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# 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

*Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; Eomes, eomesodermin; fgf, fibroblast growth factor; Fox, forkhead box; mVegT, maternal VegT; zVegT, zygotic VetT; VegT, T-box protein associated with vegetal cortex.

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embryos. In the blastula, mVegT, in cooperation with β-catenin, directly induces the zygotic expression of *Xenopus nodal-related* genes (*Xnrs*). *Xnr* genes encode signaling molecules of the transforming growth factor β (TGF-β) 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).

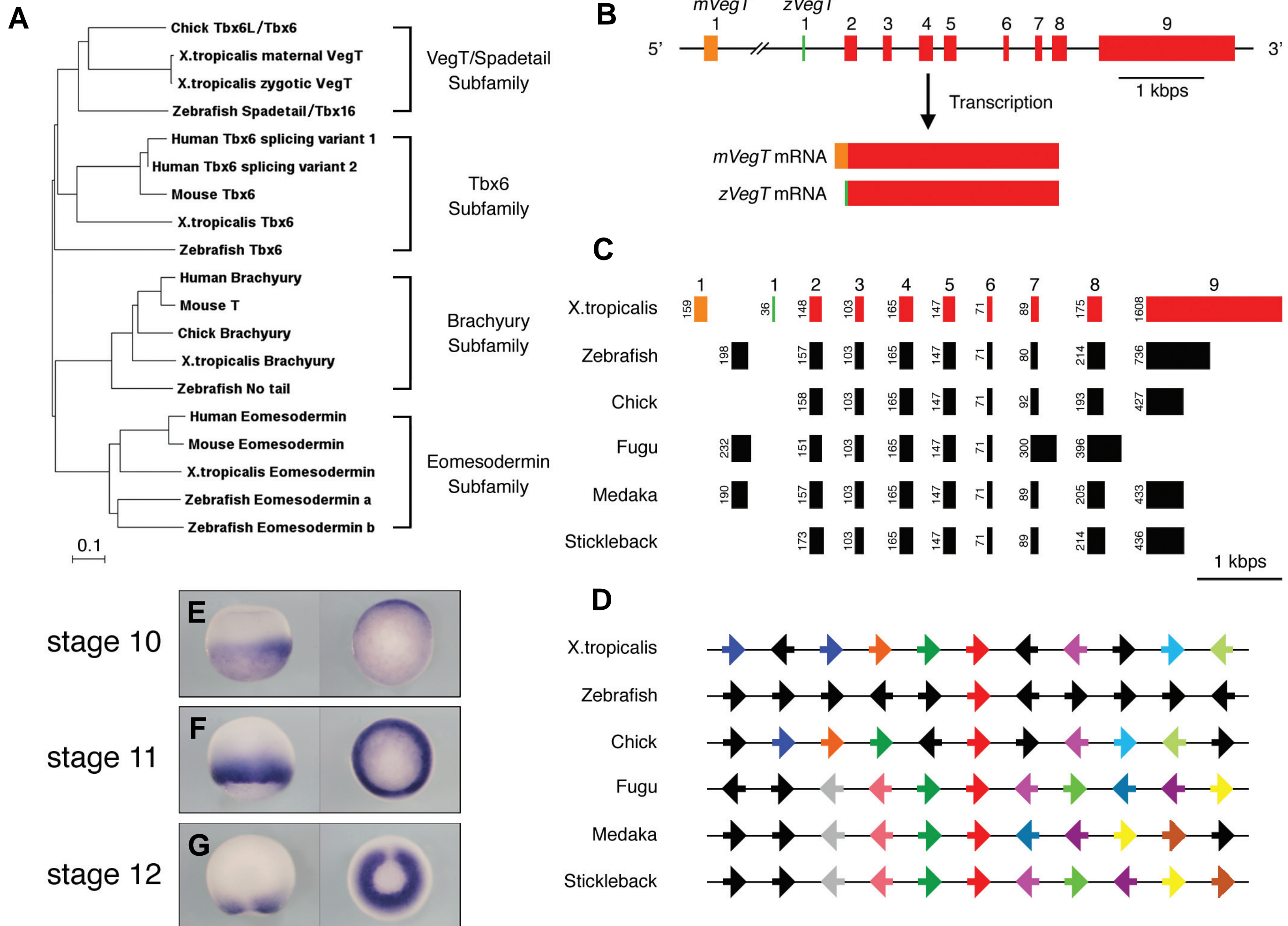


**Fig. 1. Zygotic *VegT* is the *Xenopus* ortholog of the *VegT*/*Spadetail* gene subfamily. (A)** *Phylogenetic tree of the T-box gene family associated with vertebrate mesoderm development. The phylogenetic tree was calculated by MacVector 7.2.3 software. Human Tbx6 splicing variant1 (GenBank accession no. NM_004608), human Tbx6 splicing variant2 (GenBank accession no. NM_080758), human Brachyury (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 Brachyury (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*., 1996; 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; Stennard *et al*., 1999). 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*. In addition, we identified two *cis*-regulatory regions of *zVegT* that are necessary and sufficient for mesoderm-specific expression. These *cis*-regulatory elements are directly coregulated by Nodal/FoxH1 signaling and Eomesodermin (Eomes), which are crucial transcriptional regulators for mesoderm specification and patterning (Bruce *et al*., 2003; Russ *et al*., 2000; Ryan *et al*., 1996; Watanabe and Whitman, 1999).

