Zygotic VegT is required for Xenopus paraxial mesoderm formation and is regulated by Nodal signaling and Eomesodermin

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ABSTRACT The T-box gene VegT plays a crucial role during mesendoderm specification of the amphibian embryo. While the function of maternal VegT (mVegT) has been extensively investigated, little is known about the function and transcriptional regulation of zygotic VegT (zVegT). In the present study, we used comparative genomics and a knockdown experiment to demonstrate that zVegT is the orthologous gene of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6, and has an essential role in paraxial mesodermal formation. zVegT knockdown embryos show several defects in the patterning of trunk mesoderm, such as abnormal segmentation of somites, a reduction in muscle, and the formation of an abnormal mass of cells at the tail tip. We also identified the cis-regulatory elements of zVegT that are necessary and sufficient for mesoderm-specific expression. These cis-regulatory elements are located in two separate upstream regions of zVegT, corresponding to the first intron of mVegT. The results of in vitro binding and functional assays indicate that Forkhead box H1 (FoxH1) and Eomesodermin (Eomes) are the trans-acting factors required for zVegT expression. Our results highlight the essential role of zVegT in organization of paraxial mesoderm, and reveal that zVegT is regulated by a coherent feedforward loop of Nodal signaling via Eomes.

KEY WORDS: VegT, eomesodermin, nodal, paraxial mesodermal formation, coherent feedforward loop

Introduction

In vertebrates, mesoderm, one of the three germ layers in triploblastic animals, is formed between the ectoderm and endoderm. Mesoderm gives rise to a wide range of tissues including muscle, blood, vascular system, heart, kidney, and dermis (Kimelman, 2006; Yasuo and Lemaire, 2001). The mechanism of mesoderm formation has been a central thesis of developmental biology for decades and intensive studies have elucidated that members of the T-box family play pivotal roles during mesoderm formation (Showell et al., 2004). The T-box family proteins are transcription factors with a conserved T-domain for DNA-binding and protein dimerization (Minguillon and Logan, 2003).

In Xenopus, the T-box gene VegT (also known as Antipodean, Xombi, and Brat) is expressed maternally and zygotically (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Maternal VegT (mVegT) is expressed at high levels in oogenesis, and is asymmetrically localized to the vegetal hemisphere of the unfertilized egg and cleavage-stage...
embryos. In the blastula, mVegT, in cooperation with ß-catenin, directly induces the zygotic expression of *Xenopus nodal-related* genes (*Xnrs*). *Xnr* genes encode signaling molecules of the transforming growth factor ß (TGF-ß) superfamily, which play essential roles in mesendoderm induction (Agius et al., 2000; Kofron et al., 1999; Schier, 2003; Takahashi et al., 2000; Zorn et al., 1999). mVegT also directly activates endodermal genes, including Sox7, Sox17, Bix4, and Endodermin (Casey et al., 1999; Howard et al., 2007; Taverner et al., 2005; Zhang et al., 2005). Depletion of mVegT transcripts results in severe defects in the process of primary germ layer induction (Zhang et al., 1998). Together, these studies highlight mVegT as a key player in specification and patterning of mesendoderm in *Xenopus* embryogenesis (Showell et al., 2004).

Fig. 1. Zygotic VegT is the *Xenopus* ortholog of the VegT/Spadetail gene subfamily. (A) Phylogenetic tree of the T-box gene family associated with vertebrate mesoderm development. The phylogenetic tree was calculated by MacVector 7.2.3 software. Human Tbx6 splicing variant1 (GenBank accession no. NM_004608), human Tbx6 splicing variant2 (GenBank accession no. NM_080758), human Brachury (T) (GenBank accession no. NM_003181), human Eomesodermin (GenBank accession no. NM_003181), mouse Tbx6 (GenBank accession no. NM_011538), mouse T (GenBank accession no. NM_009309), mouse Eomesodermin (GenBank accession no. NM_010136), chick TbxL/Tbx6 (GenBank accession no. AB193180), chick Brachury (GenBank accession no. U25176), X. tropicalis maternal VegT (GenBank accession no. AB451530), X. tropicalis zygotic VegT (GenBank accession no. AB451530), X. tropicalis Tbx6 (GenBank accession no. NM_001007994), X. tropicalis Xbra (GenBank accession no. BC081350), X. tropicalis Eomesodermin (GenBank accession no. NM_001128652), zebrafish Spadetail/Tbx16 (GenBank accession no. AF077225), zebrafish Tbx6 (GenBank accession no. U80951), zebrafish No tail (GenBank accession no. AB088068), zebrafish Eomesodermin a (GenBank accession no. NM_131679), and zebrafish Eomesodermin b (GenBank accession no. NM_001083575) were tested. Zebrafish Tbx5 (GenBank accession no. NM_130915) was used as the outgroup. (B) Genomic structure of the X. tropicalis VegT locus. First exon of mVegT, first exon of zVegT, and common exons of both variants are indicated as orange, green, and red boxes, respectively. (C) Comparison of the exon structure of genes in the VegT/Spadetail subfamily between six vertebrate genomes. The numbers shown on the left side of the box indicate the base pair size of each exon. (D) Conservation of synteny. By using MetaZome, the flanking upstream and downstream genes of the putative X. tropicalis VegT orthologs were compared between six vertebrates. This set of genes is comprised of common orthologous genes among five vertebrate species including X. tropicalis, chick, fugu, medaka, and stickleback. Red arrows indicate the locus of the VegT ortholog. The gene and the transcriptional orientation are indicated by the color and direction of the arrow, respectively. (E-G) X. tropicalis zVegT expression patterns at stage 10, 11, and 12. The antisense probe for WISH recognized both mVegT and zVegT. Panels at the left and right show the lateral and vegetal views with the dorsal side up, respectively.
It has been reported that zygotic VegT (zVegT)/Antipodean, which has an identical nucleotide sequence to mVegT, apart from the extreme 5' terminal sequences, is a splicing variant arising from a single VegT gene locus (Stennard et al., 1999). zVegT is initially expressed in the dorsal side of the marginal zone at the mid-blastula stage, and subsequently at high levels throughout the entire circumference of the marginal zone until the mid-gastrula stage. In late gastrula embryos, zVegT expression is excluded from the notochord. In contrast to mVegT, the function of zVegT remains unknown. Based on sequence similarity, it has been suggested that Xenopus VegT is a homolog of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6 (Griffin et al., 1998). In Spadetail mutant embryos, the trunk progenitor cells fail to move properly and accumulate at the tip of the tail (Griffin et al., 1998; Kimmel et al., 1989). Spadetail controls the movement of somite progenitors by regulating the expression of paraxial protocadherin (PAPC), encoding a cell adhesion molecule, during gastrulation (Yamamoto et al., 1998). Consequently, the trunk paraxial mesoderm of Spadetail mutant embryos is highly deficient and tail development is relatively unaffected. In chick embryogenesis, Tbx6L expression is restricted to the early paraxial mesoderm lineage and regulates somitogenesis (Knezevic et al., 1997).

