RESEARCH REPORT

A time delay gene circuit is required for palp formation in the ascidian embryo

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ABSTRACT
The ascidian larval brain and palps (a putative rudimentary placode) are specified by two transcription factor genes, ZicL and FoxC, respectively. FGF9/16/20 induces ZicL expression soon after the bi-potential ancestral cells divide into the brain and palp precursors at the early gastrula stage. FGF9/16/20 begins to be expressed at the 16-cell stage, and induces several target genes, including Otx, before the gastrula stage. Here, we show that ZicL expression in the brain lineage is transcriptionally repressed by Hes-a and two Blimp-1-like zinc finger proteins, BZ1 and BZ2, in the bi-potential ancestral cells. ZicL is precociously expressed in the bi-potential cells in embryos in which these repressors are knocked down. This precocious ZicL expression produces extra brain cells at the expense of palp cells. The expression of BZ1 and BZ2 is turned off by a negative auto-regulatory feedback loop. This auto-repression acts as a delay circuit that prevents ZicL from being expressed precociously before the brain and palp fates split, thereby making room within the neural plate for the palps to be specified. Addition of the BZ1/2 delay timer circuit to the gene regulatory network responsible for brain formation might represent a key event in the acquisition of the primitive palps/placodes in an ancestral animal.

KEY WORDS: Ciona intestinalis, Gene circuit, Palp, Brain, Placode

INTRODUCTION
The ascidian Ciona intestinalis is a simple chordate belonging to the tunicate group. The anterior animal cells (a-line) of the eight-cell Ciona embryo give rise to the trunk epidermis, brain and palps, adhesive organs containing sensory neurons (Nishida and Satoh, 1983) (Fig. 1A). FGF9/16/20, which is expressed in vegetal cells from the 16-cell to gastrula stage, then induces expression of the neural markers Otx (Hudson and Lemaire, 2001; Bertrand et al., 2003; Hudson et al., 2003) and DMRT1 (Tresser et al., 2010) in the future brain/palp (a7.9 and a7.10) and brain/epidermis (a7.13) cells at the 32- and 64-cell stages. At the gastrula stage, bi-potential brain/palp progenitor cells in the anterior part of the neural plate become further fate restricted, separating into distinct palp progenitors and brain progenitors (Nicol and Meinertzhagen, 1988) (Fig. 1B). After this fate restriction, only the brain progenitors express ZicL (Imai et al., 2004) in response to FGF9/16/20 signaling, because the brain progenitors, but not the palp progenitors, abut on FGF9/16/20-expressing vegetal cells (Wagner and Levine, 2012). Thus, even though FGF9/16/20 signals are present as early as the 16-cell stage, the brain progenitors respond to these signals to express ZicL only after the developmental fates of the brain and palps diverge. The mechanism by which this timing is coordinated is still unknown.

RESULTS AND DISCUSSION
Identification of novel zinc finger genes expressed at the 16-cell stage
During a microarray screen for zygotically activated genes in early Ciona embryos (Matsuoka et al., 2013), we identified a gene that is expressed in all anterior animal cells (a-line cells) at the 16-cell stage (Fig. 1C). Prior to the 64-cell stage, expression of this gene diminishes in all but one descendant, a7.13, and then is lost in a7.13 by the early gastrula stage. This gene is also transiently expressed in b6.5 at the 32-cell stage, and in a pair of posterior vegetal cells between the 32-cell and early gastrula stages.

The gene we identified encodes a C2H2-type zinc-finger protein with sequence similarity to Blimp-1/PRDM1 in other animals. However, it lacks the PR/SET-domain common to Blimp-1 in other animals. Whereas Blimp-1 proteins in other animals have four or five zinc fingers, this has only three. A molecular phylogenetic analysis failed to give strong support (data not shown). Therefore, we named this gene Blimp-1-like Zinc finger gene 1 [BZ1; DNA Data Bank of Japan (DDBJ) accession number AB819270], although it is possible that this gene is a divergent Blimp-1. Another gene encoding a protein with 67% overall amino acid identity to BZ1 and an identical zinc finger domain was found 15 kb upstream of BZ1, and we named this BZ2 (supplementary material Fig. 1S; DDBJ accession number AB819271).