## Results

### Xenopus *zygotic VegT is essential 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/Spadetail* subfamily that also includes zebrafish *Spadetail/Tbx16* and chick *Tbx6L/Tbx6* (Fig. 1A). This result indicates that *X. tropicalis VegT* is the putative ortholog of zebrafish *Spaedtail/Tbx6* 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, fugu, medaka, and stickleback (Fig. 1C). The exon structure of genes in the *VegT/Spadetail* 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

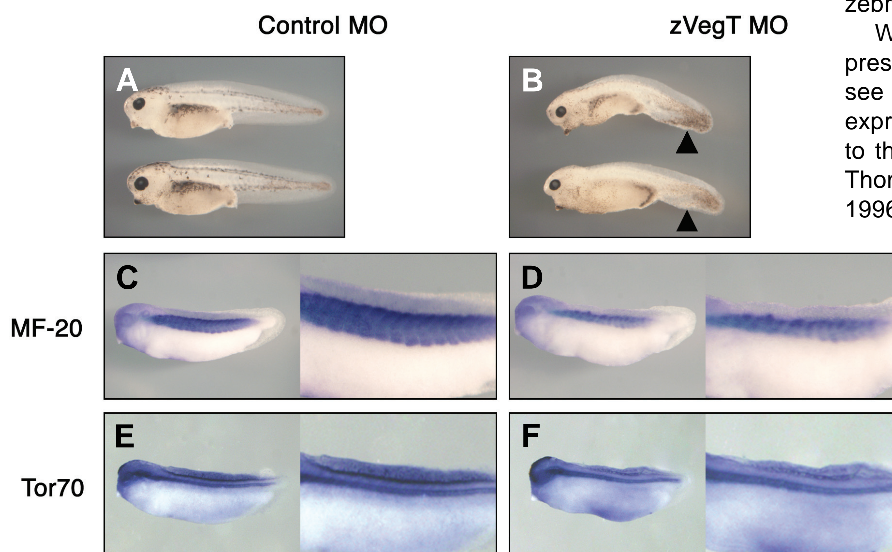


**Fig. 2. Zygotic *VegT* knockdown embryos show severe defects in the structure of the posterior mesoderm. (A,C,E)** *Control MO-injected embryos.* **(B,D,F)** *zVegT MO-injected embryos. 12 ng of MOs were injected into the marginal zone of both blastomeres at the two-cell stage in* X. tropicalis *embryos, 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.*