As previously reported, zVegT is induced by Nodal signaling (Horb and Thomsen, 1997; Lustig et al., 1998; Stennard et al., 1996) and is inhibited by overexpression of Xlefty/Xantivin, a Nodal signaling specific inhibitor (Tanegashima et al., 2000). However, the involvement of Nodal signaling in the direct regulation of zVegT expression has not been defined. Furthermore, the role of fibroblast growth factor (FGF) signaling on zVegT expression is also controversial. FGF signaling plays a crucial role in the development of trunk and tail mesoderm (Bottcher and Niehrs, 2005). In animal cap cells, Xombi and Brat, but not Antipodean, can be induced by FGF treatment (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Stennard et al., 1999). However, Xombi and Antipodean are not regulated by FGF signaling in the late gastrula, whereas they are regulated by FGF signaling in the early gastrula (Lustig et al., 1996; Stennard et al., 1996). Therefore, it is not certain whether FGF signaling affects the expression of zVegT in vivo.

In the present study, we provide evidence that Xenopus zVegT is crucial for paraxial mesoderm formation and is an ortholog of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6. To identify VegT orthologs, we performed comparative genomics analyses and functional studies. Phylogenetic analyses and homology searches of vertebrate T-box genes involved in mesodermal formation revealed that Xenopus VegT is classified into the VegT/Spedetail subfamily that also includes zebrafish Spedetail/Tbx16 and chick Tbx6L/Tbx6 (Fig. 1A). This result indicates that X. tropicalis VegT is the putative ortholog of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6. Next, we investigated the genomic exon-intron structure of X. tropicalis VegT (Fig. 1B). The X. tropicalis VegT gene was mapped to scaffold_12 of the Xenopus tropicalis genome assembly v4.1 (Joint Genome Institute, JGI). The maternal and zygotic VegT genes have 9 exons each and share exons 2 to 9. The first exon of zVegT is located within the first intron of mVegT. To assess the evolutionary relationship of VegT, we compared the exon structures of putative orthologous genes of VegT found in frog, zebrafish, chick, frog, medaka, and stickleback (Fig. 1C). The exon structure of genes in the VegT/Spedetail subfamily is strikingly similar, especially for exons 3 to 6, which encode the T-box DNA-binding domain. Next, we analyzed conserved synteny of these putative orthologous genes by Metazome v2.0.4 (http://www.metazome.net/index.php) of the JGI and the Center for Integrative Genomics (Fig. 1D). These genes were located in the region of highly conserved synteny between the five species, with the exception of zebrafish Spadetail/Tbx16.

We also determined the temporal and spatial expression of X. tropicalis VegT in detail (Fig. 1 E-G and see Fig. S1 A-R in the supplementary material). The expression pattern of X. tropicalis VegT is very similar to that in X. laevis (D’Souza et al., 2003; Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). The VegT transcript is regulated and expressed by paraxial muscle progenitors at the two-cell stage in X. tropicalis embryos, harvested at stage 34 (C-F) or 40 (A-B). (C,D) Immunochemistry with the muscle-specific antibody, MF-20. (E,F) Immunochemistry with the notochord-specific antibody, Tor70. Panels at the right indicate magnification of the trunk region.

