

# Tead proteins activate the *Foxa2* enhancer in the node in cooperation with a second factor

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## Summary

The cell population and the activity of the organizer change during the course of development. We addressed the mechanism of mouse node development via an analysis of the node/notochord enhancer (NE) of *Foxa2*. We first identified the core element (CE) of the enhancer, which in multimeric form drives gene expression in the node. The CE was activated in Wnt/ $\beta$ -catenin-treated P19 cells with a time lag, and this activation was dependent on two separate sequence motifs within the CE. These same motifs were also required for enhancer activity in transgenic embryos. We identified the Tead family of transcription factors as binding proteins for the 3' motif. Teads and their co-factor YAP65 activated the CE in P19 cells, and binding

of Tead to CE was essential for enhancer activity. Inhibition of Tead activity by repressor-modified Tead compromised NE enhancer activation and notochord development in transgenic mouse embryos. Furthermore, manipulation of Tead activity in zebrafish embryos led to altered expression of *foxa2* in the embryonic shield. These results suggest that Tead activates the *Foxa2* enhancer core element in the mouse node in cooperation with a second factor that binds to the 5' element, and that a similar mechanism also operates in the zebrafish shield.

Key words: Tead, Node, *Foxa2*, Notochord, Enhancer, Mouse

## Introduction

During mouse embryogenesis, the organizer plays a central role in the establishment of the correct body plan (Davidson and Tam, 2000; Niehrs, 2004; Tam and Behringer, 1997). The organizer is formed at the anterior end of the primitive streak of the gastrula embryo at embryonic day (E) 6.5, and is maintained throughout embryogenesis. However, the activity of the organizer changes during development, as does its constituent cell population and cell fates. The cells constituting the early gastrula organizer (EGO) and mid-gastrula organizer (MGO) later form the head mesoderm and anterior axial mesoderm, respectively, and together regulate head development (Kinder et al., 2001; Tam and Steiner, 1999). These changes in cell population are the result of a dynamic process in which cells migrate into and out of the organizer in a coordinated fashion (Kinder et al., 2001). After E7.0 or the early bud (EB) stage, organizer activity is localized to a morphologically distinctive structure, the node, which regulates trunk development, including the laterality of the embryo (Beddington, 1994; Davidson et al., 1999; Klingensmith et al., 1999). The node contains the notochord progenitor cells and continuously produces notochord cells. The notochord progenitor cells remain in the node and later localize to the posterior end of the notochord in the tailbud

(chordneural hinge) (Beddington, 1994; Cambray and Wilson, 2002; Tam et al., 1997).

Analysis of lower vertebrates revealed the mechanisms of the initial stage of organizer development. In zebrafish and *Xenopus* embryos, dorsal activation of Wnt pathway followed by TGF $\beta$ /nodal signaling was found to promote organizer development (De Robertis et al., 2000; Hibi et al., 2002; Moon and Kimelman, 1998). In chick embryos, cooperation of these two signals continuously activates organizer genes in cells passing through the anterior end of the primitive streak (Joubin and Stern, 1999). The development of the EGO/MGO in mouse embryos may also operate by a similar mechanism (Tam and Gad, 2004). However, little is known about the mechanism of node formation and maintenance in mouse embryos.

*Foxa2* (formerly known as HNF3 $\beta$ ) is a key transcription factor for the development of midline signaling centers, including the gastrula organizer, node, notochord and floor plate of the neural tube (Ang and Rossant, 1994; Sasaki and Hogan, 1994; Weinstein et al., 1994) (see Fig. S1 in the supplementary material). As part of an effort to analyze the regulation of *Foxa2* expression, we previously identified two enhancers that drive gene expression in the node/notochord and the floor plate, respectively (Sasaki and Hogan, 1996). Analysis of the node/notochord enhancer in multiple species

led to the identification of an evolutionarily conserved sequence motif, CS3, which is essential for enhancer activity (Nishizaki et al., 2001). Here, we identified the Tead family transcription factors as proteins that bind to CS3.

Tead family transcription factors all contain a DNA-binding domain called a TEA domain, and consist of four members (Tead1-Tead4) in both mouse and human (Jacquemin et al., 1998; Kaneko and DePamphilis, 1998). The founding member of this family, Tead1 [also known as transcriptional enhancer factor 1 (TEF-1)], was originally identified as an activator of simian virus 40 (SV40) enhancer (Davidson et al., 1988; Xiao et al., 1991). A *Drosophila* Tead protein, Scalloped (Sd), interacts with a co-activator protein, Vestigial (Vg), and regulates wing development (Halder et al., 1998; Simmonds et al., 1998). Vertebrate Tead proteins also require co-factors to act as activators, and the candidates are the four Vg homologs (Maeda et al., 2002; Vaudin et al., 1999) and Yes-associated protein 65 (YAP65) (Maeda et al., 2002; Vassilev et al., 2001; Vaudin et al., 1999). Several other mechanisms are also suggested for regulation of Tead activity, including interaction with other transcription factors and modification by protein kinases (Gupta et al., 2001; Gupta et al., 1997; Jiang et al., 2001; Thompson et al., 2003). *Tead* genes are expressed widely, from preimplantation embryos to various adult tissues, with distinct patterns (Jacquemin et al., 1998; Kaneko et al., 1997). Tead proteins are suggested to be involved in activation of the cardiac and skeletal muscle genes, CTP:phosphocholine cytidylyltransferase (*Pcyt* – Mouse Genome Informatics) and *Pax3* in neural crest cells (Jiang et al., 2000; Milewski et al., 2004; Stewart et al., 1994; Sugimoto et al., 2001), and *Tead1* mutant embryos die between E11 and 12 due to resulting heart defects (Chen et al., 1994). However, the roles played by *Tead* genes during early embryogenesis have not yet been revealed.

In this study, we first showed that the core element (CE) of the *Foxa2* enhancer drives gene expression in the node. Two transcription factors activate the CE in a cooperative fashion, and Tead proteins are one of these factors. The Tead-binding site in the CE was essential for node/notochord enhancer (NE) activity, and inhibition of Tead function in mouse embryos disturbed notochord development. In zebrafish embryos, manipulation of Tead activity changed the expression of *foxa2*. These results suggest that the key mechanism of *Foxa2* expression in the node/notochord is activation of the enhancer core element in the node by Tead in cooperation with an unidentified transcription factor, and that a similar mechanism also operates in the embryonic shield of zebrafish.

## Materials and methods

### Production of transgenic mice

Mutations were introduced into the NE enhancer by a PCR-based method. The mutated enhancer or eight copies of double-stranded CE oligonucleotide 5'-ccTTTGCAAGGAAGGGAGAAATTCACCA-3' 3'-AAACGTTCCCTTCCCTCTTTAAGGTGGTgg-5' was cloned into ASShspLacZpA reporter cassette (Sasaki and Hogan, 1996). Transgenic mice were produced by pronuclear injection of transgene DNA into (C57BL/6×C3H/He) F<sub>2</sub> or ICR-fertilized eggs (Hogan et al., 1994). Mouse embryos were staged on the basis of their morphology (Downs and Davies, 1993). Identification of transgenic embryos and whole-mount staining of embryos for β-galactosidase activity were performed as described previously (Sasaki and Hogan, 1996).

