes, Bon, and Gata5 can induce *cas* expression but not *foxa2* expression indicates that at endogenous levels of Cas, an additional factor (X) is essential for the proper expression of *foxa2*.

The Localization of Eomes Demarcates the Future Mesendodermal Domain

We have shown that maternally deposited *eomes* transcripts are uniquely localized soon after fertilization to blastomeres that fate mapping studies have shown are likely to remain at or near the margin (Helde et al., 1994), and which will adopt mesendodermal fates. Similarly, we have shown that the Eomes protein is also preferentially localized to the margin. In contrast, Bruce et al. (2003) reported that Eomes protein is ubiquitously distributed. However, the major protein detected by Bruce et al. (2003) is an abundant protein of 94 kDa, which is 20 kDa larger than the predicted size of

Eomes, whereas the endogenous protein we detect migrates at the predicted size, and this protein can be entirely competed away from the antibody using the Eomes peptide (Figure 1G). In contrast, they and we observe that in vitro translated Eomes, as well as Eomes from embryos injected with *eomes* mRNA, migrates at the predicted size, demonstrating that Eomes does not migrate anomalously on gels. The likely explanation for the differences in our results is that the antibody used by Bruce et al. (2003) reacts with an abundant ubiquitous 94 kDa protein, which obscures the localized Eomes staining at the margin.

An Endodermal Role for Eomes in Other Species?

It will be interesting to determine whether *eomes* plays a role in endoderm formation in other vertebrate species as well. While the investigations of Eomes function in zebrafish, *Xenopus*, and mice have established a role for Eomes in mesoderm induction and/or patterning (Ryan et al., 1996; Russ et al., 2000; Bruce et al., 2003), these studies did not examine the possible role of Eomes in endoderm formation in *Xenopus* and mice. However, a homolog of *cas* has not been reported yet in any other organism, and thus the role of Eomes as a necessary component of endoderm formation could potentially be unique to zebrafish, or at least to teleosts. Mesendoderm induction in teleosts has many unique features (Kimelman and Bjornson, 2004), and the adaptation of maternal Eomes in regulating endoderm formation may have developed as part of this mechanism. It will be interesting to now study Eomes function in other teleost fish.

Experimental Procedures

Fish Stocks and Maintenance

Zebrafish stocks were maintained as described (Westerfield, 1995), and *MZoep*^{tz57} fish (Gritsman et al., 1999) were kindly provided by Alex Schier.

In Situ Hybridization and Embryo Injections

The *eomes* probe and in situ hybridizations were as previously described (Mione et al., 2001), with the following changes. Embryos were fixed at 28°C, to improve the morphology of the early stage embryos; *eomes* probe was used at very low concentrations, and embryos were hybridized for 48 hr; the alkaline phosphatase color reaction was performed at 14°C for up to several days. All other probes are previously described. The *eomes* coding region was amplified by PCR to add specific restriction sites and inserted into the CS2+-myc vector to generate pCS-Myc-Eomes. A plasmid for expressing Myc-tagged Ntl (Myc-ntl) was a kind gift of David Grunwald. mRNAs were prepared using mMessage machine (Ambion, Austin, TX) and were injected at 100 pg per embryo, except *gata5*, which was injected at 60 pg per embryo. MOs were injected in the following amounts per embryo: *bon* MO, 4–8 ng (gift of Didier Stainier); *mezzo* MO, 10 ng (gift of Thierry Lepage); *eomes* MO1, 2.5 ng (from Bruce et al., 2003; gift of Robert Ho); *eomes* MO2, 1.5 ng (5′-CTTTCTAACTGCATTCTTCACTGTG-3′); *eomes* MO3, 2.5 ng (5′-CCAGGGAGGATGCTTTCTAACTGCA-3′). *Mesogenin* MO was used in conjunction with other MOs to maintain a constant total dose of MOs.