Fig. 2. Zygotic VegT knockdown embryos show severe defects in the structure of the posterior mesoderm. (A,C,E) Control MO-injected embryos. (B,D,F) zVegT MO-injected embryos. 12 ng of MOs were injected into the marginal zone of both blastomers at the two-cell stage in X. tropicalis embryos, harvested at stage 34 or 40. (A-D) Immunochemistry with the muscle-specific antibody, MF-20. (E,F) Immunochemistry with the notochord-specific antibody, Tor70. Panels at the right indicate magnification of the trunk region.
uniformly distributed during the early stages of oogenesis and becomes gradually localized to the vegetal hemisphere from stage III (see Fig. S1 B-G in the supplementary material). During the early gastrula stage, zygotic expression of VegT is first detected in a highly restricted pattern on the dorsal side of the marginal zone (Fig. 1E). By the mid-gastrula stage, the expression of VegT is uniformly distributed within the entire marginal zone that forms the prospective mesoderm (Fig. 1F). By the end of gastrulation, the expression of VegT is maintained, except in a narrow dorsal region where the notochord forms (Fig. 1G). In X. laevis, these two isoforms are expressed in different germ layers, mVegT in endoderm and zVegT in mesoderm (Stennard et al., 1999). We distinguished the signal of zVegT from that of mVegT by the regions expressing these genes. The expression pattern of VegT resembles the reported gene expression of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6. To examine the functional homology between X. tropicalis zVegT, zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6, we performed knockdown experiment of zVegT translation by using an antisense MO (Fig. 2 A-F and see Fig. S2 A,B in the supplementary material). zVegT MO specifically inhibited translation from microinjected myc-tagged zVegT mRNA (see Fig. S2B in the supplementary material). zVegT MO-injected embryos exhibited defects including curvature of the anterior/posterior axis, disruption of trunk mesodermal structure, and formation of an abnormal mass of cells at the tail tip at stage 40 embryos (78%, n=74) (Fig. 2 A,B). Moreover, injection of zVegT MO caused a reduction of muscle formation and disorder of somite segmentation, but did not affect notochord formation (Fig. 2 C-F). Zebrafish Spadetail/Tbx16 mutants also show severe defects in non-notochordal trunk mesoderm formation (Amacher and Kimmel, 1998; Griffin et al., 1998; Ruvinsky et al., 1998). Chick Tbx6L/Tbx6 regulates somitogenesis (Knezevic et al., 1997). The common functional roles for the formation of paraxial mesoderm suggest a close evolutionary
relationship among *Xenopus* zVegT, zebrafish Spadetail/Tbx16, and chick Tbx6L/Tbx6. These results also indicate that zVegT is the *Xenopus* ortholog of zebrafish Spadetail/Tbx16 and chick Tbx6L/Tbx6.

**The cis-regulatory regions of zygotic VegT in Xenopus**

To identify the *cis*-regulatory region of zVegT, we isolated 6156 bp upstream from the first exon of *X. tropicalis* zVegT, which also corresponds to the mVegT first intron (Figs. 1B, 3A). The 6156 bp of genomic DNA was subcloned into an EGFP reporter vector, and we investigated the transcriptional activity in *X. laevis* embryos using a transgenic technique. EGFP driven by -6156 bp upstream from the first exon of zVegT (Fig. 3A). Transgenic embryos generated with the -2035, -1769, -122, -6156Δ(-2397/-1769), and -2420Δ(-1749/-122) constructs showed mesodermal expression of the EGFP reporter gene, but transgenic embryos generated with the -2035, -1769, -122, -6156Δ(-2397/-1769), and -2420Δ(-1749/-122) constructs did not (Fig. 3A and see Fig. S3 A-D in the supplementary material). These results suggest that both the -2420/-2035 and -545/-122 upstream regions are required for mesodermal expression of zVegT. Consistently, in a luciferase reporter assay, the transcriptional activity of the -1766 was remarkably decreased, compared with that of -2420 (see Fig. S4A in the supplementary material), suggesting the presence of regulatory elements for transcriptional activation between -2420 and -1766.

Sequence analysis reveals that the distal region (-2420/-2035) and proximal region (-545/-122) contain putative binding sites for FoxH1, the Nodal signaling mediator (AAATNNACA) (Zhou et al., 1998), Smad, the transcriptional partner of FoxH1 (GTCT or GNC) (Shi and Massague, 2003; Silvestri et al., 2008), T-box (TVDCACYH) (Conlon et al., 2001), and Ets, the transcriptional factor of the FGF-Ras-MAPK signaling pathway (MGGAW) (Sharrocks et al., 1997). These transcriptional factors are essential transcriptional regulators of mesoderm induction (Fig. 3C) (Bottcher and Niehrs, 2005; Schier, 2003; Showell et al., 2004). The *X. laevis* zVegT upstream region of approximately 11 kbps was also isolated (Y. -J. Kim and C.-Y. Yeo, unpublished data). A comparison of the upstream regions of *X. laevis* and *X. tropicalis* by dot matrix analysis revealed that the distal region -2420/-2035 and proximal region -545/-122 are highly conserved between *X. laevis* and *X. tropicalis*, and the putative transcription factor binding elements for FoxH1-1, 2, 3 and T-box-2, 5 are identical (Fig. 3D and data not shown). It is noteworthy that these upstream regions of *X. laevis* zVegT and zebrafish Spadetail/Tbx16 also...
contain clusters of putative FoxH1/Smad and T-box binding sites (data not shown). These results indicate the possibility for a common regulatory system of mesodermal specification in *Xenopus* and zebrafish.

**Nodal signaling, but not FGF signaling, is essential for zygotic VegT expression**

We investigated whether Nodal signaling and FGF signaling are required for the initiation of *zVegT* gene expression in *X. laevis*. It has been reported that the expression of *zVegT*, but not *mVegT*, is inducible by Activin in animal caps (Stennard et al., 1999) and that *zVegT* expression in marginal zone is suppressed by the overexpression of *Xlefty/Xantivin*, a specific inhibitor of Nodal signaling (Schier and Talbot, 2001; Tanegashima et al., 2004; Tanegashima et al., 2000). To confirm the role of Nodal signaling on *zVegT* expression, we examined the effect of inhibiting Nodal signaling using the C-terminal fragment of Cerberus (*CerS*). Embryos injected with *CerS* exhibited severe reduction of *VegT* gene expression in the marginal region of the injected side (89%, n=18) (Fig. 4 A,B), which suggests that Nodal signaling is required for expression of *zVegT*.