### Yeast one-hybrid screening

Yeast one-hybrid screening was performed using the MATCHMAKER One-Hybrid System (Clontech) following the manufacturer's protocol. A tetramer of double-stranded CE-oligonucleotide 5'-TTTGCAAGGAAGGGAGAAATTCACCAc-3' 3'-gAAACGTTCCCTTCCCTCTTTAAGGTGGT-5' was used as a target site, and was cloned into the vectors pHisi-1 and pLacZi. Clones (4.5×10<sup>6</sup>) of a mouse 7-day embryo MATCHMAKER cDNA library (Clontech) were screened in the presence of 10 mmol/l 3-aminotriazole. The cDNA insert of the plasmid DNA was amplified from positive yeast colonies by PCR, followed by sequence determination and BLAST search against the GenBank database. Plasmid DNAs were recovered from representative clones for subsequent analysis.

### Gel mobility shift assay

Expression plasmids for Tead and Rel were constructed by cloning the coding sequences of respective cDNAs into pcDNA3.1-His (Invitrogen) or pCMV/SV-Flag1 (Kamachi et al., 2000). Tead1 and Tead3 cDNAs were gifts from Dr H. Ohkubo (Yasunami et al., 1996). The resulting plasmids were used for production of proteins via the TnT T7 coupled reticulocyte lysate system (Promega). Gel mobility shift assay was performed as described (Sasaki et al., 1997).

### Transfection assay

Reporter plasmids were constructed by cloning the NE enhancer fragment or eight copies of CE oligonucleotide sequences into pδ51-LucII (Kamachi et al., 2000). The 7×Tcf-BS reporter and stabilized β-catenin expression vector are described (Takahashi et al., 2000; Ueda et al., 2002). Wnt expression plasmids were gifts from Dr S. Nakagawa (Kubo et al., 2003). The Yap65 expression plasmid was created by cloning the coding sequence of Yap65 into pcDNA3. For transfection, P19 cells were plated into 6-well plates at a density of 2×10<sup>5</sup> cells/well 4 hours before transfection. A mixture of Fugene 6 (Roche) and DNA consisting of effector (0.4 μg), reporter (0.4 μg) and reference (pCS2-β-gal, 0.1 μg) was added to the cells and was cultured for 14 to 48 hours, depending on experiments. Preparation of lysates, luciferase and β-galactosidase assays were as described (Sasaki et al., 1999). Luciferase activities were normalized by β-galactosidase activities.

### In situ hybridization

In situ hybridization of whole-mount tissue or paraffin sections of mouse and zebrafish embryos was performed as described previously (Henrique et al., 1995; Nikaïdo et al., 1997; Wilkinson, 1992).

### Electroporation

Electroporation and in vitro culture of mouse embryos were performed based on procedures described previously (Davidson et al., 2003; Sturm and Tam, 1993). Briefly, each embryo was soaked in a 10-μl drop of Tyrode's Ringer solution containing pDISP-SEAP (an expression vector for the membrane-tethered form of human placental alkaline phosphatase; a gift from Dr T. Yamamoto) and either pCS2-Tead2-EnR or pCS2 (0.5 μg/μl each) for 10 minutes, followed by electroporation with five pulses of 15V for 50 mseconds using a square-wave pulse generator (CUY-21; BEX). The distance between electrodes was 3 mm. After 16 hours' culture, embryos were stained for both β-galactosidase and alkaline phosphatase activities (Itasaki et al., 1996).

### Zebrafish embryos

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of fish with the AB/India genetic background. Capped mRNAs, prepared as previously described (Koshida et al., 1998; Makita et al., 1998), were diluted to the appropriate concentration with MilliQ water containing 0.05% Phenol Red and injected into 1-cell embryos. Approximately 400-500 pl of RNA was injected into each embryo.

## Results

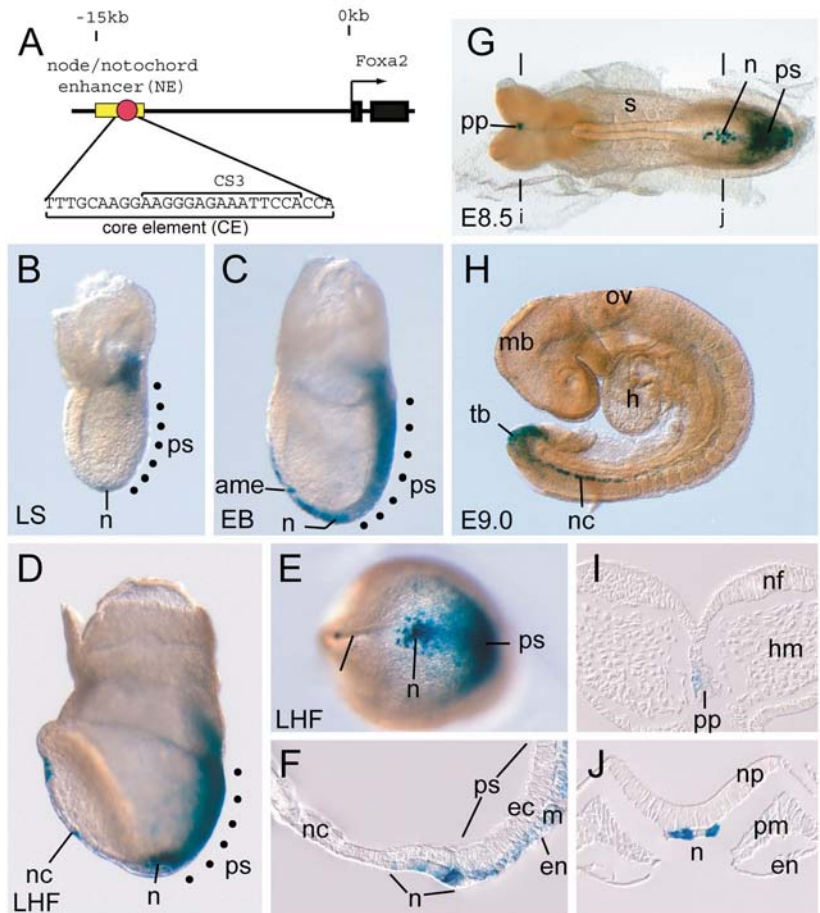
### Identification of the core region of the *Foxa2* enhancer

Previously, we identified the *Foxa2* enhancer (NE), which drives gene expression in the node and notochord but not in the EGO/MGO, and also identified a 14-bp sequence motif named CS3 that is essential for the enhancer activity (Nishizaki et al., 2001; Sasaki and Hogan, 1996) (Fig. 1A; Fig. 3C). To determine whether a short DNA fragment containing CS3 is sufficient for gene expression in the node and/or notochord, we made transgenic mouse embryos that utilize eight copies of a 27-bp DNA fragment of the enhancer straddling CS3 to drive expression of  $\beta$ -galactosidase (Fig. 1A). Initially, we produced primary transgenic E7.5-9.0 embryos, and subsequently we established a transgenic line showing essentially the same pattern of  $\beta$ -galactosidase expression at E7.5-8.5. This transgenic line was used for more detailed analyses. While *Foxa2* is expressed in the gastrula organizer at E6.5, the early streak (ES) stage (see Fig. S1A in the supplementary material),  $\beta$ -galactosidase activity was not observed in this tissue (data not shown). But upon formation of the node at the distal tip of embryos at late streak (LS) stage,  $\beta$ -galactosidase expression initiated in the node and this expression extended anteriorly at early bud (EB) stages (Fig. 1B,C). During this period, a second  $\beta$ -galactosidase expression domain, which does not correlate with the expression pattern of endogenous *Foxa2* (see Fig. S1A-D in the supplementary material), appeared at the posterior proximal portion of the primitive streak and expanded distally to cover the entire streak (Fig. 1B,C). Expression in the node and primitive streak continued up to E8.5 (Fig. 1D,E,G and data not shown).  $\beta$ -galactosidase expression in the bilayered node was restricted to the ventral layer continuous with the surrounding endoderm (Fig. 1F,J). Around the primitive streak,  $\beta$ -galactosidase activity was detected in the mesoderm and ectoderm, and in the ectoderm the expression was restricted to the region closest to

