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<tr>
<td>Citation</td>
<td>Development (2017), 144(1): 38-43</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2017-01-01</td>
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<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/217773">http://hdl.handle.net/2433/217773</a></td>
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<td>Type</td>
<td>Journal Article</td>
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Differential temporal control of Foxa.a and Zic-r.b specifies brain versus notochord fate in the ascidian embryo

Tatsuro Ikeda* and Yutaka Satou

ABSTRACT
In embryos of an invertebrate chordate, Ciona intestinalis, two transcription factors, Foxa.a and Zic-r.b, are required for specification of the brain and the notochord, which are derived from distinct cell lineages. In the brain lineage, Foxa.a and Zic-r.b are expressed with no temporal overlap. In the notochord lineage, Foxa.a and Zic-r.b are expressed simultaneously. In the present study, we found that the temporally non-overlapping expression of Foxa.a and Zic-r.b in the brain lineage was regulated by three repressors: Prdm1-r.a (formerly called BZ1), Prdm1-r.b (BZ2) and Hes.a. In morphant embryos of these three repressor genes, Foxa.a expression was not terminated at the normal time, and Zic-r.b was precociously expressed. Consequently, Foxa.a and Zic-r.b were expressed simultaneously, which led to ectopic activation of Brachyury and its downstream pathways for notochord differentiation. Thus, temporal controls by transcriptional repressors are essential for specification of the two distinct fates of brain and notochord by Foxa.a and Zic-r.b. Such a mechanism might enable the repeated use of a limited repertoire of transcription factors in developmental gene regulatory networks.

KEY WORDS: Ciona intestinalis, Transcriptional repressor, Temporal regulation

INTRODUCTION
In animal development, many transcription factors are used reiteratively in different combinations at different places and times. In embryos of the invertebrate chordate Ciona intestinalis, Foxa.a, Zic-r.b (formerly ZicL, renamed according to a recently published nomenclature rule; Stolfi et al., 2015) and Fgf signaling are used for specifying the developmental fates of the notochord and the brain. In the anterior (A-line) notochord lineage, Foxa.a and Zic-r.b are expressed simultaneously in the same cells from the 32-cell to the gastrula stage (Imai et al., 2002b). Fgf9/16/20 is expressed in the vegetal hemisphere from the 16-cell to the early gastrula stage (Imai et al., 2002a, 2004). Fgf9/16/20 is expressed in the vegetal hemisphere from the 16-cell to the early gastrula stage (Imai et al., 2002a, 2004). Foxa.a, Zic-r.b and Fgf signaling combinatorially activate Brachyury at the 44-cell stage (Imai et al., 2002a, 2006; Yagi et al., 2004; Yasuo and Hudson, 2007). Brachyury encodes a key transcription factor for specifying the notochord, and activates notochord-specific genes directly and indirectly (Chiba et al., 2009; Hotta et al., 2000; Katikala et al., 2013; Kubo et al., 2010; Takahashi et al., 1999). Indeed, in another ascidian species, Halocynthia roretzi, overlapping expression of Foxa and ZicN (an ortholog of Zic-r.b) and activation of Ets by Fgf signaling have been reported to be required for Brachyury expression (Kumano et al., 2006; Matsumoto et al., 2007; Miya and Nishida, 2003).

Similarly, Foxa.a, Zic-r.b and Fgf signaling are all required for specifying the brain fate in Ciona (Bertrand et al., 2003; Hudson et al., 2003; Imai et al., 2002a, 2006; Lamy et al., 2006; Wagner and Levine, 2012). However, in the brain lineage, Foxa.a is expressed from the 8- to the 32-cell stage, whereas Zic-r.b is expressed from the early gastrula to the neurula stage (Imai et al., 2002a, 2004; Shimauchi et al., 2001). Thus, Foxa.a and Zic-r.b are expressed sequentially, not simultaneously, in the brain lineage. In addition, the brain-lineage cells continuously receive the Fgf signal from the vegetal hemisphere from the 32-cell to the early gastrula stage (Hudson et al., 2003; Wagner and Levine, 2012).

Three transcriptional repressors, Prdm1-r.a (formerly BZ1), Prdm1-r.b (formerly BZ2) and Hes.a are important for ensuring that Foxa.a and Zic-r.b are expressed sequentially in the presumptive brain cells, as indicated by the fact that Zic-r.b is precociously expressed at the 32-cell stage in triple morphants of Prdm1-r.a, Prdm1-r.b and Hes.a (Prdm1-r.a/b/Hes.a morphants) (Ikeda et al., 2013). In the present study, we show that Prdm1-r.a also promotes termination of Foxa.a expression, and we propose a robust mechanism for temporally distinct expression of Foxa.a and Zic-r.b to ensure the brain lineage specification.