In *Xenopus*, four T-box genes, *zVegT*, *Eomes* (Ryan et al., 1996), *Brachyury* (*Xbra*) (Smith et al., 1991), and *Tbx6* (Uchiyama et al., 2001), are zygotically expressed in mesoderm. Therefore, we examined whether the T-box genes, *zVegT*, *Eomes*, *Xbra*, and *Tbx6*, induce *zVegT* expression without Nodal signaling (Fig. 4C). Expression of *zVegT* was induced in animal cap cells by the overexpression of *XVegT*, *Eomes*, or *Tbx6*, but not by *Xbra*. *Xnr5* and *Xnr2* were also induced in these animal cap cells. Coinjection with *CerS* inhibited the induction of *zVegT* expression. These results indicate that upregulation of *zVegT* expression by these T-box genes is mediated via Nodal signaling and that induction of

Fig. 5. FoxH1 and T-box proteins directly bind to the cis-regulatory region of zygotic VegT. (A-C) The loss of FoxH1 or T-box sites decreases the ratio of mesoderm-specific EGFP reporter gene expression. Diagram of the deletion (A) and mutation (B) constructs for transgenesis. The FoxH1 binding sites and the T-box binding sites are indicated as red and blue boxes, respectively. The number of transgenic embryos that express EGFP in the marginal region, and the total number of normally developing embryos obtained with each construct on transgenesis, are indicated on the right along with the percentage of EGFP-positive cases. (C) Sequences of Fox1-1, 2, 3 and T-box-1, 2, 3, 4, 5. Underlines and lower case letters show mutated residues in each putative binding site. (D-F) EMSA for binding of FoxH1 and T-box proteins to the cis-regulatory elements of zygotic VegT. Probes used for EMSA experiments (D). For EMSA, the radiotisotope-labeled probes for FoxH1-1, 2, 3, or T-box-1-5 were incubated with in vitro transcription/translation Myc-tagged proteins, FoxH1-myc, VegT-myc, Eomes-myc, Xbra-myc, and Tbx6-myc. Binding of protein to probe was eliminated using wild-type competitor (wt), whereas the mutated competitor did not inhibit protein binding to the probe (mut) (C,E,F). The arrowheads indicate the shifted band.
zVegT expression is due to T-box proteins mimicking the activity of the mVegT protein.

The effect of inhibition of FGF signaling on VegT expression has been reported previously (Fletcher and Harland, 2008; Lustig et al., 1996; Stennard et al., 1996). However, mVegT and zVegT were not distinguished in these experiments. To evaluate the contribution of FGF signaling on zVegT expression, we performed the following experiments. First, we examined the expression of various FGF ligands by different concentrations of Activin in animal cap cells. FGF3, eFGF, and FGF8 were induced by Activin in a concentration-dependent manner, simultaneously with the expression of zVegT (Fig. 4 D-M). Next, we investigated whether FGF signal is necessary for the induction of zVegT using a dominant negative FGFR1 (XFD) and a specific chemical inhibitor of FGFR (SU5402) (Fig. 4 D-M). The expression of Xbra and Tbx6 were decreased in embryos injected with XFD (Xbra: 100%, n=32; Tbx6: 84%, n=27). By comparison, XFD had no effect on the expression of zVegT and Eomes (Fig. 4 D-K). Treatment with SU5402 inhibited the expression of Xbra and Tbx6 but had no effect on the expression of zVegT and Eomes, which are induced by Activin in animal caps (Fig. 4M). These results are consistent with previous reports that showed FGF signaling is required for the gene expression of Xbra and Tbx6, and has no effect on the expression of Eomes (Fang et al., 2004; Fletcher and Harland, 2008; Isaacs et al., 1994; Schulte-Merker and Smith, 1995). These results suggest that FGF signaling is not necessary for induction of zVegT and Eomes, but is required for the expression of Xbra and Tbx6.

Taken together, our results indicate that Nodal signaling, but not FGF signaling, is essential for zVegT expression, and that T-box proteins alone cannot initiate zVegT expression. These results also suggest that the putative FoxH1 and T-box binding sites, but not the Ets binding sites, are candidates for the cis-regulatory elements of zVegT expression.

FoxH1 and T-box proteins directly interact with cis-regulatory elements to induce zygotic VegT expression

To evaluate the role of FoxH1 and T-box binding sites in the upstream region of zVegT, we generated deletion and mutation reporter constructs based on the -2420Δ(-2035/-545) construct (Fig. 5 A,B). Deletion or mutation of the FoxH1 binding sites, -2420Δ(-2176/-545), -2420Δ(-2353/-545), -2420Δ(-2035/-545+FoxH1), the T-box binding sites, -2420Δ(-2035/-290), -2420Δ(-2035/-290+T-box), or both binding sites together, -2420Δ(-2035/-545+FoxH1+T-box), greatly reduced the proportion of mesodermal specific expression of EGFP in transgenic embryos, compared with that of -2420Δ(-2035/-545) (Fig. 5 A,B and see Fig. S3 B,E-J in the supplemental material). Consistent with these results, the transcriptional activity of the zVegT promoter-luciferase reporter construct was markedly reduced with mutations in FoxH1 and T-box sites (see Fig. S4B in the supplementary material).