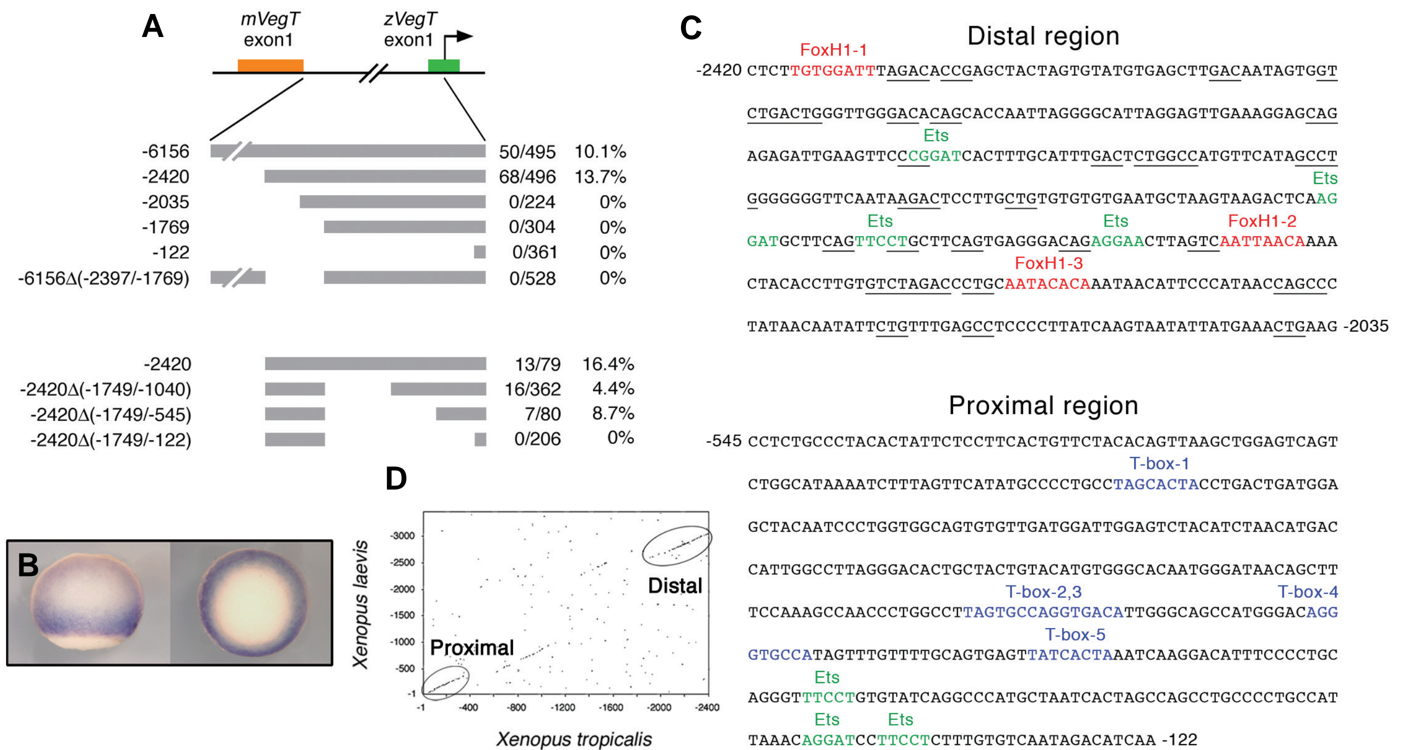


**Fig. 3. Two *cis*-regulatory regions controlling zygotic *VegT* expression in the marginal zone. (A)** *Diagram of the* X. tropicalis VegT *genome and constructs for transgenesis. The number of transgenic embryos that express* EGFP *in the marginal region, and the total number of normally developing embryos obtained with each construct, are indicated on the right along with the percentage of* EGFP*-positive cases. The 122 bp of genomic DNA upstream of* zVegT *contains an initiator motif that overlaps the transcription start site for the* zVegT *gene promoter, but does not contain a TATA motif.* **(B)** *Mesoderm-specific* EGFP *expression in transgenic* X. laevis *embryos generated with the -6156 construct. Panels at the left and right indicate lateral and vegetal views with the dorsal side up, respectively.* **(C)** *The distal region -2420/-2035 and the proximal region -545/-122 contain several putative transcriptional factor binding sites for FoxH1, Smad, T-box, and Ets. Underlines indicate Smad binding elements.* **(D)** *Comparison of upstream sequences between* X. tropicalis *and* X. laevis *by dot matrix analysis (MacVector 7.2.3 software). The distal and proximal regions are conserved between the two species. FoxH1-1, 2, 3 and T-box-2, 5 regions are highly conserved in* Xenopus.

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.* Zebrafish *Spadetail/Tbx16* is expressed in all marginal cells of the blastoderm, and during gastrulation its expression becomes restricted to the paraxial mesoderm excluding the dorsal mesoderm fated to form notochord (Griffin *et al.*, 1998; Ruvinsky *et al.*, 1998). Chick *Tbx6L/Tbx6* is expressed in the primitive streak progenitors before gastrulation and its expression becomes restricted to the paraxial mesodermal lineage excluding the Hensen's node and notochord (Knezevic *et al.*, 1997). The results of comparative analyses of genomic structures and expression patterns support the hypothesis that *Xenopus VegT* is the ortholog 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 the 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; Kimmel *et al.*, 1989; Weinberg *et al.*, 1996; Yamamoto *et al.*, 1998). Chick *Tbx6L* 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 transgene was expressed in the mesoderm region in the gastrula and the expression pattern was consistent with that of endogenous *zVegT* (Fig. 3 A,B). A series of deletion constructs was generated for screening of *cis*-regulatory regions in the 6156 bp fragment (Fig. 3A). Transgenic embryos generated with the -2420, -2420Δ(-1749/-1040), and -2420Δ(-1749/-545) 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 both the distal region (-2420/-2035) and proximal region (-545/-122) contain putative binding sites for FoxH1, the Nodal signaling mediator (AATNNACA) (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. tropicalis* and *X. laevis*, and that 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

