Maternal Eomesodermin regulates zygotic nodal gene expression for mesendoderm induction in zebrafish embryos

Pengfei Xu†, Gaoyang Zhu†, Yixia Wang, Jiawei Sun, Xingfeng Liu, Ye-Guang Chen, and Anming Meng*

State Key Laboratory of Biomembrane and Membrane Engineering, Tsinghua – Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China
† These authors contributed equally to this work.
* Correspondence to: Anming Meng, E-mail: mengam@mail.tsinghua.edu.cn

Development of animal embryos before zygotic genome activation at the midblastula transition (MBT) is essentially supported by egg-derived maternal products. Nodal proteins are crucial signals for mesoderm and endoderm induction after the MBT. It remains unclear which maternal factors activate zygotic expression of nodal genes in the ventrolateral blastodermal margin of the zebrafish blastulas. In this study, we show that loss of maternal Eomesodermin a (Eomesa), a T-box transcription factor, impairs zygotic expression of the nodal genes ndr1 and ndr2 as well as mesodermal and endodermal markers, indicating an involvement in mesendoderm induction. Maternal Eomesa is also required for timely zygotic expression of the transcription factor gene mxtx2, a regulator of nodal gene expression. Eomesa directly binds to the Eomes-binding sites in the promoter or enhancer of ndr1, ndr2, and mxtx2 to activate their transcription. Furthermore, human and mouse Nodal genes are also regulated by Eomes. Transfection of zebrafish eomesa into murine embryonic stem cells promotes mesendoderm differentiation with constant higher levels of endogenous Nodal expression, suggesting a conserved function of Eomes. Taken together, our findings reveal a conserved role of maternal T-box transcription factors in regulating nodal gene expression and mesendoderm induction in vertebrate embryos.

Keywords: Eomesodermin, Nodal, transcription, mesoderm, endoderm, embryo, zebrafish

Introduction

Large amounts of maternal products, including RNAs and proteins, are stored in animal eggs and support early embryonic development upon fertilization before the zygotic genome starts to transcribe at the midblastula transition (MBT) (Tadros and Lipshitz, 2009). The germ layers are formed during gastrulation stages, but their progenitors are specified at and after MBT by coordinated action of multiple signals. It has been found that Nodal proteins, TGF-β superfamily members, play an essential role in mesoderm and endoderm induction in vertebrate embryos (Tian and Meng, 2006). For example, Nodal-deficient mouse embryos fail to form the primitive streak and mesoderm and endoderm precursors (Conlon et al., 1991, 1994; Zhou et al., 1993); in zebrafish embryos, double mutants with simultaneous loss-of-function of two zygotic nodal genes ndr1/squint (sqt) and ndr2/cycllops (cyc) lack endodermal tissues and most of mesodermal tissues (Feldman et al., 1998), and maternal ndr1 transcripts may also be required for dorsal mesodermal specification independent of Ndr1 protein (Gore et al., 2005; Hong et al., 2011; Lim et al., 2012; Kumari et al., 2013); during Xenopus embryogenesis, Nodal signals are also essential for mesoderm induction and patterning (Jones et al., 1995; Osada and Wright, 1999; Agius et al., 2000). An interesting question is how maternal factors contribute to zygotic transcription of nodal genes during developmental course.

In Xenopus and zebrafish embryos, nodal gene expression in the dorsal organizer is induced partially by maternally supplied β-catenin (Kelly et al., 2000; Xanthos et al., 2002; Bellipanni et al., 2006). Studies in Xenopus indicate that zygotic expression of nodal genes is initiated in the entire vegetal endoderm, which also requires other maternal factors such as the vegetally localized T-box transcriptional factor VegT (Agius et al., 2000; Xanthos et al., 2002). However, fish or mammalian orthologs of VegT remain to be verified or identified. In zebrafish embryos, ndr1 and ndr2 are expressed during mid-to-late blastulation period in the yolk syncytial layer (YSL), an extraembryonic syncytial layer of cytoplasm and nuclei formed by breakdown of
blastomeres lying against the yolk cell at the midblastula transition, as well as in overlying blastodermal marginal cells that contain mesoderm and endoderm precursors reside (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998; Fan et al., 2007). The expression of ndr1 and ndr2 in the ventrolateral blastodermal margin requires YSL-derived signals (Chen and Kimelman, 2000) and their own transcripts that are present in the YSL (Fan et al., 2007). Knockdown of YSL-specific expression of mxtx2, which encodes a member of the Mix/Bix transcription factor family and is zygotically expressed (Hirot a et al., 2000), inhibits ndr1 and ndr2 expression in the ventrolateral margin of blastulas (Hong et al., 2011), indicating that Mxtx2 is an important regulator of nodal gene expression. It is unknown whether in the zebrafish embryo there exist maternal transcription factors that, like VegT in the Xenopus embryo, play a role in mesendoderm induction by promoting ndr1 and ndr2 expression.

