CORRIGENDUM

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The name of the second author was incorrectly given as Atsushi Enomoto. The correct author list appears above.

The authors apologise to readers for this mistake.

The transcription factor FoxB mediates temporal loss of cellular competence for notochord induction in ascidian embryos

Hidehiko Hashimoto*, Atsushi Enomoto, Gaku Kumano and Hiroki Nishida

SUMMARY

In embryos of the ascidian *Halocynthia roretzi*, the competence of isolated presumptive notochord blastomeres to respond to fibroblast growth factor (FGF) for induction of the primary notochord decays by 1 hour after cleavage from the 32- to 64-cell stage. This study analyzes the molecular mechanisms responsible for this loss of competence and provides evidence for a novel mechanism. A forkhead family transcription factor, FoxB, plays a role in competence decay by preventing the induction of notochord-specific *Brachyury* (*Bra*) gene expression by the FGF/MAPK signaling pathway. Unlike the mechanisms reported previously in other animals, no component in the FGF signal transduction cascade appeared to be lost or inactivated at the time of competence loss. Knockdown of FoxB functions allowed the isolated cells to retain their competence for a longer period, and to respond to FGF with expression of *Bra* beyond the stage at which competence was normally lost. FoxB acts as a transcription repressor by directly binding to the cis-regulatory element of the *Bra* gene. Our results suggest that FoxB prevents ectopic induction of the notochord fate within the cells that assume a default nerve cord fate, after the stage when notochord induction has been completed. The merit of this system is that embryos can use the same FGF signaling cascade again for another purpose in the same cell lineage at later stages by keeping the signaling cascade itself available. Temporally and spatially regulated *FoxB* expression in nerve cord cells was promoted by the ZicN transcription factor and absence of FGF/MAPK signaling.

KEY WORDS: Competence, Embryonic induction, Notochord, Nerve cord, FoxB, FGF, Ascidians

INTRODUCTION

In animal development, inductive cell interactions are crucial for fate specification of various embryonic cell types. Occurrence of inductive events depends on the presence of not only an inductive signal but also on cellular competence, i.e. the ability of cells to receive and respond to inductive signals. These properties are temporally and spatially regulated in a coordinated manner, ensuring that tissues are induced at the right time and in the proper location. One important form of competence regulation is temporal loss of competence. During development, cells do not retain signal-receiving competence for a long time, and their competence is restricted to within a specific period in order to prevent further and inadequate induction. Such loss of competence has been observed in various systems such as induction of the mesoderm and nervous system in *Xenopus* embryos (Grainger and Gurdon, 1989; Sharpe and Gurdon, 1990; Servetnick and Grainger, 1991; Grimm and Gurdon, 2002), vulval induction in *Caenorhabditis elegans* (Hopper et al., 2000; Berset et al., 2001), as well as notochord and mesenchyme induction in ascidian embryos (Nakatani et al., 1996; Nakatani and Nishida, 1999; Kim et al., 2000).

The molecular mechanisms that underlie the loss of competence to respond to the TGFβ signal during mesoderm induction in *Xenopus* have been studied. In that system, the nuclear accumulation ability of activated Smad2, an intracellular signal transducer, is abolished (Grimm and Gurdon, 2002; Abe et al.,

2005). As to the loss of competence to respond to a signal-activating EGF receptor during vulval induction in *C. elegans*, temporal upregulation of the MAPK phosphatase LIP-1 and the tyrosine kinase ARK-1 negatively regulate the EGF-receptor signaling pathway (Hopper et al., 2000; Berset et al., 2001). However, details of the molecular mechanisms operating in other animals remain elusive.

The embryos of ascidians, a group of invertebrate chordates, would be a good model system for studying the molecular mechanisms involved in loss of cellular competence because of the considerable knowledge of notochord induction events that has already been accumulated. The ascidian embryo shows an invariant cleavage pattern and a fixed cell lineage (Fig. 1) (Conklin, 1905; Nishida, 1987). The cell fates of each single cell are mostly specified by the 110-cell stage (Nishida, 1987), and inductive events are initiated as early as the 32-cell stage. Induction of the primary notochord, in particular, has been studied in detail at the single-cell level. Fibroblast growth factor (FGF) induces the primary notochord fate in the vegetal hemisphere (Fig. 1) (Nakatani and Nishida, 1994; Nakatani et al., 1996). A6.2 and A6.4 blastomeres, named notochord/nerve cord precursors at the 32-cell stage, divide into notochord precursors and nerve cord precursors at the 64-cell stage. The notochord/nerve cord precursor blastomere receives the FGF signal and is polarized at the 32-cell stage. It then divides asymmetrically into a notochord precursor with an induced fate and a nerve cord precursor with a default fate. Experiments in which the notochord/nerve cord blastomeres isolated before reception of the FGF signal were recombined with inducer cells such as endoderm at various stages, ranging from the early 32-cell stage to the 64-cell stage, and those in which isolated cells were exposed to recombinant basic fibroblast growth factor (bFGF) at various stages, have indicated that the notochord/nerve cord

Department of Biological Sciences, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka 560-0043, Osaka, Japan.

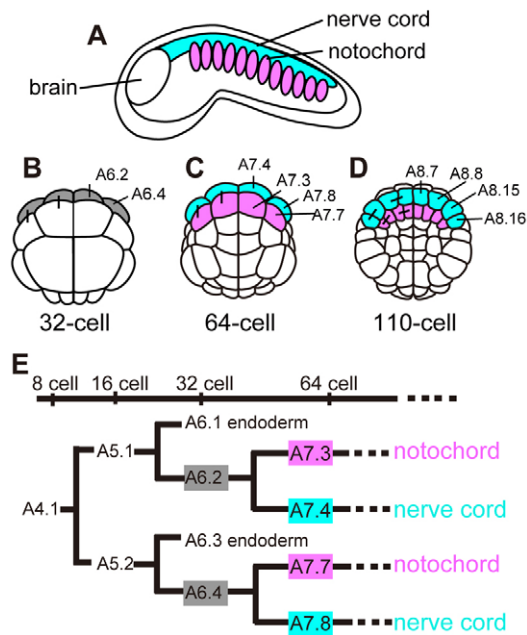*Author for correspondence (h.hashimoto@bio.sci.osaka-u.ac.jp)

Fig. 1. Cell lineages of notochord and nerve cord in ascidian embryos. Primary notochord and nerve cord lineage cells are colored pink and blue, respectively. (**A**) Tailbud embryo. Lateral view. (**B-D**) Embryos at the 32-, 64- and 110-cell-stage, respectively. Vegetal views. Anterior is upwards. Names of the blastomeres are given. Bars connecting two blastomeres indicate the orientation of the previous cleavage. The notochord/nerve cord precursors at the 32-cell stage, A6.2 and A6.4, are colored gray. (**E**) Lineage tree of the primary notochord and nerve cord.

precursors lose their competence to respond to the FGF signal at the 64-cell stage (Nakatani et al., 1996; Nakatani and Nishida, 1999). This system makes it possible to analyze the decay of competence at the single cell level.