the primitive streak (Fig. 1F and data not shown). At E8.5, the prechordal plate also expressed  $\beta$ -galactosidase (Fig. 1G,I). Between E9.0 and 9.5, when the node is not morphologically identifiable,  $\beta$ -galactosidase activity was localized to the tailbud and posterior notochord, and weak and scattered activity was observed in the notochord and somites (Fig. 1H and data not shown). Endogenous *Foxa2* is not expressed in the tailbud mesoderm (see Fig. S1E in the supplementary material). In summary, this 27-bp fragment was sufficient to drive expression in the node; thus, we refer it as the core element (CE) of the enhancer (Fig. 1A).

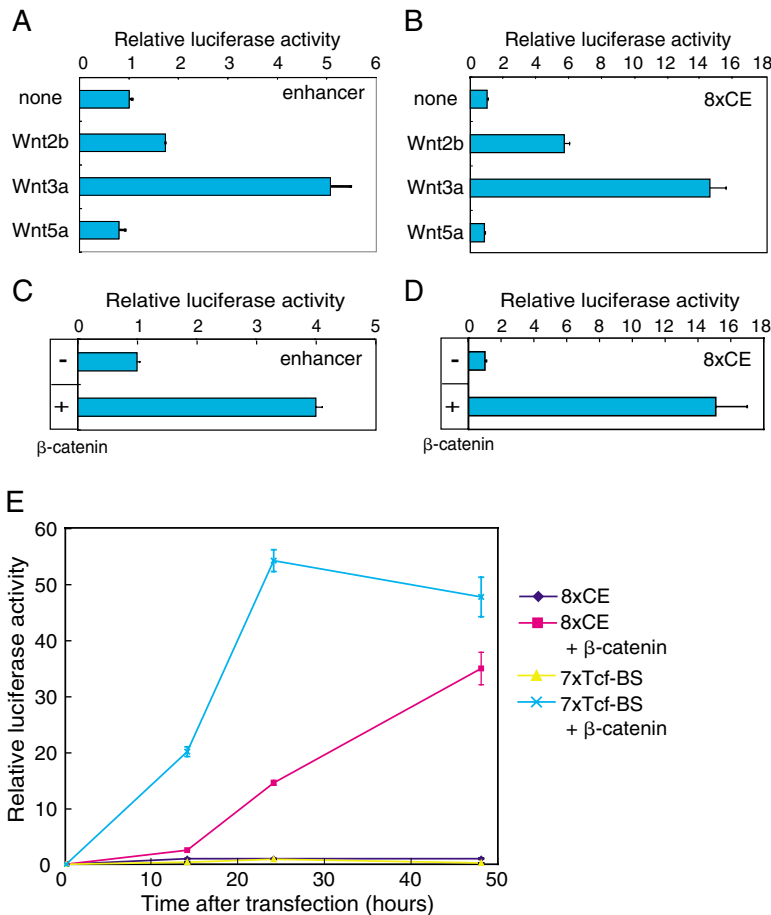
### The CE is activated in Wnt/ $\beta$ -catenin-treated P19 cells with a time lag

The  $\beta$ -galactosidase expression pattern of CE transgenic embryos in the node and primitive streak resembles those of the Wnt/ $\beta$ -catenin reporter transgenic lines TOPGal (Maretto et al., 2003; Merrill et al., 2004), suggesting a potential link between Wnt signaling and CE activation. To test this possibility, we used a co-transfection assay in a mouse embryonic carcinoma cell line, P19. Two days after transfection, a Wnt3a expression plasmid strongly activated, and a Wnt2b plasmid weakly activated, the enhancer-luciferase reporter, while Wnt5a, which does not activate the canonical Wnt pathway, did not alter reporter expression (Fig. 2A). A similar but stronger response was observed using a reporter with eight copies of CE (8 $\times$ CE, Fig. 2B). Activation of both reporters by expression of stabilized  $\beta$ -catenin, a downstream



**Fig. 1.** Activity of the core element of the *Foxa2* node/notochord enhancer in transgenic mouse embryos. (A) Schematic representation of the mouse *Foxa2* enhancer. The node/notochord enhancer is located upstream of the transcription initiation site (yellow square). CS3 (red circle) is essential for enhancer activity. A 27-bp DNA fragment straddling CS3 is the core element (CE) of the enhancer. (B-J) Distribution of  $\beta$ -galactosidase activity in transgenic mouse embryos that express *LacZ* under the control of eight copies of the CE. Whole-mount staining of LS (B), EB (C), LHF (D,E), E8.5 (G) and E9.0 (H) stage embryos. Sagittal section of an LHF embryo crossing through the node (F), and cross-sections of an E8.5 embryo (I,F). Approximate positions of sections shown in panels I and F are indicated in panel G by i and j, respectively. ame, axial mesoendoderm; ec, ectoderm; en, endoderm; h, heart; hf, headfold; hm, head mesenchyme; m, mesoderm; mb, midbrain; n, node; nc, notochord; nf, neural fold; np, neural plate; ov, otic vesicle; pm, presomitic mesoderm; pp, prechordal plate; ps, primitive streak; s, somite; tb, tailbud.





**Fig. 2.** Activation of the node/notochord enhancer and CE in Wnt/ $\beta$ -catenin treated P19 cells. Expression of reporters containing the enhancer (A) or 8 copies of CE (8 $\times$ CE) (B) at 48 hours after co-transfection of Wnt expression plasmids. Expression of reporters containing the enhancer (C) or 8 $\times$ CE (D) at 48 hours after co-transfection of stabilized- $\beta$ -catenin expression plasmid. (E) Timecourse of reporter activation after co-transfection with stabilized  $\beta$ -catenin expression plasmid. Luciferase activities were normalized to the activity of 8 $\times$ CE reporter without  $\beta$ -catenin at each time point. In Figs 2, 3, and 4, the results of luciferase assays represent the average of two samples with standard deviations.

effector of canonical Wnt signaling, further indicated that the enhancer is a downstream target of the canonical Wnt pathway (Fig. 2C,D).

The CE does not contain the recognition sequence of Tcf/Lef, direct downstream transcription factors of Wnt/ $\beta$ -catenin signaling, suggesting that the effect of Wnt on CE is either indirect or mediated by other downstream transcription factors. To distinguish these possibilities, we compared the timecourse of CE activation by stabilized  $\beta$ -catenin with the activation of a Tcf-binding site reporter construct (Tcf-BS). A reporter containing seven copies of a Tcf-binding site (7 $\times$ Tcf-BS) was activated as early as 14 hours after transfection, reaching a plateau at 24 hours. By contrast, expression of the 8 $\times$ CE reporter remained near basal levels at 14 hours post-transfection, and steadily increased from that point onward (Fig. 2E). This delay suggests that activation of the CE by Wnt/ $\beta$ -catenin signaling is an indirect event, probably

activated by a transcription factor induced in P19 cells following Wnt/ $\beta$ -catenin signaling.