Eomesodermin (Eomes) is a T-box transcription factor. Several pieces of evidence suggest a role of Eomes in mesendodermal development: mouse embryos deficient for zygotic Eomes show abnormal mesoderm and endoderm tissue formation (Russ et al., 2000; Arnold et al., 2008); zebrafish embryos overexpressing eomesa increase the expression of some endodermal and dorsal mesodermal markers (Bruce et al., 2003; Bjornson et al., 2005); and interfering with Eomes in MZmid embryos carrying a foxh1 mutation causes a loss of endodermal and nonaxial mesodermal tissues in addition to axial mesodermal defects (Slagle et al., 2011). It is believed that Eomes may act downstream and mediate the expression of Nodal signaling in mesendoderm and endoderm cell lineage development (Ryan et al., 1996; Brennan et al., 2001; Bjornson et al., 2005; Slagle et al., 2011). However, mouse Eomes and zebrafish eomesodermin (eomesa) gene are maternally expressed (Bruce et al., 2003; Bjornson et al., 2005; Mcconnell et al., 2005), implying that maternally provided Eomes may act upstream of Nodal signaling. Recently, the eomesaC(C) mutant line has been generated in zebrafish, which carries a C to A mutation at position 300 to create a premature stop codon (Du et al., 2012). MZeomesa mutant embryos, which lack both maternal and zygotic Eomesa, display delayed epiboly initiation with defects in endodermal gene expression, indicating a role in epibolic process and endoderm specification (Du et al., 2012). On the other hand, it remains inconclusive whether eomesa plays a role in regulating nodal gene expression and mesoderm specification.

In this study, we further demonstrate that maternal product of eomesa is implicated in promoting ndr1 and ndr2 expression in the ventrolateral margin during mesendoderm specification in the zebrafish embryo. Mechanistically, maternal Eomesa binds to the promoter or enhancer and activates zygotic expression of ndr1 and ndr2 in cooperation with Mxtx2 that is also activated by maternal Eomesa. Furthermore, we show that ectopic expression of zebrafish eomesa in mouse embryonic stem cells (mESCs) can enhance the expression of endogenous Nodal gene and promote their differentiation into mesendoderm precursors. Therefore, Eomesa may act on the top of the regulatory hierarchy of mesendoderm induction during embryogenesis.

Results
Loss-of-function of maternal Eomesa impairs mesendoderm specification in zebrafish embryos

Before the eomesa mutant line became available, we developed a strategy to knock down maternal eomesa by injecting eomesa-MO into immature oocytes followed by in vitro oocyte maturation and fertilization (oKD) (Supplementary Figure S1A), which was modified from previously reported protocols (Gore et al., 2005; Bontems et al., 2009; Nair et al., 2013). We showed that oKD was able to effectively inhibit function of maternal oep gene product (Supplementary Figure S1B) and to block the translation of the reporter eomesa-gfp mRNA during oocyte maturation (Supplementary Figure S1C). As revealed by in situ hybridization, knockdown of eomesa by oKD led to missing of the pan-mesodermal marker ntla and the endodermal marker sox32 in some ventrolateral blastodermal marginal areas but retaining of their expression in the dorsal margin at shield and 30% epiboly stages, whereas knockdown of eomesa in embryos after fertilization (zKD) did not cause obvious changes of these markers (Figure 1A). A marked reduction for the dorsal mesodermal marker gsc during midgastrulation and the ventral mesodermal marker eve1 at the shield stage was also observed in oKD eomesa morphants (Figure 1B). Real-time RT–PCR analyses confirmed the reduction of these marker expression levels (Supplementary Figure S2A). These results suggest that maternal Eomesa is implicated in mesodermal and endodermal fate specification.

A recent study in MZeomesa mutants has demonstrated important functions of maternal Eomesa in epiboly initiation and endoderm specification, but failed to draw a reliable conclusion about its role in mesoderm specification (Du et al., 2012). We set to re-examine the expression patterns of endodermal and mesodermal markers in MZeomesa mutants in detail. We found, by in situ hybridization, that the expression of sox32 was missing in the ventrolateral domains of the blastodermal margin in MZeomesa mutants at the 30% epiboly stage and was still undetectable in some ventrolateral domains at the shield stage in mutants while its expression occurred in the whole margin in wild-type (WT) control embryos at these two stages (Figure 1C), which were consistent with the previous finding that sox32 expression was absent in the ventrolateral margin of MZeomesa mutants at the 40% epiboly stage (Du et al., 2012). We confirmed, by real-time RT–PCR analysis, that MZeomesa mutants had reduced amounts of sox32 transcripts at 30% epiboly and shield stages compared with WT embryos (Supplementary Figure S2B). Therefore, maternal Eomesa plays a role in endoderm specification.

Our in situ hybridization results disclosed that, in WT embryos, ntla was expressed in the whole blastodermal margin at 30% epiboly and shield stages; in contrast, the majority of MZeomesa mutants exhibited an absence of ntla expression in the ventrolateral domains of the blastodermal margin at the 30% epiboly stage, and they recovered ntla expression at the shield stage in some but not all ventrolateral domains (Figure 1D). The reduction of ntla expression in MZeomesa at these stages was verified by
real-time RT–PCR results (Supplementary Figure S2C). Du et al. (2012) also noted that \textit{ntla} was expressed in fewer tiers of blastodermal marginal cells in MZeomesa mutants at the 50\% epiboly stage. These data together indicate that deficiency of maternal Eomesa is likely to impair mesoderm specification, at least in ventrolateral domains of the blastodermal margin.

We found that, compared with WT embryos, about half of MZeomesa mutants at the 30\% epiboly stage expressed the dorsal mesoderm marker \textit{gsc} in a narrower dorsal margin; at the shield stage when WT embryos expressed \textit{gsc} exclusively in the shield at high levels, 28/43 of mutants had \textit{gsc}-positive cells in the shield as well as in the surrounding areas including the dorsal margin, which might result from abnormal convergence and extension during early gastrulation, and the remaining portion (15/43) of mutants showed \textit{gsc} expression only in the shield but at lower levels (Figure 1E). Real-time RT–PCR analysis indicated that the relative mRNA expression level of \textit{gsc} in mutants was significantly reduced compared with WT embryos.