The extrinsic FGF signal is transduced during ascidian notochord induction by the FGF receptor, Ras, MEK and MAPK (ERK1/2), and then phosphorylated MAPK is transferred to the nucleus, where it activates the Hr-Ets transcription factor. Activated Ets promotes the expression of the notochord-specific gene *Brachyury* (*Hr-Bra*) at the 64-cell stage (Yasuo and Satoh, 1993; Nakatani et al., 1996; Kim and Nishida, 2001; Shimauchi et al., 2001; Imai et al., 2002; Miya and Nishida, 2003). Transcriptional activation of the *Bra* gene involves not only activation of Ets by the extrinsic FGF signal but also of two intrinsic competence factors, Hr-FoxA and Hr-ZicN (Kumano et al., 2006; Matsumoto et al., 2007; Kumano and Nishida, 2009), which are members of the Fox and Zic transcription factor families, respectively (Shimauchi et al., 1997; Shimauchi et al., 2001; Wada and Saiga, 2002). *Bra* activates various downstream genes that are essential for notochord formation, which include the Not-1 antigen, which we have used as a notochord differentiation marker at the tail bud stage (Nishikata and Satoh, 1990; Takahashi et al., 1999; Hotta et al., 2000). This detailed knowledge of notochord induction at the molecular level makes it possible to analyze the specific step of signal transduction that is inactivated during the temporal decay of cellular competence.

To understand the mechanisms that underlie loss of competence, we molecularly investigated the decay of competence to respond to the FGF signal during notochord induction in *Halocynthia*

roretzi. Unlike the mechanism revealed in *Xenopus laevis* and *C. elegans*, the FGF signaling pathway can still be activated after loss of competence. Here, we provide evidence for a novel mechanism that facilitates the decay of competence. In notochord/nerve cord precursors, the transcription factor *Hr-FoxB* is expressed at the right time for attenuation of competence. FoxB mediates the loss of competence by suppression of *Bra* gene expression through direct binding of FoxB protein to the specific cis-regulatory element of the *Bra* gene.

FoxB is a member of the Fox family of transcription factors, which have a forkhead DNA-binding domain. Previous studies have reported the expression pattern of the *FoxB* gene in hydra, sea urchin, frog and zebrafish (Cheavalier et al., 2006; Gamse and Sive et al., 2001; Clayton et al., 2008; Tu et al., 2006; Minokawa et al., 2004; Grinblat et al., 1998). In vertebrates, the *FoxB* gene has been used as a marker gene for hindbrain. Only limited information on the functions of FoxB is currently available. During vulval development in *C. elegans*, FoxB promotes the expression of *CKI-1*, a gene encoding a cyclin-dependent kinase inhibitor, to mediate cell-cycle quiescence in vulval precursor cells (Clayton et al., 2008). In the present study, we show that, in ascidians, FoxB is involved in the attenuation of cellular competence, and present evidence for a novel function of FoxB.

MATERIALS AND METHODS

Animals and embryos
Adult individuals of *Halocynthia roretzi* were collected near the Asamushi Research Center for Marine Biology, Aomori, Japan, or were purchased from fisherman at Otsuchi International Coastal Research Center, Iwate, Japan. Spawned eggs were fertilized with non-self sperm and cultured in filtered seawater containing 50 μg/ml streptomycin and 50 μg/ml kanamycin at 9-10°C.

Blastomere isolation
Vitelline membranes were removed with tungsten needles, and eggs were raised in 1% agar-coated plastic dishes filled with filtered seawater before blastomere isolation. At the early 32-cell stage, target blastomeres were isolated with a fine glass needle under a stereomicroscope (SZX-12; Olympus). Isolated blastomeres were cultured separately in agar-coated dishes.

Treatment with bFGF and MEK inhibitor
Presumptive nerve/notochord blastomeres (A6.2, A6.4) that had been isolated at the early 32-cell stage were treated with seawater containing 0.1% bovine serum albumin (BSA) and 10 ng/ml recombinant human bFGF (Sigma) at various stages. To inhibit FGF intracellular signaling, whole embryos were treated with 2 μM MEK inhibitor (U0126, Promega, Madison, WI, USA) (Kim and Nishida, 2001).

Immunohistochemistry and whole-mount in situ hybridization
The monoclonal antibody Not-1 was used for monitoring notochord formation (Nishikata and Satoh, 1990). Imunohistochemistry for the Not-1 antigen was carried out as described previously (Kobayashi et al., 2003). Indirect immunofluorescence detection was performed by standard methods using a TSA fluorescein system (PerkinElmer Life Sciences, Waltham, MA, USA) in accordance with the manufacturer's protocol. Immunostaining for activated MAPK with anti-diphosphorylated ERK1/2 antibody (M8159; Sigma) and nuclear staining with DAPI were performed as described by Nishida (Nishida, 2003). Detection of *Hr-FoxB*, *Hr-Brachyury* (Yasuo and Satoh, 1993) and *Hr-FGF9/16/20* (Kumano et al., 2006) mRNAs by whole-mount in situ hybridization with digoxigenin (DIG)-labeled antisense RNA probes was performed according to Miya et al. (Miya et al., 1997).

Cloning of *Hr-FoxB*

To isolate the cDNA fragment of *Halocynthia FoxB*, we used a degenerate upstream oligonucleotide (5′-GAYCARAARCCNCCNTA-3′) and a downstream oligonucleotide (5′-TTYTGCCANCKYTGNGT-3′) that were designed on the basis of the conserved FoxB Forkhead Domain (FHD) sequences of zebrafish, *Xenopus*, mouse, human and *Ciona*. Using cDNA generated from poly (A)+ mRNA, we amplified a 150 bp cDNA fragment of *Halocynthia* FoxB FHD. To obtain the full-length cDNA, 5′ and 3′ RACE was carried out using the Gene Racer Kit (Invitrogen) and the SMART RACE cDNA Synthesis Kit (BD Bioscience, San Jose, CA, USA), respectively. The nucleotide sequences employed were 5′-GATTGGATTGCCATTGCCGTCAAGGCG-3′ for 5′ RACE, and 5′-ATCGCCTTGACGCAATGGCAATCCAAT-3′ for 3′ RACE. We obtained the 1946 bp cDNA of *Hr-FoxB* with the 5′UTR and 3′UTR sequences (Accession Number, AB593009) (see Fig. S1 in the supplementary material). The entire ORF was cloned into pBS-RN3 injection plasmids.

cDNA encoding FoxB-VP16, which consists of the VP16 transcription activation domain (amino acids 78) fused to the C terminus of Hr-FoxB FHD (91 amino acids), and that of FoxB-En^R, which was created by fusing the engrailed transcription repressor (amino acids 298) to the C terminus of Hr-FoxB FHD, were made by PCR and cloned into the pBS-RN3 injection plasmids (Fig. 7B). A plasmid containing Venus YFP (Kumano et al., 2006) was used as a control for mRNA synthesis.

Injection of MOs and synthetic mRNAs

To suppress the functions of Hr-FoxB by inhibiting its translation, we used antisense morpholino oligonucleotides (MOs) (Gene Tools, Philomath, OR, USA). Among three Hr-FoxB MOs we purchased, MO1 and MO3 produced in similar phenotypes (described in Results section). The sequences of the MOs were: Hr-FoxB MO1 (5′-GACGAGGCATTTTCAGATTTTGTTC-3′), which covers the starting methionine; and Hr-FoxB MO3 (5′-GATACAGCAATCGCTATTTATACTC-3′), which cover the 5′UTR. Hr-FoxB MO1 (700 pg) and 1000 pg of Hr-FoxB MO3 were injected into fertilized eggs. The sequences of the MOs against Hr-FGF9/16/20 and ZicN were 5′-TACCATTTGTACTGAAGGCATTTTC-3′ and 5′-GCTGTT-GCGTATGCCATTTTTGCTT-3′, respectively, both of which cover the starting methionine (Wada and Saiga, 2002; Kumano et al., 2006). Hr-FGF9/16/20 MO (500 pg) and 400 pg of ZicN MO were injected into fertilized eggs. In control experiments, we used standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′, Gene Tools). The amount of control MO injected was equal to that used in each of the knockdown experiments.