### Two transcription factors cooperate in the activation of the CE by $\beta$ -catenin and in gene expression in the node and notochord

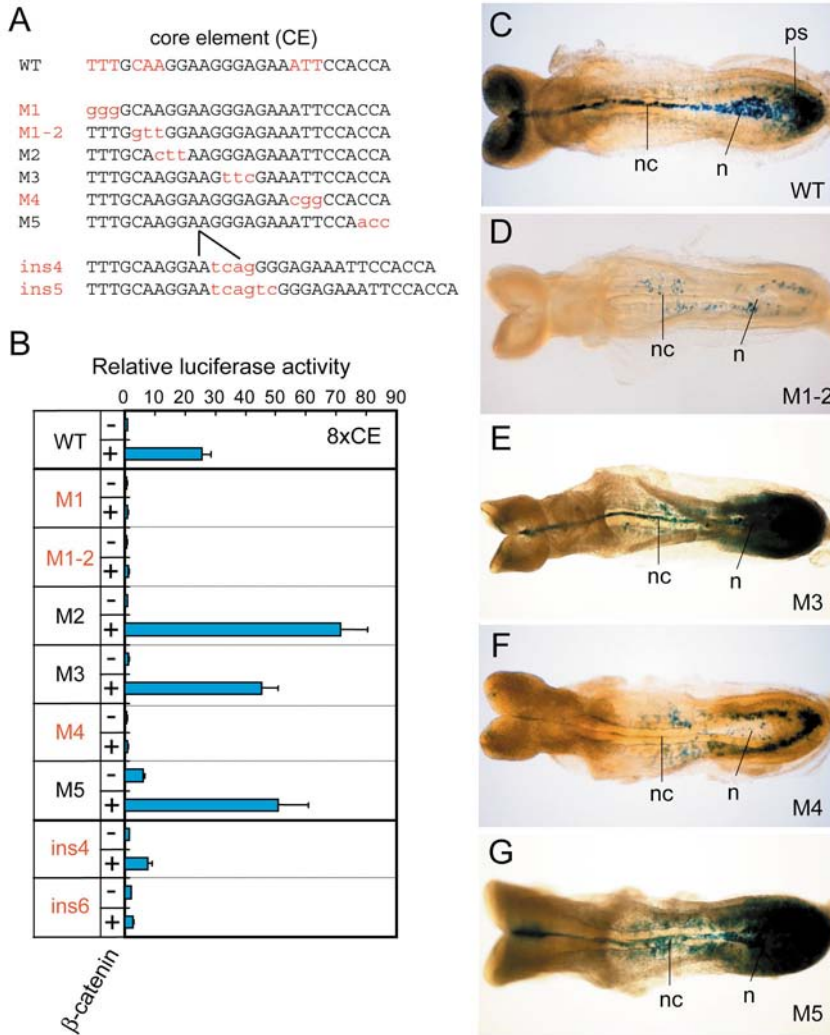
As the CE is activated efficiently in Wnt/ $\beta$ -catenin-treated P19 cells, we used this system to analyze the mechanism of CE activation. For this purpose we first studied the effect of altering the CE sequence on its activation in P19 cells. The CE mutants (M1, M2, M3, M4 and M5) are evenly-spaced trinucleotide mutants spanning the CE and beginning at its 5' end (Fig. 3A). Two disjunct mutations, M1 and M4, abolished activation, while the others had little or no effect on activation rate and/or basal expression (Fig. 3B). As confirmation, an additional mutant, M1-2, also failed to respond to  $\beta$ -catenin, confirming the importance of the 5' flanking region of CE (Fig. 3A,B). These results suggest that two transcription factors bind to the CE to activate it. To understand the relationship between these two transcription factors, we altered the distance between the two binding sites by inserting four or six nucleotides between them (ins4 and ins6: Fig. 3A). These alterations significantly reduced  $\beta$ -catenin-mediated activation of the CE (Fig. 3B), indicating the importance of the distance and/or topological relationship between the binding sites.

To understand the functional significance of these binding sites for in vivo gene expression, we constructed mutant enhancers containing the M1, M1-2 or M4 mutations, and tested their activities in  $\beta$ -catenin-treated P19 cells. M1-2 and M4 mutated enhancers lost their activity, while the M1 mutated enhancer was activated at a reduced level (data not shown). Thus, we selected the M1-2 and M4 mutations as representative mutations canceling enhancer activity in Wnt-treated P19 cells, and tested their effects on in vivo enhancer activity. No transgenic embryos harboring M1-2 or M4 mutated enhancers expressed the  $\beta$ -galactosidase transgene in the node or notochord (Fig. 3D,F; wild type, Fig. 3C). Instead, the  $\beta$ -galactosidase expression pattern in these embryos displayed a modified pattern, in that

the transgene-expressing cells were confined to the mediolateral portion of the posterior endoderm, resembling mutants with a deletion of CS3 (Nishizaki et al., 2001). Introduction of M3 and M5 mutations, which retained CE activity in P19 cells, to the enhancer led to normal transgene expression in the node/notochord (Fig. 3E,G). Taken together, these results suggest that activation of the CE through the cooperation of two transcription factors is essential for NE-mediated *Foxa2* gene expression in the node and notochord.

### Identification of Tead as a CE-binding protein

To identify the transcription factors acting on the CE, we performed yeast one-hybrid screening of an E7.0 mouse embryo cDNA library using the CE as a probe. Among the 70 positive clones obtained, 34 clones encoded Tead4, three clones encoded Tead2, and 22 clones encoded RelA. The remaining 11 clones did not encode transcription factors,



**Fig. 3.** Activation of CE in  $\beta$ -catenin-treated cells and node/notochord enhancer activity require two separate sequences within the CE. (A) Sequences of wild-type (WT) and mutated CEs (M1-5 and ins4/5). In the mutated CEs, the altered or inserted nucleotides are shown in red lower case. The nucleotides required for the activation are summarized in wild type sequence and denoted with red. (B) Expression of reporters containing wild-type and mutated 8 $\times$ CEs 48 hours after co-transfection of the stabilized  $\beta$ -catenin expression plasmid. (C) Transgenic embryos containing wild-type enhancer express  $\beta$ -galactosidase in the node and notochord, reflecting endogenous *Foxa2* expression accompanied by ectopic expression in the primitive streak region ( $n=9/11$ ). (D,F) Transgenic embryos with mutated enhancers lacking activity in  $\beta$ -catenin-expressing P19 cells did not show  $\beta$ -galactosidase expression in the node or notochord (D: M1-2;  $n=0/8$ , F: M4;  $n=0/10$ ). (E,G) Transgenic embryos with mutated enhancers with activity in P19 cells retained  $\beta$ -galactosidase expression in the node and notochord (E: M3;  $n=3/7$ , G: M5;  $n=6/8$ ). n, node; nc, notochord; ps, primitive streak.