The expression pattern of BZ2 was almost identical to that of BZ1, except that BZ2 expression at the 16-cell stage was undetectable and that BZ1 and BZ2 were expressed in non-overlapping tissues of tailbud embryos (Fig. 1D).

BZ1 and BZ2 are required for proper differentiation of the brain and palp lineages
We knocked down BZ1 and BZ2 by injecting specific morpholino oligonucleotides (MOs) into fertilized eggs. All BZ1 morphant larvae show clear brain defects, with 6% also losing palps (n=51), whereas BZ2 morphant larvae appear normal (n=7) (supplementary material Fig. S2A,B). However, when both MOs are simultaneously injected, the brain and palps are severely disrupted compared with normal larvae (n=34, 100%; Fig. 2A-B), indicating functional redundancy between BZ1 and BZ2. Indeed, the palp markers islet and ZF220 are absent in BZ1 and BZ2 double morphants (BZ1/2 morphants) at the tailbud stage (Fig. 2C-F). EphrinA-d, one of the earliest markers for the palp lineage (Imai et al., 2004; Wagner and Levine, 2012), is lost at the late gastrula stage in BZ1/2 morphants, whereas FoxC, another earliest marker for the palp lineage, is not affected (Fig. 2G-J). Six3/6, which is normally expressed in the anterior row of the two rows of the brain progenitor cells at the late gastrula stage (Imai et al., 2004; Moret et al., 2005), is ectopically expressed in the palp progenitor cells in BZ1/2 morphants, whereas
MYTF, which is normally expressed in the posterior row, is not affected (Fig. 2K-N). ZicL, which is normally expressed in the brain lineage at the early gastrula stage, is ectopically expressed in the palp lineage in BZ1/2 morphants (Fig. 2O,P). Thus, BZ1 and BZ2 are necessary for proper specification of the brain and palp lineages.

Previous studies revealed essential roles for ZicL and FoxC in specification of the brain and palps, and that ZicL regulates Six3/6, but not FoxC and MYTF (Imai et al., 2006; Wagner and Levine, 2012). EphrinA-d is also regulated by ZicL, because EphrinA-d expression is expanded to the brain lineage in ZicL morphants (supplementary material Fig. S3A). Next, we made a construct in which the ZicL coding sequence was linked to the DMRT1 upstream sequence. With this construct, ZicL is expected to be expressed in the brain/palp lineage from the 64-cell to late gastrula stages. When this construct is injected into fertilized eggs, EphrinA-d, islet and ZF220 are lost, and Six3/6 is ectopically expressed in the palp lineage (supplementary material Fig. S3B,C; Fig. 2Q,R). Thus, overexpression of ZicL generates a similar phenotype to knockdown of BZ1 and BZ2, suggesting that repression of ZicL might be the primary function of BZ1 and BZ2.

**BZ1 and BZ2 temporally control ZicL expression**

To understand how BZ1 and BZ2 regulate ZicL expression, we made constructs in which either of the BZ1 or BZ2 coding sequences was linked to the DMRT1 upstream sequence. Following injection of these constructs into an anterior animal cell at the eight-cell stage, ZicL expression is completely or partially lost in the injected octant (Fig. 3A,B). Thus, BZ1 and BZ2 repress ZicL expression in the brain/palp lineage. In BZ1/2 morphants, ZicL is precociously expressed at the 64-cell stage in the bi-potential progenitors a7.9 and a7.10 (Fig. 3C,D). FGF signaling is required for this precocious expression, because ectopic ZicL expression is rarely observed in BZ1/2 morphants treated with the MEK inhibitor U0126 at the 44-cell stage (Fig. 3E). In addition, BZ1/2 morphant embryos treated with recombinant human basic fibroblast growth factor (bFGF) at the 44-cell stage ectopically express ZicL in a7.9/a7.10 and in neighboring epidermal/brain progenitor cells (a7.13/a7.14), whereas similarly treated uninjected embryos do not show ectopic ZicL expression (Fig. 3F,G). Therefore, FGF signaling acts positively on a7.9/a7.10 to induce ZicL expression at the 64-cell stage, and BZ1 and BZ2 repress the precocious expression of ZicL in normal embryos at this stage.