Hr-FoxB, *Hr-FoxB-VP*, *Hr-FoxB-En^R* and *YFP* mRNAs were synthesized with the mMessage mMachine kit (Ambion, Austin, TX, USA) and subsequently Poly (A) was added with a Poly (A) Tailing kit (Ambion). *Hr-FoxB* mRNA (7.5, 15 and 50 pg) and 50 pg of *Hr-FoxB-VP*, *Hr-FoxB-En^R* and *YFP* mRNAs were injected. MOs and mRNAs were dissolved in sterile distilled water and injected into fertilized eggs as described by Miya et al. (Miya et al., 1997).

The effect of mRNA injected into fertilized eggs was often limited to the left or right half of the embryo owing to slow diffusion of the mRNAs. To ensure that isolated cells had inherited the injected mRNA, we co-injected *FoxB* mRNA (15 pg) with *YFP* mRNA (100 pg) into fertilized eggs. At the early 32-cell stage, blastomeres exhibiting YFP fluorescence were isolated. For the control experiment, 115 pg of *YFP* mRNA was injected into a fertilized egg. In the rescue experiment, we injected FoxB MO1 (700 pg) together with *FoxB* mRNA (15 pg) and *YFP* mRNA (100 pg). FoxB MO1 (700 pg) and *YFP* mRNA (115 pg) were co-injected in the control experiment. To make the YFP fluorescence appear as early as possible, we used an Ampliscribe T3-Flash Transcription kit for more efficient translation of YFP. Subsequently, the synthetic mRNA was capped with the ScriptCap m7G Capping System and Script Cap 2′-o-Methyltransferase, and finally, poly (A) was added with an A-Plus Poly (A) Polymerase Tailing Kit (Epicentre Biotechnologies, Madison, WI, USA). YFP fluorescence was detected in living embryos at blastomere isolation, but was faint in embryos after fixation with methanol. Therefore, green fluorescence in the immunodetection for Not-1antigen was not interfered by YFP fluorescence in fixed embryos.

Recombinant proteins and electrophoretic mobility shift assay (EMSA)

The amplified fragments of the open reading frame (1737 bp) of full-length *Hr-FoxB* cDNA were digested with *Xho*I and *Eco*RI, and inserted into the multicloning site of plasmid pET32a containing His-tag sequences. Expression of the His-tag fused recombinant FoxB protein was induced by treatment with 0.1 mM IPTG at 15°C in BL21 (DE3)pLysS *E. coli* and incubation overnight with Ni-NTA agarose beads (Qiagen, Valencia, CA, USA). The recombinant protein was eluted from the agarose beads in PBS buffer containing 500 mM imidazole and 10% glycerol, and then dialyzed at 4°C for 24 hours in PBS containing 10% glycerol. Oligonucleotides (21 bp) of sense and antisense strands containing the Fox BS1 sequence were labeled with biotin 3′ end DNA labeling kit (Pierce, Rockford, IL, USA) and annealed (Fig. 7G). The recombinant proteins (750 ng) and biotin-labeled double-stranded oligonucleotides (1 pmol) were incubated in the binding buffer containing 10 mM Tris, 1 mM DTT, 2.5% glycerol, 100 mM KCl, 0.05% NP-40 and 50 ng/µl poly dA-dT for 30 minutes at room temperature. To examine the specificity of binding, supershift EMSA was performed with 0.4 µg of mouse antibody against the His-tag (Qiagen). The binding mixtures were electrophoresed in 7.5% polyacrylamide gel in TGE buffer [10 mM Tris-HCl (pH 8.5), 0.4 M glycine, and 0.2 mM EDTA], and the electrophoresed oligonucleotides were transferred to a nylon membrane. Detection of biotin-labeled nucleotides was performed using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA).

RESULTS

Activation of MAPK even after loss of competence

The notochord/nerve cord precursors (A6.2, A6.4 in Fig. 1) that are isolated at the 32-cell stage before induction occurs lose the competence to respond to the FGF signal at the 64-cell stage (Nakatani et al., 1996; Nakatani and Nishida, 1999). In the previous studies, notochord differentiation was evaluated by observing notochord morphology and expression of the notochord-specific Not-1 antigen. In the present study, we also monitored the notochord markers, notochord morphology in hatched larvae, the expression of Not-1 antigen at the tailbud stage, as well as the expression of the *Hr-Bra* gene at the 110-cell stage. Notochord/nerve cord precursors (A6.2 and A6.4) were isolated at the early 32-cell stage before notochord induction, and treated with 10 ng/ml bFGF starting at various times before and after they had divided, i.e. –10 minutes to 60 minutes (with time 0 representing the timing of the 6th cleavage) (Fig. 2A). Isolated cells that were treated with FGF at –10 minutes and +10 minutes developed into partial embryos, in which the cells were elongated (Fig. 2B,C; Fig. 3). This elongation is typical of differentiated notochord cells in partial embryos (Nakatani and Nishida, 1994). By contrast, when FGF treatment was initiated at 30 minutes or later, the frequency of partial embryos exhibiting the elongated cell shape was significantly decreased (Fig. 2D,E; Fig. 3). Occurrence of partial embryos that expressed the Not-1 antigen and the *Hr-Bra* gene was then examined. We observed a similar and gradual reduction in the proportion of positive partial embryos (Fig. 2G-J,L-O; Fig. 3). Partial embryos that were not treated with FGF did not express these features (Fig 2F,K,P). These results confirm the previous ones, and indicate that the decay of competence occurred within 1 hour at the 64-cell stage for all of the notochord features tested. These results also suggest that the decay of competence occurred upstream of the activation of notochord-specific transcription of the *Bra* gene.

To further investigate which step of the induction cascade is inactivated upon loss of competence, we examined intracellular transduction of the FGF signal by FGFR-RAS-MEK-MAPK. First, we investigated MAPK (ERK) activation with an antibody against diphosphorylated MAPK. This antibody stains the nuclei of
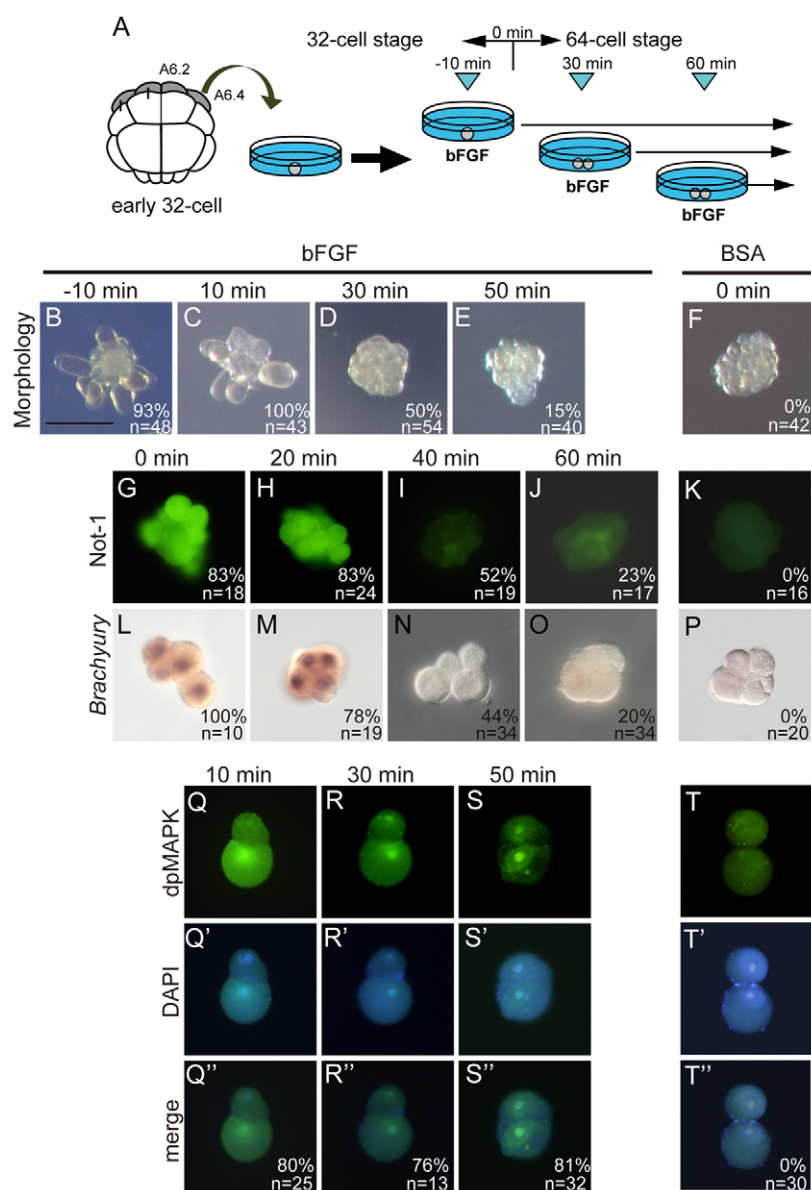
Fig. 2. Expression of notochord-specific features and activation of MAPK in partial embryos. (**A**) Diagram showing the design of the experiments. Notochord/nerve cord precursors were isolated at the early 32-cell stage, and then treated with bFGF starting at various times. Isolated notochord/nerve cord precursors divide at time 0. (**B-F**) Morphology of partial embryos at the hatching stage. Time of initiation of FGF treatment is indicated above each photo. Partial embryo in F was not treated with FGF. (**G-K**) Expression of Not-1 antigen. (**L-P**) Expression of *Hr-Bra* mRNA stained by in situ hybridization at the 110-cell stage. (**Q-T**) Embryos stained with anti-dually phosphorylated (dp) MAPK antibody. (**Q'-T'**) Nuclear staining with DAPI. (**Q"-T"**) The merger of Q-T and Q'-T'. Percentages of partial embryos that showed the notochord features are indicated with the total number of specimens in the bottom right-hand corners. Scale bar: 100 μm.

