Developmental Biology 355 (2011) 358–371

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

# Onecut is a direct neural-specific transcriptional activator of Rx in *Ciona intestinalis*

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ARTICLE INFO

*Article history:*
Received for publication 18 November 2010
Revised 21 April 2011
Accepted 4 May 2011
Available online 12 May 2011

*Keywords:*
*Ciona intestinalis*
Ascidian
Sensory organs
Pigment cells
Rx enhancer

ABSTRACT

Retinal homeobox (Rx) genes play a crucial and conserved role in the development of the anterior neural plate of metazoans. During chordate evolution, they have also acquired a novel function in the control of eye formation and neurogenesis. To characterize the Rx genetic cascade and shed light on the mechanisms that led to the acquisition of this new role in eye development, we studied Rx transcriptional regulation using the ascidian, *Ciona intestinalis*. Through deletion analysis of the *Ci*-Rx promoter, we have identified two distinct enhancer elements able to induce *Ci*-Rx specific expression in the anterior part of the CNS and in the photosensory organ at tailbud and larva stages. Bioinformatic analysis highlighted the presence of two Onecut binding sites contained in these enhancers, so we explored the role of this transcription factor in the regulation of *Ci*-Rx. By *in situ* hybridization, we first confirmed that these genes are co-expressed in the same cells. Through a series of *in vivo* and *in vitro* experiments, we then demonstrated that the two Onecut sites are responsible for enhancer activation in *Ci*-Rx endogenous territories. We also demonstrated *in vivo* that Onecut misexpression is able to induce ectopic activation of the Rx promoter. Finally, we demonstrated that *Ci*-Onecut is able to promote *Ci*-Rx expression in the sensory vesicle. Together, these results support the conclusion that in *Ciona* embryogenesis, *Ci*-Rx expression is under the control of the Onecut transcription factor and that this factor is necessary and sufficient to specifically activate *Ci*-Rx through two enhancer elements.



## Introduction

Rx genes belong to the paired-like homeobox gene family. The developmental role of Rx genes has been widely studied in both vertebrate and invertebrate species. It has been found that they play a conserved role in the development of the anterior neural plate in metazoans. In chordates, they have also acquired a novel later function in the organogenesis of the vertebrate eye (Mathers et al., 1997). Moreover, a growing series of studies suggest that Rx genes are essential for the proliferation and specification of retinal progenitor cells. Mouse Rx null embryos have no eyes and variable reduction of the forebrain and midbrain structures. A similar phenotype has been found in *Xenopus* embryos injected with a dominant negative form of the XRx1 gene. Therefore, this suggests a conserved and essential role of Rx genes in the anterior neural development of *Xenopus* embryos (Andreazzoli et al., 1999). In zebrafish, the *chock*$^{s399}$ mutant, which contains a nonsense mutation in the homeodomain of Rx3, generates embryos missing eye structures. Specifically, the neuroretina fails to differentiate and the retinal pigmented epithelium is not visible (Loosli et al., 2003). In humans, mutations in the Rx locus cause severe ocular malformations, such as anophthalmia (absence of the eye) and microphthalmia (very small eye) (Voronina et al., 2004).

Analysis of Rx dependent genes in vertebrates is consistent with its roles in anterior neural and eye development. In *Xenopus*, Rx inhibits neural differentiation by repressing X-ngnr-1, XDelta1 and N-tubulin (Andreazzoli et al., 2003; Chuang and Raymond, 2001) and activating Zic2 and Xhairy2 (Zilinski et al., 2004). During retinal development in *Xenopus*, Rx induces cellular proliferation through repressing expression of the p27Xic1 gene, a cell cycle inhibitor gene (Andreazzoli et al., 2003). In Rx mutant mouse embryos, the initial activation of *Otx*2, *Six*3 and *Pax*6 gene expression in the anterior neural plate is Rx dependent, although their downregulation could be explained by the absence or lack of proliferation of the retinal progenitor cells (Zhang et al., 2000; Zilinski et al., 2004). However, the finding that ectopic expression of Rx in mouse embryonic stem cells induces their differentiation into retinal progenitor cells supports the hypothesis that Rx genes have a more direct role in retinal differentiation (Tabata et al., 2004). Furthermore, it has been reported that Rx can activate two specific photoreceptor markers, Arrestin and IRBP, by binding specific and conserved elements (PCE/RET1) present in their promoters (Kimura et al., 2000).