Figure 1 Deficiency of maternal Eomesa results in a partial loss of the mesendoderm markers \textit{ntla} and \textit{sox32}. (A and B) Mesendodermal marker expression revealed by \textit{in situ} hybridization following \textit{eomesa} knockdown. Embryos treated differently were collected at morphologically matchable stages. Stages for \textit{in situ}: \textit{ntla} and \textit{eve1}, shield stage; \textit{sox32}, 30\% epiboly; \textit{gsc}, midgastrulation. Orientation: animal-pole views for \textit{ntla}, \textit{sox32}, and \textit{eve1} with dorsal to the right; dorsal views for \textit{gsc} with animal pole to the top. Ctr, naturally fertilized, uninjected WT embryos; zKD, embryos injected with 5 ng eomesa-MO at the one-cell stage; oCtr, embryos derived from oocytes maturated and fertilized \textit{in vitro}; oKD, embryos derived from oocytes injected with 5 ng eomesa-MO. (C–E) \textit{sox32} (C), \textit{ntla} (D), and \textit{gsc} (E) expression revealed by \textit{in situ} hybridization at 30\% epiboly and shield stages. All embryos were in animal-pole view with dorsal to the right. MZeomesa, mutants lacking maternal and zygotic Eomesa. (F) \textit{boz} expression revealed by \textit{in situ} hybridization at indicated stages. All embryos were laterally viewed with dorsal to the right. Mutant embryos in C–F were fixed at time points when the control embryos were collected at indicated stages. The ratio of embryos with the representative expression pattern is indicated. Scale bar, 200 μm. Corresponding real-time RT–PCR results are shown in Supplementary Figure S2.
(Supplementary Figure S2D). Our results support a previous finding that eomesa knockout in WT embryos led to a slight reduction of gsc and flh expression at the shield stage (Bruce et al., 2003). However, Du et al. (2012) claimed, based on in situ hybridization results, that the dorsal mesendodermal markers gsc and flh were normally expressed in MZeomesa mutants at the 50% epiboly stage. The discrepancy may lie in different developmental stages of the examined embryos and variations of the gsc expression pattern among individuals.

To test whether abnormal expression of the mesendodermal markers in MZeomesa mutants was due to a possible developmental delay, we examined the expression of bozozok (boz), a direct target of canonical Wnt signaling (Leung et al., 2003). Both in situ hybridization and real-time RT–PCR analyses failed to detect alterations of boz expression in MZeomesa embryos at various stages (Figure 1F, and Supplementary Figure S2E). Thus, defects in mesendoderm specification are unlikely to be caused by any epibolic defects in MZeomesa embryos.

**Eomesa acts upstream of Nodal signaling**

A previous report demonstrated that injection of eomesa mRNA into WT embryos induced ectopic expression of the dorsal mesodermal markers at the shield stage (Bruce et al., 2003). Given that Nodal signaling plays an essential role in mesendoderm induction (Feldman et al., 1998; Gritsman et al., 1999), we wondered whether the induction activity of Eomesa was dependent on Nodal signaling. To address this issue, we compared the effect of eomesa overexpression between WT and MZoe embryos devoid of Nodal signaling (Gritsman et al., 1999). Results showed that overexpression of myc-eomesa mRNA induced ectopic expression of gsc, flh and chd in WT embryos, but failed to do so in MZoe mutants (Figure 2). Therefore, we speculate that eomesa functions upstream or parallel of Nodal signaling.

**Deficiency of maternal Eomesa impairs nodal gene expression during mesendodermal specification**

To further investigate the relationship between Eomesa and Nodal signaling, we first examined ndr1 and ndr2 expression in oKD eomesa morphants. As shown in Figure 3A and B, their expression was restricted to the dorsal blastoderm margin of oKD embryos at the dome stage while in the control embryos ndr1 and ndr2 were expressed throughout the blastodermal margin; the reduced expression was still obvious at the 30% epiboly stage. These preliminary results raised the possibility that nodal gene expression was regulated by Eomesa.

Du et al. (2012) recently reported that ndr1 expression was normal in MZeomesa mutants at the sphere stage but both ndr1 and ndr2 expressions were reduced in a small portion of mutants at the 40% epiboly stage, suggesting that regulation of nodal gene expression by maternal Eomesa is stage- or individual-dependent. Then, we analyzed the time-course expression of ndr1 and ndr2 in MZeomesa and WT embryos (Figure 3C–F). As shown in Figure 3C, like WT embryos, MZeomesa mutants at the high stage expressed ndr1 in a restricted dorsal margin; at the oblong stage, WT embryos showed an expansion of ndr1 expression domain in the dorsal margin, but MZeomesa mutants had a narrower ndr1 expression domain; from dome to 30% epiboly stages, ndr1 was expressed in the entire blastodermal margin in WT embryos, but its expression in MZeomesa mutants was still restricted to the dorsal margin though laterally expanded; at the 40% epiboly stage, MZeomesa embryos had ndr1 expression in the ventrolateral margin, resembling WT embryos; at the shield stage, ndr1 expression was very weak in WT embryos, while its expression was retained throughout the blastodermal margin at high levels in MZeomesa mutants; at the 60% epiboly stage, its expression in WT embryos was undetectable, but still occurred in a few domains of the blastodermal margin in MZeomesa mutants. The dynamic alterations of ndr1 expression were similarly observed in Meomesa mutants devoid of maternal Eomesa only, implying that ndr1 expression is regulated by maternal Eomesa. To quantify the changes in ndr1 mRNA levels, we performed real-time RT–PCR analysis. Results showed that, compared with WT embryos, MZeomesa mutants had significantly reduced amounts of ndr1 transcripts from oblong to 30% epiboly stages, increased

