Developmental Biology 445 (2019) 245–255

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Spatio-temporal regulation of *Rx* and mitotic patterns shape the eye-cup of the photoreceptor cells in *Ciona*

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ARTICLE INFO

Keywords:
Rx
Photoreceptor cell
Ascidian
Ciona
Morphogenesis

ABSTRACT

The ascidian larva has a pigmented ocellus comprised of a cup-shaped array of approximately 30 photoreceptor cells, a pigment cell, and three lens cells. Morphological, physiological and molecular evidence has suggested evolutionary kinship between the ascidian larval photoreceptors and vertebrate retinal and/or pineal photoreceptors. *Rx*, an essential factor for vertebrate photoreceptor development, has also been suggested to be involved in the development of the ascidian photoreceptor cells, but a recent revision of the photoreceptor cell lineage raised a crucial discrepancy between the reported expression patterns of *Rx* and the cell lineage. Here, we report spatio-temporal expression patterns of *Rx* at single-cell resolution along with mitotic patterns up to the final division of the photoreceptor-lineage cells in *Ciona*. The expression of *Rx* commences in non-photoreceptor a-lineage cells on the right side of the anterior sensory vesicle at the early tailbud stage. At the mid tailbud stage, *Rx* begins to be expressed in the A-lineage photoreceptor cell progenitors located on the right side of the posterior sensory vesicle. Thus, *Rx* is specifically but not exclusively expressed in the photoreceptor-lineage cells in the ascidian embryo. Two *cis*-regulatory modules are shown to be important for the photoreceptor-lineage expression of *Rx*. The cell division patterns of the photoreceptor-lineage cells rationally explain the generation of the cup-shaped structure of the pigmented ocellus. The present findings demonstrate the complete cell lineage of the ocellus photoreceptor cells and provide a framework elucidating the molecular and cellular mechanisms of photoreceptor development in *Ciona*.

1. Introduction

Vertebrates have paired eyes and extraocular photoreceptor organs, such as the pineal and parapineal organs and deep brain photoreceptors (Vigh et al., 2002; Lamb, 2013; Nakane et al., 2014). All of these photoreceptor organs develop from the diencephalon, and although the development and evolution of the photoreceptor organs have been examined (Vigh et al., 2002; Lamb, 2013; Kusakabe, 2017), it is not yet unknown how these photoreceptor organs originate during the evolution of early vertebrates.

Tunicates, including ascidians, constitute a group of marine invertebrates that are phylogenetically closest to vertebrates (Bourlat et al., 2006; Delsuc et al., 2006). The larva of ascidians, such as *Ciona* and *Halocynthia*, has a vertebrate-like body plan containing the dorsal central nervous system with photoreceptor cells (Meinertzhagen and Okamura, 2001; Satoh, 2003; Kusakabe, 2017). The brain photoreceptor cells of the ascidian larva show morphological and physiological properties resembling the ciliary photoreceptor cells of the vertebrate retina and pineal organ (Eakin and Kuda, 1971; Gorman et al., 1971; Kusakabe et al., 2001, 2009; Kusakabe and Tsuda, 2007; Horie et al., 2008). Thus the photoreceptor cells of the ascidian larva clearly show evolutionary affinity with the ciliary photoreceptor cells found in vertebrate diencephalic photoreceptor organs (Kusakabe, 2017). Therefore, elucidation of the developmental mechanism of the ascidian photoreceptor cells can be expected to provide insights into the origin and early evolution of the vertebrate diencephalic photoreceptor organs.

The *Ciona* larva has two photoreceptor organs, the pigmented and the non-pigmented ocelli, in the brain vesicle (Horie et al., 2008). The pigmented ocellus shows the cup-shaped structure and is comprised of approximately 30 photoreceptor cells, a pigment cell, and three lens cells (Eakin and Kuda, 1971; Nicol and Meinertzhagen, 1991; Horie et al., 2008). We previously showed that the photoreceptor cells in the pigmented ocellus are derived from one of the A-lineage neural plate cells, the right A9.14 cell (right blastomeres are underlined to distinguish them from left blastomeres; Conklin, 1905), but not from a-lineage cells (Oonuma et al., 2016). It is not known how the cup structure of photoreceptor cells in the pigmented ocellus is generated

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https://doi.org/10.1016/j.ydbio.2018.11.011
Received 9 October 2018; Received in revised form 18 November 2018; Accepted 18 November 2018
Available online 28 November 2018

from a neural plate cell during development and what molecular mechanisms generate the photoreceptor cells.

Rx (retinal homeobox)/*Rax* (retina and anterior neural fold homeobox) genes are essential for development of the eye and the pineal organ of vertebrates (Mathers et al., 1997; Casarosa et al., 1997; Furukawa et al., 1997; Bailey et al., 2004; Muranishi et al., 2012). *Rx* encodes a paired-type homeodomain transcription factor, and the knockout of *Rx* genes in vertebrates results in deficient eyes (Loosli et al., 2001, 2003; Mathers et al., 1997). In addition, knockdown of *Rx* causes a reduction in the expression of the genes encoding arrestin and rhodopsin (Pan et al., 2010). It has been proposed that the requirement of *Rx* is characteristic of the development of ciliary photoreceptor cells (Arendt et al., 2004). Thus, whether *Rx* is involved in the development of the ascidian photoreceptor cells is an important question to address the evolutionary relationship between the ocelli of ascidians and the diencephalic photoreceptor organs of vertebrates.

The *Ciona* genome contains a single *Rx* gene (Imai et al., 2004; D'Aniello et al., 2006). A previous study suggested that *Rx* is required for differentiation of the larval photoreceptor cells in *Ciona* (D'Aniello et al., 2006). However, the same study revealed that *Ciona Rx* was expressed in the a-lineage cells at the early-middle tailbud stages, but not in the A-lineage cells from which the photoreceptor cells are derived. It thus remains unclear whether *Rx* directly or indirectly functions to regulate the differentiation of the photoreceptor cells in *Ciona*. Because *Ciona Rx* may begin to be expressed in the photoreceptor-lineage at later stages, it is necessary to examine the detailed expression patterns of *Rx* throughout photoreceptor cell development.

Removal of the chorion (egg membrane), which is a commonly used technique for the experimental manipulation of *Ciona* embryos, disrupts the left-right asymmetry of the brain vesicle, including the left-right asymmetric formation of the ocelli (Shimeld and Levin, 2006; Yoshida and Saiga, 2011; Oonuma et al., 2016). Nodal signaling plays a key role in the formation of left-right asymmetry during embryogenesis in various animal species (Capdevila et al., 2000; Shiratori et al., 2006; Boorman and Shimeld, 2002; Hamada et al., 2002; Morokuma et al., 2002; Yu et al., 2002; Duboc and Lepage, 2008; Yoshida and Saiga, 2008; Grande and Patel, 2009). In *Ciona* embryos, Nodal signaling down-regulates *Rx* expression on the left side of the brain vesicle (Yoshida and Saiga, 2011). Therefore, the normal expression and regulation of *Rx* during photoreceptor cell development in *Ciona* should be examined without removing the chorion.

In the present study, we determined the detailed expression patterns of *Rx* at single-cell resolution in *Ciona* embryos with the chorion. The transcription of *Rx* is first activated in non-photoreceptor a-lineage cells at the early tailbud stage. At the mid-tailbud stage, *Rx* begins to be expressed in the A-lineage photoreceptor cell progenitors. Two upstream *cis*-regulatory modules are shown to be required for the *Rx* expression in the photoreceptor-lineage cells. We also determined the complete cell division patterns of the photoreceptor lineage cells. These findings provide us with a framework for the elucidation of molecular and cellular mechanisms of photoreceptor development in *Ciona*.

