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RESEARCH REPORT

Differential temporal control of *Foxa.a* and *Zic-r.b* specifies brain versus notochord fate in the ascidian embryo

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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., 2002a, 2004). *Fgf9/16/20* is expressed in the vegetal hemisphere from the 16-cell to the early gastrula stage (Imai et al., 2002b). *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.

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Received 12 July 2016; Accepted 14 November 2016

Development (2017) 144, 38-43 doi:10.1242/dev.142174

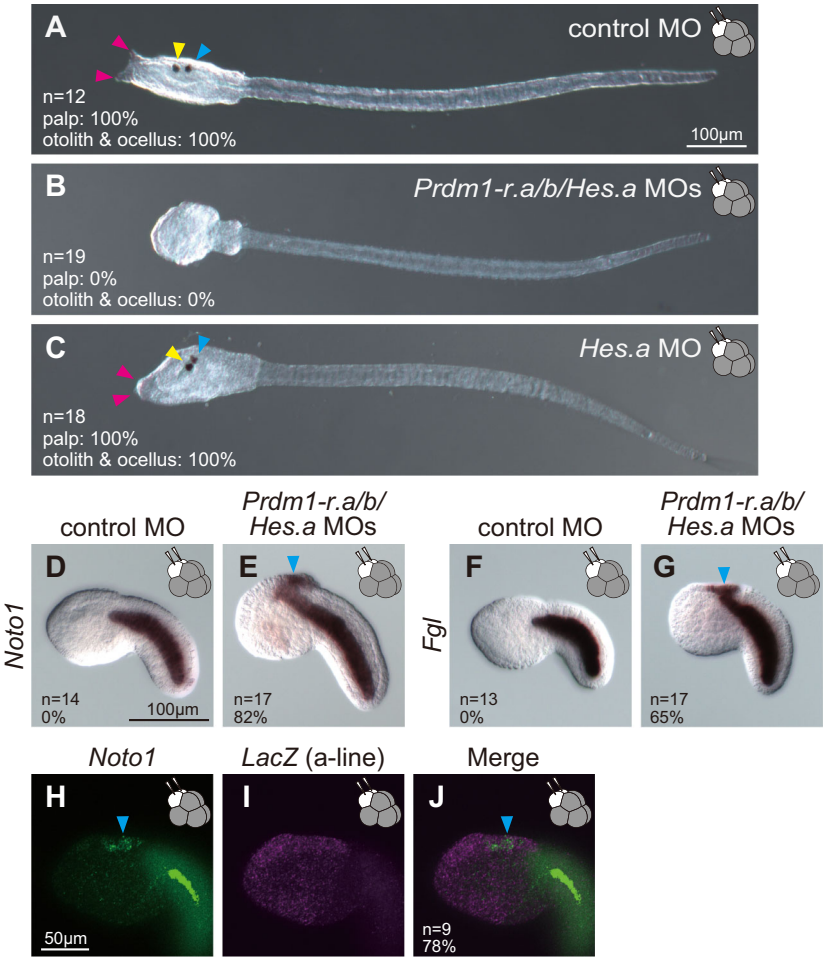

Fig. 1. *Prdm1-r.a*/*b*/*Hes.a* morphants show ectopic activation of the notochord developmental program. (A-C) Tadpole larvae developed from embryos injected with a control MO (A), *Prdm1-r.a*/*Prdm1-r.b*/*Hes.a* MOs (B) or *Hes.a* MO (C). Magenta, yellow and cyan arrowheads indicate palps, otolith 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.