**Figure 2** Dependence of eomesa function on Nodal signaling. The expression of the dorsal markers gsc, flh, and chd was examined by in situ hybridization at the shield stage. In WT embryos, injection of 50 pg myc-eomesa mRNA caused ectopic expression of the markers in some areas of the blastodermal margin (indicated by arrows) (B, D, and F). In MZoe mutant embryos deficient for Nodal signaling, myc-eomesa mRNA (50 pg per embryo) overexpression failed to induce ectopic expression of the markers (H, J, and L). All embryos were orientated in animal-pole view with dorsal to the right. The ratio of embryos with the representative expression pattern is indicated. Scale bar, 200 μm.
Figure 3 Deficiency of maternal Eomesa reduces *ndr1* and *ndr2* expression during germ layer specification. (A and B) *In situ* hybridization patterns of *ndr1* (A) and *ndr2* (B) in embryos derived from oocytes maturated and fertilized *in vitro*. Injected and uninjected embryos were harvested at matchable stages based on morphology. oCtr, uninjected control embryos; eomesa oKD, embryos derived from oocytes injected with 5 ng eomesa-MO. (C and E) *In situ* hybridization detection of *ndr1* (C) and *ndr2* (E) expression in WT control (Ctr), MZeomesa and Meomesa mutant embryos. Mutant embryos were fixed at time points when the control embryos were collected at indicated stages. All embryos were shown in animal-pole view with dorsal to the right. The ratio of embryos with representative pattern is indicated in the right corner of each image. Scale bar, 200 μm. Note that weak expression of *ndr2* at the oblong stage could be detected after staining for a longer time. (D and F) Relative expression levels of *ndr1* (D) and *ndr2* (F) mRNAs in WT (Ctr) and MZeomesa embryos detected by real-time RT–PCR at indicated stages.
amounts at the shield stage, and comparable amounts at high and 40% epiboly stages (Figure 3D), which all were consistent with in situ hybridization results. Therefore, we conclude that maternal Eomesa may not be required for initiation of ndr1 expression in the dorsal margin, but required for its timely activation in the ventrolateral blastodermal margin.

An interesting question is why ndr1 expression, upon activated, lasts a longer time in MZ(eomesa or Meomesa) mutants (Figure 3C). We hypothesize that maternal Eomesa may also positively regulate the expression of Nodal signaling repressors. Then, we examined expression of the Nodal antagonist genes lefty1 and lefty2 (Tian and Meng, 2006). As shown in Supplementary Figure S3A, lefty1 expression appeared normal in MZ(eomesa) mutants at the dome stage, was absent in the ventrolateral margin at the 30% epiboly stage and occurred in the whole blastodermal margin at the 40% epiboly stage, which resembled the change of ndr1 expression in mutants. At the shield stage, the majority of mutants showed a much weaker expression of lefty1 in the dorsal margin, contrasting retained strong expression in WT embryos. lefty2 expression appeared to be absent in the ventrolateral margin in MZ(eomesa) mutants at all examined stages ranging from dome to shield stages, which strikingly differed from its expression in the whole blastodermal margin in WT embryos (Supplementary Figure S3B).

Thus, the up-regulated expression of ndr1 after the onset of gastrulation in MZ(eomesa) mutants may be related, at least in part, to the abnormal expression of lefty2 and lefty1.

The expression of ndr2 was also reduced in MZ(eomesa) and Meomesa mutant embryos, more strikingly in the ventrolateral margin, from oblong to 30% epiboly stages (Figure 3E and F). Like ndr1, ndr2 expression was recovered in the ventrolateral margin in mutants at the 40% epiboly stage. It appeared that ndr2 expression in the dorsal margin of mutants at 40% epiboly and shield stages was stronger than in WT embryos, which might be also related to the abnormal expression of lefty1 and lefty2.

We further tested the relatedness of ndr1 and ndr2 reduction in MZ(eomesa) mutants to loss of Eomesa by overexpressing synthetic eomesa mRNA in mutants. We found that the missing expression of ndr1 and ndr2 in ventrolateral margin of MZ(eomesa) embryos could be effectively rescued by injection of myc-eomesa mRNA (Supplementary Figure S4).

Eomesa binds to and activates transcription of ndr1 and ndr2 loci

We next asked whether Eomesa directly regulated nodal gene expression in zebrafish embryos. Based on the consensus Eomesa-binding motif (T/C)(C/A)/(A/G)CAC(C/T)(T/C) identified in Xenopus embryos by Conlon et al. (2001), we identified two putative Eomesa-binding sites, EBS1ndr (from −258 to −251) and EBS2ndr (from −99 to −92), in the proximal promoter of the ndr1 locus (Figure 4A). Chromatin immunoprecipitation (ChIP) analysis disclosed that overexpressed Myc-Eomesa bound to the DNA region containing these two sites in embryos at the 30% epiboly stage (Figure 4B). As reported before (Fan et al., 2007), a distal enhancer (a) plus proximal promoter (p) of ndr1 in the construct pndr1ap:gfp could drive gfp expression in the blastodermal margin at 30%–40% epiboly stages (Figure 4C and D). When the EBS1ndr and EBS2ndr in the proximal promoter were individually or simultaneously mutated, the percentage of GFP-positive embryos and the GFP intensity were reduced (Figure 4D). These results imply that both Eomesa-binding sites are required for ndr1 expression. Using luciferase as a reporter for testing ndr1 promoter/enhancer activity, we also showed that injection of eomesa mRNA or eomesa-enR mRNA coding for a dominant negative form of Eomesa (Bjomson et al., 2005) could increase or reduce the luciferase expression level in WT embryos, respectively (Figure 4E), and that the luciferase expression was inhibited by mutations of EBS1ndr and EBS2ndr (Figure 4F). Taking these data together, we conclude that Eomesa activates ndr1 transcription dependent on binding to the promoter.