Although the function of Rx genes and downstream consequences has been analyzed in vertebrate and invertebrate species, the genetic networks that regulate Rx expression have not received the same attention. Analysis of the XRx1 promoter in *Xenopus* demonstrated that three distinct upstream regions, which contain a high degree of

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doi:10.1016/j.ydbio.2011.05.584

conservation with the Rx upstream region of human, mouse, and opossum, are able to drive expression of a reporter gene in the same territories as the endogenous transcript (Danno et al., 2008). Moreover, the authors demonstrate that *Otx*2 and *Sox*2 are responsible for recapitulating endogenous Rx gene expression. Therefore, given the little that is known about gene regulatory networks responsible for Rx expression, understanding these networks is likely to tell us about the evolutionary and developmental mechanisms by which Rx genes have come to regulate eye development.

Ascidian larvae and vertebrate embryos share anatomical and molecular homologies, making them an excellent model to study the origin of developmental mechanisms, such as Rx regulation and function. At the larval stage, *Ciona intestinalis* embryos have a CNS with many chordate features. The simple ascidian nervous system has four main regions along the anteroposterior axis: the sensory vesicle, the neck, the visceral ganglion and the caudal nerve cord (Meinertzhagen and Okamura, 2001). The sensory vesicle is thought to be homologous to the forebrain of vertebrates and contains two pigmented cells: the anterior spherical otolith, which has a gravity-sensing function and is on the left ventral side of the sensory vesicle, and the photosensing ocellus, which has a half-moon shape and is in the right posterior wall of the sensory vesicle (Lemaire et al., 2002). The ascidian ocellus is composed of a cup-shaped pigmented cell, around 30 photoreceptors and three lens cells (Horie et al., 2005). Photoreceptor cells in *Ciona* exhibit both morphological (ciliary type) (Gorman et al., 1971) and electrophysiological characteristics (hyperpolarization in response to the light) similar to those of vertebrate photoreceptor cells (unpublished data). Furthermore, the ocellus has a photosensitive function during the larval stage, which has been proposed to be ancestral to vertebrate eyes (D'Aniello et al., 2006; Horie et al., 2008; Sakurai et al., 2004; Tsuda et al., 2003). That the *Ciona* ocellus and its photoreceptors are homologous to the vertebrate eye is also suggested by the similarity of *Ci*-Opsin1 to the vertebrate ciliary opsin subfamily (Kusakabe et al., 2001).

In addition to the homology of the ascidian larvae to vertebrates, the compact organization of genes in the ascidian genome (one gene every 7.5 kb of euchromatic DNA) makes it an ideal organism to study conserved gene regulatory networks. The core promoters and associated enhancers are often located within the first 1.5 kb upstream of the transcription start site (Satoh et al., 2003). Using phylogenetic footprinting between the genomic sequences of the ascidians *C. intestinalis* and *Ciona savignyi* allows rapid identification of *cis*-regulatory DNA sequences that mediate tissue-specific patterns of gene expression during *Ciona* embryogenesis. In addition, the ability to rapidly make transgenic larvae offers the opportunity to verify if *Ciona* regulatory elements have been conserved during chordate evolution (Di Gregorio and Levine, 2002; Locascio et al., 1999; Ristoratore et al., 1999). Altogether, the similarities of the ocellus to the vertebrate eye and the compact genome render *C. intestinalis* an optimal and simplified model to study Rx developmental functions and to identify its *cis*-regulatory sequences in chordate embryos (Passamaneck and Di Gregorio, 2005; Satoh et al., 2003).

We have previously studied the function of *Ciona* Rx during development. Similar to what has been demonstrated in vertebrates, we found that *Ciona* ocellus development is dependent on Rx gene function (D'Aniello et al., 2006). Electrophysiological measurements under variable light conditions indicate these Rx-deficient *Ciona* larvae did not show any light dependent changes in their electrical activity and had a corresponding altered ability to swim spontaneously with respect to control larvae (D'Aniello et al., 2006). Furthermore, knockdown of *Ci*-Rx specifically inhibited the expression of two photoreceptor-specific genes, *Ci*-Opsin1 and *Ci*-Arrestin, suggesting the lack of functional photoreceptor cells underlies their inability to sense light stimuli (D'Aniello et al., 2006). In the present study, we describe the analysis of a 2.9 kb non-coding sequence upstream of the *Ciona* Rx gene. Using deletion analysis, we identified a 197 bp region, (−603 to −407 bp) able to recapitulate the endogenous *Ci*-Rx expression pattern in transient transgenic embryos. Bioinformatic analysis indicated that there are putative *Ci*-HNF6/Onecut binding sites within this region of the promoter. We go on to provide evidence that *Ci*-Onecut is directly involved in *Ci*-Rx activation *in vivo* and *in vitro*. Together, these results extend our understanding of the evolutionary regulatory mechanisms underlying eye development through providing the first evidence of a key and direct role of a Onecut gene in the regulation of chordate Rx transcriptional activation.