The brain/palp progenitors at the 64-cell stage are derived from a pair of anterior animal cells (a6.5) in the 32-cell embryo. Although no precocious ZicL expression was detected in BZ1/2 morphants at the 32-cell stage, we found ectopic and precocious ZicL expression in the anterior animal cells of BZ1, BZ2 and Hes-a triple morphants (Fig. 3H-J). Hes-a transcriptional repressor gene is normally expressed in all of the blastomeres except for the posterior-most pair of cells at the 16-cell stage, and the expression in the animal hemisphere diminishes by the 32-cell stage (Imai et al., 2004). By contrast, in embryos injected with only the Hes-a MO, ectopic expression is observed only weakly in a small number of cells.
Therefore, at the 32-cell stage, ZicL is repressed by Hes-a, BZ1 and BZ2. Ectopic expression of ZicL in BZ1/2/Hes-a morphants at the 32-cell stage is dependent on FGF signaling, because this ectopic expression is lost in BZ1/2/Hes-a morphants treated with U0126 (Fig. 3L) and because bFGF treatment evokes ectopic expression in all anterior ectodermal cells of BZ1/2/Hes-a morphants (Fig. 3M,N).

BZ1 and BZ2 expression is transient, owing to a negative-feedback loop

The results described above indicate that transient expression of BZ1 and BZ2 is important for ZicL expression in the brain lineage. This transient expression is controlled mainly by BZ1, because BZ1 and BZ2 expression is strongly upregulated in BZ1 morphants (Fig. 4A-D), but weakly in BZ2 morphants (Fig. 4E,F).

We injected synthetic BZ1 and BZ2 mRNAs into embryos. Because the synthetic BZ1 and BZ2 mRNAs lack the endogenous 3′-UTRs, we were able to measure the amount of the endogenous mRNAs of BZ1 and BZ2 by RT-qPCR with primers designed to their 3′-UTRs. Whereas levels of the maternal control mRNA macho-1 are unchanged by injection of BZ1 and BZ2 mRNAs, BZ1 and BZ2 expression levels are greatly reduced by injection of synthetic BZ1 mRNA (Fig. 4G). By contrast, injection of BZ2 mRNA rarely reduces BZ1 and BZ2 expression. Therefore, feedback repression of BZ1 and BZ2 expression by BZ1 results in transient expression of these two repressor genes.

Conclusions

FGF9/16/20 is expressed as early as the 16-cell stage, and is used repeatedly to induce different genes in different cells at different times by functioning in combination with different transcription factors (Imai et al., 2002; Bertrand et al., 2003; Imai et al., 2003; Yagi et al., 2004; Imai et al., 2006; Kumano et al., 2006; Hudson et al., 2007; Picco et al., 2007; Shi and Levine, 2008; Hashimoto et al., 2011). The present study shows that the same signaling pathway can evoke differential outputs (ZicL expression or not) in a single lineage of cells at different times by functioning in combination with Hes-a and BZ1/BZ2 repressors (supplementary material Fig. S4). Without these repressors, the brain/palp progenitors precociously express ZicL, and therefore the palms are not properly specified. After the brain/palp progenitors divide, the palp precursor cells do not abut on FGF9/16/20-expressing cells (Wagner and Levine, 2012), and therefore ZicL is not induced in the palp lineage.

The duration of this repression is probably determined by a negative-feedback circuit of BZ1/2, the kinetics of which are likely to depend on mRNA and protein synthesis and turnover rates. Our results suggest that this duration coincides with cell division of the brain/palp progenitors, and thus that the feedback loop acts as a ‘delay timer’ to temporally control ZicL expression in the brain lineage without regulating the cell division directly.

This situation contrasts with instances in which cell cycle genes are directly controlled by regulatory gene circuits. In vertebrates, the key
transcription factors MyoD and Myf5 control cell cycle withdrawal and induction of differentiation in skeletal muscle cells (Kitzmann et al., 1998). This type of regulation is likely to be more robust. Nevertheless, evolutionary processes might favor delay timers in early embryos, probably because gene regulatory networks in early animal embryos typically have a cascade-like structure (Davidson et al., 2002; Levine and Davidson, 2005; Imai et al., 2006).