We performed EMSAs to investigate whether the FoxH1 and T-box binding sites in the zVegT cis-regulatory regions have the capacity for FoxH1 and T-box proteins to bind directly (Fig. 5 E,F). We examined the DNA fragments FBS1, FBS2, and FBS3, corresponding to FoxH1 binding sites for FoxH1-1, 2, and 3, respectively, and the DNA fragments TBS1-5, corresponding to the T-box binding sites for T-box-1, 2, 3, 4, and 5 (Fig. 5 C,D). The FoxH1 protein bound specifically to FBS1, FBS2, and FBS3 (Fig. 5E). The FoxH1 protein bound to FBS3 strongly compared with FBS1 and FBS2. Recently, the ideal consensus FoxH1 binding site was revealed (Silvestri et al., 2007).

Fig. 6. Comparative gene expression patterns of the T-box genes in X. laevis embryos. (A-Q) Spatial expression of zVegT (A-E), Eomes (F-J), Xbra (K-N), and Tbx6 (O-Q) was detected by WISH from stage 9 to stage 11. Arrowheads indicate the initial zygotic expression of individual genes. The VegT antisense probe recognized both mVegT and zVegT. The top and bottom panels show the vegetal side view with dorsal side up and dorso lateral view, respectively. (R) RT-PCR reveals temporal expression of T-box genes.
The difference in binding affinity between these three FoxH1 binding sites seems to relate to DNA sequence similarity with the ideal consensus FoxH1 binding site. The T-box proteins, zVegT, Eomes, and Tbx6, bound specifically to TBS1-5 (Fig. 5F), but Xbra did not (data not shown). These results indicate that FoxH1, zVegT, Eomes, and Tbx6 can directly bind to the corresponding binding sites located in the distal and proximal cis-regulatory regions of zVegT.

Eomesodermin is the upstream regulator of zygotic VegT

To determine which T-box genes regulate zVegT expression, we compared the expression patterns of T-box genes from the blastula to gastrula stages of *Xenopus* (Fig. 6). Eomes is first expressed at stage 9 in the dorsal mesoderm and it is the earliest zygotically expressed gene among the T-box genes (Fig. 6 F,R). zVegT was initially expressed at stage 9.5 in the same dorsal region as Eomes (Fig. 6 B,G,R). The expression of Eomes and zVegT preceded that of Xbra and Tbx6 (Fig. 6 B,F,L,O,R). The temporal and spatial expression patterns suggest that Eomes, among the T-box proteins, is the best candidate for the trans-acting factor of zVegT.

To confirm our hypothesis that Eomes regulates the expression of zVegT, we performed a loss-of-function study of Eomes (Fig. 7). We designed two antisense MOs, Eomes MO1 and Eomes MO2, to prevent proper splicing of Eomes transcripts in *X. tropicalis* (Fig. 7A). The temporal and spatial expression patterns suggest that Eomes, among the T-box proteins, is the best candidate for the trans-acting factor of zVegT. For these experiments, 12 ng of Eomes MO1 or Eomes MO2 was injected into the marginal zone of both blastomeres at the two-cell stage in *X. tropicalis* embryos and then harvested at stage 10+. Eomes MO1 or Eomes MO2 effectively inhibited the splicing of Eomes pre-mRNA and the expression of zVegT transcripts. Sequence analysis confirmed that Eomes MO1 and Eomes MO2 caused marked premature termination of Eomes transcripts (arrowheads). Arrows show the band corresponding to normal Eomes transcripts. (C-H) VegT expression is eliminated in the Eomes MOs-injected region. Eomes MOs or control MO (6 ng) and β-gal mRNA (100 pg) were coinjected into the marginal zone of one blastomere at the two-cell stage, and were fixed at stage 10 for WISH. Red β-gal staining indicates the injected side. Lateral views are shown.

Fig. 7. Eomesodermin is essential for expression of zygotic VegT. (A) Diagram of the exon-intron structure of Eomes and design of two Eomes splice-inhibiting antisense MOs. The *X. tropicalis* Eomes gene has 6 exons. The splicing sites targeted by the Eomes MO1 and Eomes MO2 (purple) were the boundary of intron1-exon2 and exon2-intron2, respectively. The light blue arrows indicate the PCR primers to confirm Eomes splicing. (B) Eomes MOs effectively inhibited the proper splicing of Eomes, and suppress expression of zVegT. For these experiments, 12 ng of Eomes MO1 or Eomes MO2 was injected into the marginal zone of both blastomeres at the two-cell stage in *X. tropicalis* embryos and then harvested at stage 10+. Eomes MO1 or Eomes MO2 effectively inhibited the splicing of Eomes pre-mRNA and the expression of zVegT. Sequence analysis confirmed that Eomes MO1 and Eomes MO2 caused marked premature termination of Eomes transcripts (arrowheads). Arrows show the band corresponding to normal Eomes transcripts. (C-H) VegT expression is eliminated in the Eomes MOs-injected region. Eomes MOs or control MO (6 ng) and β-gal mRNA (100 pg) were coinjected into the marginal zone of one blastomere at the two-cell stage, and were fixed at stage 10 for WISH. Red β-gal staining indicates the injected side. Lateral views are shown.

Fig. 8. Model of zygotic VegT regulation by Eomesodermin and Nodal signaling. mVegT induces expression of Xnrs before stage 9. Nodal signaling induces expression of Eomes at stage 9. Then, Eomes and Nodal signaling function in a coordinated, simultaneous manner to activate the expression of zVegT at stage 9.5. This inductive mechanism of regulation forms a coherent feedforward loop.
or Eomes MO2 severely reduced the level of zVegT expression (MO1: 60%, n=33; MO2: 64%, n=20) (Fig. 7 B-H).