notochord blastomeres after induction by FGF in ascidians, and its specificity has been confirmed (Nishida, 2003). Partial embryos did not show the diphosphorylated MAPK signal if they were not treated with FGF (Fig. 2T-T"). Unexpectedly, the diphosphorylated MAPK signal was detected within the nuclei of either or both daughter blastomeres of the isolated cells following FGF treatment starting at 10, 30 and 50 minutes in ~80% of cases (Fig. 2Q-S", Fig. 3). Therefore, components of the FGF signaling pathway as far downstream as MAPK can be activated after the loss of competence for notochord formation. The mechanisms responsible for the loss of competence seem to operate in the cascade downstream of MAPK activation and upstream of *Bra* gene expression.

Involvement of FoxB in the decay of competence for *Brachyury* expression

We hypothesized that the appearance of a certain repressor may account for inability to express the *Bra* gene after loss of competence. A candidate gene was sought on the basis of two criteria. First, the gene of the candidate repressor had to be expressed in nerve cord precursor cells. This is because when

isolated notochord/nerve cord precursors do not receive the FGF signal, the cells assume the default nerve cord fate (Minokawa et al., 2001) and thus lose their competence to receive the FGF signal. Second, expression of the gene would need to be first evident at the 64-cell stage, when attenuation of competence occurs. To find genes that would fit these criteria, we searched for genes of transcription factors that have been recorded in the genome of the ascidian *Ciona intestinalis* (Imai et al., 2004; Imai et al., 2006). Appropriate spatial and temporal expression was observed for *Ci-FoxB*, which encodes a forkhead transcription factor. Therefore, we cloned *Halocynthia FoxB* cDNA to examine whether the expression pattern of *Hr-FoxB* is similar to that in *Ciona*. *Hr-FoxB* expression was observed in nerve cord precursors but not in notochord precursors of 64-cell-stage embryos (Fig. 4A,B). In addition to nerve cord cells, muscle cells had weaker expression. Next, we examined the temporal profile of *Hr-FoxB* expression in isolated blastomeres. Initiation of *Hr-FoxB* expression was evident in isolated cells from 20 minutes, coinciding with the beginning of competence attenuation (Fig. 4C-F). Thus, Hr-FoxB appeared to be a good candidate.
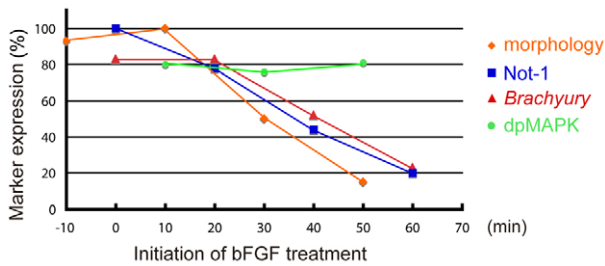
Fig. 3. Percentage of partial embryos that expressed notochord-specific features and activated MAPK. The horizontal axis represents time in minutes after division of the isolated cells.

Fig. 4. Spatiotemporal expression pattern of *Hr-FoxB* in whole embryo and isolated notochord/nerve cord precursor cells. (**A**,**B**) Expression of *Hr-FoxB* is shown in vegetal views at the 64- and 110-cell stages. Anterior is upwards. (**C-F**) *Hr-FoxB* expression becomes evident at 20 minutes in both daughter cells derived from isolated notochord/nerve cord cells. Percentages of partial embryos that showed *Hr-FoxB* expression are indicated with the total number of specimens in the bottom right-hand corners. Scale bars: 100 μm.

To examine the role of FoxB in the decay of competence for notochord induction, FoxB functions were knocked down by injection of antisense morpholino oligonucleotide (MO1, MO3) (Fig. 5A, Table 1). Treatment with FGF was initiated at 0 minutes and 70 minutes before and after the loss of competence. Cellular responses were evaluated by observing the expression of the Not-1 antigen and *Bra* mRNA. Isolated cells derived from embryos injected with control MO lost their competence during this period (Fig. 5B,C,H,I). Those injected with FoxB MO1 or MO3 expressed Not-1 antigen and *Bra* in ~80% of cases, even when FGF treatment was initiated at 70 minutes (Fig. 5E,F,L,M). MO1 and MO3 had similar effects on *Bra* expression (Table 1), supporting specificity of these MOs. To further confirm specificity of MO1, rescue experiment was carried out by injecting *FoxB* mRNA. In the experiment, partial embryos that were derived from eggs injected with FoxB MO together with *FoxB* mRNA and *YFP* mRNA expressed Not-1 and *Bra* in only 25 and 10% of cases, respectively, when FGF treatment was initiated at 70 minutes. The result indicates that the phenotype caused by MO1 was rescued by mRNA. By contrast, co-injection of FoxB MO1 and *YFP* mRNA did not rescue the phenotype (Table 1). We noticed that rates of Not-1 and *Bra* expression were significantly reduced to 20 and 31%, respectively, even when FGF treatment was started 0 minutes before loss of competence. This could be accounted for by the observation that precocious expression of FoxB by injection of the mRNA without MO1 caused total suppression of *Bra* expression as described in last part of this section and in a later section. Given the similar effects of both MO1 and MO3, in addition to the result of rescue experiment, the specificity of the MOs was confirmed. These results show that cells retained their competence for a longer time in the absence of FoxB function.
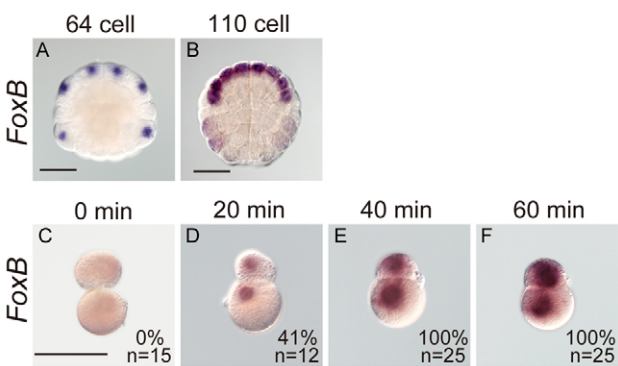
Unexpectedly, injection of FoxB MO1 and MO3 resulted in an increase of Not-1 and *Bra* expression in the absence of exogenously applied FGF in control BSA-treated partial embryos (50%, 37%, 12% and 30%, respectively in Table 1; Fig. 5D,G,J,N). The number of partial embryos that expressed *Bra* was further increased when they were fixed at the 194-cell stage (47% and 43%, respectively; Fig. 5K,O). We noticed that in normal embryos, *Hr-FGF9/16/20* gene was expressed in notochord (A7.3, A7.7) and nerve cord (A7.4, A7.8) precursor cells of 64-cell embryos, and that thereafter expression was restricted to the nerve cord precursor cells after cell division to the 110-cell stage (Fig. 5P,Q). *FGF* gene expression was also observed at the 110-cell stage in isolated cells (Fig. 5R). *FGF* expression was not altered in embryos injected with FoxB MO (data not shown). It is possible that the cells in partial embryos injected with FoxB MOs may fail to lose their competence, and may inadequately respond to endogenously expressed FGF protein by expression of the notochord features, even after completion of the notochord-induction stage, in normal embryos. This possibility was assessed by co-injection of FGF 9/16/20 MO, together with FoxB MO. Knockdown of endogenous FGF resulted in loss of *Bra* expression in FoxB knocked down partial embryos (Fig. 5S, Table