in vivo. When the sequence surrounding the Tead-binding site of the CE was altered into that of the SV40 enhancer, GT-IIC (Fig. 4B), Teads, but not Rels, bound to this mutated CE (CE/GT-IIC) (Fig. 4C lanes 6, 12). Introduction of this CE/GT-IIC alteration into the NE led to retention of enhancer activity in transgenic mouse embryos (Fig. 4D). By contrast, alteration of the sequence surrounding the Rel-binding site of the CE into that of an NF- $\kappa$ B-binding site of the Ig- $\kappa$  enhancer (NF- $\kappa$ B-BS) produced a mutated CE (CE/NF- $\kappa$ B, Fig. 4B)

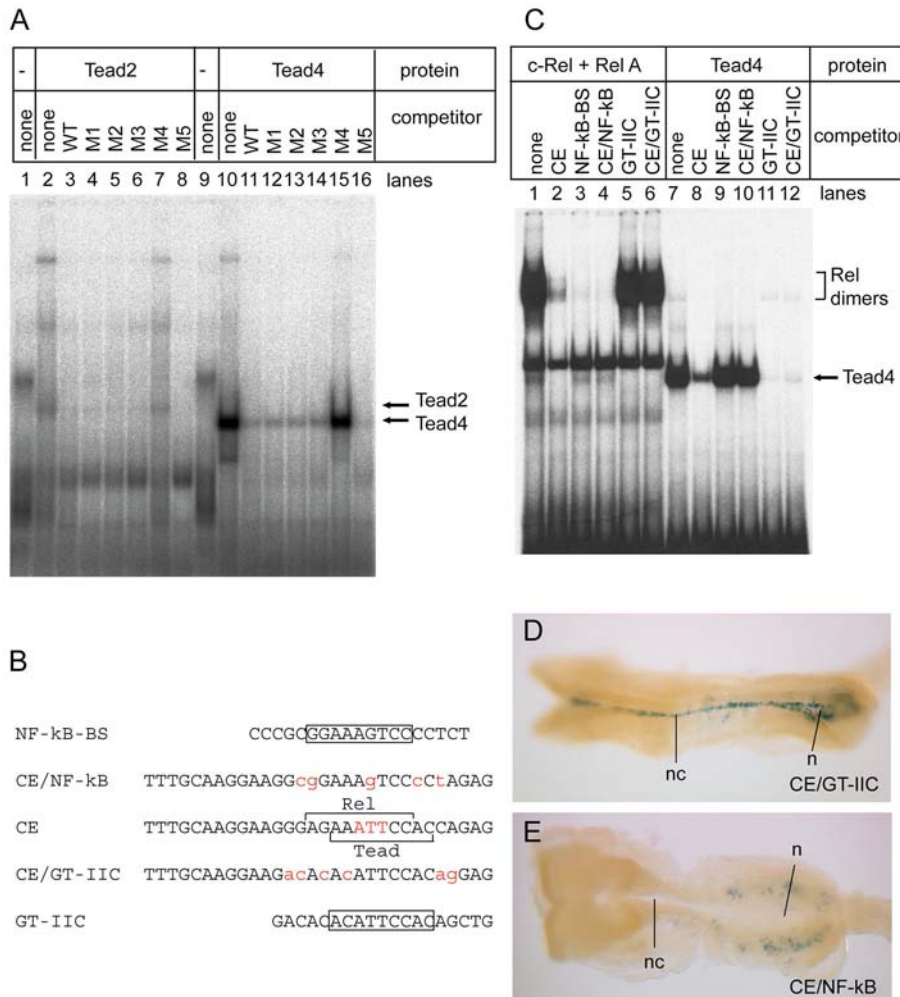
specifically bound by Rels (Fig. 4C, lanes 4,10). This mutated NE was not capable of driving expression in the node/notochord (Fig. 4E). Taken together, these results clearly indicate that Tead proteins are one of two factors that bind to the CE and drive expression from the *Foxa2* enhancer, both in Wnt/ $\beta$ -catenin-treated P19 cells and in the node/notochord in vivo.

### Expression of Tead in gastrulating mouse embryos

To understand which of the four mouse Tead family members is responsible for activation of *Foxa2*, we studied the expression of these genes between E6.5 and 9.0 by in situ hybridization. At E6.5, *Tead2* was expressed in the entire epiblast and mesoderm, but not in the extraembryonic ectoderm or visceral endoderm (Fig. 5B,C). *Tead3* and *Tead4* were expressed throughout the embryo in all germ layers, but with stronger expression in the extraembryonic region and proximal portion of the embryo than in the distal portion (Fig. 5D,E). Yap65, a co-factor of Tead, was expressed throughout the embryo. Expression of *Tead1* was not observed in the embryonic portion at this stage, either by in situ hybridization or RT-PCR (Fig. 5A and data not shown). At E7.5 and 8.5, in situ hybridization on both whole-mount and sections showed wide expression of all four *Tead* genes and *Yap65* (Fig. 5G-K and data not shown),

suggesting that they are pseudo-positives. A gel mobility shift assay showed that Tead4 and Tead2 proteins bound to CE in a sequence-specific manner, as shown by competition with an excess amount of unlabeled CE oligonucleotide (Fig. 4A, lanes 2, 3, 10, 11). Competition with a series of unlabeled mutant CEs (M1-M5, Fig. 3A) showed that the M4 mutation abolished binding of Tead2/4 (Fig. 4A, lanes 4-8, 12-16). Tead1 binds to the two unrelated sequence motifs of SV40 enhancers, GT-IIC and Sph-I/II (Davidson et al., 1988). The similarity of the wild-type sequence straddling the M4 mutation [5'-AAATTCAC-3' (complementary strand: 5'-GTGGAATTT-3')] with that of GT-IIC (5'-GTGGAATGT-3') suggests that Tead proteins recognize this sequence. The signal of the Tead2-DNA complex was weaker than that of the Tead4-DNA complex (Fig. 4A), probably reflecting the weaker DNA-binding activity of Tead2 as reported previously (Kaneko and DePamphilis, 1998).

Rel consists of a family of related proteins (Li and Verma, 2002), and various combinations of Rel protein dimers were also found to bind to the CE in a sequence-specific manner. However, this binding site overlapped with that of Tead (Fig. 4B,C lanes 1,2 and data not shown), and the following analyses revealed that Rel is not involved in activation of the enhancer



**Fig. 4.** Tead proteins bind the CE in a sequence-specific manner. (A) Gel mobility shift assay showing that Tead2 and Tead4 bind to the CE. Wild-type CE was used as a probe. Positions of the Tead-DNA complexes are indicated by arrows. Combination of protein and competitor are indicated above each lane. Sequences of the probe and competitors are described in Fig. 3A. (B) Sequences of the competitors used in panel C. Sequences of NF-kB-BS and GT-IIC were adapted from those described previously (Davidson et al., 1988; Fujita et al., 1992). Altered residues in CE/NF-kB and CE/GT-IIC are indicated in red lower case. Core recognition sequences of Rel and Tead are indicated by boxes. Position of the M4 mutation that is essential for NE activity is shown in red in the CE sequence. (C) Comparison of DNA-binding activities of Rel and Tead. Combinations of proteins and competitors are indicated above each lane. Positions of protein-DNA complexes are indicated on the right. (D) Transgenic embryos carrying a mutant enhancer in which CE was altered to CE/GT-IIC retained gene expression in the node and notochord ( $n=6/7$ ). (E) Transgenic embryos carrying a mutant enhancer in which CE was altered to CE/NF-kB lost gene expression in the node/notochord ( $n=0/4$ ). n, node; nc, notochord.

except for the extraembryonic visceral endoderm at E7.5 and the heart at E8.5, where *Tead2* was not expressed. The *Tead2* signal in the node and notochord was weaker compared with the surrounding tissues, but clearly stronger than non-expressing tissues (Fig. 5I,K). The expression of the other *Tead* genes was essentially uniform at these stages (data not shown). Thus, all *Tead* and *Yap* genes are expressed widely in the mouse embryo between E6.5 and 8.5, including the expression domain of *Foxa2*, suggesting that all of them may be involved in the activation of the *Foxa2* enhancer.