2. Materials and methods

2.1. Animals and embryos

Mature adults of *Ciona intestinalis* type A (*Ciona robusta*) were provided by the Maizuru Fisheries Research Station of Kyoto University and by the Misaki Marine Biological Station of the University of Tokyo through the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The adults were also collected from the pond of the Fukae campus of Kobe University, Kobe, Japan. These adults were maintained in indoor tanks of artificial seawater (ASW) (Marine Art BR, Tomita Pharmaceutical Co., Tokushima, Japan) at 18 °C under constant light to induce the maturation of oocytes and spermatocytes. Eggs and sperm were obtained surgically from the gonoducts. After insemination, embryos were raised in ASW containing 0.005% streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 18 °C.

2.2. Preparation of reporter constructs and microinjection

To make the −7212/−1 construct, we amplified the 7.2-kb upstream region of *Ciona Rx* (D'Aniello et al., 2006; KH Gene Model ID KH.C12.152) from genomic DNA by polymerase chain reaction (PCR) and inserted it into the *Sal*I/*Sma*I site of the pBluescript-EGFP vector (Yoshida et al., 2004). The −3700/−1 construct was made by excising the upstream region between −7212 and −3701 by digestion with *Hin*dIII followed by self-ligation.

DNA constructs −2000/−1, −1500/−1, −1000/−1, −1500/−1(Δ−1000/−701), −1500/−1(Δ−1000/−301), and −1500/−1(Δ−700/−301) were made by PCR using the primers shown in Supplementary Table S1. The −1500/−1001MA4basal construct was made by replacing the −1000/−1 upstream region of the −1500/−1 construct with the basal promoter of a *Halocynthia roretzi* muscle actin gene (*HrMA4a*; Kusakabe et al., 1995). The point mutations were introduced into the consensus sequences (G/AACAAT) of the putative Sox binding sites (BSs) on the *cis*-regulatory region of *Rx* by PCR with mutagenic primers (Supplementary Table S2). Mutagenesis of putative BSs for Fox (TA/GTTT) or homeodomain transcription factors (HDTF; ATTA) was achieved by using a commercial artificial gene synthesis service (Eurofins Genomics, Tokyo). Mutations introduced in the Fox and HDTF BSs are shown in Supplementary Fig. S7.

For microinjection, plasmid DNAs and FITC-dextran (D1820; Thermo Fisher Scientific, Waltham, MA) were dissolved in the microinjection solution (140 mM KCl, 1 mM $MgCl_2$, 8 mM NaCl and 10 mM Hepes, pH 7.2) at the concentration of 10 ng/μl (DNA constructs) or 1.0 μg/μl (FITC-dextran). Plasmid DNA constructs were injected into fertilized eggs with the chorion. The FITC-dextran solution was injected into the right a4.2 or A4.1 blastomeres with the chorion at the 8-cell stage. The microinjection was carried out with the aid of holding and microinjection pipettes, as described by Kusakabe et al. (1996) and Oonuma et al. (2016).

2.3. Whole-mount in situ hybridization (WISH)

Embryos with the chorion were fixed in 4% paraformaldehyde in 0.1 M MOPS (pH 7.5) and 0.5 M NaCl at 4 °C for 16 h, prior to storage in 80% ethanol at −30 °C. After fixation, the chorion was removed using a pair of tungsten needles. Immunohistochemical whole-mount *in situ* hybridization (WISH) was performed using NBT/BCIP as described by Ikuta et al. (2004). Fluorescent WISH was carried out by the method described by Ikuta and Saiga (2007) with following modifications.

After hybridization, washes, and blocking, the FITC-conjugated RNA probe or FITC-dextran was detected by anti-FITC-HRP (NF7100001EA; Perkin Elmer, San Jose, CA) (1:200 dilution). Signal amplification was performed with the TSA system using Alexa488-conjugated tyramide (T20912; Thermo Fisher Scientific) (1:100 dilution) for 40 min at room temperature. After the washes and the inactivation of anti-FITC-HRP with 50% formamide, 5 ×SSC, and 0.1% Tween 20, the DIG-conjugated RNA probe was detected by using anti-DIG-POD (11207733910; Sigma-Aldrich) (1:1000 dilution). Signal amplification was performed using Alexa594-conjugated tyramide (T20950; Thermo Fisher Scientific) (1:100 dilution) for 40 min at room temperature. Fluorescent images were obtained by using a laser scanning confocal microscope (FV1200 IX83; Olympus, Tokyo, Japan). Confocal images were collected at 1-μm intervals in the z-axis.

We obtained the template DNA for probe synthesis by amplifying a genomic DNA fragment of a part of the first exon sequence of *Rx* (forward primer, 5'-GCTGGATCCACACGGATAGTGGGAGCAAC-3'; reverse primer, 5'-TTAGTCGACCTTGCTGTTCTGGTCGGTG-3'; restriction

sites are underlined). The amplified genomic region was inserted into the *Bam*HI/*Sal*I sites of pBluescript II SK(+). We also used the pBluescript-EGFP vector (Yoshida et al., 2004) as the probe template of EGFP.

Antisense digoxigenin and/or fluorescein-labeled RNA probes were synthesized using T7 RNA polymerase from plasmid DNA linearized with *Bam*HI for *Rx* and *EGFP*. A digoxigenin-labeled antisense RNA probe for *Ciona FoxB* was synthesized using a plasmid clone obtained from the *Ciona* Gene Collection release 1 (Gene Collection ID GC28o19) (Satou et al., 2002).

Critical resources and reagents are listed in the Key Resources Table.

3. Results

3.1. Later commencement of Rx expression in A-lineage cells

We recently demonstrated that the photoreceptor cells of *Ciona* develop solely from A-lineage cells of the neural plate (Oonuma et al., 2016), and an earlier study suggested that *Rx* was expressed in a-lineage cells at the tailbud stages (D'Aniello et al., 2006). We therefore hypothesized that the expression of *Rx* shifts from a-lineage to A-lineage cells during development. To assess this hypothesis, we investigated the temporal changes of *Rx* expression from the mid- to late-tailbud stages by WISH (Fig. 1). At 12 h post-fertilization (hpf), *Rx* mRNA was detected on the right side of the anterior brain vesicle at a location anterior to the presumptive otolith pigment cell (Fig. 1A). The expression was expanded posteriorly at 13 hpf (Fig. 1B). At 14 hpf, transcripts of *Rx* were detected posterior to the otolith pigment cell (Fig. 1C). At 15 hpf, a strong signal of *Rx* mRNA was observed on the right side of the posterior brain vesicle (Fig. 1D).

To further examine whether the posterior shift of *Rx* expression is due to the change of cells expressing *Rx* from a-lineage to A-lineage, we labeled all of the right a- or A-line cells with FITC. The two lineages segregate at the third cleavage (Fig. 1E). We introduced FITC-conjugated dextran into a single blastomere of 8-cell embryos with the chorion. When we introduced FITC-conjugated dextran into the right a4.2 blastomere at the 8-cell stage, the *Rx* expression detected by WISH at 12 hpf was overlapped with the FITC signal (Fig. 1F–H). By contrast, FITC staining was not overlapped with *Rx* mRNA localization at the same stage when the right A4.1 blastomere was labeled (Fig. 1L–N). At 15 hpf, localization signals of FITC and *Rx* mRNA were overlapped in both a4.2-labeled embryos and A4.1-labeled embryos (Fig. 1I–K,O–Q, Supplementary Fig. S1).

These results suggest that *Rx* is first expressed only in a-lineage cells but is also expressed in A-lineage cells at later stages during the development of the *Ciona* larval brain. *Rx* seems to continue to be expressed in a-lineage cells.

3.2. Ciona Rx begins to be expressed in the photoreceptor lineage cells after the 12th cell division

All of the photoreceptor cells of the pigmented ocellus derive from the right A9.14 cell (Oonuma et al., 2016). Although the above-described results showed that *Rx* is expressed in A-lineage cells, it was unclear whether and when the developing photoreceptor cells express *Rx* during the embryogenesis.