Eomes Protein Analysis

Affinity-purified Eomes antibody was obtained from rabbits immunized with the Eomes carboxy-terminal peptide DASPTIKCEDL SSEEYN (Quality Controlled Biochemicals, Camarillo, CA). Both control and treated embryos were solubilized in a Triton X-100 lysis buffer and incubated with α-Eomes antibody (1:100; 24 μg/ml) ± Eomes immunization peptide (100 μg/ml) overnight at 4°C and immunoprecipitated the following day using protein-G Sepharose beads (Amersham, Piscataway, NJ). Proteins were resolved using an 8.5% acrylamide gel and transferred to a nitrocellulose membrane for Western analysis. Blots were probed with α-Eomes antibody (1:1000) followed by IRDye800-conjugated goat α-rabbit secondary antibody (Rockland Immunochemicals, Gilbertsville, PA; 1:5000) and visualized using an Odyssey Imager (Li-Cor, Lincoln, NE) as per the manufacturer's specifications.

EMSA Analysis

Gel shift analysis was carried out as previously described (Himeda et al., 2004).

The primer sequences used are: wild-type site 5′-TGAAACTTCT CACGCTTCACACCTTTAATCCACTCCTCCG-3′ (T site underlined), and mutant site 5′-TGAAACTTCTCACGCTgCACgCtTTTAATCCAC TCCTCCG-3′ (mutated bases in lower case).

Luciferase Assays

The 1476 bp of sequence upstream of the *cas* coding region was subcloned into the pGL3-enhancer vector (Clontech, Palo Alto, CA) to generate *p(1476)cas-luc*. One-cell embryos were injected with 40 pg of *p(1476)cas-luc* and 5 pg of pCMV *Renilla*-luciferase, which acted as an injection control. A subset of embryos was coinjected with combinations of mRNA encoding Eomes, Bon, and Gata5 at a concentration of 50, 50, and 30 pg, respectively. At 6 hpf (shield stage), 30 embryos were homogenized in 600 μl passive lysis buffer (Promega, Madison, WI). Approximately five to six samples were collected per condition and quantified twice using the Dual Luciferase Assay kit (Promega) and an E&G Berthold luminometer (Bad Wildbad, Germany) as per the manufacturer's specifications. All data are reported as the fold change in luciferase activity compared with the condition where no mRNA was coinjected (control), and reported with the standard error.

GST-Pull-Down Assay

The minimal Eomes T box domain (residues 199–406), the extended Eomes T box (residues 149–406), the No Tail T box (residues 1–219), and the complete coding sequences of Bon and Gata5 were subcloned into the pGEX-2T vector (Amersham). Rosetta (DE3) pLysS bacteria (Novagen, San Diego, CA) overexpressing the GST-fusion proteins were lysed in binding buffer (Stennard et al., 2003) and the lysates were incubated with glutathione 4B beads (Amersham). ^{35}S-labeled Eomes, Bon, and Gata5 were synthesized in vitro (SP6 Quick Coupled TnT kit; Promega) and incubated with beads that had been preloaded with the GST-fusion proteins. Beads were washed and then stripped using SDS-sample buffer. The products were resolved using SDS-PAGE on a 12% gel.

Supplemental Data

Figures S1–S3 are available online at http://www.developmentalcell.com/cgi/content/full/9/4/523/DC1/.

Acknowledgments

We wish to thank Le Trinh and Didier Stainier, Robert Ho, Thierry Lepage, Alex Schier, and David Grunwald for generously supplying reagents, Wilson Clements for experimental help, David Grunwald for experimental advice, and Eddy de Robertis for comments on an earlier version of the manuscript. This work was supported by NSF grant IBN-0315123 to D.K. and by grants from Grant-in-Aid for Scientific Research from MEXT (KAKENHI 16027223) and JSPS (KAKENHI 15370094) to Y.K.

Received: October 25, 2004
Revised: August 22, 2005
Accepted: August 24, 2005
Published: October 3, 2005

References

Alexander, J., and Stainier, D.Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. Curr. Biol. *9*, 1147–1157.

Alexander, J., Rothenberg, M., Henry, G.L., and Stainier, D.Y. (1999). casanova plays an early and essential role in endoderm formation in zebrafish. Dev. Biol. *215*, 343–357.