Collectively, our results indicate that Eomes is the upstream regulator acting on the proximal cis-regulatory element of zVegT.

Discussion

Maternal VegT and zygotic VegT

There are two isoforms of Xenopus VegT: mVegT, a maternal determinant, and zVegT, a zygotic mesoderm gene. In a prior report on X. laevis, these variants were reported to have identical nucleotide sequences, except those encoding the N-terminal region of the proteins (Stennard et al., 1999). Our study on the genomic structure of VegT in X. tropicalis revealed that the mVegT and zVegT variants are expressed from the same gene locus (Fig. 1B). Moreover, we demonstrated that the cis-regulatory regions containing the promoter of zVegT are located within the first intron of mVegT (Fig. 3 A,B). These results suggest that the mVegT and zVegT variants are controlled by distinct transcriptional regulation mechanisms, and not generated by alternative mRNA splicing. In addition, other vertebrate VegT orthologs are not expressed maternally (Griffin et al., 1998; Knezevic et al., 1997). From these findings, we infer that mVegT gene is probably emerged by insertion of a set of mVegT promoter into the region upstream of zVegT locus during amphibian evolution.

Xenopus zygotic VegT is essential for the patterning of mesoderm

In the present study, we define zVegT as a member of the VegT/Spadetail subfamily, which includes zebrafish Spadetail/ Tbx16 and chick Tbx6/Tbx6, based on genomic and functional analyses. Functional knockdown of zVegT by MO disrupts formation of the somites but not notochord (Fig. 2), indicating that zVegT is essential for paraxial mesoderm formation.

Previous studies, especially in zebrafish, have demonstrated interactions among three genes of the T-box subfamily, Spadetail, No tail (an ortholog of Brachyury), and Tbx6, which perform multiple region-specific functions to regulate the expression of many mesodermal genes and characterize subdivision of presumptive mesoderm into various cell fates, notochord, trunk, and tail mesoderm (Goering et al., 2003; Showell et al., 2004). It has been assumed that Spadetail and Tbx6 perform common functions for paraxial mesoderm formation (Showell et al., 2004). In Xenopus gastrula, the expression patterns of zVegT and Tbx6 are different from that of Xbra. The expression of zVegT and Tbx6 is maintained in the future paraxial mesoderm but downregulated in the future notochord, whereas the expression of Xbra is maintained in the future notochord but downregulated in the future paraxial mesoderm (see Fig. S1 in the supplementary material) (Smith et al., 1991; Uchiyama et al., 2001). In addition, the phenotypes of the zVegT knockdown embryo and Tbx6 knockdown embryo were strikingly similar (Fig. 2) (Lou et al., 2006). Thus, VegT/Spadetail and Tbx6 may have overlapping functions for paraxial mesoderm specification. In this work, we revealed the genomic structure of the VegT variants, mVegT and zVegT, the evolutionarily conserved function of zVegT, and the relationship between three T-box genes, mVegT, zVegT, and Eomes. These findings will provide the basis for elucidating the mechanism by which T-box genes define mesodermal regionalization.

Eomesodermin is an essential trans-acting factor for the initiation of zygotic VegT expression

In the isolated cis-regulatory region of zVegT, T-box binding sites are crucial for the expression of the EGFP reporter (Fig. 5 A,B). Besides zVegT, four T-box genes, mVegT, Eomes, Xbra, and Tbx6, are expressed during early Xenopus embryogenesis. mVegT transcripts are asymmetrically deposited in the vegetal region of the Xenopus egg and it was considered as a candidate for the upstream regulator of zVegT. However, it has been reported that mVegT mRNA and protein are restricted to the presumptive endoderm, while zVegT mRNA and protein are localized in the presumptive mesoderm (Stennard et al., 1999).

Furthermore, it has been proposed that mVegT-induced genes promote endodermal fate and repress mesodermal fate in the vegetal cells (Zorn and Wells, 2007). Therefore, mVegT is not likely to act as a trans-acting factor for zVegT expression in mesoderm.

Eomes, Xbra, and Tbx6 are zygotically expressed in mesodermal region (Fig. 6 F-R). Xbra and Tbx6 are expressed at later stages as compared with zVegT (Fig. 6 B,L,O,R). Furthermore, Xbra and Tbx6 were not expressed, in contrast to zVegT and Eomes, when FGF signaling was inhibited in whole embryos or in Activin-stimulated animal caps (Fig. 4 D,K,M). These results suggest that the expression of zVegT does not require Xbra or Tbx6.

Eomes is appropriately expressed spatiotemporally to activate the initial expression of zVegT (Fig. 6 B,F,R) (Ryan et al., 1996; Stennard et al., 1996). EMSA revealed that the Eomes protein can directly bind to the cis-regulatory region of zVegT (Fig. 5F). Moreover, functional knockdown of Eomes severely reduced the expression of zVegT (Fig. 7 B,C). These results indicate that Eomes is an essential trans-acting factor for the initiation of zVegT expression.