As a probe to chase the photoreceptor lineage cells, we used *FoxB*, which was expected to be continuously expressed in the A9.14 descendants from the late gastrula stage (Imai et al., 2004). We confirmed that *FoxB* transcripts were detected in the A10.27 and A10.28 cells, daughters of A9.14 cells, at the early neurula stage (Supplementary Fig. S2A–C) [see Conklin (1905) and Nishida and Stach (2014) for a detailed explanation of the naming system of cells in ascidian embryos]. These 10th generation cells divided along the anterior-posterior axis at the initial tailbud stage (Supplementary Fig. S2D–F; in the cell naming system, the first digit following the letter denotes the cell generation, counting the egg as the first). By 11 hpf, each of the 11th generation cells (A11.53, A11.54, A11.55, A11.56) divided along the dorsal-ventral axis (Supplementary Fig. S3A–C", Supplementary Video S1). These observations were consistent with those of a previous study (Navarrete and Levine, 2016).

Rx transcripts were not detected in the A9.14-derived 11th- and 12th-generation cells (A12.105–A12.112) (Fig. 2A–C"). When the pigmentation started in the otolith pigment cell, the 12th-generation cells divided along the left-right axis, and subsequently, *Rx* began to be expressed in the anteriorly located cells among the newborn 13th-generation cells derived from the A9.14 cell (Fig. 2D–E", Supplementary Fig. S3D–I"). After 20 min, the rest of the A9.14-derived 13th-generation cells (A13.209–A13.224) also expressed *Rx* (Fig. 2F–G", Supplementary Video S2). Then, strong *Rx* expression continued in the right A9.14-derived 13th-generation cells (Fig. 2H–I"). These results revealed that *Rx* expression begins in the photoreceptor precursor cells of the pigmented ocellus after the 12th cell division.

3.3. Spatio-temporal mitotic patterns shape the cup-like arrangement of the photoreceptor cells

We continued to track the descendants of the A9.14 cell by detecting *Rx* mRNA (Fig. 3). The dorsal 13th-generation cells, except for the two anterior-most cells (i.e., A13.221 and A13.222), divided along the left-right and/or the dorsoventral axes at 15 h 20 min after fertilization (Fig. 3A–A",C–C",E–E"). Interestingly, the A13.221 and A13.222 cells did not divide during further observations up to the late tailbud stages. By 30 min after the dorsal cells divided, the ventral 13th-generation cells completed dividing along the left-right and/or the dorsoventral axes (Fig. 3B–B",D–D",F–F", Supplementary Video S3). All of the A9.14-derived cells continued to express *Rx* mRNA at this stage.

The total number of the A9.14-derived cells is 30, which is consistent with the reported number of photoreceptor cells of the pigmented ocellus reported by previous studies (Horie et al., 2005, 2008; Oonuma et al., 2016). These findings suggest that all 30 of these cells are postmitotic terminally differentiating photoreceptor cells of the pigmented ocellus.

Reconstructed transverse section images of the brain vesicle revealed a cup-shaped arrangement of the nuclei of the A9.14-derived cells (Fig. 3I,I'). Migration of photoreceptor cell precursors was not observed. Instead, the orderly division patterns seem to result in the cup-shaped arrangement of the photoreceptor cells (Fig. 3G–I'). We also observed movement of the nucleus of the ocellus pigment cell from the dorsal side of the neural tube to the cavity of the brain vesicle during the tailbud stages (Fig. 3G–I'). Thus, the orderly regulated cell divisions are an important mechanism for generating the cup-like structure of the pigmented ocellus.

3.4. Cis-regulatory regions for Rx expression in the A-lineage cells

D'Aniello et al. (2011) identified a genomic region important for the expression of *Ciona Rx* in the tailbud and larval stages, but they did not distinguish *Rx* expression in a- and A-lineage cells. It has thus been unclear which part of the genome regulates the *Rx* expression in the A-lineage cells giving rise to photoreceptor cells. To identify the *cis*-regulatory region generating the expression of *Rx* in the A-lineage, we searched for genomic regions that can drive the expression of a reporter gene in the photoreceptor-lineage cells at 15 hpf (Fig. 4, Supplementary Fig. S4). D'aniello et al. (2006) suggested that the upstream region 7.2 kb from the translation start site of *Rx* recapitulates the endogenous expression of *Rx* at the tailbud and larval stages. They used a *lacZ* reporter and detected the reporter expression with β-galactosidase activity. We confirmed that a *GFP* reporter gene driven by the 7.2-kb upstream region (−7212/−1; from the nucleotide position −7212 to −1, relative to the translation start site) can recapitulate the endogenous *Rx* expression by detecting transcripts of