## Methods

### *Ascidian eggs and embryos*

*C. intestinalis* adults were collected in the bay of Naples, Italy. Eggs and sperm were collected from the gonoducts of several animals and used for *in vitro* fertilization. Fertilized eggs and embryos were used in electroporation or *in situ* hybridization experiments. Embryos were raised in Millipore-filtered seawater at 18–20 °C. Only the batches in which at least 60% of the embryos developed normally were selected for the experiments. Samples at appropriate stages of development were also collected by low speed centrifugation and used for RNA preparation or fixed for *in situ* hybridization.

### *Constructs preparation*

pBlueScript II KS containing the *GFP* reporter gene and the SV40 polyadenylation sequence (Alfano et al., 2007) was used to prepare the B1.6 and C1.3 constructs. The B1.6 construct contains a 0.2 kb DNA fragment corresponding to the basal promoter of the human β-globin. This insertion was necessary to provide the TATA and CAAT boxes necessary for the basal transcription machinery activation. pBlueScript 1230 (gift of R. Krumlauf, Stowers Institute, Kansas City, USA), which contains the *LacZ* reporter gene and SV40 polyadenylation sequence with and without the human β-globin basal promoter, was used for all other constructs. The desired fragments were amplified using polymerase chain reaction (PCR) and inserted in the 5′–3′ orientation upstream of the reporter genes. PCR primers were designed according to the sequence of *C. intestinalis* genome (http://genome.jgi-psf.org/ciona4/ciona4.home.html) and are listed in Table S1.

The constructs from J-A to J-F and OC2 were prepared using a different strategy. Pairs of synthetic purified oligonucleotides (Primm Company; Table S2) were designed containing flanking *Kpn*I and *Xho*I restriction sites at the 5′ and 3′ end, respectively. Oligonucleotides were annealed by boiling 5 min in a hybridization buffer (1× TE and 150 mM NaCl) and slowly cooling to RT. The annealed products were cloned in the pBlueScript 1230 vector.

The 3 kb Etr promoter was amplified by PCR using primers indicated in Table S1. The Etr-GFP construct was prepared by replacing the B1.6 fragment with the Etr promoter in the B1.6 construct. For Etr-OC-VP16 and Etr-OC-WRPW constructs the Etr promoter fragment and the *Ci*-Onecut coding sequence replaced the MESP promoter and the coding sequence of Ets in the MESP>Ets:VP16 and MESP>Ets:WRPW (gift of Brad Davidson, University of Arizona, Tucson, AZ, USA) respectively.

### *In silico analysis of putative trans-acting factors*

The J-A and the J-F sequences were submitted to the MatInspector software of the Genomatix Database (http://www.genomatix.de/cgi-bin/eldorado.main.pl), which is a database of transcription factors binding sites and DNA-binding profiles from many organisms (Cartharius et al., 2005).

*Mutagenized Ci-Rx/LacZ constructs*

Two different strategies have been used to prepare the J0.2 MUT and J-A MUT constructs. The first was prepared by site-directed mutagenesis from the J0.2 construct with Quik Change Site-Directed Mutagenesis Kit (Stratagene). The putative Onecut-binding site was replaced by a sequence that reduced the binding affinity by using the mutagenic oligonucleotides listed in Table S3. The second was prepared using a pair of synthetic purified oligonucleotides, containing the flanking *Kpn*I and *Xho*I restriction sites at the 5′ and 3′ end, respectively, and replacing the core sequence of the Clox site with a sequence with a reduced binding affinity (Table S3).

*Isolation of the Ci-Onecut cDNA*

The full-length cDNA of *Ci*-Onecut has been amplified by PCR using as template cDNA from mRNA poly(A)$^+$ isolated at tailbud stage of *C. intestinalis*. The forward oligonucleotide was designed overlapping the ATG start codon (Onecut up) and the reverse oligonucleotide overlapping the stop codon (Onecut down) (Table S3). The full-length *Ci*-Onecut PCR fragment was cloned in the TOPO TA vector and sequenced.