Although we do not have solid evidence that BZ1 and BZ2 are Blimp-1 orthologs, it should be noted that Blimp-1 is required for proper anterior neural tissues in a variety of animals including amphioxus, zebrafish and Xenopus (de Souza et al., 1999; Roy and Ng, 2004; Onai et al., 2010). In zebrafish, blimp-1 (prdm1a – Zebrafish Information Network) is expressed at the boundary of the neural plate and non-neural ectoderm. Loss of blimp-1 activity inhibits specification of neural crest cells and the primary sensory neurons, whereas misexpression of blimp-1 generates supernumerary primary sensory neurons. It has been suggested that the ascidian palps represent a rudimentary placode and might be derived from an ancestral structure that evolved into separate anterior placodes in the vertebrate lineage (Manni et al., 2004; Mazet et al., 2005; Wagner and Levine, 2012). Addition of the BZ1/2 delay timer circuit to the gene regulatory network responsible for brain formation might represent a key event in the acquisition of the primitive palps/placodes in an ancestral animal, because this circuit makes room within the neural plate for the palps to be specified by a FoxC-dependent mechanism.

**Fig. 3. Precocious expression of ZicL.** BZ1, BZ2 and Hes-a. (A,B) ZicL expression at the early gastrula stage in embryos expressing BZ1 (A) and BZ2 (B) under the DMRT1 upstream sequence. The overexpression constructs were injected into one anterior animal cell of eight-cell embryos. Descendants of the blastomere injected with the constructs are encircled by pink dashed lines. The expression is illustrated schematically at the bottom right in blue (brain). (C-G) ZicL expression at the 64-cell stage in control (C,F) and BZ1/2 morphant (D,E,G) embryos (shown in anterior views; animal pole up). The embryo shown in C is treated with U0126, and the embryos in F and G are treated with bFGF. (H-N) ZicL expression at the 32-cell stage in control (H,M), BZ1/2 morphant (I), BZ1/2/Hes-a morphant (J,L,N) and Hes-a morphant (K) embryos (shown in animal views). The embryo shown in L is treated with U0126, and the embryos in M and N are treated with bFGF. Ectopic expression is shown by yellow arrowheads. Black arrowheads indicate the expression of ZicL.

**MATERIALS AND METHODS**

**Animals**

Ciona intestinalis adults were obtained from the National Bio-Resource Project of MEXT. An inhibitor of MEK signaling (U0126, Sigma) (Hudson et al., 2003) and human recombinant bFGF (Sigma) were used at 10 μM, and 100 ng/ml, respectively.

**Microarray screen**

We manually isolated individual blastomeres from 16-cell embryos, and obtained gene expression profiles at single-cell resolution with microarrays (Matsuoka et al., 2013). We injected synthetic mRNAs for genes that were activated in specific cells at the 16-cell stage, and found that injection of BZ1 mRNA severely disrupted morphology. We therefore examined the function of BZ1 in the present study. Data from the microarray screen will be available in Gene Expression Omnibus under accession number GSE45575.

**Knockdown and overexpression studies**

We used the same MOs (Gene Tools) for Hes-a [E(spl)/Hairy-a] and ZicL that we used in a previous study (Imai et al., 2006). We designed two MOs for BZ1 (BZ1a: 5’-CAGAAGATCGTAAATTACCTCGATGTC-3’; and BZ1b: 5’-CGTAACCTTTCCGCGGTATTCCTCCT-3’) and one MO for BZ2 (5’-GTCGAAACACATGATCTCCGAC-3’). Both combinations of BZ1a/BZ2 MOs and BZ1b/BZ2 MOs gave the same morphological phenotype and elicited precocious expression of ZicL in the neural lineage. Although we could not design additional MOs for BZ2 because of its short 5’-UTR, the BZ2 MO gave specific phenotypes only when it was injected with the BZ1a or BZ1b MO, strongly suggesting specificity of this BZ2 MO. We describe results obtained from injection of the BZ1a and BZ2 MOs.
Fig. 4. Negative-feedback loops of BZ1 and BZ2 expression. (A-F) Expression of BZ1 (A,C,C,E) and BZ2 (B,D,D,F) at the early gastrula stage in normal embryos (A,B), BZ1 morphants (C,D) and BZ2 morphants (E,F). Embryos in A-F are shown in lateral views (animal pole right). C′ and D′ show animal views of the embryos in C and D, respectively. Arrowheads in C-F indicate upregulated expression. (G) The amount of endogenous BZ1 and BZ2 mRNAs is measured by qPCR in embryos injected with GFP (control), BZ1 and BZ2 mRNAs. The relative amount of mRNA in the experimental embryos compared with control embryos is shown. A maternal mRNA, macho-1, is used as a control. Error bars indicate standard errors between two biological duplicates.