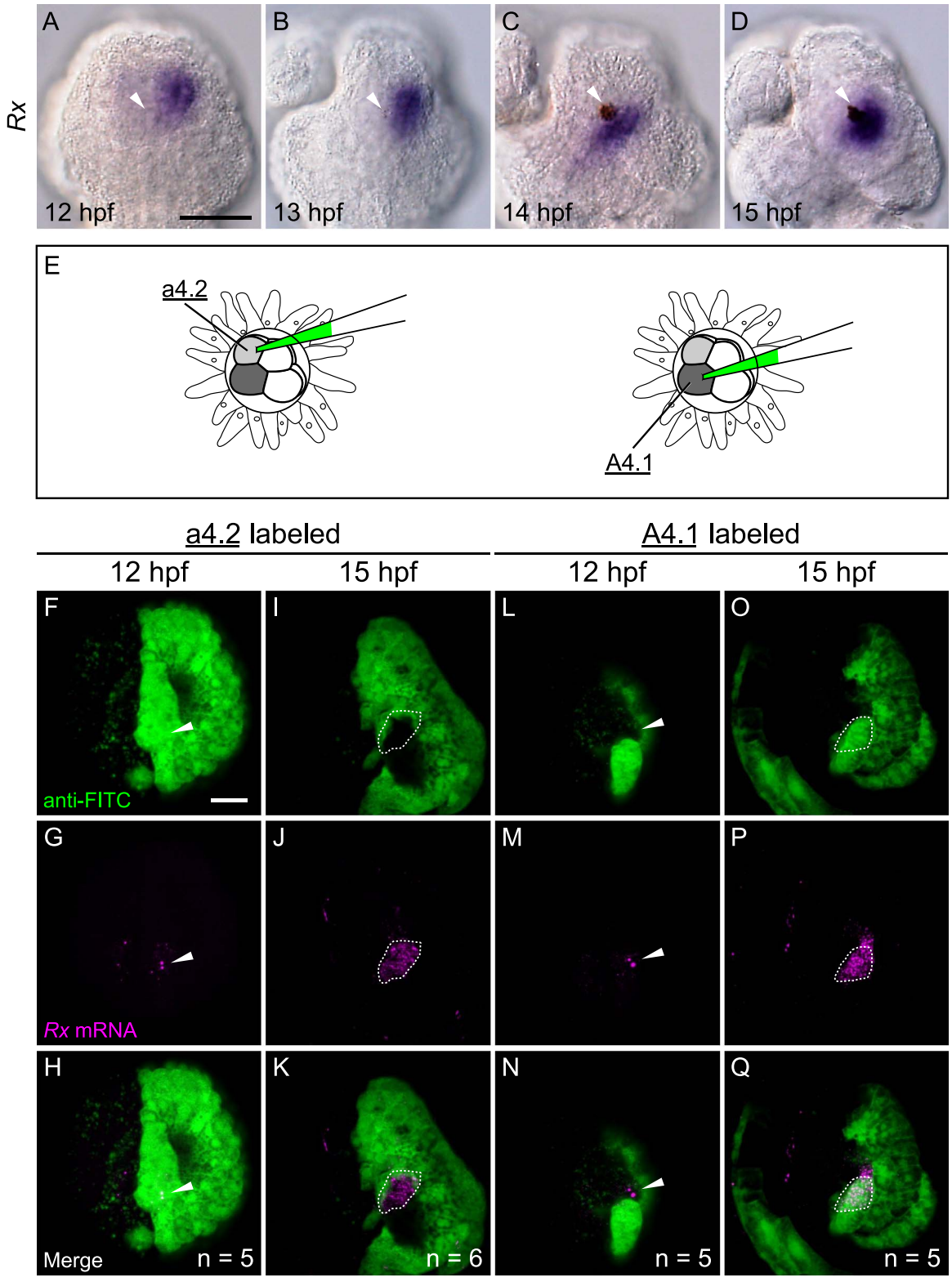


Fig. 1. *Ciona Rx* is expressed in both a- and A-lineage cells of the developing central nervous system. (A–D) Localization of *Rx* mRNA detected by whole-mount *in situ* hybridization (WISH) from 12 h post-fertilization (hpf) through 15 hpf. Anterior is to the top. Dorsal view. *White arrowheads* indicate the position of the otolith pigment cell. (E) Schematic diagram showing the method of labeling the descendants of a single blastomere of 8-cell embryos. A single blastomere in the embryo with the chorion was labeled by microinjecting an FITC-dextran solution. The a4.2 and A4.1 blastomeres are shown in light and dark gray, respectively. *Scale bars* represent 50 μm. (F–Q) FITC (F, I, L, O) and *Rx* mRNA (G, J, M, P) were detected in tailbud embryos in which either a single a4.2 or A4.1 blastomere had been labeled with FITC-dextran. H, K, N, and Q show merged images. Anterior is to the top. Dorsal view. *White arrowheads* indicate the *Rx* mRNA signal. *White dotted lines* indicate the A-lineage cells expressing *Rx* mRNA. *Scale bars* represent 20 μm.

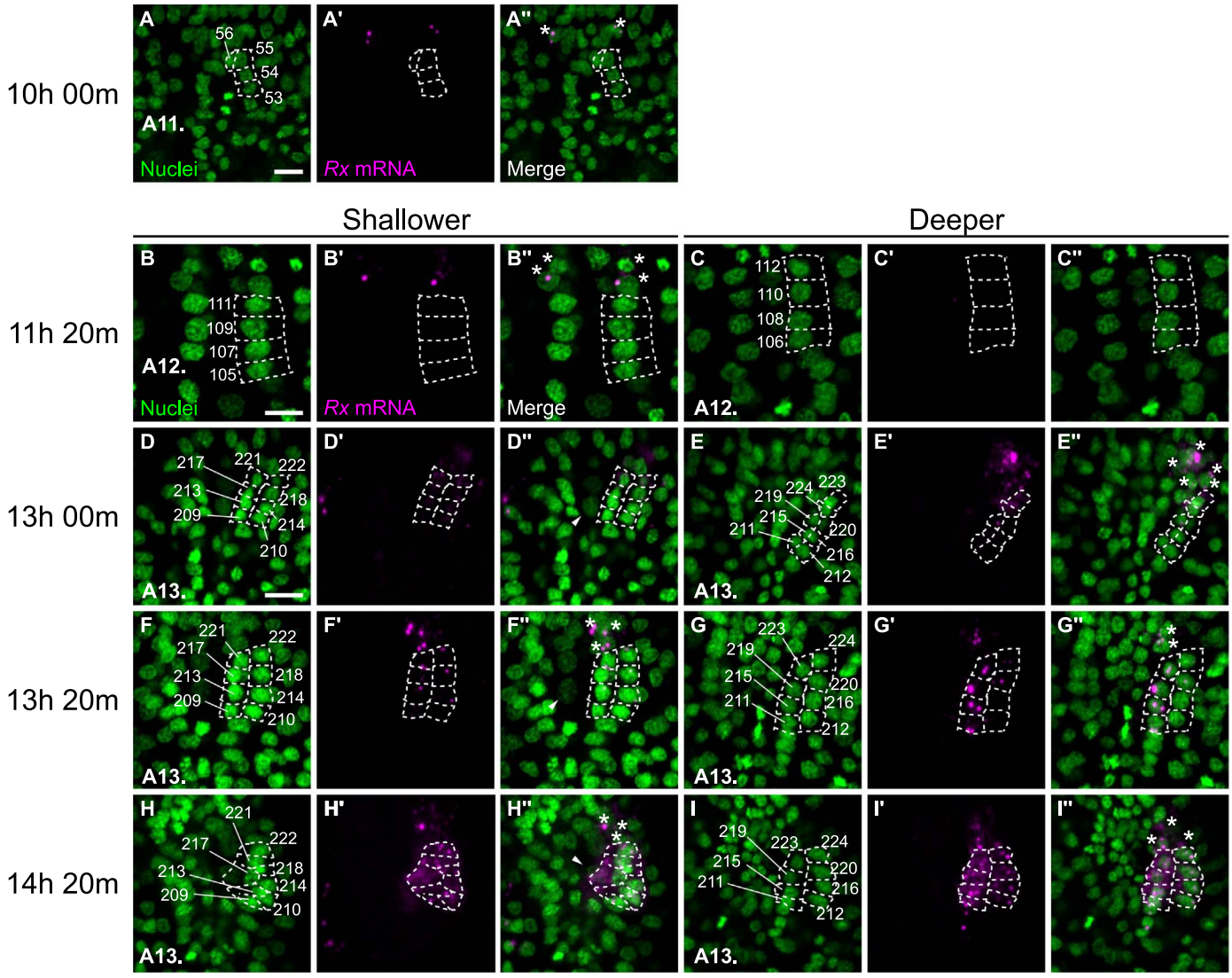


Fig. 2. Developmental expression profile of *Rx* in the A-lineage photoreceptor precursor cells at single-cell resolution. Localization of *Rx* mRNA was visualized by fluorescent WISH in embryos fixed at the indicated time after fertilization. Nuclei were counterstained with DAPI. Anterior is to the top. Dorsal views at a relatively shallow level (B–B", D–D", F–F" and H–H") and at a relatively deep level (C–C", E–E", G–G" and I–I") are shown. A9.14-derived cells are outlined by *white dots*. *Asterisks* indicate the expression of *Rx* in the a-lineage cells. *Arrowheads* indicate the nucleus of the ocellus pigment cell. *Scale bars* represent 10 μm.