Aoki, T.O., David, N.B., Minchiotti, G., Saint-Etienne, L., Dickmeis, T., Persico, G.M., Strahle, U., Mourrain, P., and Rosa, F.M. (2002). Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. Development *129*, 275–286.

Blokzijl, A., ten Dijke, P., and Ibanez, C.F. (2002). Physical and functional interaction between GATA-3 and Smad3 allows TGF-β regulation of GATA target genes. Curr. Biol. *12*, 35–45.

Bruce, A.E., Howley, C., Zhou, Y., Vickers, S.L., Silver, L.M., King, M.L., and Ho, R.K. (2003). The maternally expressed zebrafish T-box gene eomesodermin regulates organizer formation. Development *130*, 5503–5517.

Bruce, A.E., Howley, C., Dixon Fox, M., and Ho, R.K. (2005). T-box gene eomesodermin and the homeobox-containing Mix/Bix gene mtx2 regulate epiboly movements in the zebrafish. Dev. Dyn. *233*, 105–114.

Chen, S., and Kimelman, D. (2000). The role of the yolk syncytial layer in germ layer patterning in zebrafish. Development *127*, 4681–4689.

Ciruna, B.G., and Rossant, J. (1999). Expression of the T-box gene Eomesodermin during early mouse development. Mech. Dev. *81*, 199–203.

Conlon, F.L., Fairclough, L., Price, B.M., Casey, E.S., and Smith, J.C. (2001). Determinants of T box protein specificity. Development *128*, 3749–3758.

Dickmeis, T., Mourrain, P., Saint-Etienne, L., Fischer, N., Aanstad, P., Clark, M., Strahle, U., and Rosa, F. (2001). A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. Genes Dev. *15*, 1487–1492.

Feldman, B., Dougan, S.T., Schier, A.F., and Talbot, W.S. (2000). Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. Curr. Biol. *10*, 531–534.

Fuchikami, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Hosomi, T., Watanabe, T., Kurokawa, D., Kataoka, M., Harada, Y., Satoh, N., Kusunoki, S., et al. (2002). T-brain homologue (HpTb) is involved in the archenteron induction signals of micromere descendant cells in the sea urchin embryo. Development *129*, 5205–5216.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., et al. (2003). GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature *424*, 443–447.

Germain, S., Howell, M., Esslemont, G.M., and Hill, C.S. (2000). Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. Genes Dev. *14*, 435–451.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., and Schier, A.F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell *97*, 121–132.

Hancock, S.N., Agulnik, S.I., Silver, L.M., and Papaioannou, V.E. (1999). Mapping and expression analysis of the mouse ortholog of *Xenopus* Eomesodermin. Mech. Dev. *81*, 205–208.

Helde, K.A., Wilson, E.T., Cretekos, C.J., and Grunwald, D.J. (1994). Contribution of early cells to the fate map of the zebrafish gastrula. Science *265*, 517–520.

Himeda, C.L., Ranish, J.A., Angello, J.C., Maire, P., Aebersold, R., and Hauschka, S.D. (2004). Quantitative proteomic identification of six4 as the trex-binding factor in the muscle creatine kinase enhancer. Mol. Cell. Biol. *24*, 2132–2143.

Kikuchi, Y., Trinh, L.A., Reiter, J.F., Alexander, J., Yelon, D., and Stainier, D.Y. (2000). The zebrafish *bonnie and clyde* gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors. Genes Dev. *14*, 1279–1289.

Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B., and Stainier, D.Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev. *15*, 1493–1505.

Kimelman, D., and Bjornson, C. (2004). Vertebrate mesoderm induction: From frogs to mice. In Gastrulation: From Cells to Embryo, C.D. Stern, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 363–372.

Lunde, K., Belting, H.G., and Driever, W. (2004). Zebrafish pou5f1/pou2, homolog of mammalian Oct4, functions in the endoderm specification cascade. Curr. Biol. *14*, 48–55.