RESULTS AND DISCUSSION
The notochord developmental program was ectopically activated in Prdm1-r.a/b/Hes.a morphants
We previously showed that Zic-r.b is precociously expressed in bi-potential brain/palp progenitors at the 64-cell stage in double-morphant embryos of Prdm1-r.a and Prdm1-r.b (Prdm1-r.a/b morphants), and this ectopic activation of Zic-r.b converts palp fate into brain fate (Ikeda et al., 2013). We also showed that Zic-r.b expression begins earlier (at the 32-cell stage) in Prdm1-r.a/b/Hes.a triple morphants. However, Prdm1-r.a/b/Hes.a morphant larvae are severely disorganized (probably because Hes.a is expressed in the endomesodermal lineages in addition to the ectodermal lineages), and therefore we cannot analyze their morphology. To overcome this problem, in the present study, we injected morpholino oligonucleotides (MOs) into the pair of anterior animal (a-line) blastomeres of 8-cell embryos, from which ectodermal tissues, including the brain and the palps, are derived.

Prdm1-r.a/b/Hes.a morphant larvae lost not only palps but also the otolith and ocellus in the brain (Fig. 1A,B), whereas Prdm1-r.a/b morphant larvae lost only palps (Ikeda et al., 2013). In addition, Hes.a single-morphant larvae did not lose palps, otolith or ocellus (Fig. 1C). Thus, Prdm1-r.a/b/Hes.a morphant larvae showed a more severe phenotype than Prdm1-r.a/b morphant larvae and Hes.a morphant larvae.
In some Prdm1-r.a/b/Hes.a morphants, the notochord appeared to be longer than that in normal embryos. Indeed, Noto1 and Fgl (formerly Fibrinogen-like), which are markers for notochord (Hotta et al., 2000), were expressed ectopically in the trunk region of Prdm1-r.a/b/Hes.a morphant tailbud embryos (Fig. 1D-G). Cells that ectopically expressed Noto1 and Fgl were derived from the anterior animal blastomeres of 8-cell embryos, as indicated by our finding that lacZ mRNA, injected as a tracer together with

Fig. 1. Prdm1-r.a/b/Hes.a morphants show ectopic activation of the notochord developmental program. (A-C) Tadpole larvae developed from embryos injected with a control MO (A), Prdm1-r.a/Prdm1-r.b/Hes.a MOs (B) or Hes.a MO (C). Magenta, yellow and cyan arrowheads indicate palps, otolith and ocellus, respectively. (D-G) The expression of Noto1 (D,E) and Fgl (F,G) at the tailbud stage in embryos injected with a control MO (D,F) or Prdm1-r.a/Prdm1-r.b/Hes.a MOs (E,G). Cyan arrowheads indicate ectopic expression. Percentages of embryos with ectopic expression are shown. (H-J) Double fluorescence in situ hybridization of tailbud embryos injected with Prdm1-r.a/Prdm1-r.b/Hes.a MOs concomitantly with lacZ mRNA. Green and magenta indicate expression of Noto1 and lacZ, respectively. Percentage of embryos with simultaneous expression of Noto1 and lacZ is shown. All MOs were injected into the pair of anterior animal cells at the 8-cell stage. n, number of embryos examined.

Fig. 2. Brachyury was ectopically expressed in Prdm1-r.a/b/Hes.a morphants. (A-I) Brachyury expression at the 64-cell (A,D,G), early gastrula (B,B',E,E',H,H') and late gastrula (C,F,I) stages in embryos injected with a control MO (A-C), Prdm1-r.a/Prdm1-r.bl Hes.a MOs (D-F) or Prdm1-r.a/Prdm1-r.b MOs (G-I). Cyan arrowheads indicate ectopic expression in the a-line. Black arrowheads indicate expression in the notochord progenitors. Percentages of embryos with ectopic expression are shown. n, number of embryos examined.
Prdm1-r.a, Prdm1-r.b and Hes.a MOs, was detected in the same cells that expressed Noto1 (Fig. 1H-J). Thus, Prdm1-r.a, Prdm1-r.b and Hes.a suppress the developmental program of the notochord in the anterior animal cells.

Brachyury was ectopically activated in the brain progenitors of Prdm1-r.a/b/Hes.a morphants

Brachyury is a key gene for notochord differentiation (Chiba et al., 2009; Yasuo and Satoh, 1993, 1998). We found that Brachyury was expressed ectopically in the presumptive brain/palp cells of Prdm1-r.a/b/Hes.a morphants from the 64-cell to the gastrula stage (Fig. 2A-F).