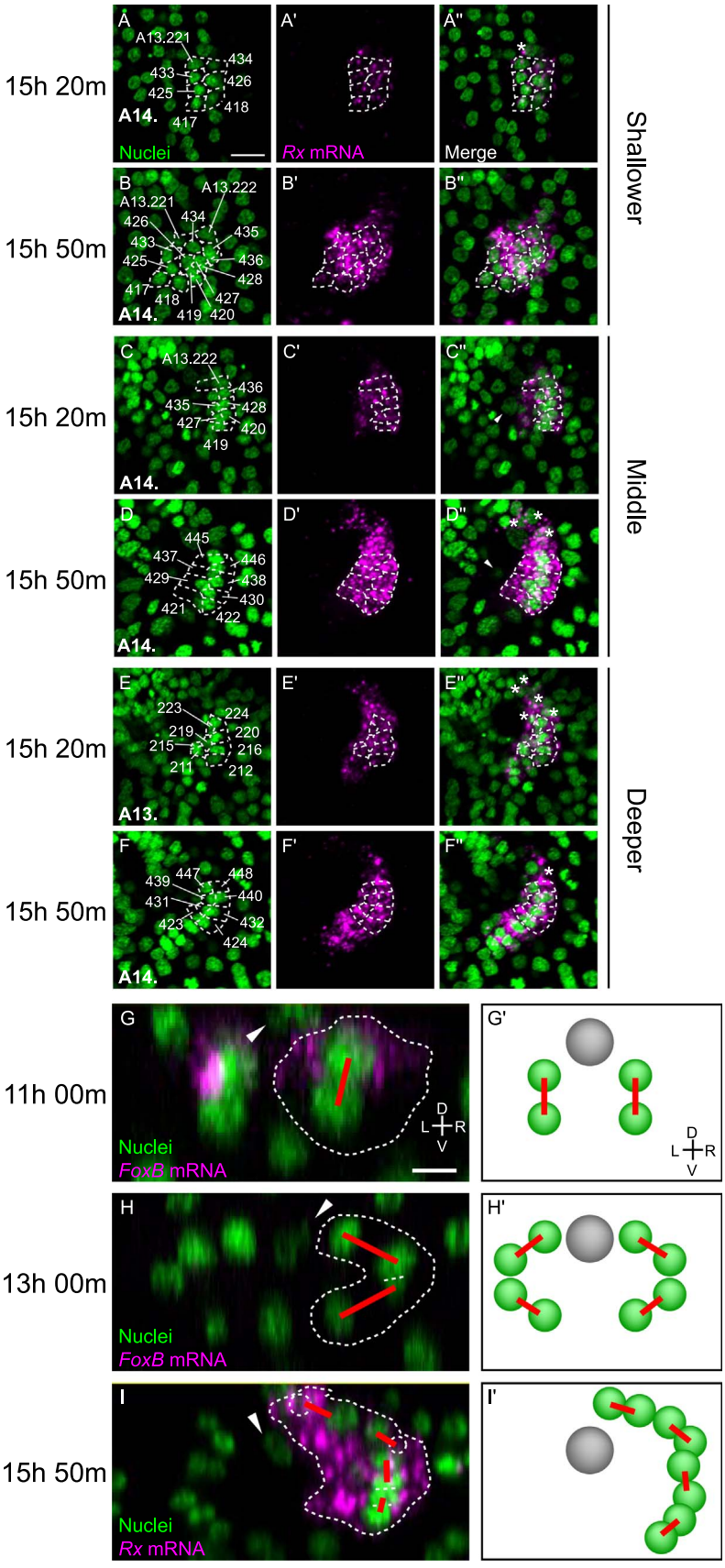
GFP and the endogenous *Rx* by double-fluorescent WISH (Fig. 4, Supplementary Fig. S4). We expect our approach to provide better spatial and temporal resolution by avoiding the detection of reporter proteins carried over from previous stages.

We next examined the transcriptional activity of a deletion series of the 7.2-kb upstream region. When the upstream region was deleted from the 5' side down to 3.7 kb, 2.0 kb and 1.5 kb, these upstream regions drove the expression of *GFP* mRNA in the lineage of photoreceptor cells with a frequency similar to that of the 7.2-kb upstream region (Fig. 4, Supplementary Fig. S4). In contrast, transgene expression was detected at low frequency when a construct harboring the 1.0-kb upstream region (−1000/−1) was introduced into embryos. These results suggest that the −1500/−1001 upstream region contains an enhancer.

However, *GFP* mRNA was not detected when a construct containing the −1500/−1001 region connected with the basal promoter of a *Halocynthia roretzi* muscle actin gene (*HrMA4a*) was introduced (Fig. 4, Supplementary Fig. S4). These results suggest that the −1500/−1001 region alone is not sufficient to drive gene expression in photoreceptor lineage cells. Thus, an additional *cis*-regulatory sequence(s) located in the region between the nucleotide positions −1000 and −1 seems to be required for the *Rx* expression in photoreceptor-lineage cells.

We further functionally dissected the −1000/−1 region by making an internal deletion from the 1.5 kb-upstream region. When the region between −1000 and −701 was deleted, the reporter construct drove the *GFP* expression similarly to the −1500/−1 construct (Fig. 4, Supplementary Fig. S4). In contrast, deletion of the region between −1000 and −301 resulted in the loss of *GFP* expression.

Expressed sequence tags (ESTs) of *Ciona* indicate that the mature transcripts of *Rx* are *trans*-spliced (Satou et al., 2006), so the 5' end of the *Rx* mRNA does not represent the transcription start site (TSS). The bona fide TSS determined by using the comprehensive TSS mapping data (Yokomori et al., 2016) is 214 bp upstream (−214) from the translation start site, indicating that the TSS is not deleted in the DNA construct (Supplementary Fig. S5). Ectopic expression of *GFP* in mesenchyme cells (data not shown) also suggests that the basal promoter remains intact after deletion of the region between −1000 and −301. These results suggest that the −700/−301 region contains an important *cis*-regulatory sequence for the *Rx* expression in the photoreceptor-lineage cells. Indeed, the deletion of the −700/−301 region resulted in the loss of *GFP* expression (Fig. 4). Thus, both the −1500/−1001 and −700/−301 regions are important for the transcription of *Rx* in the photoreceptor lineage cells at 15 hpf.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11h 00m
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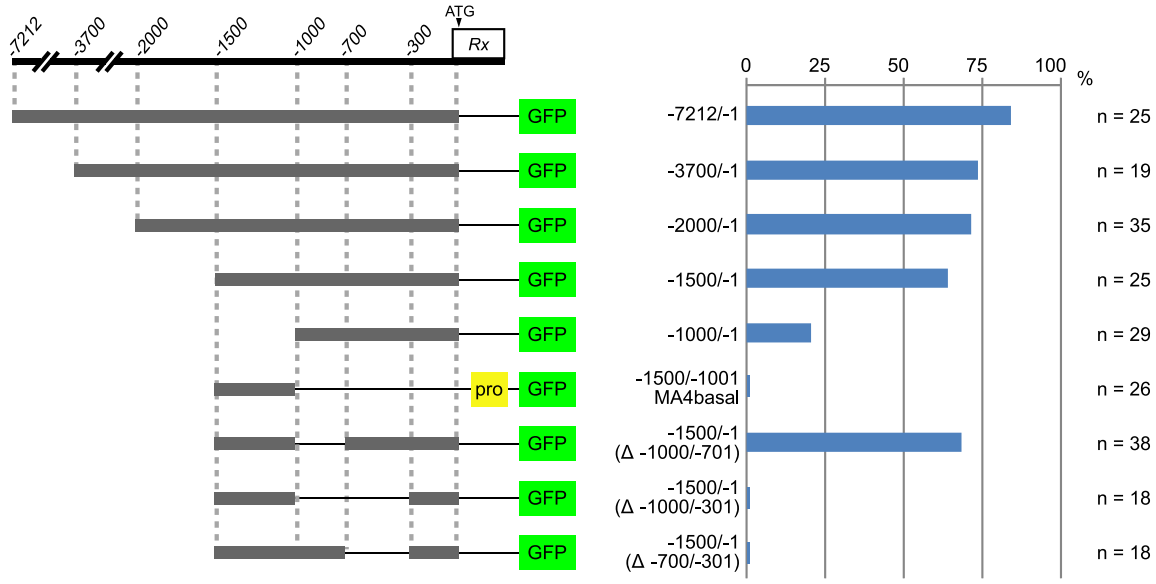

Fig. 4. *Cis*-regulatory activity of the upstream region of *Ciona Rx* in the developing photoreceptor cells. Expression of *GFP* mRNA was detected by fluorescent WISH in the photoreceptor-lineage cells of late tailbud (15 hpf) embryos that developed from fertilized eggs microinjected with *Ciona Rx > GFP* fusion constructs. A diagram on the left side indicates organization of *GFP* constructs harboring upstream region sequences of *Rx*. Numbers at the top of the diagram indicate nucleotide positions relative to the translation start site. Green boxes indicate the coding sequence of *GFP* and the yellow box indicates the basal promoter of a *Halocynthia roretzi* muscle actin gene. On the right side of the drawing representing each construct, bars indicate the percentage of positive embryos, i.e., individuals with *GFP* expression in photoreceptor lineage cells. Numbers on the right side indicate the number of embryos scored for each construct.