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

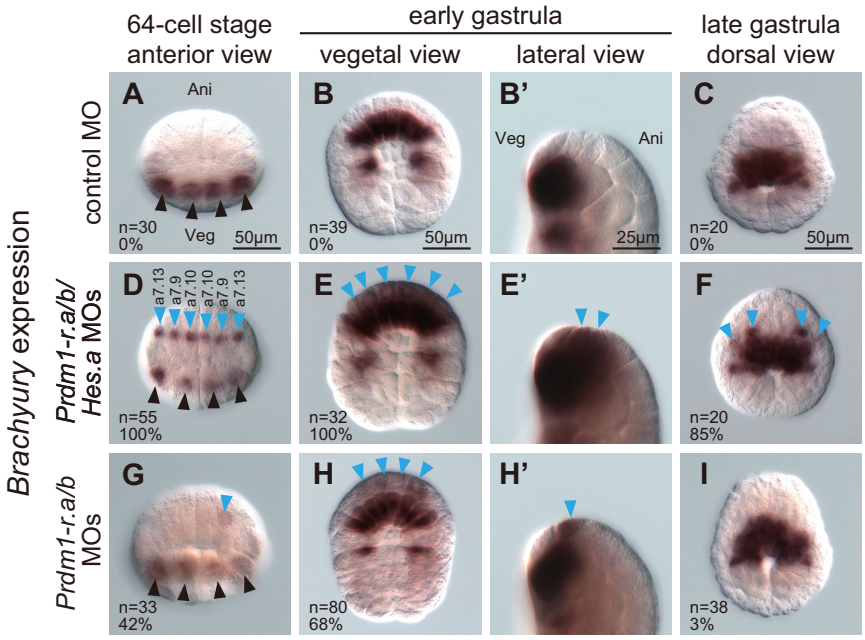

Fig. 2. *Brachyury* was ectopically expressed in *Prdm1-r.a*/*b*/*Hes.a* morphants. (A-I) *Brachyury* expression at the 64-cell (A,D,G), early gastrula (B,B′,E, E′,H,H′) and late gastrula (C,F,I) stages in embryos injected with a control MO (A-C), *Prdm1-r.a*/*Prdm1-r.b*/*Hes.a* MOs (D-F) or *Prdm1-r.a*/*Prdm1-r.b* 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.

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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. 2G-I), they rarely expressed *Noto1* or *Fgl* ectopically (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 *Fox a.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