### Tead activates the CE in P19 cells

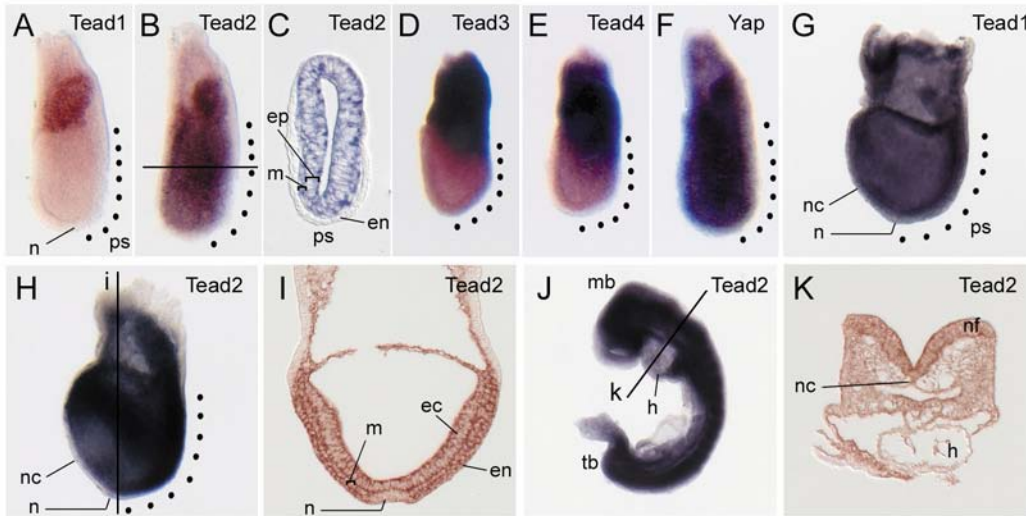
To study the regulatory activity of the Tead proteins, we used a co-transfection assay in P19 cells to determine their respective ability to activate a CE reporter construct. Tead1 or Tead4 activated the 8×CE reporter weakly, while Tead2 or Tead3 showed no activity (Fig. 6A). However, in the presence of Yap65, all four Tead proteins strongly activated the 8×CE reporter but not a control reporter without CE, suggesting that the Tead-Yap complex activates CE (Fig. 6A). When a construct was used containing M4 mutated CE, to which Tead proteins do not bind, Tead-Yap was not able to drive expression of the reporter, indicating that direct binding of Tead to CE is necessary for activation (Fig. 6B). The M1-2 mutated CE that is not activated in  $\beta$ -catenin-treated P19 cells was weakly activated by the Tead-Yap complex, indicating that, when overexpressed, the activator function of Tead-Yap becomes partially independent of the partner transcription factor that acts on the 5' side of the CE. These results together with the expression pattern of the *Tead* genes suggest that all Tead proteins may be involved in the activation of the CE in the node.

Activation of the CE in Wnt/ $\beta$ -catenin-treated P19 cells could be the result of upregulation of Tead activity. To test this possibility, we used a reporter containing eight copies of a Tead-binding site, GT-IIC. This reporter was not activated by  $\beta$ -catenin treatment of P19 cells, suggesting that  $\beta$ -catenin treatment does not affect the activity of Tead (Fig. 6C). Therefore, the expression and/or activity of the other transcription factor acting on the 5' side of CE is regulated in P19 cells in response to this stimulus. Considering the wide expression of *Tead* in embryos, this other factor is likely to play an important role in the activation of the CE in the node. We refer to this unidentified transcription factor as 'Partner Of Tead' (POT).

### The activator function of Tead is required for notochord development and NE activation in vivo

To directly access the role of Tead in regulating enhancer activity, we inhibited Tead activator function by locally expressing a repressor-modified Tead (Tead-EnR: a fusion protein of the DNA-binding domain of Tead2 and the repression domain of *Drosophila* Engrailed) in enhancer-*LacZ* transgenic mouse embryos by electroporation. When a control (empty) plasmid was electroporated into the distal tip of LHF stage embryos (the location of the node), the notochord and





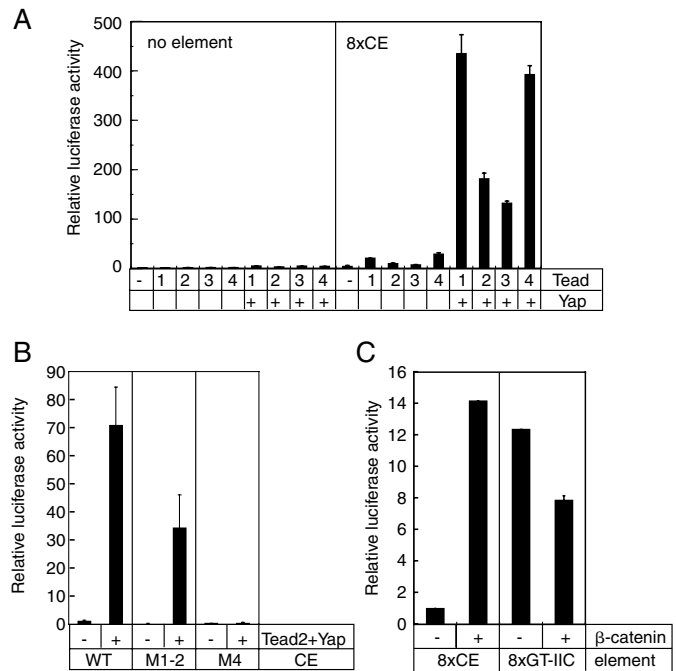
**Fig. 5.** *Tead* and *Yap65* are widely expressed during mouse embryogenesis. Whole-mount in situ hybridization of *Tead1* (A,G), *Tead2* (B,H,J), *Tead3* (D), *Tead4* (E) and *Yap65* (F) in mid- to late-streak (A-F), early head-fold (G,H) and E8.5 (J) stage embryos. (C) A section of a whole-mount stained embryo shown in panel B. Approximate position is indicated by the line in panel B. (I,K) In situ hybridization of *Tead2* on sectioned embryos. Approximate positions of sections are indicated in panels H and J.

endoderm cells efficiently incorporated the plasmid at E8.5, as revealed by the activity of a co-electroporated marker, membrane-tethered alkaline phosphatase (Fig. 7A). The notochord cells of these control embryos expressed both  $\beta$ -galactosidase and alkaline phosphatase (Fig. 7B). However, when the Tead-EnR expression plasmid was electroporated into the same site,  $\beta$ -galactosidase expression in the midline was significantly reduced (Fig. 7C). Eventual recovery of  $\beta$ -galactosidase expression in the posterior notochord may reflect the fact that notochord progenitors in the ventral node are replenished from the anterior primitive streak, as suggested by cell lineage analyses (Cambray and Wilson, 2002; Robb and Tam, 2004). Consistent with this notion, at 4 hours after electroporation, electroporated cells were observed in the notochord and the anterior portion of the node, but not in the posterior portion of the node (data not shown). In Tead-EnR-expressed embryos, most of the notochord cells were absent, suggesting that inhibition of Tead activator function disturbed proper notochord development as well as expression of the enhancer transgene (Fig. 7D; see Table S1 in the supplementary material). These results suggest that Tead activity at the *Foxa2* NE enhancer is necessary for expression of endogenous *Foxa2*, which is essential for notochord development.