Table 1. Expression of notochord-specific features in descendents of isolated notochord/nerve cord precursors

	Not-1			Brachyury			
	bFGF+BSA		BSA	bFGF+BSA		BSA	
	0 minutes *	70 minutes *	*	0 minutes †	70 minutes †	†	‡
Control MO	90% (32)	18% (33)	6% (32)	78% (23)	15% (33)	4% (25)	6% (15)
FoxB MO1	96% (31)	84% (46)	50% (48)	89% (28)	81% (37)	12% (41)	47% (21)
FoxB MO3	96% (26)	84% (45)	37% (29)	87% (32)	70% (42)	30% (23)	43% (23)
FoxB mRNA+FoxB MO1+*YFP* mRNA	20% (53)	25% (43)	8% (47)	31% (35)	10% (39)	2% (34)	
FoxB MO1+*YFP* mRNA	90% (32)	77% (45)	51% (39)	88% (35)	71% (28)	13% (22)	
FoxB MO1+FGF MO							0% (42)
FoxB mRNA+*YFP* mRNA	6% (30)		0% (31)	14% (27)		0% (30)	
YFP mRNA	87% (41)		0% (41)	90% (32)		0% (28)	

*Results obtained at tailbud stage.
†Results obtained at 110-cell stage.
‡Results obtained at 194-cell stage.
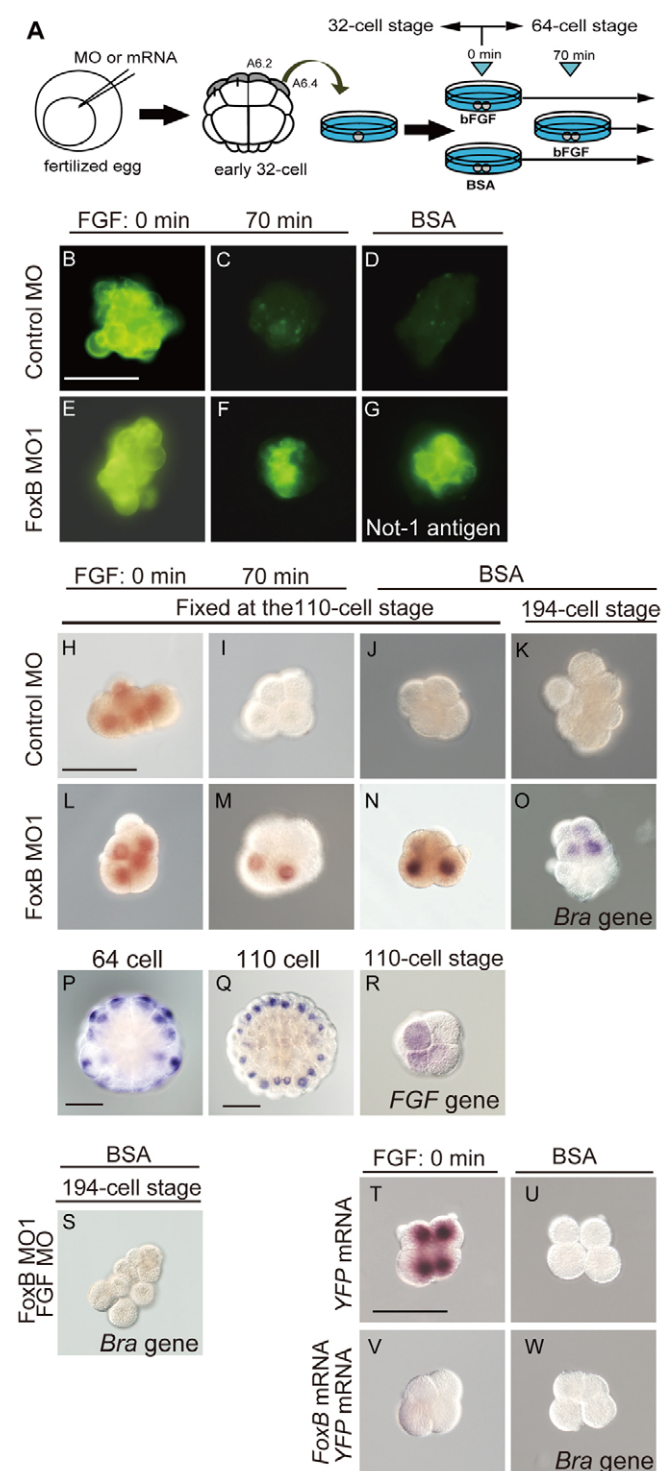Number of specimens is given in parentheses.

Fig. 5. Involvement of Hr-FoxB in decay of competence.
(**A**) Diagram illustrating the experimental design. MOs or mRNA were injected into fertilized embryos. Notochord/nerve cord precursors were isolated at the early 32-cell stage, and then treated with FGF at 0 minutes or 70 minutes. (**B-G**) Expression of Not-1 antigen in partial embryos. Injected MOs are indicated on the left. (**H-O**) Expression of *Bra* mRNA visualized by in situ hybridization. The partial embryos were fixed at the stage indicated above. (**P-R**) Expression of *Hr-FGF* mRNA at the 64- and 110-cell stages, and that in partial embryos fixed at the 110-cell stage. (**S**) *Bra* gene expression in partial embryos that had been co-injected with FoxB and FGF MOs. (**T-W**) *Bra* gene expression in partial embryos injected with the indicated mRNAs. Scale bars: 100 μm.

1). These results support the contention that FoxB is required for suppression of inadequate activation of *Bra* expression after the 110-cell stage by endogenously produced FGF protein.

We then examined the consequences of precocious expression of FoxB. We injected *FoxB* mRNA into eggs, isolated the notochord/nerve cord blastomeres, then treated them with FGF at 0 minutes (Fig. 5T-W; Table 1). They did not express Not-1 and *Bra*, in contrast to the control *YFP* mRNA-injected partial embryos. Thus, precocious expression of FoxB suppressed the induction of *Bra* expression.