Although a small fraction of Prdm1-r.a/b double morphants also expressed Brachyury weakly in the presumptive brain/palp cells (Fig. S1A,B). Single morphants of either Prdm1-r.a, Prdm1-r.b or Hes.a did not express Brachyury ectopically (Fig. S1C-E). These data suggest that strong ectopic activation of Brachyury led to ectopic expression of Noto1 and Fgl in the brain progenitors of Prdm1-r.a/b/Hes.a morphants.

Prdm1-r.a causes Foxa.a expression to be transient

Previous studies showed that Foxa.a, Zic-r.b and Fgf signaling are required for activating Brachyury expression (Imai et al., 2002a, 2006; Yagi et al., 2004; Yasuo and Hudson, 2007). The cells with the brain/palp fates receive Fgf signaling continuously from the 32-cell to the early gastrula stage, and Zic-r.b begins to be expressed at the 32-cell stage in Prdm1-r.a/b/Hes.a morphants (Hudson et al., 2003; Ikeda et al., 2013; Wagner and Levine, 2012). Based on these observations, we examined whether Foxa.a expression was also changed in Prdm1-r.a/b/Hes.a morphants. In the brain/palp lineage, Foxa.a is expressed between the 8- and the 32-cell stages in normal embryos. At the 32-cell stage, Foxa.a was expressed normally in embryos injected with either control, Prdm1-r.a, Prdm1-r.b or Hes.a MO (Fig. 3A-E). At the 64-cell stage, Foxa.a expression disappeared normally in the a-line cells of embryos injected with

Fig. 3. Prdm1-r.a causes termination of Foxa.a expression.

(A) Schematics of embryos at the 32-cell, 64-cell and early gastrula stages in lateral views. The a-line cells are colored in magenta. (B-E) Foxa.a expression at the 32-cell, 64-cell and early gastrula stages in embryos injected with control (B), Prdm1-r.a (C), Prdm1-r.b (D) or Hes.a (E) MO (shown in lateral views; animal pole right). Cyan arrowheads indicate expression in the a-line. Percentages of embryos with Foxa.a expression in the a-line cells are shown. (F-I) Foxa.a expression in 16-cell embryos injected with control Gfp (F), Prdm1-r.a (G), Prdm1-r.b (H) or Hes.a (I) mRNA (shown in lateral views; animal pole right). Magenta arrowheads indicate loss of expression. Percentages of embryos that showed the wild-type expression pattern are shown. n, number of embryos examined.
either control, Prdm1-r.b or Hes.a MO (Fig. 3B,D,E). However, in Prdm1-r.a morphants, Foxa.a continued to be expressed even at the early gastrula stage (Fig. 3C). Consistent with this, Foxa.a expression was drastically decreased in 16-cell embryos injected with Prdm1-r.a mRNA (Fig. 3G), but not in embryos injected with mRNA of Gfp (control) or Hes.a (Fig. 3F,I). Because 59% of embryos injected with Prdm1-r.b mRNA lost Foxa.a expression in one or more cells, Prdm1-r.b could repress Foxa.a expression (Fig. 3H). Thus, Prdm1-r.a negatively regulates Foxa.a expression, and therefore Foxa.a expression ceases at the 64-cell stage. Prdm1-r.b might also contribute to this repression.

**Simultaneous expression of Foxa.a and Zic-r.b leads to ectopic Brachyury expression**

The preceding results indicated that simultaneous expression of Foxa.a and Zic-r.b activated Brachyury in the brain/palp lineage, in which cells continuously receive Fgf signaling. The ectopic expression of Brachyury in Prdm1-r.a/b/Hes.a morphants did indeed depend on Foxa.a, Zic-r.b and Fgf signaling, as indicated by the following two findings. First, Prdm1-r.a/b/Hes.a morphants treated with U0126 (which inhibits the Fgf signaling pathway) from the 44-cell stage did not express Brachyury (Fig. 4A,B). Second, ectopic Brachyury expression was lost when we injected either Foxa.a or Zic-r.b MO concomitantly with Prdm1-r.a, Prdm1-r.b and Hes.a MOs (Fig. 4C-E). Because Foxa.a begins to be expressed earlier than Zic-r.b in the brain lineage of normal embryos, there is a possibility that Foxa.a activates Zic-r.b expression. However, when we injected Foxa.a MO into the pair of anterior animal cells of 8-cell embryos, Zic-r.b expression was not lost (Fig. S2). Thus, Foxa.a does not activate Zic-r.b expression in the brain lineage, and Foxa.a and Zic-r.b are required for the ectopic Brachyury expression.