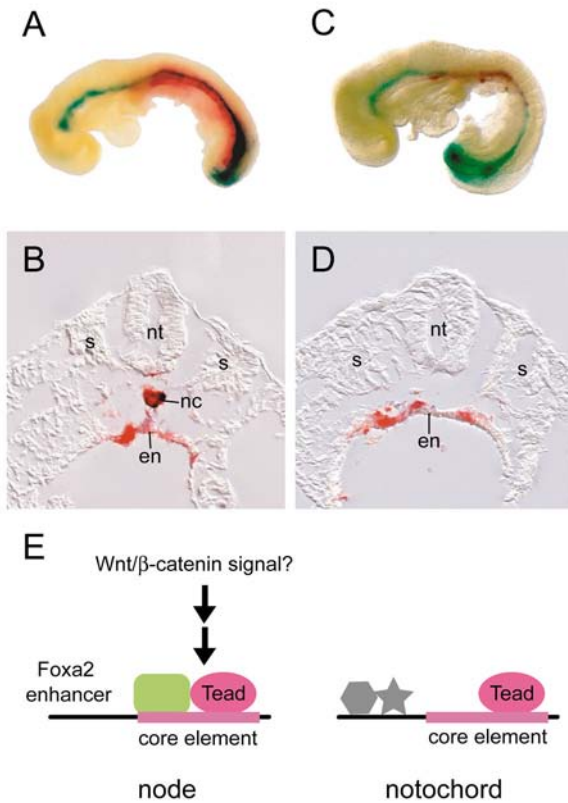
**Modified Tead alters *foxa2* expression domain in zebrafish embryos**

We previously showed that CS3, which contains the Tead-binding site, is evolutionarily conserved among the node/notochord enhancers of mouse, chicken and fish (Nishizaki et al., 2001). Therefore, we asked if Tead also regulates *Foxa2* in other species. To address this question, we used zebrafish embryos, and manipulated Tead activity by injecting variously modified forms of mouse *Tead2* RNA. We chose to adopt an overexpression strategy, because at least four *tead* genes are expressed in shield stage embryos (data not shown), making knockdown experiments with antisense morpholinos difficult to perform because of toxicity caused by high doses of morpholinos. Injection of *Tead2* RNA into 1-cell stage embryos did not disturb development for up to 2 days, even when 100 pg of RNA was injected (data not shown). As

various regulatory mechanisms of Tead proteins have been suggested, we hypothesized that the activity of the Tead proteins are also regulated during embryogenesis, and thus overexpression of wild-type Tead protein might not be expected to disturb development. To exaggerate the activator

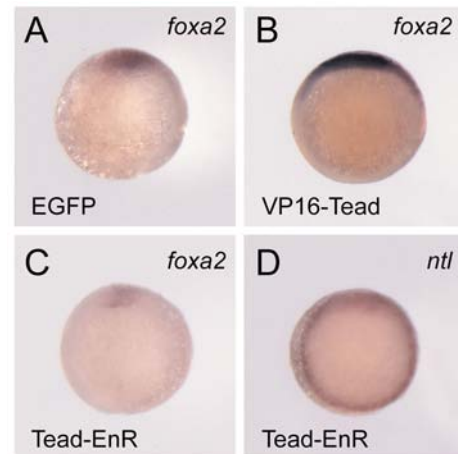


**Fig. 6.** Tead-Yap complex activates the CE in P19 cells. (A) Transfection assay showing activation of 8xCE reporter by co-transfection of Tead and Yap expression vectors. The reporter is indicated in the upper left corner of each panel. Combinations of Tead and Yap effectors are indicated below the panel. (B) The Tead binding site is required for activation of the CE by Tead-Yap complex. Mutation of the Tead-binding site (M4) abolished activation by Tead-Yap. (C) Transcriptional activity of Tead is not increased in  $\beta$ -catenin-expressed P19 cells. By contrast to the activation of 8xCE, the authentic Tead-binding site (8xGT-IIC) was not activated in  $\beta$ -catenin-expressed cells.



**Fig. 7.** Tead regulates NE enhancer activity and notochord development in vivo. Electroporation of control (A,B) and Tead-EnR expression plasmid (C,D) in enhancer-*LacZ* transgenic mouse embryos. Representative cross-sections of embryos in A and C are shown in B and D, respectively. Green and red represent NE enhancer activity and electroporated cells, respectively. (A,B) Control electroporation did not affect NE expression. (C,D) Tead-EnR repressed NE activity and notochord development. en, endoderm; nc, notochord; nt, neural tube; s, somite. (E) Model of *Foxa2* regulation in the node and notochord. In the node, a Wnt signal promotes formation of the Tead-POT complex on the core element of the enhancer. Once the cells migrate from node to notochord, POT disappears from the CE, and Tead together with other transcription factors (gray objects) continue the activation of the enhancer.

function of Tead proteins, we injected an RNA encoding VP16-Tead (a fusion protein of the activation domain of herpes simplex virus VP16 protein and the DNA-binding domain of mouse Tead2). This resulted in lateral expansion of the *foxa2* expression domain at the shield stage (Fig. 8B). To inhibit the activator function of Tead proteins, we injected the *Tead-EnR* RNA, which resulted in reduced expression of *foxa2* at the shield stage (Fig. 8C). Expression of the pan-mesodermal marker *Brachyury/no tail (ntl)* was not significantly affected, indicating that general mesoderm induction took place normally (Fig. 8D). These results suggest that Tead proteins regulate *foxa2* in the zebrafish shield. At later stages, both types of RNA-injected embryos showed various abnormalities unrelated to organizer/notochord development (data not shown). This may reflect the fact that Tead proteins also regulate multiple developmental processes other than organizer development after the shield stage.



**Fig. 8.** Tead regulates *foxa2* in the zebrafish shield. Expression of *foxa2* (A-C) or *ntl* (D) in shield-stage embryos injected with various RNAs. Injection of EGFP RNA (100 pg) did not alter *Foxa2* expression. Injection of VP16-Tead (B; 100 pg) expanded *Foxa2* expression ( $n=102/125$ ), while injection of Tead-EnR (C,D; 25 pg) reduced *Foxa2* expression (C;  $n=26/36$ ) without affecting expression of *ntl* (D;  $n=13/13$ ).