3.5. Putative transcription factor binding sites (TFBSs) required for Rx transcription in A-lineage cells

To obtain insights into the molecular mechanisms that generate the expression of *Rx* in the photoreceptor lineage cells, we looked for putative transcription factor binding sites (TFBSs) in the upstream region of *Ciona Rx*. As shown in Fig. 5, the two upstream regions identified as *cis*-regulatory regions (−1500/−1001 and −700/−301) contain a number of putative TFBSs, including those for Sox, Fox, and homeodomain transcription factors.

We examined functional importance of these TFBSs by introducing point mutations into these sequences in the GFP reporter constructs (Fig. 6 and Supplementary Fig. S6). When all of the homeodomain transcription factor (HDTF) binding sites (BSs) or Fox BSs in the −1500/−1001 region were mutated, these mutations did not affect the frequency of *GFP* expression in embryos at 15 hpf. By contrast, the elimination of Sox BSs from the −1500/−1001 region led to a significant increase in the frequency of embryos that express *GFP* in the right A-lineage cells (Fig. 6). Moreover, the frequency of the *GFP* expression was significantly decreased when all of the HDTF BSs in the −700/−301 region were mutated (Fig. 6). These results suggest that Sox factor(s) and HDTF(s) downregulate and upregulate, respectively, the expression of *Rx* in the photoreceptor-lineage cells.

4. Discussion

4.1. Cell lineage and morphogenetic cell division of the photoreceptor cells

In our earlier study, we demonstrated that the A9.14 cell of the neural plate gives rise to the photoreceptor cells in the pigmented ocellus (Oonuma et al., 2016). However, it remained unclear how these cells divide to form the eye cup structure. In the present study, we observed the cell division patterns of the A9.14 descendants leading to the formation of the cup structure during the tailbud stages (Fig. 7). Our observations revealed that the A9.14 descendants undergo an orderly series of longitudinal and transverse divisions to form a cup-like organization of the retina comprised of 30 photoreceptor cells.

The total number of the A9.14 descendants generated by the late tailbud stage is 30, which is consistent with the reported number of the photoreceptor cells in the pigmented ocellus (Horie et al., 2005, 2008; Oonuma et al., 2016). Therefore, these 30 cells seem to be postmitotic cells, which then terminally differentiate into photoreceptor cells. Before the terminal differentiation, the A9.14 cell goes through five rounds of divisions with the exception of the two anterior-most dorsal cells, A13.221 and A13.222. These two cells may differentiate into photoreceptor cells earlier than other photoreceptor precursor cells. Indeed, we observed the expression of *opsin1* in a few anterior-most cells among the A9.14 descendants at the middle-late tailbud stage (data not shown).

The regulation of the timing and the orientation of cell divisions must be an important mechanism of the morphogenesis of the pigmented ocellus of the ascidian larva. At present, however, the mechanism is completely unknown. Another unsolved question is how the process from the final division through the terminal differentiation of the photoreceptor cells proceeds and is controlled. Because the arrangement of the photoreceptor nuclei of the ocellus in the larva is different from that of the premature postmitotic ocellus in the late tailbud embryo (Horie et al., 2005, 2008), further rearrangement of the photoreceptor cells must occur during the maturation of the ocellus.

Fig. 3. Spatio-temporal mitotic patterns of the A9.14 descendants during mid and late tailbud stages. (A–F") Expression of *Rx* in the A9.14 descendants around the 13th cell division. Localization of *Rx* mRNA was detected by fluorescent WISH in embryos fixed at the indicated time after fertilization. Nuclei were counterstained with DAPI. Anterior is to the top. Dorsal views at relatively shallow (A–B"), relatively deep (E–F"), and intermediate (C–D") levels are shown. A9.14-derived cells are outlined by *white dots*. *Asterisks* indicate the expression of *Rx* in the a-lineage cells. *Scale bars* represent 10 μm. (G–I') Transverse images reconstructed from confocal optical sections, showing dorso-ventral patterning of the photoreceptor precursor cells. *FoxB* (G, H) and *Rx* (I) transcripts were detected by fluorescent WISH. Nuclei were counterstained with DAPI. Photoreceptor precursor cells are outlined by *white dots*. Diagrams in G', H', I' are schematic representations of the images shown in G, H, I, respectively. The photoreceptor-lineage cells are indicated in green and the ocellus pigment cell is indicated in gray. Red lines indicate cell division patterns. *Arrowheads* indicate the nucleus of the ocellus pigment cell. *Scale bars* represent 5 μm.

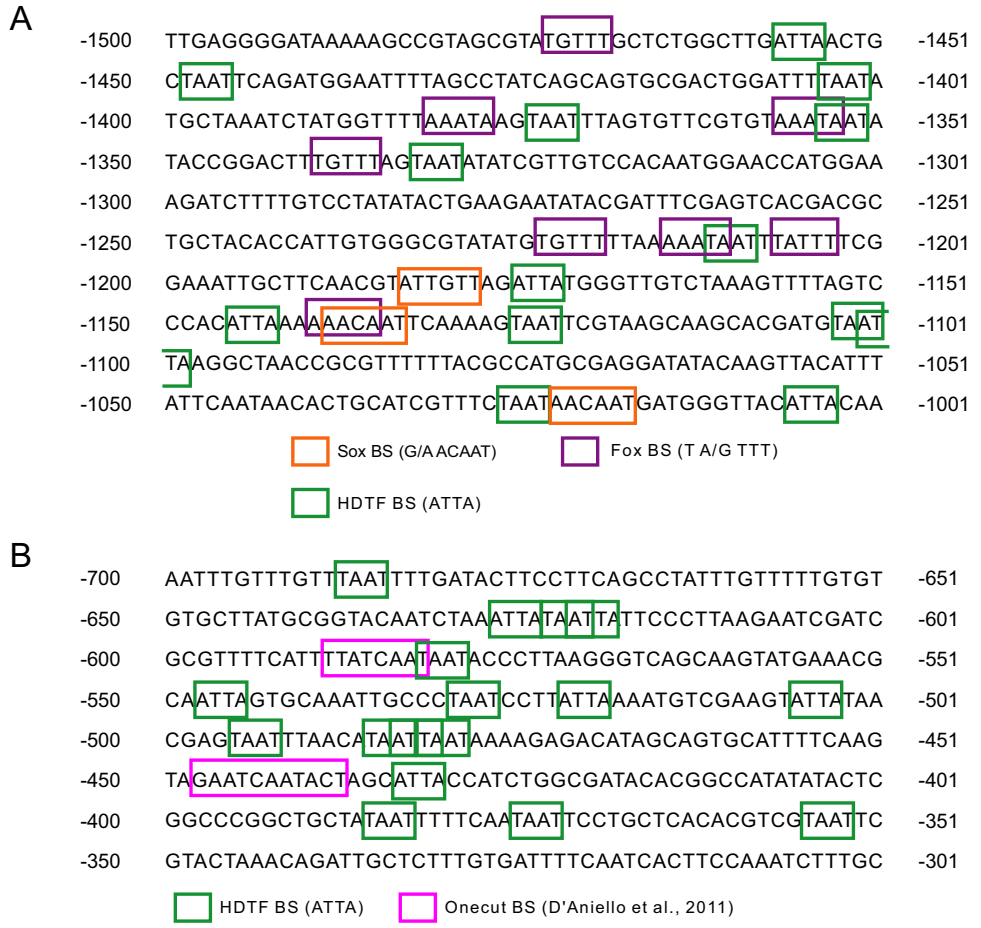

Fig. 5. Putative transcription factor binding sites (BSs) in the *cis*-regulatory regions of *Ciona Rx*. (A) The nucleotide sequences of the −1500/−1001 region. (B) The nucleotide sequences of the −700/−301 region. Numbers on each side of the sequence represent nucleotide positions relative to the translation start site. The putative BSs for Sox, Fox, and homeodomain transcription factors are boxed in orange, purple, and green, respectively, as indicated below the *cis*-regulatory region sequences. Two Onecut BSs described in D'Aniello et al. (2011) are also boxed in magenta.