*Electrophoretic mobility shift assay (EMSA)*

Electrophoretic mobility shift assays (EMSAs) were carried out under the following conditions. The *Ci*-Onecut full-length protein was synthesized *in vitro* using the TNT Coupled Reticulocyte Lysate System (Promega). Each reaction contained $^{32}$P labeled substrate DNA fragments and 2 μl of *Ci*-Onecut *in vitro* translated protein in 20 μl of binding mixture, consisting of 10 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA pH 8.0, 1 mM DTT, 20% glycerol and 6 pmol poly [dIdC]. DNA fragments were prepared by annealing complementary oligonucleotides. Briefly, 6 pmol single-strand oligonucleotides (Table S3) were radiolabeled by 5′ termini phosphorylation and annealed with cold complementary strand by boiling 5 min and slowly cooling it up to RT. The mixture was incubated 10 min on ice before the addition of radiolabeled DNA probe ($10^5$ cpm) in the presence or absence of specific, mutated or random (Table S3) double strand competitor oligonucleotides corresponding to a 100× or 200× molar excess. After addition of the labeled DNA, the binding mixtures were placed 20 min on ice and subjected to electrophoresis on a 5% native polyacrylamide/0.5× TBE gel, at 150 mV for 3 h. The products were visualized by autoradiography of the dried gel.

*Preparation of co-electroporation construct*

The pBS/pBra700/GFP/SV40 construct (gift of Dr. A. Spagnuolo, Stazione Zoologica A. Dohrn, Napoli, Italy) (Corbo et al., 1997) was used to prepare the construct used for the coelectroporation experiments. It consists of a BlueScript vector containing 700 bp of *Ciona* promoter region of Brachyury gene (*Ci*-Bra), GFP as reporter gene and SV40 polyadenylation signal. The GFP reporter gene was replaced with the full-length *Ci*-Onecut cDNA using *Eco*RI to make the Bra-Onecut construct.

*Electroporation*

Different fusion gene constructs were electroporated into fertilized eggs as described in Locascio and collaborators (1999). Each electroporation was performed using eggs from several different batches, and each construct was tested in two or more electroporations.

*Histochemical detection of β-galactosidase activity*

Transgenes expression was visualized by histochemical detection of β-galactosidase activity. In brief, embryos at the desired developmental stage were fixed for 15 min in 1% glutaraldehyde in FSW, washed twice with 1× PBS and stained at 37 °C in staining solution (3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, 0.1% Tween 20 and 250 μg/ml X-gal in 1× PBS). After incubation, embryos were washed in 1× PBS and imaging capture was made with a Zeiss Axio Imager M1 microscope. For each construct, a minimum of 100 embryos were analyzed in at least five different electroporations.

*In situ hybridization*

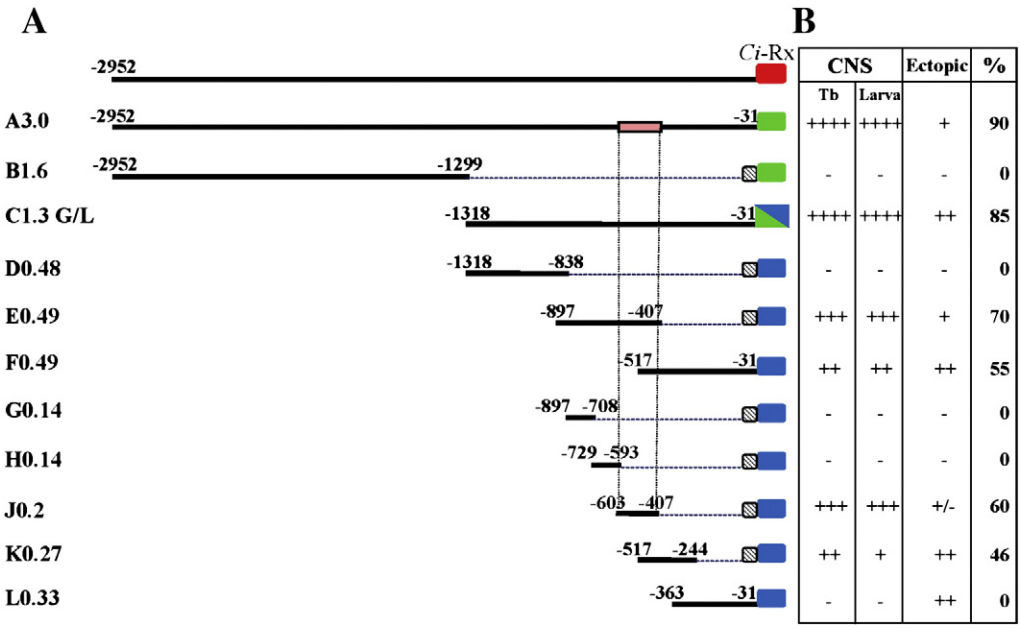
Single whole mount *in situ* hybridization was carried out as previously described (Locascio et al., 1999). Double fluorescent *in situ* hybridization was performed as described by Dufour and collaborators (2006). Embryo imaging capture was made with a Zeiss Axio Imager M1 and a Zeiss LSM 510 META confocal microscopes.