Synthetic transcripts of BZ1, BZ2 and Gfp were prepared from cDNA cloned into the pBluescript RN3 vector (Lemaire et al., 1995) using the mMESSAGE mMACHINE T3 Kit (Ambion), and injected into fertilized eggs (1 mg/ml). To overexpress ZicL, BZ1 and BZ2 under the DMRT1 upstream sequence, we injected DNA constructs (20 μg/ml). We amplified the DMRT1 enhancer with the following primers: 5'-ATGGGCCCTTTCGCCGGAGAGTTAAAATC-3' and 5'-ATGGAGCTTCCATGCCAGTTAACACGAACT-3' (restriction sites used for cloning are underlined). The amplified region was slightly longer than that used in a previous report (Wagner and Levine, 2012). All knockdown and overexpression phenotypes were confirmed in at least two independent injections.

Whole-mount in situ hybridization
The detailed procedure for whole-mount in situ hybridization was described previously (Imai et al., 2004). To discriminate between BZ1 and BZ2 transcripts, we used 437-bp and 658-bp portions of the 5′-regions of cDNAs for BZ1 and BZ2 cDNAs.

RT-qPCR
For RT-qPCR, we extracted RNA from wild-type embryos and embryos injected with BZ1, BZ2 and Gfp mRNAs. The RNA was reverse-transcribed with an oligo-dT primer. The obtained cDNA samples were then analyzed by quantitative PCR with the SYBR green method. For each qPCR, the amount of cDNA used was equivalent to two-thirds of an embryo. Primers used were: BZ1, 5′-GAATTGGACATGAGAATAAACAACTACA-3′ and 5′-TGCTTTGAAGATGCGCATATAAA-3′; BZ2, 5′-AAAAACACGTCAGATGACATGACATGACGAT-3′; Macho-1, 5′-CCCCAGTGATCTCAGAATTATTACGTAGAAT-3′; and D, 5′-GGTTGGAATTGGACATGAGAATAAACAACTACA-3′. The amount of endogenous BZ1 and BZ2 mRNAs is measured by qPCR in embryos injected with GFP (control), BZ1 and BZ2 mRNAs. The relative amount of mRNA in the experimental embryos compared with control embryos is shown. A maternal mRNA, macho-1, is used as a control. Error bars indicate standard errors between two biological duplicates.

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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.100339/-/DC1

References


**Figure S1. An alignment of BZ1 and BZ2 proteins.** Identical residues are highlighted in black, and similar residues are highlighted in gray. Three zinc finger domains are underlined. Amino acid positions are shown in left in each line.
Figure S2. *BZ1* and *BZ2* are required for proper brain and palp formation. Tadpole larvae developed from eggs injected with (A) the *BZ1* MO and (B) the *BZ2* MO. Palps and the brain region are shown by red and blue arrowheads, respectively. Compare these embryos with the embryos shown in Figure 2A and B.
Figure S3. ZicL suppresses the palp fate. The expression of (A, B) EphrinA-d and (C) Six3/6, in (A) a ZicL morphant embryo, and (B, C) embryos expressing ZicL under the DMRT1 upstream sequence at the late gastrula stage. The expression is illustrated schematically in the lower right by red (palps) and blue (brain). In the experiment shown in (A), we injected the ZicL MO into one anterior animal cell at the 8-cell stage, and in the experiments shown in (B, C), we injected the DMRT>ZicL construct into fertilized eggs.
Figure S4. Summary of gene regulatory circuits for ZicL expression in the brain progenitors.

Gene regulatory circuits from the 32-cell to early gastrula stages are illustrated by the BioTapestry software (Longabaugh, 2012). See the text for details.