## Discussion

### *Foxa2* regulation in the node is distinct from that in gastrula organizers

We have shown that *Foxa2* expression in the node is determined by the core element (CE) of the node/notochord enhancer. Although *Foxa2* is expressed in both gastrula organizers (EGO and MGO) and the node, the enhancer and CE are active only in the node but not in the EGO/MGO, indicating that *Foxa2* regulation in the node is distinct from that in gastrula organizers (Sasaki and Hogan, 1993; Sasaki and Hogan, 1996). This difference may be a reflection of the differences between these cell populations. Lineage-tracing studies showed that the EGO/MGO is composed of a transient population of cells passing through the anterior end of the primitive streak, and it has been suggested that Wnt and TGFβ/nodal signals synergistically activate organizer genes in this dynamic population of cells, as shown for chicken embryos (Joubin and Stern, 1999; Tam and Gad, 2004). By contrast, the node is composed of a rather static population of cells, which includes notochord progenitors and continuously produces notochord cells (Beddington, 1994; Cambrey and Wilson, 2002; Tam et al., 1997). Activation of CE in Wnt/β-catenin-treated P19 cells, but not in nodal/smad2/FAST1-treated cells (data not shown), raises the possibility that *Foxa2* expression in the node is a downstream product of Wnt signaling. Although the effect of Wnt signaling is not direct, Wnt1- or Wnt3a-expressing P19 cells maintain an undifferentiated state unless exposed to differentiation conditions (Petropoulos and Skerjanc, 2002; Tang et al., 2002), suggesting that the effect of Wnt observed with P19 cells does not involve multiple steps of gene activation accompanying differentiation. Positive expression from a Wnt-responsive transgene, TOPGal, in the mouse node also suggests active Wnt signaling (Merrill et al., 2004). These observations suggest that *Foxa2* expression in the node is actively maintained by continuous Wnt/β-catenin



signaling rather than autoregulation of *Foxa2*, which is a known mechanism for formation of the floor plate (Sasaki and Hogan, 1994). The *Foxa2* enhancer in the node may be activated by Wnt proteins diffusing from the primitive streak, where Wnt3a, Wnt5a and Wnt5b are continuously expressed (Takada et al., 1994). Alternatively, Wnt8 and Wnt11, which are transiently expressed in the node between E7.0 and 8.0 (Bouillet et al., 1996; Kispert et al., 1996) and which activate the  $\beta$ -catenin pathway in *Xenopus* embryos (Tao et al., 2005), may be responsible.

### Tead and POT cooperatively activate *Foxa2* expression in the node

We showed that Tead and the unidentified transcription factor POT cooperatively activate the CE, and that this is the key mechanism of enhancer activation. Inhibition of Tead activity by Tead-EnR resulted in failure of notochord formation. This is consistent with the idea that the NE enhancer is the major driver of *Foxa2* expression in the node/notochord, and that *Foxa2* is essential for node/notochord development (Ang and Rossant, 1994; Weinstein et al., 1994). Evolutionary conservation of the Tead-binding site among the node/notochord enhancers of *Foxa2* in mouse, chicken and fish (Nishizaki et al., 2001) and mis-expression of *foxa2* in Tead manipulated zebrafish embryos suggest that *Foxa2* regulation by Tead is evolutionarily conserved and thus a fundamental mechanism. Whether or not POT is also involved in *Foxa2* expression in other species is a question best addressed following the molecular identification of POT.

The widespread expression of Tead and Yap suggests that spatially restricted activation of the CE is achieved by localized expression of POT. The most probable mechanism of CE activation is the induction of POT by Wnt expressed in the primitive streak. A number of transcription factors expressed in the node and primitive streak and/or induced by Wnt signaling in these tissues, e.g. Sp5, Cdx, Brachyury, and Evx (Dush and Martin, 1992; Ikeya and Takada, 2001; Yamaguchi et al., 1999), failed to activate CE in P19 cells or to bind to the CE in vitro (data not shown), suggesting that POT is likely to be a novel transcription factor acting in the node and primitive streak downstream of Wnt signaling.

### A model of *Foxa2* enhancer activation in the node and the notochord

The NE enhancer of *Foxa2* drives gene expression in both the node and the notochord while CE drives expression only in the node, suggesting that distinct mechanisms operate in these tissues. To summarize our results, we would like to propose a model of *Foxa2* enhancer activation in the node and the notochord (Fig. 7E). In the node, Wnt signaling promotes binding of Tead and POT to the CE, where they cooperatively activate the enhancer. Once the cells exit from the node to form the notochord, the CE is not sufficient to drive gene expression, probably because of the absence of POT. Tead may continue to bind to the CE and may cooperate with other transcription factors that bind outside the CE to activate the NE enhancer. Although we could not experimentally address the question of whether Tead activates the enhancer only in the node or in both the node and notochord, we prefer the latter idea, because Tead is expressed in both tissues.

One interesting observation is that, although the CE drives

gene expression only in the node, disruption of CE (either the Tead site or POT site) in the enhancer resulted in a loss of gene expression in both the node and notochord. This raised the suggestion of a possible link between enhancer activation in the notochord and its preceding activation in the node. If this is the case, activation of CE by the Tead-POT complex in the node may lead to altered chromatin structure and the recruitment of other transcription factors to the enhancer. In the notochord, these transcription factors and Tead would then cooperatively continue the activation. A similar two-step system of regulation was recently determined for left-side-specific expression of the *Pitx2* enhancer, which is transiently activated by nodal-stimulated FAST1 followed by maintenance by the widely expressed Nkx2 (Shiratori et al., 2001). Verification of this two-step model for NE enhancer regulation awaits future analysis.

### Evolutionarily conserved function of Tead in gene regulation

In *Drosophila*, the Tead protein Scalloped (Sd) forms a complex with its co-activator protein Vestigial (Vg) to regulate wing development (Halder et al., 1998; Simmonds et al., 1998). The Sd-Vg complex and other transcription factors cooperatively achieve wing-field-specific gene expression. For example, the Sd-Vg complex cooperates with Su(H) or Mad/Med to achieve Notch- or Dpp-signaling-regulated gene expression in the wing field, but none of these transcription factors drives gene expression by themselves in vivo (Guss et al., 2001). This is reminiscent of the role of Tead in *Foxa2* enhancer activation in mouse embryos. A multimer of the CE, which contains the binding sites of Tead and POT, efficiently activated gene expression in the node and primitive streak, but a multimer of Tead-binding sites (GT-IIC) (Davidson et al., 1988) alone did not produce reporter gene expression in transgenic mouse embryos (H.S., R.N. and H.S. unpublished). These observations suggest that these widely expressed Tead transcription factors play crucial roles to achieve spatiotemporally regulated gene expression by promoting the activity of other transcription factors acting downstream of specific morphogenetic signals. Considering the functional conservation of Tead between mouse and fly, it is of interest to know if any of the four mouse homologs of fly Vg (Chen et al., 2004; Maeda et al., 2002; Vaudin et al., 1999) are involved in the regulation of *Foxa2* enhancer and Tead function in mouse development.

### Conclusion

We showed that activation of the CE by Tead and POT in the node is the key mechanism of *Foxa2* enhancer activation in the node and notochord, and that CE activation is likely to be a downstream-regulated target of Wnt signaling. Synergistic action of Tead and POT suggests that the function of Tead transcription factors in in vivo gene regulation is highly evolutionarily conserved. Molecular identification of POT should help to address the mechanism of axis formation regulated by Wnt in the node, primitive streak and tailbud.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/21/4719/DC1>

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**Table S1. Effects of Tead-EnR on notochord development**

Plasmids	Total number	Notochord		
		Normal	Normal	Absent
		AP expression		
		Endoderm	Endoderm, Notochord	Endoderm
Control	6	2 (33.3%)	4 (67.7%)	0
Tead-EnR	16	6 (37.5%)	0	10 (62.5%)