Our results clearly show that the combination of Foxa.a, Zic-r.b and Fgf signaling can activate Brachyury even in the brain/palp lineage. Thus, the temporal control of expression of Foxa.a and Zic-
**Foa.a and Fgf signaling enhance Prdm1-r.a expression at the 32-cell stage**

*Prdm1-r.a* begins to be expressed at the 16-cell stage (Ikeda et al., 2013). Although it was therefore expected that *Prdm1-r.a* protein would repress *Foxa.a* at the 32-cell stage, we found that *Foxa.a* was not repressed before the 64-cell stage (Fig. 3B). To understand this delay, we measured the amount of *Prdm1-r.a* mRNA at the 16- and 32-cell stages by reverse transcription followed by quantitative PCR (RT-qPCR), and found that the amount of *Prdm1-r.a* mRNA was 19-fold greater at the 32-cell stage than at the 16-cell stage (Fig. 4F).

*Foxa.a* and Fgf signaling were necessary for this increase between the 16- and 32-cell stages. RT-qPCR showed that *Prdm1-r.a* expression was significantly reduced in embryos treated with U0126, *Foxa.a* morphants, and *Foxa.a* morphants treated with U0126 (Fig. 4G). We confirmed this result by *in situ* hybridization (Fig. S4).

**Conclusions**

Knockdown of *Prdm1-r.a* and *Prdm1-r.b* resulted in precipitous expression of *Zic-r.b* at the 64-cell stage, and expansion of the brain region at the expense of anterior placode-like cells (Ikeda et al., 2013). Triple knockdown of *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* evoked precipitous *Zic-r.b* expression even at the 32-cell stage, and subsequently evoked ectopic expression of notochord marker genes in the brain/palp lineage. This difference in phenotypes between *Prdm1-r.a/b* and *Prdm1-r.a/b/Hes.a* morphants was likely due to a difference of the duration of overlap of expression of *Foxa.a* and *Zic-r.b*.

Our results showed that temporal overlap of the expression of *Foxa.a* and *Zic-r.b* activates *Brachyury* and its downstream pathways for notochord differentiation under the control of Fgf signaling even in the brain/palp lineage. Thus, temporal control of gene expression by the transcriptional repressors *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* is important for proper function of the gene regulatory network. *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* enable the same combination of *Foxa.a*, *Zic-r.b* and Fgf signaling to be used repeatedly, but with different timings, for specification of the brain versus the notochord (Fig. 4H). Temporal control by transcriptional repressors might have played an important role in the evolution of gene regulatory networks, because animal embryos develop a variety of cell types by reiteratively using a limited repertoire of transcription factors.

The extended notochord in *Prdm1-r.a/b/Hes.a* morphants is evocative of the notochord in cephalochordates, in which the notochord extends into the head. Acquisition of the temporal control by *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* might represent a key event that excluded the notochord program from the head region after the divergence of ascidians and amphibians.

**Materials and Methods**

**Animals, Fgf inhibition and gene identifiers**


**Gene knockdown and overexpression**

For gene knockdown, we used the same MOs (Gene Tools) for *Prdm1-r.a*, *Prdm1-r.b*, *Foxa.a*, *Hes.a* and *Zic-r.b* that we used in previous studies (Ikeda et al., 2013; Imai et al., 2006). We also used a standard control MO (5′-CCTCTTACCTGCATTATA-T3) purchased from Gene Tools.

Synthetic transcripts of lacZ, Gfp, *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* were prepared from cDNA cloned into the pBluescript RN3 vector (Lemaire et al., 1995) using an mMESSAGE mMACHINE T3 Kit (Thermo Fisher Scientific), and injected into fertilized eggs (1 mg/ml). All knockdown and overexpression phenotypes were confirmed in at least two independent injections.

**RT-qPCR**

For RT-qPCR, RNA extracted from 20-51 embryos was reverse-transcribed with an oligo-dT primer. The cDNA samples thus obtained were then analyzed by quantitative PCR with the SYBR-Green method. For each qPCR, the amount of cDNA used was equivalent to that in one embryo. The amount of maternal *Zic-r.a* mRNA was measured as an endogenous control. We used the same primers that we used previously (Ikeda et al., 2013).

**In situ hybridization**

The detailed procedure for whole-mount *in situ* hybridization was described previously (Ikuta and Saiga, 2007; Satoh et al., 1995). We synthesized a probe for *Prdm1-r.a* using the same cDNA that we used previously (Ikeda et al., 2013).

**Acknowledgements**

We thank Reiko Yoshida and Chikako Imaizumi and all the staff members of the Maizuru Fisheries Research Station of Kyoto University for collecting and cultivating *Ciona intestinalis* under the National Bio-Resource Project (NBRP) of MEXT, Japan.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

T.I. and Y.S. designed the study and wrote the paper. T.I. performed the experiments.

**Funding**

This research was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) (15J01153 to T.I.) and the CREST program of the Japan Science and Technology Agency to Y.S.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.142174.supplemental

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