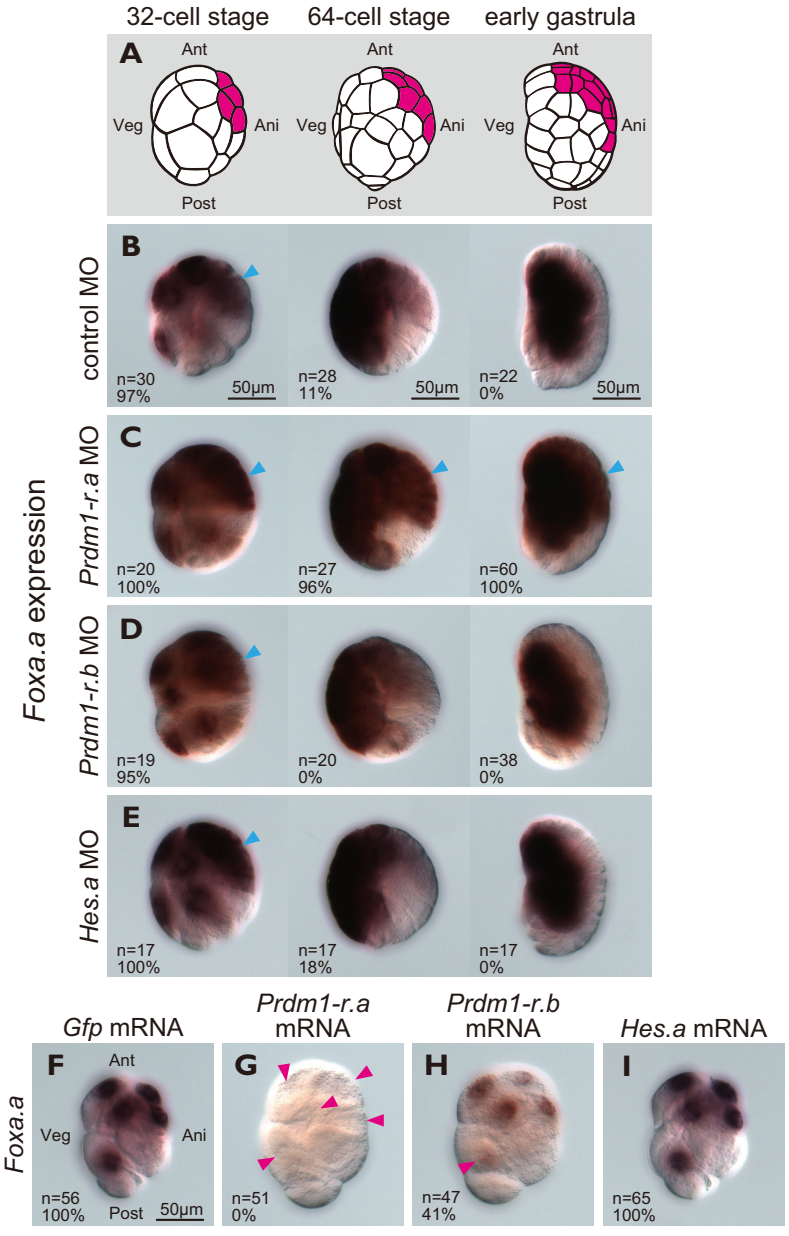

Fig. 3. *Prdm1-r.a* causes termination of *Foxa.a* expression. (A) Schematics of embryos at the 32-cell, 64-cell and early gastrula stages in lateral views. The a-line cells are colored in magenta. (B-E) *Foxa.a* expression at the 32-cell, 64-cell and early gastrula stages in embryos injected with control (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.

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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-*