McConnell, J., Petrie, L., Stennard, F., Ryan, K., and Nichols, J. (2005). Eomesodermin is expressed in mouse oocytes and preimplantation embryos. Mol. Reprod. Dev. *71*, 399–404.

Messenger, N.J., Kabitschke, C., Andrews, R., Grimmer, D., Nunez Miguel, R., Blundell, T.L., Smith, J.C., and Wardle, F.C. (2005). Functional specificity of the *Xenopus* T-domain protein Brachyury is conferred by its ability to interact with Smad1. Dev. Cell *8*, 599–610.

Mione, M., Shanmugalingam, S., Kimelman, D., and Griffin, K. (2001). Overlapping expression of zebrafish T-brain-1 and eomesodermin during forebrain development. Mech. Dev. *100*, 93–97.

Peyrieras, N., Strahle, U., and Rosa, F. (1998). Conversion of zebrafish blastomeres to an endodermal fate by TGF-β-related signaling. Curr. Biol. *8*, 783–786.

Poulain, M., and Lepage, T. (2002). Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. Development *129*, 4901–4914.

Randall, R.A., Germain, S., Inman, G.J., Bates, P.A., and Hill, C.S. (2002). Different Smad2 partners bind a common hydrophobic pocket in Smad2 via a defined proline-rich motif. EMBO J. *21*, 145–156.

Reim, G., Mizoguchi, T., Stainier, D.Y., Kikuchi, Y., and Brand, M. (2004). The POU domain protein spg (pou2/Oct4) is essential for endoderm formation in cooperation with the HMG domain protein casanova. Dev. Cell *6*, 91–101.

Reiter, J.F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N., and Stainier, D.Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. Genes Dev. *13*, 2983–2995.

Reiter, J.F., Kikuchi, Y., and Stainier, D.Y. (2001). Multiple roles for Gata5 in zebrafish endoderm formation. Development *128*, 125–135.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J.C., Patient, R., and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-β family signals and discrimination of mesoderm and endoderm by FGF. Development *126*, 3067–3078.

Russ, A.P., Wattler, S., Colledge, W.H., Aparicio, S.A., Carlton, M.B., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehls, M.C., et al. (2000). Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature *404*, 95–99.

Ryan, K., Garrett, N., Mitchell, A., and Gurdon, J.B. (1996). Eomesodermin, a key early gene in *Xenopus* mesoderm differentiation. Cell *87*, 989–1000.

Sakaguchi, T., Kuroiwa, A., and Takeda, H. (2001). A novel sox gene, 226D7, acts downstream of Nodal signaling to specify endoderm precursors in zebrafish. Mech. Dev. *107*, 25–38.

Satoh, G., Takeuchi, J.K., Yasui, K., Tagawa, K., Saiga, H., Zhang, P., and Satoh, N. (2002). Amphi-Eomes/Tbr1: an amphioxus cognate of vertebrate Eomesodermin and T-Brain1 genes whose expression reveals evolutionarily distinct domain in amphioxus development. J. Exp. Zool. *294*, 136–145.

Shivdasani, R.A. (2002). Molecular regulation of vertebrate early endoderm development. Dev. Biol. *249*, 191–203.

Stainier, D.Y. (2002). A glimpse into the molecular entrails of endoderm formation. Genes Dev. *16*, 893–907.

Stennard, F.A., Costa, M.W., Elliott, D.A., Rankin, S., Haast, S.J., Lai, D., McDonald, L.P., Niederreither, K., Dolle, P., Bruneau, B.G., et al. (2003). Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. Dev. Biol. *262*, 206–224.

Tagawa, K., Satoh, N., and Humphreys, T. (2001). Molecular studies

of hemichordate development: a key to understanding the evolution of bilateral animals and chordates. Evol. Dev. *3*, 443–454.

Warga, R.M., and Nusslein-Volhard, C. (1999). Origin and development of the zebrafish endoderm. Development *126*, 827–838.

Westerfield, M. (1995). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), Third Edition (Eugene: University of Oregon Press).

Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. Cell *94*, 515–524.