We found that there is one putative Eomesa-binding site (EBSndr) at positions from 3090 to 3097) in the first intron of the ndr2 locus (Figure 5A). ChIP assay demonstrated an association of overexpressed Myc-Eomesa with this site in fish embryos (Figure 5B). When the EBSndr-containing fragment derived from the first intron of ndr2 was placed into pndr1ap:m1+2:gfp, in which both EBS1ndr and EBS2ndr were mutated, an increasing proportion of injected embryos could express GFP, and the mutation of the EBSndr led to a relatively smaller proportion of GFP-positive embryos (Figure 5C). These results indicate that the EBSndr possesses an enhancer activity. We made luciferase reporter constructs pmEBSndrGL3 and pmEBSndrGL2 by inserting an EBSndr, containing fragment from intron 1 of ndr2 and a control fragment without EBS from intron 2 of ndr2 into the vector pmGL3, respectively (Figure 5D). The construct pmEBSndrGL3 was derived from pmEBSndrGL3 but contained the mutated EBSndr. These constructs were injected into embryos and the relative luciferase activity was measured. Compared with pmEBSndrGL3, pmEBSndrGL3 injection resulted in a significantly lower level of the luciferase activity (Figure 5E). Co-injection of myc-eomesa or eomesa-enR mRNA with pmEBSndrGL3 caused a marked increase or reduction of the luciferase activity, respectively; however, myc-eomesa or eomesa-enR mRNA injection had no effect on the luciferase activity expressed by pmn2ndrGL3 (Figure 5F). These results further confirmed the responsiveness of the EBSndr to Eomesa.

Maternal eomesa directly activates transcription of the Nodal upstream activator mxtx2

The expression of mxtx2 occurs in blastodermal cells at and before the oblong stage and then in both marginal blastoderm and YSL at the sphere stage, but is restricted to the YSL at the dome stage (Hirata et al., 2000). Mxtx2 is reported to regulate ndr1 and ndr2 expression in the ventrolateral margin of the blastoderm during mesendoderm induction (Hong et al., 2011). We found that knockdown of maternal Eomesa led to partial or complete loss of mxtx2 expression at the dome stage (Figure 6A). We confirmed the previous finding that mxtx2 expression was temporally inhibited in MZ(eomesa) mutants (Du et al., 2012), and disclosed the same changes in Meomesa mutants (Figure 6A and B). Importantly, over-expression of myc-eomesa mRNA in MZ(eomesa) could efficiently initiate mxtx2 expression as early as the dome stage (Figure 6C).

To test whether Eomesa directly regulates mxtx2 expression, we isolated a 1-kb promoter region of mxtx2, which contains a putative
Eomes-binding site (EBS\textsuperscript{mxtx}, −189 AGGTGTTGA−182) (Figure 6D), and made a luciferase reporter by placing it upstream of the luc coding sequence. The expression of this reporter in WT embryos was enhanced by eomesa overexpression but inhibited by eomesa-enR overexpression (Figure 6E, left panel). Furthermore, mutation of the EBS\textsuperscript{mxtx} in the reporter construct caused a reduction of the reporter expression (Figure 6E, right panel). ChIP assay demonstrated an association of overexpressed Myc-Eomesa with endogenous EBS\textsuperscript{mxtx} element (Figure 6F). These data together support the idea that maternal Eomesa activates mxtx2 transcription.

**Eomesa and Mxtx2 cooperatively activate nodal gene expression**

We hypothesized, based on the above findings and the previous report (Hong et al., 2011), that Eomesa regulated ndr1 and ndr2 expression directly by binding to their promoters/enhancers and indirectly through induction of Mxtx2. To further test this hypothesis, we studied functional interaction between Eomesa and Mxtx2 on nodal gene expression. Injection of myc-eomesa mRNA alone into WT embryos could not cause ectopic expression of ndr1 and ndr2, but its co-injection with mxtx2 mRNA enhanced mxtx2-induced ectopic expression of ndr1 and ndr2 in the animal-pole area (Figure 7A). The ectopic expression of the Nodal target genes ntlα and sox32 in the animal-pole area was also more efficiently induced by co-overexpression of myc-eomesa and mxtx2 compared with mxtx2 overexpression alone (Figure 7A). These results suggest that Eomesa and Mxtx2 cooperatively activate nodal gene expression during mesendoderm induction.

We next investigated epistatic interaction between Eomesa and Mxtx2. As shown in Figure 7B, mxtx2 knockdown in WT or MZeomesa embryos, using a specific antisense morpholino (mxtx2-MO) (Hong et al., 2011), inhibited ndr1 and ndr2 expression in the ventrolateral blastodermal margin at the 30% epiboly stage, which is consistent with the previous finding by Hong et al. (2011). In contrast, mxtx2 knockdown in MZeomesa embryos did not cause a further reduction of the ndr1 and ndr2 expression domains (Figure 7B), which could be explained by the absence of mxtx2 transcripts in the ventralateral blastodermal margin of mutants (Figure 6B). Co-injection of myc-eomesa mRNA with mxtx2-MO into WT or MZeomesa embryos led to certain levels of recovery of ndr1 and ndr2 expression in the ventrolateral margin compared with mxtx2-MO injection alone (Figure 7B). These results indicate that Eomesa regulates ndr1 and...
ndr2 expression in both Mxtx2-dependent and Mxtx2-independent fashions.