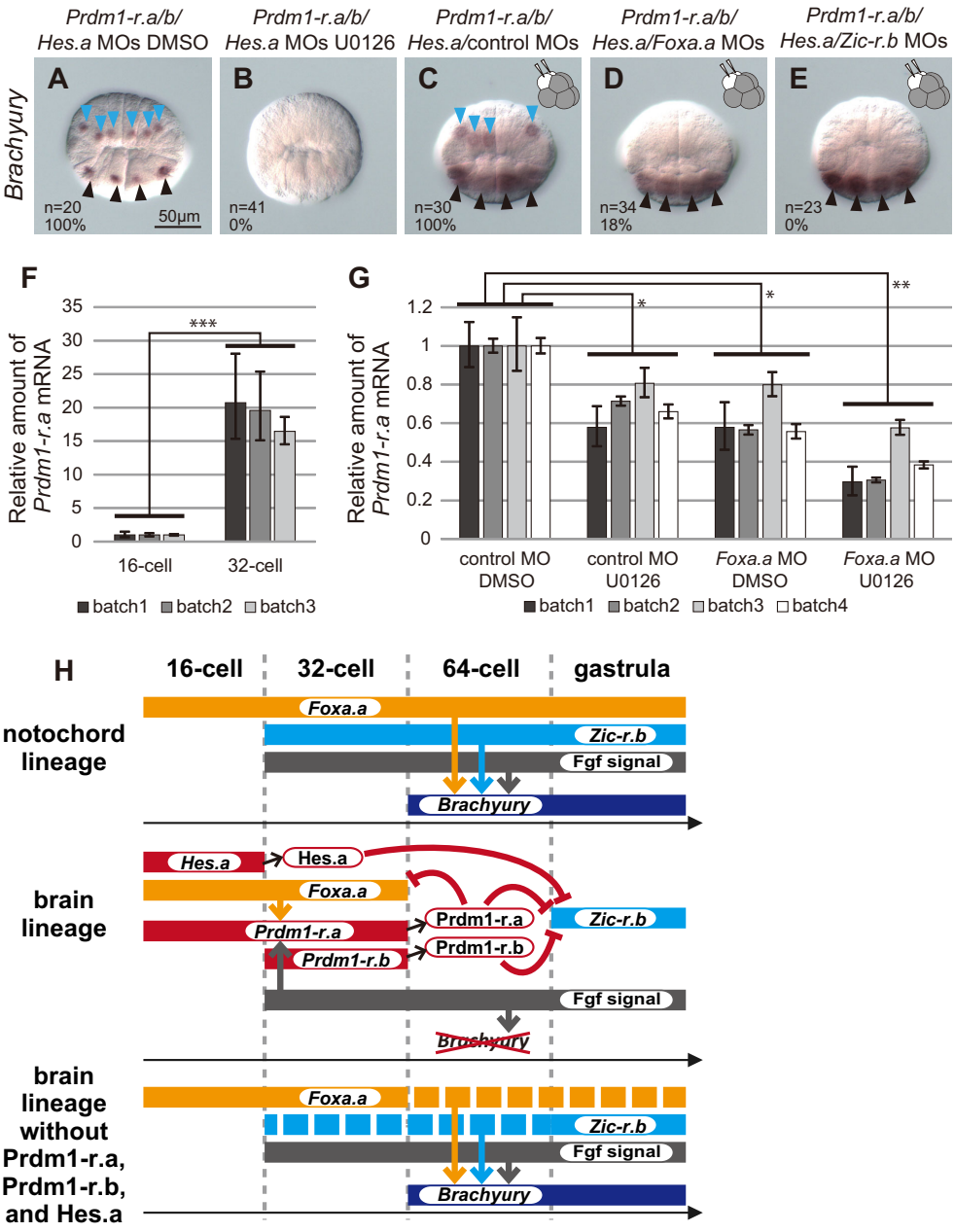

Fig. 4. Temporal misregulation leads to ectopic *Brachyury* expression. (A-E) *Brachyury* expression at the 64-cell stage in *Prdm1-r.a*/*b*/*Hes.a* morphant embryos treated with DMSO (A) or U0126 (B), and in embryos injected with *Prdm1-r.a*/*Prdm1-r.b*/*Hes.a* MOs (C-E) concomitantly with control (C), *Foxa.a* (D) or *Zic-r.b* (E) MO. In C-E, all MOs were injected into the pair of anterior animal cells at the 8-cell stage. Percentages of embryos with ectopic expression are shown. All embryos are shown in anterior views. n, number of embryos examined. (F) The amount of *Prdm1-r.a* mRNA was measured by RT-qPCR in wild-type embryos at the 16- and 32-cell stages. The level of a maternal mRNA, *Zic-r.a* (*Macho-1*), was used as an endogenous control. The *y*-axis shows relative expression compared with expression at the 16-cell stage. ***P<0.001; two-tailed paired *t*-test. Data are mean±s.d. (G) The amount of *Prdm1-r.a* mRNA at the 32-cell stage was measured by RT-qPCR in controls or *Foxa.a* morphants treated with DMSO or U0126 from the 16-cell stage. The level of *Zic-r.a* was used as an endogenous control. The *y*-axis shows relative expression compared with expression in embryos injected with control MO and treated with DMSO. Error bars indicate mean±s.d. between two technical duplicates. *P<0.05, **P<0.01; two-tailed paired *t*-test. In F and G, the results of three and four independent experiments are shown in different colors. (H) Summary of temporal regulation of *Foxa.a*, *Zic-r.b* and Fgf signaling. This regulation enables these three factors to be used repeatedly for specification of the brain and the notochord fates.

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r.b is essential for preventing *Brachyury* from being expressed in the brain/palp lineage. Interestingly, expression of early neural marker genes, *Otx*, *Dmrt1* and *Celf3* (formerly *Etr*) (Hudson and Lemaire, 2001; Hudson et al., 2003; Imai et al., 2004), was not lost in the brain/palp lineage of *Prdm1-r.a*/*b*/*Hes.a* morphants (Fig. S3). This suggests that *Prdm1-r.a*, *Prdm1-r.b* and *Hes.a* are not required for activating the early neural program.

Foxa.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 precocious 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 precocious *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 amphioxus.

MATERIALS AND METHODS

Animals, Fgf inhibition and gene identifiers

Ciona intestinalis adults (type A) were obtained from the National Bio-Resource Project for this animal. U0126 (Sigma) was used at 10 µM as described in a previous study (Hudson et al., 2003). Identifiers for genes examined in the present study are as follows: CG.KH2012.C11.313 for *Foxa.a*, CG.KH2012.C12.493 for *Prdm1-r.a*, CG.KH2012.C12.105 for *Prdm1-r.b*, CG.KH2012.C1.159 for *Hes.a*, CG.KH2012.S1404.1 for *Brachyury*, CG.KH2012.S816.1 for *Zic-r.b*, CG.KH2012.L20.18 for *Noto1*, CG.KH2012.C1.832 for *Fgl*, CG.KH2012.C1.727 for *Zic-r.a* (formerly *Macho-1*), CG.KH2012.C4.84 for *Otx*, CG.KH2012.S544.3 for *Dmrt1* and CG.KH2012.C6.128 for *Celf3*.

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′-CCTCTTACCTCAGTTACAATTTATA-3′) 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; Satou 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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