Hr-FoxB prevents *Hr-Bra* expression by nerve cord precursor cells in whole embryos

FoxB is involved in the decay of competence for *Brachyury* expression in partial embryos. However, the roles played by FoxB in the whole embryo are unknown. *FGF* expressed in nerve cord precursors is required for brain induction at the anterior edge of the animal hemisphere (Bertrand et al., 2003; Miyazaki et al., 2007). Thus, there is a possibility that the FGF signal induces a notochord fate in nerve cord precursor cells if the competence persists, because nerve cord precursor cells receive FGF in an autocrine manner. Therefore, we observed the expression of *Bra* in nerve cord precursors in whole embryos injected with FoxB MO1. Ectopic expression of *Hr-Bra* in likely descendent cells of A7.8 was evident in only three out of 26 cases of 110-cell embryos injected with FoxB MO (Fig. 6A,D, arrowhead). Similarly, no ectopic expression was observed in most embryos at the 194-cell stage (only one case of ectopic expression among 14 cases).
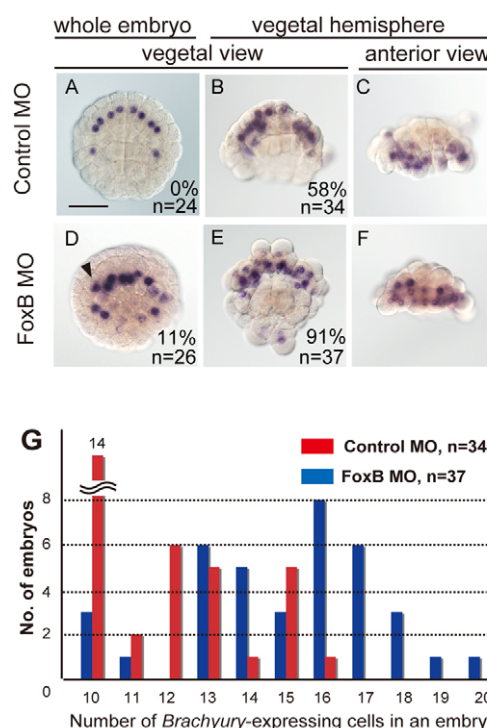

Fig. 6. The role of Hr-FoxB in whole embryos. (**A-F**) The expression of *Hr-Bra* mRNA was visualized at the 110-cell stage. Embryos in C,F are shown in anterior view. (A,D) Whole embryos. (B,C,E,F) Vegetal half of embryos. Injected MOs are indicated on the left. Percentages of embryos showing ectopic expression are indicated with the total number of specimens in the bottom right-hand corners. (**G**) Number of *Bra*-positive cells in an embryo is indicated on the x-axis; y-axis represents the number of embryos that had the indicated number of *Bra*-positive cells. Scale bar: 100 μm.
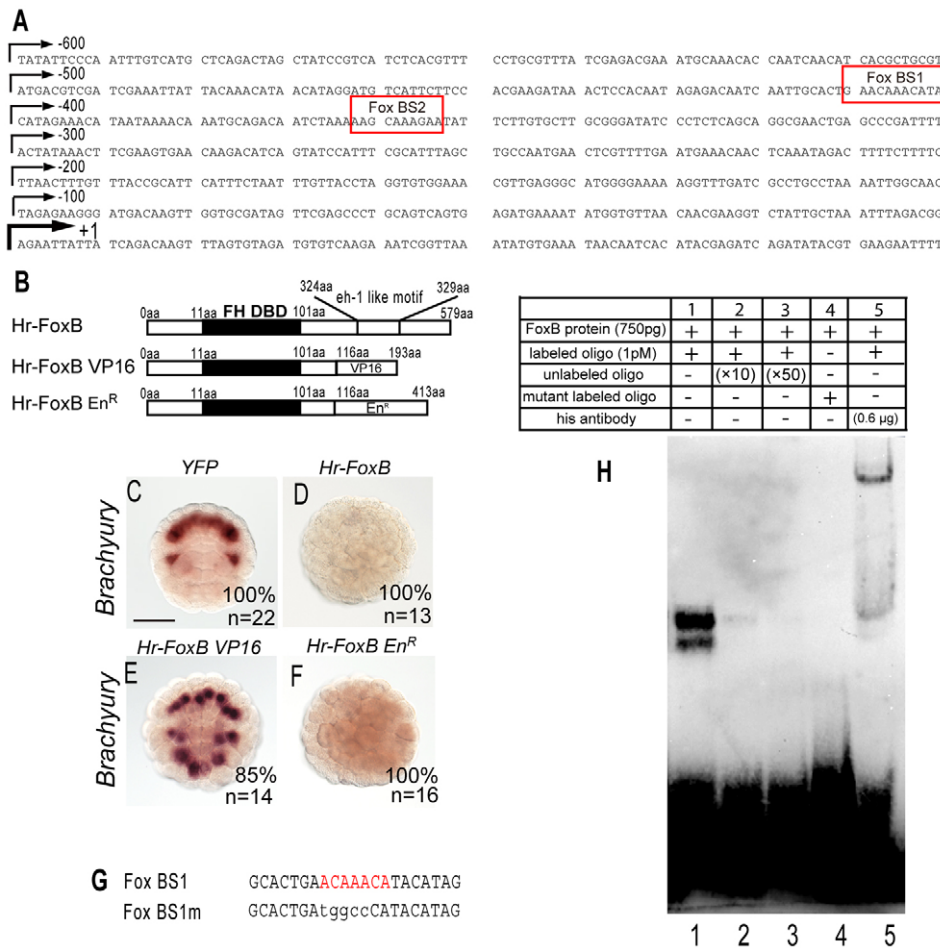
Fig. 7. FoxB protein binds directly to the cis-regulatory element of the *Bra* gene. (A) The 5'-upstream sequence of the *Hr-Bra* gene. Red boxes indicate the two putative Fox-binding sites (Fox BS1, Fox BS2) suggested in the previous study (Matsumoto et al., 2007). Thick arrow indicates the transcription initiation site (+1). (B) Schematic drawing of Hr-FoxB protein and its variants. Black box indicates the forkhead DNA-binding domain (FH DBD). (C-F) Expression of *Bra* at the 110-cell stage. Fertilized eggs were injected with 50 pg of control *YFP* mRNA (C), 7.5 or 50 pg synthetic *Hr-FoxB* mRNA (D), 50 pg of mRNA encoding Hr-FoxB conjugated with the VP16 transcription activation domain (E) and with the En[R] transcription repression domain (F). Scale bar: 100 μm. (G) Probe sequences of Fox BS1 and its mutant (Fox BS1m). The putative consensus core sequence, which is adopted from Carlsson and Mahlapuu (Carlsson and Mahlapuu, 2002), is in red. (H) EMSA performed with the recombinant full-length Hr-FoxB protein. The shifted band decreased gradually upon addition of a 10-fold or 50-fold excess of unlabeled competitor (lanes 2, 3, respectively). The band was not observed with the mutated probe (Fox BS1m, lane 4), and was supershifted by addition of the his-antibody (lane 5).