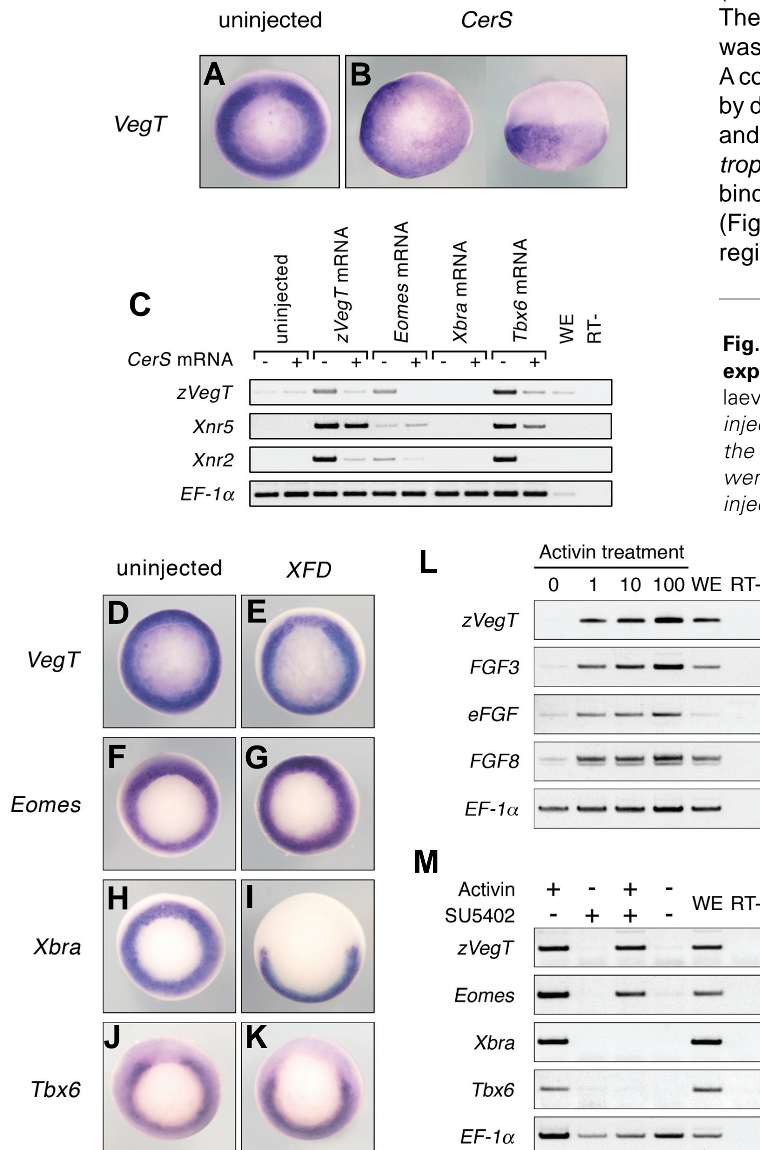


**Fig. 4. Nodal signaling, but not FGF signaling, is necessary for expression of zygotic *VegT*. (A,B)** CerS *inhibits* zVegT *expression in* X. laevis *embryos. WISH of the* zVegT *gene was performed on* CerS*-injected or uninjected embryos. 300 pg of* CerS *mRNA was injected into the marginal zone of one blastomere at the two-cell stage, and embryos were harvested at stage 11. Panels at the left and right for the* CerS*-injected embryo show the vegetal view with the dorsal side up and lateral view, respectively.* **(C)** *Expression of the* zVegT *gene is inhibited by coinjection of* CerS *mRNA in animal caps.* CerS *(600 pg) was coinjected with* zVegT, Eomes, Tbx6, *or* Xbra *(1 ng) into the animal pole of both blastomeres at the two-cell stage. Animal cap cells were explanted at stage 9 and then cultured until stage 11 for RT-PCR. Injected mRNAs were not detected by these experiments.* **(D-K)** *FGF signaling is not required for* zVegT *gene expression. The overexpression of* XFD *inhibited the expression of* Xbra **(H,I)** *and* Tbx6 **(J,K)***, but not the expression of zygotic* VegT **(D,E)** *and* Eomes **(F,G)** *in* X. laevis *embryos. Embryos were injected with 1 ng of* XFD *mRNA into the dorsal marginal zone of two blastomeres at the four-cell stage, and were fixed at stage 11 for WISH. Vegetal views are shown with dorsal side up.* **(L)** *Expression of* zVegT *and* FGFs *is induced dose-dependently by Activin in animal cap cells. The animal caps dissected at stage 9 were treated with 1, 10, or 100 ng/ml Activin, and harvested at stage 11 for RT-PCR analysis.* **(M)** *Expression of* zVegT *is induced by Activin independent of FGF signaling. The animal caps dissected at stage 9 were treated with or without Activin (10 ng/ml) and SU5402 (80 μM), and were harvested at stage 11 for RT-PCR analysis. The induction of expression of* zVegT *and* Eomes *by Activin was not affected by inhibition of FGF signaling. The expression of* Xbra *and* Tbx6 *was downregulated by SU5402 in animal cap cells.*