Transcriptional coregulation of Xenopus zygotic VegT by Eomesodermin and Nodal signaling

We have demonstrated that both Nodal signaling and Eomesodermin are required for the expression of zVegT. In the isolated cis-regulatory elements of zVegT, both the FoxH1 binding sites and the T-box binding sites are crucial for the full activation of the transgenic EGFP (Fig. 5 and Fig. S3 in the supplementary material), suggesting that each type of binding site is necessary, but insufficient, for activation of the isolated zVegT cis-regulatory region. This is also true for the expression of endogenous zVegT. In animal cap assay, ectopic expression of T-box protein alone or exogenous stimulation of Nodal (Activin) alone could induce the expression of zVegT (Fig. 4 C,L,M). However, this could be due to the mutual inductive activities of T-box and Nodal genes and each of them alone, without the activation of the other, is not sufficient for zVegT expression. Overexpression of zVegT, Eomes, or Tbx6 in animal caps induced the expression of zVegT but they also induced the expression of Xnr2 and Xnr5 (Fig. 4C). However, the T-box-induced expression of zVegT was significantly reduced when Nodal signaling was blocked by coinjection of CerS, indicating that T-box proteins require concomitant Nodal signaling to induce zVegT expression. This also suggests that a subset of T-box genes can
mimic the endogenous function of mVegT to induce Nodal ligands. Conversely, exogenous Nodal/Activin signaling is able to induce the expression of subset of T-box genes, including zVegT, in animal caps (Fig. 4 L,M). However, the expression of endogenous zVegT, presumably induced by endogenous Nodal signaling, was severely reduced by Eomes knockdown (Fig. 7). These results indicate that Eomes and Nodal signaling are required simultaneously for initial zVegT expression.

**A new model of paraxial mesoderm formation**

Based on the present observations, we propose a model of how T-box genes and Nodal signaling regulate the expression of zVegT and the formation of paraxial mesoderm formation in *Xenopus* (Fig. 8). *mVegT* initially induces the expression of *Xnrs*, then Nodal signaling subsequently induces the expression of *Eomes*, and finally *Eomes* and Nodal signaling act in a coordinated, simultaneous manner to activate the expression of *zVegT*. *Eomes* is an immediate-early mesoderm gene expressed prior to *Xbra* (Ryan et al., 1996). The direct regulation of *Eomes* occurs in a concentration-dependent manner by Nodal/Activin signaling mediated by two FoxH1 (also known as FAST2) sites within the promoter region (Ryan et al., 2000). The induction of *Eomes* by Nodal signaling, and the subsequent activation of *zVegT* by both *Eomes* and Nodal signaling, generates the "coherent feedforward loop", which is known as a network motif in transcriptional regulation (Alon, 2007). A coherent feedforward loop consists of three genes. Gene X regulates gene Y, and gene Z is coregulated by X and Y. Thereby, the network motif creates a delay between the expression of gene Z and the activation of gene Y to prevent the misexpression of gene Z. Indeed, there is a delay between the expression of zVegT and the expression of Eomes (Fig. 6 B,F,R). The regulation of VegT by the coherent feedforward loop may be highly effective in controlling appropriate mesoderm regionalization. In conclusion, we demonstrated that zVegT plays essential roles in paraxial mesoderm formation and that the expression of zVegT is established by the coherent feedforward loop of Nodal signaling via Eomes.

**Materials and Methods**

*Xenopus* embryo manipulation, microinjection and animal cap assay

- Manipulation of *Xenopus laevis* (*X. laevis*) and *Xenopus tropicalis* (*X. tropicalis*) embryos, microinjection, and animal cap assays were performed according to Haramoto et al. (2004). *Xenopus* embryos were staged according to Nieuwkoop and Faber (1956). For lineage tracing, β-galactosidase (β-gal) mRNA was coinjected and visualized by Red-Gal (Research Organics) staining.
- Human recombinant Activin A was kindly provided by Dr. Yuzuru Eto of Ajinomoto co., Inc.

**DNA constructs and mRNA**

*X. tropicalis* genomic DNA, 6156 bp upstream sequences of zVegT locus, was amplified by polymerase chain reaction (PCR) using the following primers:
- Forward, 5'-ggggagctcGTAACACTCACCTGGGTAGGGAG-3' and reverse, 5'-ggggcagctgTTCCTCCTAGCAGCTAACCCACC-3'.
- The restriction enzyme site added to the 5' end of each primer is shown in lower case letters. The PCR product was subcloned into pGEM-T (Easy) vector (Promega) and named pGEM-6156. The pGEM-6156 construct was digested with *Mlu*I and inserted into *Mlu*I-digested pd2EGFP+s+s+N+M that had two restriction sites, NotI and *Mlu*I, added to pd2EGFP+s (Hitachi et al., 2008). The following constructs containing 5' sequence subdomain combinations, deletions or point mutations, were created from pd2EGFP-6156 (-6156) as shown in figures 3A and 5A-C:
  - -2420, -2035, -1769, -122, -6156, 2397/-1769, -2420, -1749/-1040, -2420, -1749/-545, -2420, -1749/-122, -2420, -2035/-545, -2420Δ(-2035/-290), -2420Δ(-2035/-264), -2420Δ(-2167/-545), -2420Δ(-2353/-545), -2420Δ(-2035/-545+FoxH1), -2420Δ(-2035/-545+T-box), and -2420Δ(-2035/-545+FoxH1+T-box).
  - To generate pCS2-zVegT, pCS2-FoxH1-myc, pCS2-zVegT-myc, pCS2-Eomes-myc, pCS2-Xbra-myc, and pCS2-Tbx6-myc, the coding regions of zVegT, FoxH1, Eomes, Xbra, and Tbx6 were amplified by PCR then subcloned into pCS2+ or pCS2-MT (http://sitemaker.umich.edu/dlturner.vectors). The correct sequence of resultant constructs was verified by DNA sequencing. An upstream DNA fragment of 11 kbps was amplified by inverse PCR using primers specific to the 5'-UTR sequence of *X. laevis* zVegT and was sequenced. Capped RNAs were synthesized using SP6 and T3 mMESSAGE mMACHINE (Ambion) with the following plasmids as templates: pCS2-zVegT, pCS2-Cer5 (Takahashi et al., 2000), pXFD/Xss (Amaya et al., 1993), pEomes/RN3-3 (Ryan et al., 1996), pCS2-Xbra (Tanegashima et al., 2000), pCS2-Tbx6 (Uchiyama et al., 2001), and pCS2-nuclear localizing signal-β-galactosidase (Takahashi et al., 2000).