Previous studies have shown that an antagonistic signal from the animal hemisphere inhibits nerve cord precursor cells from expressing *Bra* before *FoxB* expression in nerve cord precursor cells (Kim et al., 2007). It is possible that the antagonistic signal lasts even beyond the 64-cell stage and prevents nerve cord cells from expressing *Bra*. To confirm this, we injected FoxB MO, and then ablated animal hemisphere cells at the eight-cell stage to remove the antagonistic effects emanating from them. In this experiment, embryos showed ectopic expression in most cases (Fig. 6B,C,E,F), although the cleavage pattern was slightly perturbed. We counted the number of *Bra*-positive cells in the embryos. In control MO-injected and animal hemisphere-ablated embryos, the numbers exceeded that in non-ablated normal embryos because the antagonistic effect from the animal hemisphere had been removed. However, the numbers in FoxB MO-injected and animal hemisphere-ablated embryos was significantly higher than that in control MO-injected and ablated embryos (Fig. 6G; $P<0.001$ by t-test). These results suggest that the antagonistic signal from the animal hemisphere and FoxB redundantly prevent nerve cord precursor cells from ectopically expressing *Bra* at the 64-cell stage and beyond.

FoxB protein binds to the cis-regulatory element of the *Hr-Bra* gene

FoxB is a transcription factor, and may directly suppress expression of the *Bra* gene by binding to its cis-regulatory element. A previous study has indicated that there are two Fox-binding consensus sequences, Fox BS1 (–411/401) and Fox BS2 (–363/354), in the *Hr-*

Bra gene upstream sequence (Fig. 7A) (Matsumoto et al., 2007). These two candidates for Fox BS were suggested on the basis of their sequence homology with previously identified Fox protein binding consensus sequences in other animals (Overdier et al., 1994; Ruminy et al., 2001). FoxA protein, a competence factor for notochord induction, weakly binds to the two Fox BS sequences of the *Hr-Bra* gene. However, reporter analysis of the 5' deletion series has shown that injection of reporter DNAs lacking FoxBS1 (Bra398-*lacZ*) led to ectopic expression of the *lacZ* reporter in nerve cord precursor cells (Matsumoto et al., 2007). Similarly, reporter analysis involving point mutations showed that injection of reporter constructs bearing mutations in both FoxB BS1 and BS2 caused ectopic expression in nerve cord precursors. Therefore, it has been proposed that at least Fox BS1 is required for the suppression of ectopic *Bra* expression in nerve cord precursor cells (Matsumoto et al., 2007). On this basis, we speculated that Fox BS1 might be the binding site for FoxB protein.

To test whether Hr-FoxB is a transcription repressor, we expressed fusion proteins in which the transcription-activation (VP16) or transcription-repression (En[R]) domains were conjugated with the forkhead domain of FoxB (Fig. 7B). The results are shown in Fig. 7C-F. Expression of wild-type FoxB protein completely suppressed the expression of *Bra*, confirming the results for isolated blastomeres described above. VP16-conjugated FoxB (FoxB-VP16) activated the ectopic expression of *Hr-Bra* in many cells in the vegetal hemisphere. By contrast, En[R]-conjugated FoxB (FoxB-En[R]) suppressed *Bra* expression. These results suggest that Hr-FoxB acts as a transcriptional suppressor for *Bra*.
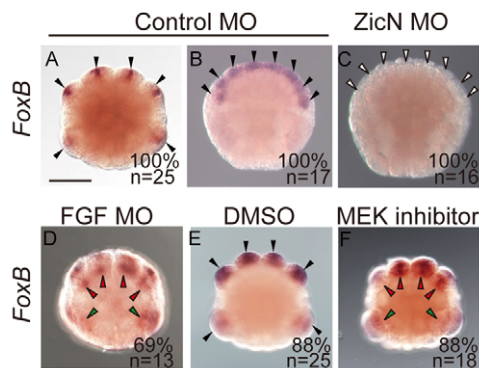
Fig. 8. Regulation of *FoxB* expression. The expression of *Hr-FoxB* was visualized at the 64- (**A**,**D-F**) and the 110-cell (**B**,**C**) stages in vegetal views. Fertilized eggs were injected with standard control MO (A,B), ZicN MO (C) or FGF MO (D). Treatments with DMSO (E) or MEK inhibitor dissolved in DMSO (F) were started from the eight-cell stage. Black arrowheads indicate normal expression of *Hr-FoxB* in nerve cord precursors and muscle precursors. White arrowheads indicate loss of the expression. Red arrowheads indicate ectopic expression in notochord precursors. Green arrowheads indicate ectopic expression in mesenchyme precursors. Scale bar: 100 μm.

In order to investigate whether FoxB protein binds directly to Fox BS1 of the *Bra* gene in vitro, an electrophoretic mobility shift assay (EMSA) was carried out. FoxB full-length recombinant protein with a histidine tag was used in the binding assay with oligonucleotides corresponding to Fox BS1 (Fig. 7G). FoxB protein was shown to bind to Fox BS1 (Fig. 7H, lane 1). The specificity of the binding was confirmed by addition of unlabeled oligonucleotides (lanes 2, 3) and by mutated Fox BS1 oligonucleotides (lane 4). Moreover, the band was supershifted by addition of the his-antibody (lane 5). These results support that FoxB binds directly to the Fox BS1 cis-element of the *Bra* gene, although the binding was not shown in vivo at the moment.

Regulation of *FoxB* expression

Spatiotemporal regulation of *FoxB* expression is of crucial importance in embryo development. We found that the Hr-ZicN transcription factor (Wada and Saiga, 2002) is required for *FoxB* expression, as the expression of *FoxB* was abrogated in ZicN MO-injected embryos (Fig. 8B,C). *ZicN* is expressed in both nerve cord precursors and notochord precursors (Wada and Saiga, 2002; Kumano et al., 2006); however, expression of *FoxB* is spatially restricted to nerve cord precursors. When the isolated cells were treated with FGF, neither of the daughter cells expressed *FoxB* (data not shown), suggesting that FGF signaling inhibit *FoxB* expression. Consistently, embryos expressed *FoxB* ectopically in notochord precursors when the FGF signaling is inhibited by injection of FGF MO or treatment with MEK inhibitor (Fig. 8A,D-F). Thus, *FoxB* is expressed in nerve cord precursors where ZicN is present and FGF signaling is inactive (see Fig. 9).

DISCUSSION

We analyzed the molecular mechanism responsible for the decay of competence to respond to FGF signals during notochord induction. The mechanism differs from those reported previously for mesoderm induction in *Xenopus* and vulval induction in *C. elegans*, where intracellular signal transduction was inactivated. We showed that a transcription repressor, FoxB, prevents
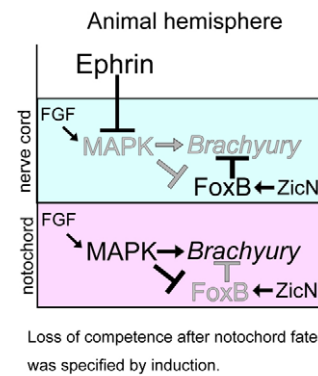

Fig. 9. A model for loss of competence for expression of the notochord-specific gene *Bra* by zygotic expression of the transcription repressor FoxB. Nerve cord and notochord precursors are colored blue and pink, respectively, as in Fig. 1. Black lines and letters indicate the interactions and the activation or expression of the factors, respectively. Gray lines and letters indicate that these events do not occur. After separation of notochord and nerve cord fates into two daughter cells, FGF/MAPK signaling cascade inhibits *FoxB* expression in notochord cells. An antagonistic ephrin signal emanating from the animal hemisphere prevents MAPK from becoming activated in nerve cord precursors and, consequently, *FoxB* is likely to be expressed. FoxB protein directly represses expression of the *Bra* gene in nerve cord cells after completion of notochord induction. The presence of the transcription factor ZicN is required for *FoxB* expression in the absence of FGF/MAPK signaling.

transcriptional activation of the *Bra* gene, which is the key gene for notochord formation, and that the FGF signaling pathway is still available after loss of competence. FoxB directly inhibits the expression of *Bra* by binding to the cis-regulatory element. Expression of FoxB protein in nerve cord precursor cells prevents inadequate ectopic notochord induction after segregation of the notochord and nerve cord fates.