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 *zVegT, 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

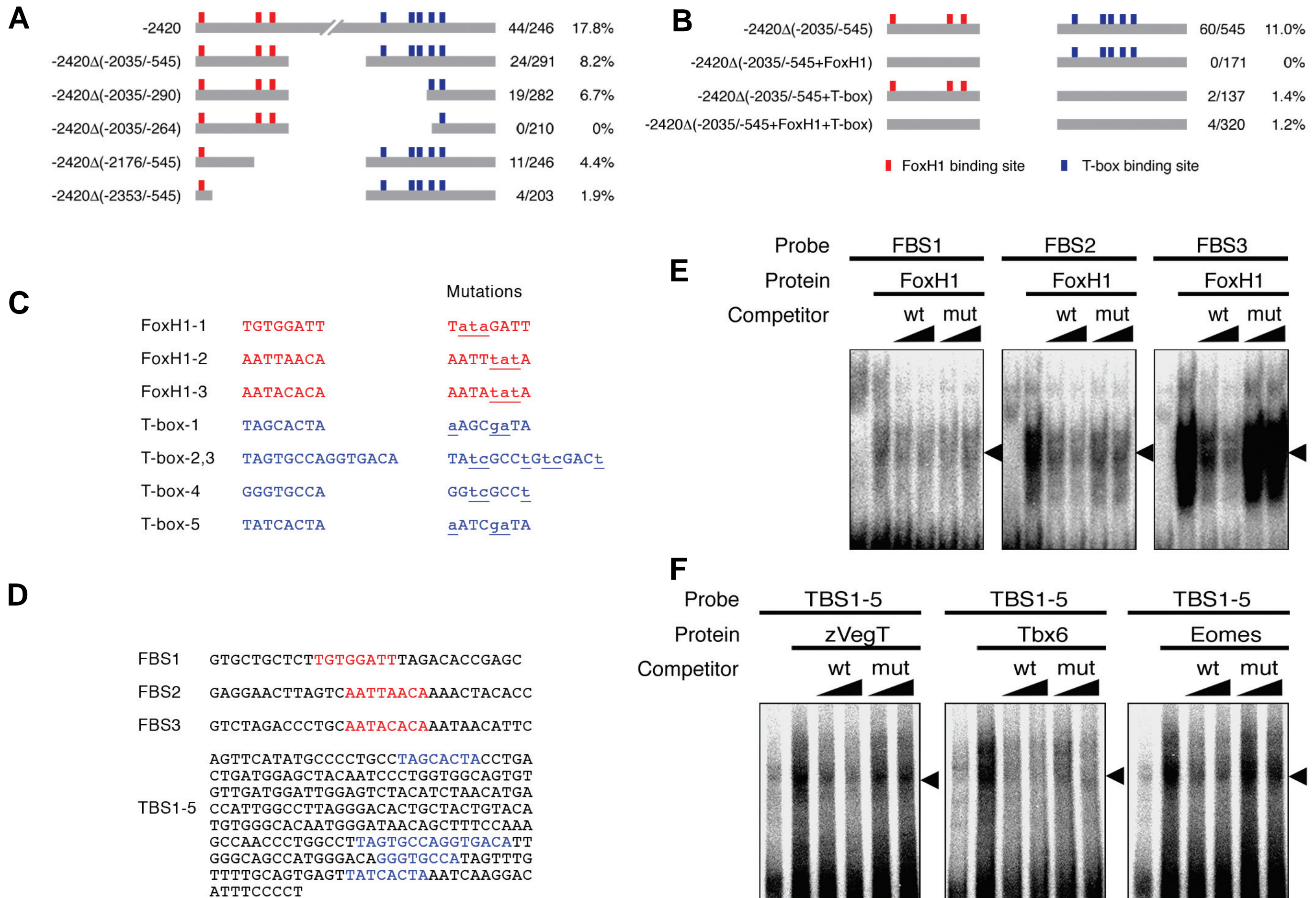


**Fig. 5. FoxH1 and T-box proteins directly bind to the *cis*-regulatory region of zygotic *VegT*. (A-C)** *The loss of FoxH1 or T-box sites decreases the ratio of mesoderm-specific* EGFP *reporter gene expression. Diagram of the deletion* **(A)** *and mutation* **(B)** *constructs for transgenesis. 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 made for transgenesis and EMSAs.* **(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 radioisotope-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. 4L). 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/-264), -2420Δ(-2035/-545+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.*,