4.2. *Ciona Rx is expressed in the developing photoreceptor cells*

The present study determined the detailed expression patterns of *Rx* during the development of the central nervous system of the *Ciona* tailbud embryo. Our results clearly revealed that *Ciona Rx* is expressed in the A9.14-derived developing photoreceptor cells. As shown by D'Aniello et al. (2006), *Rx* is first expressed on both the left and right sides of the anterior parts of the brain vesicle derived from a-lineage cells at the early tailbud stage (Fig. 2A–B"). At this stage, *Rx* is transiently expressed in a part of the ventral brain vesicle (D'Aniello et al., 2006). At the early-late tailbud stage, *Rx* continues to be expressed in the right anterior part of the brain vesicle, whereas the left side of the brain vesicle loses the *Rx* expression (Fig. 2B–E"; D'Aniello et al., 2006). The loss of the expression on the left side has been suggested to be due to Nodal signaling on the left side of the brain vesicle (Yoshida and Saiga, 2011). At the middle-late tailbud stage, *Rx*

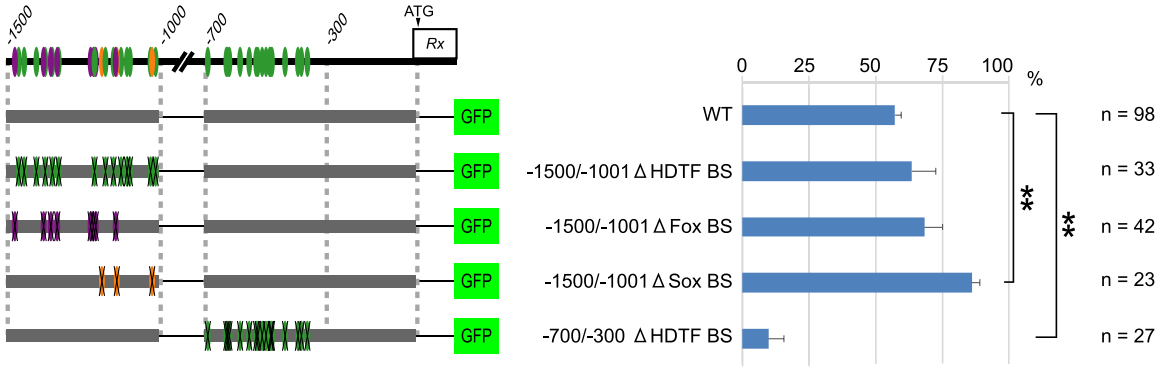

Fig. 6. Functional analysis of putative transcription factor BSs in the *cis*-regulatory regions of *Ciona Rx*. Expression of *GFP* mRNA was detected by fluorescent WISH in the photoreceptor-lineage cells of late tailbud (15 hpf) embryos that developed from fertilized eggs microinjected with *Ciona Rx > GFP* fusion constructs. Substitution mutations were introduced into putative BSs as indicated in the left diagram. Green boxes indicate the coding sequence of GFP. Numbers at the top of the diagram indicate nucleotide positions relative to the translation start site. Colored ovals indicate the putative BSs and black crosses indicate mutated BSs. On the right side of the drawing representing each construct, bars indicate the percentage of positive embryos, i.e., individuals with *GFP* expression in photoreceptor lineage cells. Error bars represent SEM from at least three independent experiments. Numbers on the right side indicate the total number of embryos scored for each construct. Statistical analysis was carried out using the standard Student *t*-test (**$P < 0.01$).

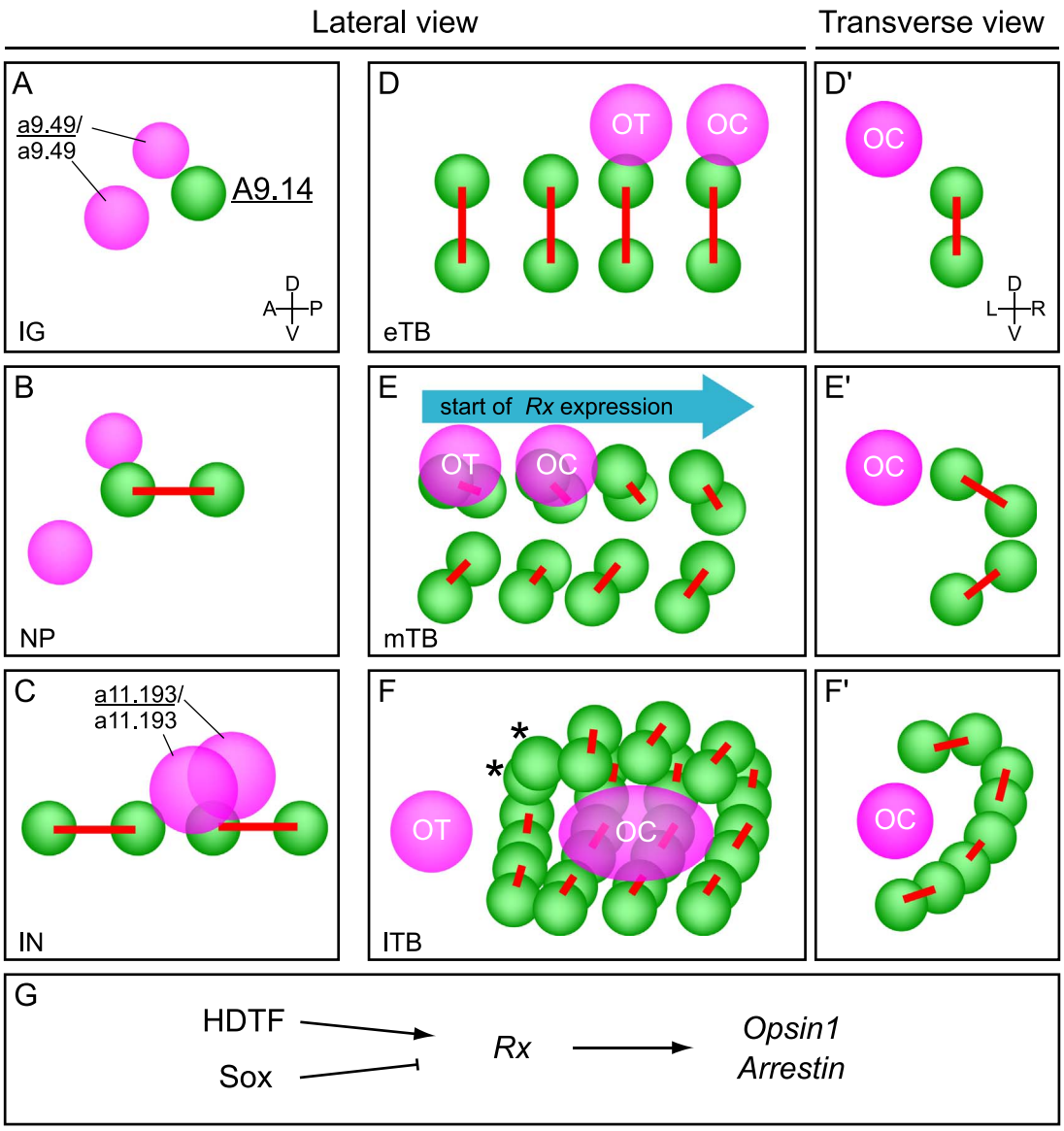

Fig. 7. Schematic diagram showing the developmental process of the photoreceptor cells of the pigmented ocellus. (A–F') Spatial patterns of cell divisions of the A9.14 descendants during the late gastrula (A), neural plate (B), late neurula (C), early tailbud (D, D'), mid tailbud (E, E'), and late tailbud (F, F') stages. The photoreceptor-lineage cells are indicated in green and the pigment-lineage cells are indicated in magenta. The expression of *Rx* begins in the anterior cells earlier than in the posterior cells as indicated by a blue arrow in (E). *Red lines* indicate the division pattern of sister cells. *Asterisks* in (F) indicate post-mitotic cells. eTB, early tailbud; lG, late gastrula; lN, late neurula; lTB, late tailbud; mTB, middle tailbud; NP, neural plate; OC, ocellus pigment cell; OT, otolith pigment cell. (G) Regulation and a role of *Rx* in the photoreceptor-lineage cells inferred by this study and previous findings. *Arrows* and a *T-bar* indicate positive and negative effects, respectively. HDTF, homeodomain transcription factor.