**RT-PCR assay**

Total RNA extraction and RT-PCR were performed according to Takahashi et al. (2000). *Xenopus* embryos or animal cap explants were cultured to the mid-gastrula stage and harvested. Primers specific for *Xnr5*, *Xnr2*, and *FGF8* of *X. laevis* were as previously described (Fletcher et al., 2006; Takahashi et al., 2000). Primers specific for *Xbra*, Ornithine decarboxylase (ODC), and Elongation factor-1 alpha (EF-1α) of *X. laevis* were described in the Xenopus Molecular Maker Resource (XMMR) (http://www.xenbase.org/WWW/Welcome.html). The primers designed for this study are as follows:
- For *X. laevis*, zVegT forward, 5'-TGATTAGTTTAGGAAAGC-3' reverse, 5'-CGGAGTCTTACTGAGAAG-3';
- FG3 forward, 5'-GGGTTTACGAACATCTTGGAAGG-3' reverse, 5'-CCACGAACTCTACATCGGTTG-3';
- eFG forward, 5'-CGGACGGGAAAGATAATGCTC-3';
- CeG forward, 5'-CGTGGCAAGAAATGGTGGCAG-3';
- Eomes forward, 5'-CGGGTGCTAGTAAAGGAGGAG-3' reverse, 5'-GGCTTTAACCCTTCTCATGGC-3';
- Tbx6 forward, 5'-CCAGAAGCAAAAAGCTACACGGC-3' reverse, 5'-CAATGAGAAGAGCAGCCAGTTG-3';
- For *X. tropicalis*, zVegT forward, 5'-GCTGTGAGGACACAGTGGACTC-3' reverse, 5'-TGAACCTGGCGTCTAGGCG-3';
- Eomes forward, 5'-TGCTAGTGGTAATGTTCCC-3' reverse, 5'-CAATGCTACCCGCGAGGAGGAGA-3';
- EF-1α forward, 5'-TGTAGGAGTTCTCAAGGGCGGTC-3' reverse, 5'-ACAGATTTGCTGCTAATGGTCCC-3';
- PCR products were verified by DNA sequencing. ODC and EF-1α were used as internal controls. Reverse transcriptase negative (RT-) reactions showed no contamination of genomic DNA.

**Transgenesis, whole mount in situ hybridization (WISH) and immunochemistry**

Transgenic embryos were generated by a sperm nuclear transplantation method as previously described (Kroll and Amaya, 1996). Plasmids used for transgenesis were linearized by digestion with *Mlu*I and inserted into *Mlu*I-digested pd2EGFP+s+N+M that had two restriction sites, NotI and *Mlu*I, added to pd2EGFP+s (Hitachi et al., 2008). The following constructs containing 5' sequence subdomain combinations, deletions or point mutations, were created from pd2EGFP-6156 (-6156) as shown in figures 3A and 5A-C:
- -2420, -2035, -1769, -122, -6156, 2397/-1769, -2420, -1749/-1040, -2420, -1749/-545, -2420, -1749/-122, -2420, -2035/-545, -2420Δ(-2035/-290), -2420Δ(-2035/-264), -2420Δ(-2167/-545), -2420Δ(-2353/-545), -2420Δ(-2035/-545+FoxH1), -2420Δ(-2035/-545+T-box), and -2420Δ(-2035/-545+FoxH1+T-box).
- To generate pCS2-zVegT, pCS2-FoxH1-myc, pCS2-zVegT-myc, pCS2-Eomes-myc, pCS2-Xbra-myc, and pCS2-Tbx6-myc, the coding regions of zVegT, FoxH1, Eomes, Xbra, and Tbx6 were amplified by PCR then subcloned into pCS2+ or pCS2-MT (http://sitemaker.umich.edu/dlturner.vectors). The correct sequence of resultant constructs was verified by DNA sequencing. An upstream DNA fragment of 11 kbps was amplified by inverse PCR using primers specific to the 5'-UTR sequence of *X. laevis* zVegT and was sequenced. Capped RNAs were synthesized using SP6 and T3 mMESSAGE mMACHINE (Ambion) with the following plasmids as templates: pCS2-zVegT, pCS2-Cer5 (Takahashi et al., 2000), pXFD/Xss (Amaya et al., 1993), pEomes/RN3-3 (Ryan et al., 1996), pCS2-Xbra (Tanegashima et al., 2000), pCS2-Tbx6 (Uchiyama et al., 2001), and pCS2-nuclear localizing signal-β-galactosidase (Takahashi et al., 2000).
specialized for the FoxH1 protein [20 mM HEPES (pH 7.9), 8% glycerol, T4 polynucleotide kinase (TaKaRa). The radioisotope-labeled probes were purified using a DyeEx 2.0 spin kit (Qiagen) and then annealed.

References


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