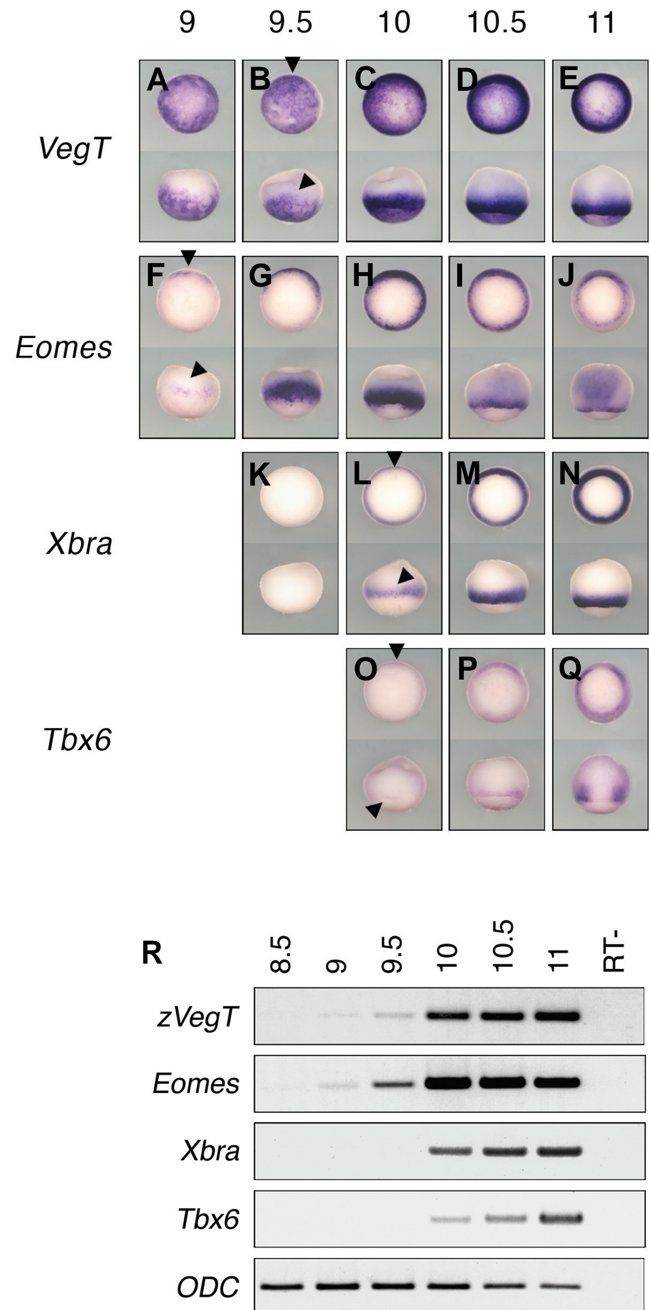


**Fig. 6. Comparative gene expression patterns of the T-box genes in *X. laevis* embryos. (A-Q)** *Spatial expression of* zVegT **(A-E)**, Eomes **(F-J)**, Xbra **(K-N)**, *and* Tbx6 **(O-Q)** *was detected by WISH from stage 9 to stage 11. Arrowheads indicate the initial zygotic expression of individual genes. The* VegT *antisense probe recognized both* mVegT *and* zVegT*. The top and bottom panels show the vegetal view with dorsal side up and dorsolateral view, respectively.* **(R)** *RT-PCR reveals temporal expression of T-box genes.*