Eomes promotes Nodal expression and mesendodermal differentiation of murine embryonic stem cells

It has been reported that Eomes in human embryonic stem cells (ESCs) is required for definitive endodermal differentiation induced by extrinsic signals such as Activin, BMP4 and FGF2 (Teo et al., 2011) and that Eomes overexpression in murine ESCs promotes cardiac differentiation in the absence of Activin (van den Ameele et al., 2012). However, the regulatory relationship between Eomes and Nodal remains elusive during ESCs differentiation. To investigate their relationship and to test whether the regulatory mechanism of Nodal expression by Eomes is evolutionarily conserved, we established a murine R1 ESCs line with stable expression of zebrafish eomesa fused to gfp, i.e. R1-eomesa. In ESC culture medium with LIF, R1-eomesa cells expressed several stemness and differentiation markers at levels comparable to R1-GFP cells that were stably expressing GFP (Figure 8A), suggesting that overexpression of eomesa is not sufficient to induce differentiation of ESCs under self-renewal conditions. ESCs can form embryoid body (EB) and autonomously differentiate into multiple lineages in the absence of LIF (Fei et al., 2010). Using the EB differentiation system, we found that, compared with the R1-GFP cells, the

Figure 5 Eomes directly regulates ndr2 transcription. (A) Illustration of ndr2 locus with Eomes-binding sites (EBS) indicated. (B) ChIP-PCR results show occupancy of EBSndr2-containing region by Myc-Eomesa. The amplified EBSndr2-containing region is indicated by arrows in A. See Figure 4B for PCR result of the control, as the same batch of immunoprecipitated DNA pools was used. (C) A 994-bp EBSndr2-containing fragment shows the enhancer activity. All three constructs were made based on pndr1ap:gfp (Figure 4C). After injection with 80 pg GFP reporter DNA at the one-cell stage, GFP was observed at 30% – 40% epiboly stage. The ratios of embryos with different GFP intensities are shown in the bar graph. n, number of observed embryos. (D) Illustration of luciferase reporter constructs. In1(EBSndr2) is a 994-bp EBSndr2-containing fragment derived from intron 1, while In2 is a 961-bp fragment without any EBS derived from intron 2 of the ndr2 locus. In pmEBSndr2GL3, the EBSndr2 was mutated as shown in C. (E and F) Relative luciferase activity expressed by different reporter constructs. WT embryos were injected at the one-cell stage with 80 pg reporter DNA alone or together with 50 pg myc-eomesa or 200 pg eomesa-enR mRNA and harvested at 30% – 40% epiboly stage for luciferase assay.

Maternal Eomes for zygotic nodal gene expression | 279

Downloaded from https://academic.oup.com/jmcb/article-abstract/6/4/272/2886286 by guest on 29 November 2018
R1-eomesa cells expressed significantly higher levels of the mesendodermal markers Brachyury (T), GATA4 and Eomes, but lower levels of the ectodermal marker Sox17 at Day 6 of differentiation (Figure 8B), and the expression of the endodermal marker FGF5 were also increased and decreased, respectively, though not statistically significant. These results indicate that eomesa overexpression causes preferential differentiation of ESCs toward the mesendodermal fates.

The R1-eomesa cells maintained higher levels of Nodal expression than the R1-GFP cells during differentiation (Figure 8C), implying that eomesa overexpression may directly promote the expression of endogenous Nodal gene. We identified a putative EBS (\(^{-168}_{+163}\)TCACCT) in the promoter region of the mouse Nodal locus. In the R1-eomesa cells, this EBS was bound by overexpressed Eomesa-GFP (Figure 8D). The human NODAL locus also contains a putative EBS in the promoter region (\(^{-728}_{-722}\)TAACACCT). The EBS derived from either mouse or human Nodal locus could act as an enhancer to promote GFP expression.

**Figure 6** Maternal Eomesa is involved in activation of mxtx2 expression. (A) eomesa knockdown in oocytes inhibited mxtx2 expression at the dome stage. (B) mxtx2 expression was impaired in MZoeomesa and Meomesa embryos. Mutant embryos were fixed at time points when the control embryos were collected at indicated stages. (C) myc-eomesa overexpression rescued mxtx2 expression in MZoeomesa mutants. MZoeomesa embryos were injected with 50 pg myc-eomesa mRNA at the one-cell stage and examined at indicated stages for mxtx2 expression by in situ hybridization. Embryos were laterally viewed. The ratio of embryos with representative pattern is indicated. Scale bar, 200 μm. (D) Illustration of the mxtx2 locus. (E) Luciferase reporter expression driven by the mxtx2 promoter harboring EBS. The EBS was mutated to AAGCGTG in the mxtx2-luc construct. Embryos were injected with 50 pg mxtx2-luc plasmid DNA and 5 pg renila DNA, with 50 pg myc-eomesa or 200 pg eomesa-enR mRNA co-injected when needed, and harvested at 30%–40% epiboly stage for luciferase assay. (F) Occupancy of the EBS of mxtx2 by Myc-Eomesa in embryos, as assayed by ChiP-PCR. The amplified EBS\(^{mxtx2}\)-containing region indicated by arrows in D. See Figure 4B for ChiP-PCR result of the control region, as the same batch of immunoprecipitated DNA pools was used.
expression when it was placed upstream of pndr1ap^m1;2:gfp (Figure 8E). Therefore, the mouse or human Nodal gene may require Eomes for activation during embryonic development.