begins to be expressed in the A9.14-derived photoreceptor lineage cells (Fig. 2D–E").

Interestingly, the *Rx* expression in the A9.14 descendants was first detected in a few anterior cells, and in the following stages the expression became evident in the other cells (Fig. 2D–G"). An extracellular cue may generate the differential activation of *Rx* along the anteroposterior axis. Alternatively, an intrinsic polarity in the precursor cells may produce different properties between anterior and posterior photoreceptor cells. The *Rx* expression in the photoreceptor cells probably persist through the larval stage (D'Aniello et al., 2006). The expression patterns of *Rx* suggest roles of *Rx* in both the developing photoreceptor cells and the non-photoreceptor a-lineage cells.

What is the role of *Ciona Rx* expressed in the non-photoreceptor a-lineage cells? In addition to the retinal anlagen, vertebrate *Rx* genes are expressed in the primordia of both the pineal gland and the hypothalamus (Chuang et al., 1999; Furukawa et al., 1997; Muranishi et al., 2012). *Rx* regulates the specification and patterning of the hypothalamic brain regions in the mouse (Lu et al., 2013; Orquera et al., 2016). In *Ciona*, a population of cells in the ventral brain vesicle has been proposed to be homologous to the vertebrate hypothalamus (Moret et al., 2005a, 2005b; Razy-Krajka et al., 2012). Therefore, the *Rx*-positive a-lineage cells may be involved in the development of a brain region homologous to the hypothalamus.

4.3. *Ciona Rx* expression may be generated by the interaction between two cis-regulatory regions

The dynamic and transient expression patterns of *Ciona Rx* during the central nervous system development suggest a complex regulation of its transcription. We identified two upstream *cis*-regulatory regions necessary for the expression of *Rx* in the photoreceptor-lineage cells. The proximal region is required for the reporter gene expression in both the a-lineage cells and the A-lineage cells. The deletion of this region resulted in the complete loss of the reporter gene expression in the central nervous system. Because ectopic reporter expression in

mesenchyme cells was observed (data not shown), the basal promoter seemed to be intact after this deletion.

These results suggest that the proximal region acts as an enhancer that is necessary for the *Rx* expression in the central nervous system. This region includes the region previously identified as the enhancer in the a-lineage cells at the early tailbud stage (D'Aniello et al., 2011). Our present result suggests that the proximal region acts as an enhancer at the late tailbud stage as well as in both the a- and A-lineages.

In contrast to the proximal enhancer, we observed that the distal region alone cannot activate transcription in the central nervous system when it is combined with a basal promoter. Instead, the distal region seems to enhance the transcriptional activity of the proximal enhancer in the A-lineage. The presence or absence of this region did not alter the expression pattern of the reporter driven by the proximal enhancer. This mode of *cis*-regulatory activity of the distal region is similar to the "booster" region known for the transcriptional regulation of the sea urchin *Endo16* gene (Yuh and Davidson, 1996). The distal booster region of *Endo16* interacts with a proximal enhancer (Yuh and Davidson, 1996). By analogy, an interaction between the two *cis*-regulatory modules probably generates the expression of *Ciona Rx* in the photoreceptor-lineage cells.

4.4. Developmental and evolutionary diversity of transcriptional regulatory mechanisms of Rx

It has been suggested that a homeodomain transcription factor Onecut directly activates *Rx* transcription in the anterior central nervous system of *Ciona* tailbud embryos (D'Aniello et al., 2011). *Ciona Onecut* is co-expressed with *Rx* in the a-lineage-derived anterior region of the developing brain vesicle (Moret et al., 2005a; D'Aniello et al., 2011). However, expression of *Onecut* has not been reported in the A-lineage photoreceptor progenitor cells. Therefore, *Ciona* Onecut may regulate the *Rx* expression in the a-lineage cells, but not in the A-lineage cells.

Here we revealed that HDTF BSs and Sox BSs are important for the *cis*-regulatory activity of the upstream region of *Ciona Rx*. When we introduced mutations into HDTF BSs in the proximal enhancer region, the *cis*-regulatory activity was significantly reduced in the photoreceptor-lineage cells. In this mutant DNA construct, the Onecut BSs that were reported to be important for *Rx* expression in the a-lineage cells (D'Aniello et al., 2011) were largely unperturbed (the core sequences remained intact), suggesting that HDTFs other than Onecut activate *Rx* in the A-lineage cells. In amphibian embryos, transcriptional regulation of *Rx* by homeodomain and Sox transcription factors has been reported (Danno et al., 2008; Martinez-de et al., 2010; Kelly et al., 2016). Otx2 and Sox2 directly activate *Rx* transcription in the neuroectoderm of *Xenopus laevis* (Danno et al., 2008). These transcription factors have also been suggested to be involved in the repression of ectopic *Rx* expression (Martinez-de et al., 2010).

Interestingly, our present results suggest an inhibitory role of Sox BSs in the distal booster region. In addition to regulation by Otx2 and Sox2, autoregulation by *Rx* itself has been reported in *Xenopus*. In *Ciona*, Otx, Sox1/2/3 (Sox2 ortholog, also called SoxB1), and Rx can be candidate regulators of *Rx*, since these transcription factors are expressed in the anterior central nervous system, including the progenitors of the photoreceptor cells, during development (Hudson and Yasuo, 2005; Imai et al., 2009).

Collectively, these results suggest that a similar set of transcription factors regulate the transcription of *Rx* in ascidians and vertebrates. At present, it is not known to what extent their modes of action are similar or dissimilar. Because our present findings clearly revealed that the expression of *Rx* is a conserved feature between ascidian larval photoreceptors and vertebrate retinal photoreceptors, the future elucidation of detailed transcriptional mechanisms and the role of *Rx* in the photoreceptor-lineage cells of *Ciona* should deepen our understanding of the origin and evolution of the photoreceptive organs in chordates.

Acknowledgements

We thank Chikako Imaizumi, Reiko Yoshida, Yutaka Satou (Kyoto University), Megumi Koutsuka, Satoe Aratake, Manabu Yoshida (The University of Tokyo), and the National BioResource Project (NBRP) of MEXT for providing us with *Ciona* adults; and Yuki Miyamoto, Yoko Ikeda and Yasuko Terashima for their technical assistance. We also thank the Graduate School of Maritime Sciences, Kobe University for generously allowing us to collect *Ciona* on the campus. This study was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to K.O. (no. 17K15130) and T.G.K. (nos. 25290067, 16H04724). This study was also supported in part by research grants from the Hirao Taro Foundation of the Konan University Association for Academic Research and the Takeda Science Foundation (no. 2015021209) to T.G.K. and by a Sasakawa Scientific Research Grant from The Japan Science Society to K.O.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.11.011.

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