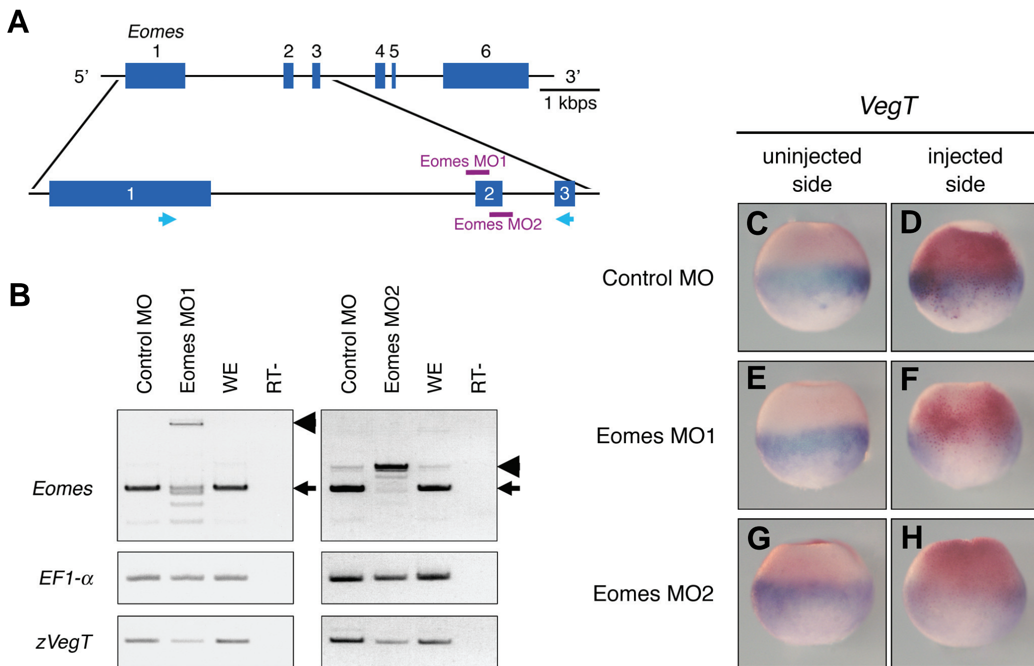


**Fig. 7. Eomesodermin is essential for expression of zygotic *VegT*. (A)** *Diagram of the exon-intron structure of* Eomes *and design of two* Eomes *splice-inhibiting antisense MOs. The* X. tropicalis Eomes *gene has 6 exons. The splicing sites targeted by the Eomes MO1 and Eomes MO2 (purple) were 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 *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 gal staining indicates the injected side. Lateral views are shown.*

2008). 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). To determine the efficiency of Eomes MO1 and Eomes MO2, embryos were injected with MOs, and collected at stage 10+ for RT-PCR analysis (Fig. 7B). In Eomes MOs injected embryos, the levels of normal splicing product were greatly reduced. The expression of *zVegT* was also remarkably repressed in these embryos (Fig. 7B). We also used WISH to examine whether Eomes MOs cause downregulation of *zVegT* expression *in vivo*. Injection of Control MO had no effect on *zVegT* expression, whereas injection of Eomes MO1

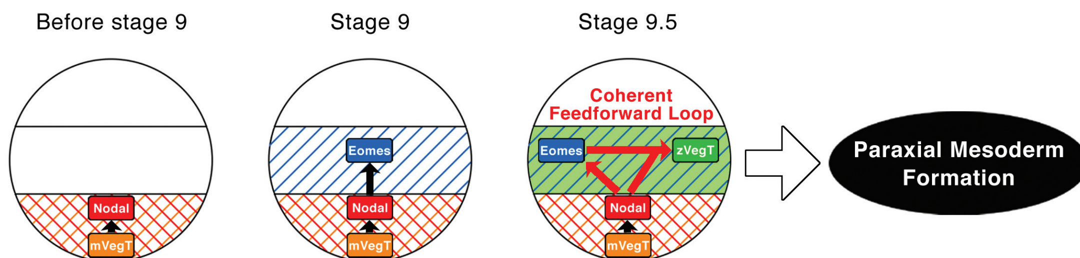


**Fig. 8. Model of zygotic *VegT* regulation by Eomesodermin and Nodal signaling.** mVegT *induces expression of* Xnrs *before stage 9. Nodal signaling induces expression of* Eomes *at stage 9. Then, Eomes and Nodal signaling function in a coordinated, simultaneous manner to activate the expression of* 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 *Tbx6L/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 evolutionally 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. SU5402 (Calbiochem) was obtained commercially.

### *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'-gggacgcgtGTTCCTCACAGCTAATCCACC-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+N+M that had two restriction sites, *Not*I 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, -6156Δ(-2397/-1769), -2420Δ(-1749/-1040), -2420Δ(-1749/-545), -2420Δ(-1749/-122), -2420Δ(-2035/-545), -2420Δ(-2035/-290), -2420Δ(-2035/-264), -2420Δ(-2176/-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-CerS (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'-TGGATTAGTTTAGGAAC-3'
reverse, 5'-CGGATCTTACACTGAGGA-3';
*FGF3* forward, 5'-GGGTTTACGAACATCTTGGAGG-3'
reverse, 5'-CCACGAACTCACATTCAGGGTTG-3';
*eFGF* forward, 5'-CGGACGGAAGGATAAATGGC-3'
reverse, 5'-CGTGGCAAGAAATGGGTCAG-3';
*Eomes* forward, 5'-CGGGTGCATGTAAGAGAAGG-3'
reverse, 5'-GGCTTTAACACTTTCATGGC-3';
*Tbx6* forward, 5'-CCAGAAGCAAAAGTCACAGCG-3'
reverse, 5'-CAATGAGAAAGACCCAGCAGTTG-3';