The molecular mechanism of competence decay

Loss of competence could involve cellular changes that inactivate the signal transduction cascade of extracellular induction molecules. However, in ascidian notochord induction, MAPK can be activated by FGF, even after the loss of the competence for notochord formation. There is possibility, however, that a small decline of responsiveness to FGF could be significant because the current data, based on whole-mount immunostaining, do not allow for quantification of the response. Downstream of MAPK activation, phosphorylation of the transcription factor Hr-Ets is essential for transcriptional activation of *Bra* (Miya and Nishida, 2003). In addition, two intrinsic competence factors, Hr-FoxA and Hr-ZicN, are required for *Bra* expression (Kumano et al., 2006; Matsumoto et al., 2007). Among these three transcription factors, ZicN is still present when loss of competence occurs, because ZicN is also required for *FoxB* expression. When FoxB functions were knocked down, the cells retained their competence for a longer period and were able to respond to FGF through expression of *Bra* beyond the stage at which competence was lost in normal embryos. This observation suggests that Ets, FoxA and ZicN, are still present after competence for notochord induction has decayed. Thus, it appears that nothing is lost or inactivated in the FGF signal transduction cascade as far as *Bra* gene activation at the time of competence loss.

Loss of competence is probably mediated by the forkhead transcription factor FoxB, which is newly expressed in nerve cord precursors at the 64-cell stage in normal embryos. *FoxB* expression is also evident in isolated cells when they lose their competence. When FoxB was expressed precociously, the cells did not express *Bra* upon treatment with FGF. We showed that FoxB protein functions as a transcriptional repressor of the *Bra* gene, binding directly with the cis-regulatory element. FoxB proteins of various organisms possess a conserved eh-1-like motif (Yaklichkin et al., 2007) (FSIDNIM in *H. roretzi*), which is also conserved in the middle of the Hr-FoxB protein (Fig. 7B). The eh-1-like motif has been shown to interact with transcriptional co-repressors of the TLE/Groucho family (Smith and Jaynes., 1996; Tolkunova et al., 1998), indicating that FoxB is able to act as a transcriptional repressor. This is consistent with the results obtained using the VP16 and En^R fusion proteins.

Hr-FoxB prevents ectopic induction of notochord fate in nerve cord precursors after completion of notochord induction

Loss of competence has been thought to be significant in that it maintains further suppression and ensures inadequate induction after the induction stage. During embryogenesis, *FoxB* expression starts at the 64-cell stage in nerve cord precursors. In isolated notochord/nerve cord precursors, both daughter cells begin *FoxB* expression. When FoxB function is inhibited in isolated blastomeres, they eventually express *Bra* without exogenous FGF treatment. It seems plausible that *Bra* expression is induced by endogenously expressed FGF in the nerve cord, because FGF MO suppressed the inadequate activation of *Bra*. In whole embryos, endogenous FGF expressed by nerve cord precursor cells is required for induction of the brain at the anterior edge of the animal hemisphere (Miyazaki et al., 2007). In contrast to the blastomere isolation experiments, when FoxB was knocked down in whole embryos we did not observe ectopic expression of *Bra* in most specimens at the 110- and 194-cell stage. This is because the antagonistic influence on FGF signaling emanates from the animal hemisphere. The signal is ephrin in *Ciona intestinalis* (Picco et al., 2007). The antagonistic signal, which is also likely to be ephrin in *Halocynthia,* redundantly functions to prevent nerve cord cells from being induced to notochord by blocking a component of the FGF signal pathway: MAPK activation (Kim et al., 2007; Picco et al., 2007). Consistently, when the animal hemisphere was removed from FoxB-knockdown embryos, ectopic *Bra* expression was observed in most cases.

The repression of transcription by FoxB and the inhibition of FGF signaling by ephrin were functionally redundant during suppression of the notochord fate in nerve cord precursors (Fig. 9). However, the role of ephrin is more important for spatial restriction of *Bra* expression when notochord is induced, while FoxB mainly mediates temporal restriction of *Bra* induction beyond the stage when notochord induction has been completed. Ephrin is required for suppression of notochord induction in nerve cord precursors when notochord is induced at the 32- to 64-cell stage (Picco et al., 2007). Consistent with the functional distinction between ephrin and FoxB, MAPK was activated again in posterior descendants of nerve cord precursor blastomeres of the gastrula embryo, while inactivation of MAPK was retained in anterior descendents that were in contact with the animal hemisphere. (Nishida, 2003; Hudson et al., 2007). After the 110-cell stage (Fig. 1D), nerve cord precursors divide along the anteroposterior axis, and FGF/MAPK signaling is used again for diversification of the cell fates between the anterior row and posterior row of the nerve cord by activating MAPK only in the posterior row (Hudson et al., 2007). Thus, the components of the FGF/MAPK signaling cascade must be kept available in ascidian embryos to execute this process after decay of the competence for notochord induction. Even upon later activation of MAPK in the posterior row of the nerve cord, *Bra* expression is not induced, probably because of the presence of FoxB, and MAPK activation results in a different outcome, highlighting the distinct role of FoxB relative to that of ephrin.

Regulation of *FoxB* expression

The expression of *FoxB* requires ZicN and absence of FGF signaling (Fig. 9). This was proved experimentally by observation of *FoxB* expression in ZicN- and FGF MO-injected embryos, as well as MEK inhibitor-treated embryos. The regulatory mechanism is consistent with the *FoxB* expression pattern in normal embryos. *ZicN* is expressed in nerve cord, notochord, mesenchyme and muscle blastomeres at the 64-cell stage (Wada and Saiga, 2002). Among these blastomeres, FGF signaling is activated in notochord and mesenchyme blastomeres, thus limiting *FoxB* expression to nerve cord and muscle (Fig. 8A,D-F).

ZicN is expressed in notochord/nerve cord precursor blastomeres at the 32-cell stage, and is a competence factor for notochord induction (Kumano et al., 2006). Its expression is retained at later stages in nerve cord blastomeres and in notochord blastomeres, being required for nerve cord-specific expression of genes such as *ETR-1* (Wada and Saiga, 2002) and *FoxB*. In spite of the presence of ZicN in both types of blastomere, *FoxB* is expressed only in the nerve cord blastomeres. Our results indicate that FGF signaling, the same signal that induces notochord, prevents *FoxB* expression in notochord blastomeres. This is rational because if FoxB is expressed in notochord precursors, it would interfere with the maintenance of *Bra* expression there.

Loss of competence to respond to the FGF signal during mesenchyme induction

The FGF signal from endoderm blastomeres also induces a mesenchyme fate in the posterior region of the vegetal hemisphere at the same stage as notochord induction (Kim and Nishida, 2001; Kim et al., 2007). Similar to notochord induction, temporal decay of competence for mesenchyme induction was observed during the 64-cell stage (Kim et al., 2000). The expression pattern of *FoxB* implies that FoxB is also involved in the loss of competence for mesenchyme induction (Fig. 4A,B). Consistently, knockdown of FGF and inhibition of MEK activity lead to ectopic expression of *FoxB* in mesenchyme precursors (Fig. 8A,D-F).

Conclusion

Our results indicate that FoxB is involved in the temporal decay of competence for notochord induction. In this system, the signal transduction cascade remains available after loss of competence, and expression of a transcription repressor, FoxB in this case, is activated to repress the cellular response by inhibiting the target gene of the tissue-specific key transcription factor. *FoxB* expression is negatively regulated by the same signaling cascade to ensure that it is not expressed in the induced cells. The merit of this system is that embryos can use the same signaling cascade again for another purpose in the same cells at later stages.

DEVELOPMENT

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