Discussion

We have demonstrated in this study that maternal Eomesa is an upstream regulator of zygotic nδr1 and nδr2 expression during mesendoderm induction in the zebrafish embryo. We found that deficiency of maternal Eomesa causes a failure of nδr1 and nδr2 expression in the ventral and lateral blastodermal margins from mid- to late-blastula stages. This finding suggests that maternal Eomesa is essential for timely activation of nδr1 and nδr2 in the ventrolateral margin but other factors are required for activating their expression in the dorsal margin. Given that nδr1 expression in the dorsal margin is absent in ichabod/β-catenin 2 mutant embryos (Kelly et al., 2000; Bellipanni et al., 2006), it is most likely that the activation of nδr1 and nδr2 expression in the dorsal margin is dependent on maternal Wnt/β-catenin signaling.

Based on our and others’ findings (Kelly et al., 2000; Dougan et al., 2003; Bellipanni et al., 2006; Hong et al., 2011), we propose a simplified model for transcription of nδdal genes during zebrafish mesendoderm induction (Supplementary Figure 55). Briefly, maternal Eomesa activates zygotic mxtx2 expression; in the ventrolateral margin, Eomesa and synthesized Mxtx2 cooperate to activate nδr1 and nδr2 expression; in the dorsal margin, maternal β-catenin 2 activates nδr1 and nδr2 expression, which may be enhanced by maternal Eomesa and zygotic Mxtx2; Nodal proteins then transduce the signals intracellularly, ultimately inducing the expression of downstream mesendodermal genes. It is worth noting that Mxtx2 has been reported to regulate nδr2 expression directly but may regulate nδr1 expression indirectly (Hong et al., 2011). Nevertheless, maternal Eomesa contributes to mesendoderm induction in zebrafish embryos in a way similar to maternal VegT in Xenopus embryos. The high-level expression of Eomes in mouse eggs (McConnell et al., 2005) suggests its possible involvement in activating zygotic Nodal gene expression during embryogenesis. It appears that the specification of mesendodermal lineages during embryogenesis by the maternal T-box transcription factors/Nodal signals is conserved across vertebrate species.

Our reporter and ChIP assays revealed that Eomesa may directly regulate nδr1, nδr2 and mxtx2 expression. However, the missing expression of these genes during mid- to late-blastulation can be largely recovered at later stages (~40% epiboly stage) (Figures 3 and 6), reflecting the complexity of underlying regulatory mechanisms. The possible explanations for the rescue of nδr1 and nδr2 expression in the ventrolateral blastodermal margin of MZeomesa mutants at late blastula stages may include: (i) ventrolateral propagation of existing Nodal signals
in the dorsal side, which involves positive autoregulation of Nodal signaling; (ii) unknown activators of nodal genes, which may be expressed or activated during late blastulation in the absence of Eomesa; (iii) incomplete recovery of the expression of the Nodal antagonists such as lefty1 and lefty2 (Supplementary Figure S3). We noted that ndr2 expression in the dorsal margin of MZeomesa and Meomesa mutants at 30% epiboly and shield stages (Figure 3E) was stronger than in WT embryos, which concurred with stronger expression of mxtx2 expression in the dorsal margin of mutants (Figure 6B). Since Mxtx2 is a direct regulator of ndr2 (Hong et al., 2011), more abundant Mxtx2 in the dorsal margin of mutants at late blastula stages may also account for enhanced ndr2 expression.

The expression of mxtx2 was undetectable in mutants depleted of maternal Eomesa before the 30% epiboly stage (Figure 6B), indicating an essential role of maternal Eomesa in timely activation of mxtx2 expression. At and after the 30% epiboly stage, mxtx2 started to be expressed in the dorsal YSL of mutants. There might be unknown factors that are responsible for late activation of mxtx2 expression in the dorsal side in the absence of maternal Eomesa.

Previous studies have found that maternal Eomesa protein exist throughout cleavage period in zebrafish embryos (Bruce et al., 2003; Bjornson et al., 2005; Du et al., 2012). However, its target genes mxtx2, ndr1 and ndr2 start to transcribe at midblastula stages. Two mechanisms may account for this transcriptional delay: (i) chromatin structures at those loci are not poised for
transcription; (ii) cofactors required for their transcription are not available at earlier stages. These mechanisms are worthy of investigation through experiments in the future.

**Materials and methods**

**Zebrafish lines**

WT embryos were obtained from AB strain. The mutant line *eomesa*<sup>h105</sup> (Du et al., 2012) was obtained from the Zebrafish International Resource Center and the line *oepp<sup>2557</sup>* (Gritsman et al., 1999) was a gift from Dr Alex Schier. Embryos derived from *eomesa*<sup>h105</sup>/<sup>h105</sup> heterozygous intercrosses were raised to adulthood and then homozygous female and male were identified by PCR genotyping. Homozygous *eomesa*<sup>h105</sup>/<sup>h105</sup> female were unable to naturally produce eggs when mated to male. Therefore, eggs squeezed from homozygous *eomesa*<sup>h105</sup>/<sup>h105</sup> female in *vitro* fertilized by sperms squeezed from *eomesa*<sup>h105</sup>/<sup>h105</sup> or WT male to produce MZ*eomesa* or Meomesa mutant embryos, respectively. Less than 10% of MZ*eomesa* or Meomesa mutants could grow up to adulthood. MZ*oepp* embryos were produced by crossing *oepp<sup>2557</sup>/<sup>2557</sup>* female with *oepp<sup>2557</sup>/<sup>2557</sup>* male. Embryos were staged according to Kimmel et al. (1995). Ethical approval was obtained from the Animal Care and Use Committee of Tsinghua University.