for *X. tropicalis*,
*zVegT* forward, 5'-GCTGTGAGGAACATGCACTC-3'
reverse, 5'-TGAAACCTGGGCTTGTAGCG-3';
*Eomes* forward, 5'-TGGTGAGTTTGAGTTTCCCG-3'
reverse, 5'-CAGTGTATCCCGCAGGAGGA-3';
*EF-1α* forward, 5'-TGTAGGAGTCATCAAGGCGGTC-3'
reverse, 5'-ACAGATTTTGGTCAAGTTGCTTCC-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 *Sfi*I. Transgenic embryos were fixed at stage 11 and expression of *EGFP* was detected by WISH as described by Harland (1991). DIG-labeled antisense RNA probes were synthesized with following plasmids as templates: pBS(SK-)-Apod (*zVegT*) (Tanegashima *et al.*, 2000), pNTM2/Bsc (*Eomes*) (Ryan *et al.*, 1996), pSP73-Xbra (Smith *et al.*, 1991), pCS2-Tbx6 (Uchiyama *et al.*,

2001), pBSIISK+d2EGFP. The DNA fragment containing d2EGFP-1 was excised from pd2EGFP-1 with *BamH*I and *Not*I, and inserted into *BamH*I and *Not*I-double digested pBluescript II SK- and named pBSIISK+d2EGFP. For immunochemistry, the anti-sarcomeric myosin monoclonal antibody, MF-20 (Bader *et al.*, 1982), and the notochord specific monoclonal antibody, Tor70 (Kushner, 1984), were diluted 1:2 and 1:5 of the initial concentration, respectively. The secondary antibody was a Goat Anti-Mouse IgG+IgM, Alkaline Phosphatase Conjugate (BIOSOURCE) diluted 1:2000 of the initial concentration, and BM-purple (Roche) was used for staining. The embryos were cleared using Murray's clearing medium (benzyl alcohol:benzyl benzoate, 1:2).

***Morpholino oligonucleotide (MO)***

Antisense MOs (Gene Tools, LLC) were designed for the blocking translation or nuclear processing of *X. tropicalis* transcripts. The sequences of MOs were as follows; zygotic VegT MO (zVegT MO), 5'-CATCCGGCAGAGAGTGCATGTTCCT-3'; Eomesodermin MO1 (Eomes MO1), 5'-GAACATCCTCCTGCAAAGCAAAGAC-3'; and Eomesodermin MO2 (Eomes MO2), 5'-TGCGCGGCACCCACCTTGCATATTA-3'; standard control MO (Control MO), 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

***In vitro transcription/translation and electrophoretic mobility shift assay (EMSA)***

Proteins for the EMSA were transcribed and translated from pCS2-FoxH1-myc, pCS2-zVegT-myc, pCS2-Eomes-myc, pCS2-Xbra-myc, and pCS2-Tbx6-myc by using the TNT SP6 Quick Coupled Transcription/ Translation Systems (Promega). The expression of these proteins was confirmed by Western blot analysis (data not shown). DNA probes used for EMSA were radioisotope-labeled with [$\gamma$-$^{32}$P]ATP (GE Healthcare) by T4 polynucleotide kinase (TaKaRa). The radioisotope-labeled probes were purified using a DyeEx 2.0 spin kit (Qiagen) and then annealed. Competitors of wild-type (wt) and mutant-type (mut) were incubated without T4 polynucleotide kinase and annealed. The DNA-protein binding reactions were performed at room temperature for 30 min in binding buffer specialized for the FoxH1 protein [20 mM HEPES (pH 7.9), 8% glycerol, 2 mM EDTA, 2 mM DTT, 7.2 mM $MgCl_2$, 0.5 mg/ml BSA] or T-box protein [25 mM HEPES (pH 7.0), 10% glycerol, 0.25 mM EDTA, 1 mM DTT, 75 mM KCl, 1 mM $MgCl_2$, 0.5 mg/ml BSA, 0.1% NP-40]. The DNA-protein mixture was separated by electrophoresis and analyzed by using BAS-5000 (Fujifilm). The sense strand sequences for probe or competitor (FBS1, FBS2, FBS3, and TBS1-5) used are shown in Fig. 4C and D. The mutated TBS1-5 competitor contained mutations in all five T-box binding sites. The polyanionic polymer poly(dI-dC) was used as the nonspecific competitive probe.

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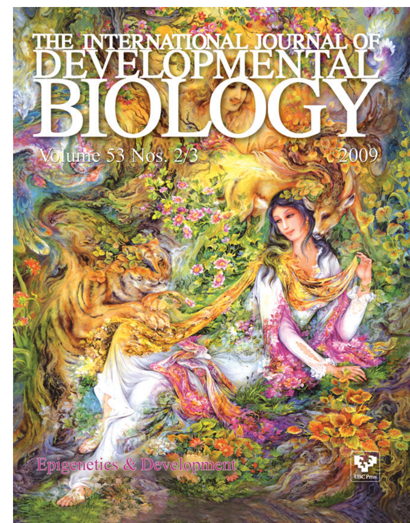


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