**In vitro oocyte maturation and fertilization**

The procedures were modified from previous reports (Gore et al., 2005; Seki et al., 2008; Bontems et al., 2009; Nair et al., 2013). Briefly, ovaries taken out from sacrificed female were placed in oocyte culture medium (OCM; 90% Leibovitz’s L-15 medium (Gibco), pH9.0, 0.5 mg/ml BSA (Amresco)) at room temperature, and stage III oocytes were chosen to culture in OCM with 1 μg/ml 17α-20β-dihydroxy-4 pregnen-3-one (DHP, Sigma-Aldrich) at 26°C. About 5 h later when proceeded to stage V, oocytes were defolliculated manually and then fertilized in a fresh plate by adding squeezed sperms. When needed, morpholino or mRNA was injected into oocytes after first 2 h maturation when they proceeded close to stage IV. Using this method, the fertilization rate was usually 10–15%, which might further drop if oocytes were injected.

**Constructs, microinjection, and embryonic assays**

mRNAs were synthesized *in vitro* using mMESSAGE mMACHINE Kit (Ambion). Transgene constructs were generated by PCR-based cloning. Sequences of the used cloning primers and morpholinos (MO) were listed in Supplementary Tables S1 and S2. Unless otherwise stated, DNA, MO, or mRNA was injected into one-cell stage embryos. Whole-mount *in situ* hybridization was performed using Digoxigenin-labeled RNA probes as usual. For real-time RT–PCR, ~15 embryos derived from oocytes matured and fertilized *in vitro* or 50 embryos derived from natural fertilization were used to extract total RNAs, and RT–PCR analysis was done as before (Jia et al., 2009). Primers for RT–PCR were listed in Supplementary Table S3. GFP in embryos were observed by fluorescence microscopy. For luciferase activity assays, 50 pg reporter DNA was co-injected with 5 pg renilla DNA (an internal control) and luciferase activity was measured in embryos at 30%–40% epiboly stages as before (Liu et al., 2013).

ChiP assay was performed as before (Liu et al., 2011; Jia et al., 2012). In brief, ~2000 embryos injected with *myc-eomesa* mRNA were collected at the 30% epiboly stage and dechorionated. The embryos were incubated for crosslinking in 1% formaldehyde for 15 min with occasional inversion at room temperature, followed by adding 1/20 volume of 2.5 M glycine and incubating for 5 min to stop crosslinking. After washed with pre-cooled PBS three times, embryos were resuspended in 2 ml lysis buffer (10 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.5% NP-40) for 15 min with gentle rocking at 4°C, and the lysate was precipitated after spinning at 1000 rpm and resuspended in 1 ml of pre-cooled nuclei lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% SDS). After incubation for 10 min with gentle rocking at 4°C, 6 ml pre-cooled IP dilution buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.01% SDS) was added and mixed well. The lysate was sonicated using a sonifier to generate chromatin fragments of 200–1500 bp in length with an enrichment of 200–500 bp fragments, and the supernatant was collected after spinning at 14000 rpm for 10 min at 4°C. The chromatin solution was diluted by adding 3 ml IP dilution buffer and 400 μl 10% Triton X-100. To 5 ml of the chromatin solution, 100 μl pre-equilibrated Protein A Sepharose beads was added for pre-cleaning. Following incubation with rotation at 4°C for 30 min and spin at 1000 rpm, the supernatant was collected. About 20 μg anti-Myc antibody or mouse IgG was added to the supernatant and incubated overnight with rotation at 4°C and the incubation was extended for additional 2 h after addition of 100 μl pre-equilibrated Protein A Sepharose beads. After several rounds of wash, DNA was eluted from the beads and the elute was treated with proteinase K and RNase A. Finally, the DNA was purified by phenol/chloroform extractions. The purified DNA was used for PCR using specific primers (Supplementary Table S4). The same batch of the immunoprecipitated DNA pool was used for amplifying corresponding regions of *ndr1*, *ndr2* and *mxtx2* loci as well as the control sequence spanning the second intron and the third exon of *ndr1* locus, which does not contain Eomes-binding sites. The control region for ChiP assay in murine ESCs is located in the 3’ UTR of the *Nodal* locus.

**Murine stem cell culture and differentiation**

Zebrafish Eomesa with GFP tag was cloned into the lentiviral vector p2K7 and the resulted construct was used to transfect HEK293FT cells to make lentiviruses. The virus-containing supernatant was used to infect murine R1 ES cells. The stable R1 cell lines were established by selection with 250 μg/ml G418 (Invitrogen) for 5 days and maintained in normal ES culture medium in the presence of feeder cells and 1000 units/ml LIF (Millipore). For differentiation in embryoid bodies (EB), single cells were plated and cultured in the feeder-free, KSR-containing medium in the absence of LIF to allow generation of floating EBs with cell differentiation (Fei et al., 2010). EB cells were collected at different days for analyzing markers expression by RT–PCR.

**Statistical analysis**

Significance between the means was analyzed using Student’s *t*-test. Significance levels were indicated by *(*P < 0.05) and **(*P < 0.01).
Supplementary material
Supplementary material is available at Journal of Molecular Cell Biology online.

Acknowledgements
We thank Drs Alex Schier and Susan Mango (Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA) for discussion and suggestions, Dr David Kimelman (Department of Biochemistry, University of Washington, Seattle, WA, USA) for myc-eomesa construct, and members of the Meng lab for discussion and technical assistance.

Funding
This work was financially supported by grants from the Major Science Research Programs of China (2011CB943800) and the National Natural Science Foundation of China (31221064).

Conflict of interest: none declared.

References
specification requires the combinatorial activities of FoxH1 and Eomesodermin.