## Results

*Ci-Rx promoter analysis in electroporated embryos*

In order to identify transcription factors (TFs) that control *Ci*-Rx expression in the ocellus and photoreceptor cells, we sought to characterize the regulatory region responsible for *Ci*-Rx tissue specific expression. Previously, we isolated a 2.9 kb genomic fragment (named A3.0, Fig. 1A) extending from the position −2952 to −31 from the translation start site of the *Ci*-Rx gene that was able to reproduce its endogenous expression pattern at tailbud and larva stages (D'Aniello et al., 2006). In order to narrow *Ci*-Rx regulatory elements, we performed a promoter deletion analysis of the 2.9 kb DNA fragment followed by electroporation using GFP or LacZ reporter genes. Initially, we subdivided this fragment into two overlapping fragments: the 1654 fragment, extending from position −2952 to −1299 (B1.6 construct, Fig. 1A), and the 1288 fragment extending from position −1318 to −31 (C1.3 construct, Fig. 1A). The electroporated *Ciona* embryos were allowed to develop until the stage of interest, anesthetized, and were mounted on slides and observed with a fluorescent microscope. The B1.6 construct was unable to induce any specific GFP expression at both tailbud and larva stages (data not shown). However, the C1.3 G construct showed a clear fluorescent signal at the tailbud stage in two anterior groups of cells of the CNS and in one more posterior cell along the dorsal midline of the CNS (Fig. 2A). At the larva stage, the fluorescent signal was detected in the sensory vesicle in the cells surrounding the ocellus (Fig. 2B). The GFP expression in the transgenic embryos completely reproduces the expression pattern of the endogenous *Ci*-Rx transcript (Figs. 2C, D).

In order to identify the regulatory elements responsible for the specific reporter activation, we prepared a series of overlapping DNA fragments covering the entire C1.3 sequence. The C1.3 G construct was subdivided into three smaller and partially overlapping fragments called D0.48, E0.49 and the F0.49 (Fig. 1A). Because of the presence of endogenous GFP auto-fluorescence, particularly evident at the level of the sensory vesicle, we decided to test these new fragments using LacZ as the reporter gene. Moreover, LacZ permits the unambiguous detection of very faint signals due to visualization with histochemical staining. To be able to directly compare the signal intensity of these fragments with that of construct C1.3, we also tested this enhancer using LacZ as reporter gene (called C1.3 L; Figs. 3A, B). This *Ci*-Rx enhancer also induces ectopic expression in the trunk mesenchyme and in some epidermal cells of the tail (Figs. 3A, B). We next investigated the ability of the smaller constructs to induce expression of the reporter gene. We never observed specific expression when embryos were electroporated with the D0.48 construct. However, both




| | CNS | | Ectopic | % |
|---|---|---|---|---|
| | Tb | Larva | | |
| A3.0 | ++++ | ++++ | + | 90 |
| B1.6 | - | - | - | 0 |
| C1.3 G/L | ++++ | ++++ | ++ | 85 |
| D0.48 | - | - | - | 0 |
| E0.49 | +++ | +++ | + | 70 |
| F0.49 | ++ | ++ | ++ | 55 |
| G0.14 | - | - | - | 0 |
| H0.14 | - | - | - | 0 |
| J0.2 | +++ | +++ | +/- | 60 |
| K0.27 | ++ | + | ++ | 46 |
| L0.33 | - | - | ++ | 0 |

**Fig. 1.** (A) Diagrams of transgene constructs. Non-coding sequences are represented by black bars. LacZ and GFP reporter genes are represented by a blue and green box, respectively. Red box indicates the Rx gene sequence. The dashed box indicates the human β-globin basal promoter. The pink bar represents a non-coding sequence with high level of homology between *Ciona intestinalis* and *Ciona savignyi.* (B) Scoring chart for expression driven by each transgene in *C. intestinalis* embryos at tailbud and larva stages. Number of "+" symbols denotes relative intensity and penetrance of LacZ and GFP expression; % indicates number of embryos showing expression of the reporter genes. CNS, central nervous system; tb, tailbud stage.

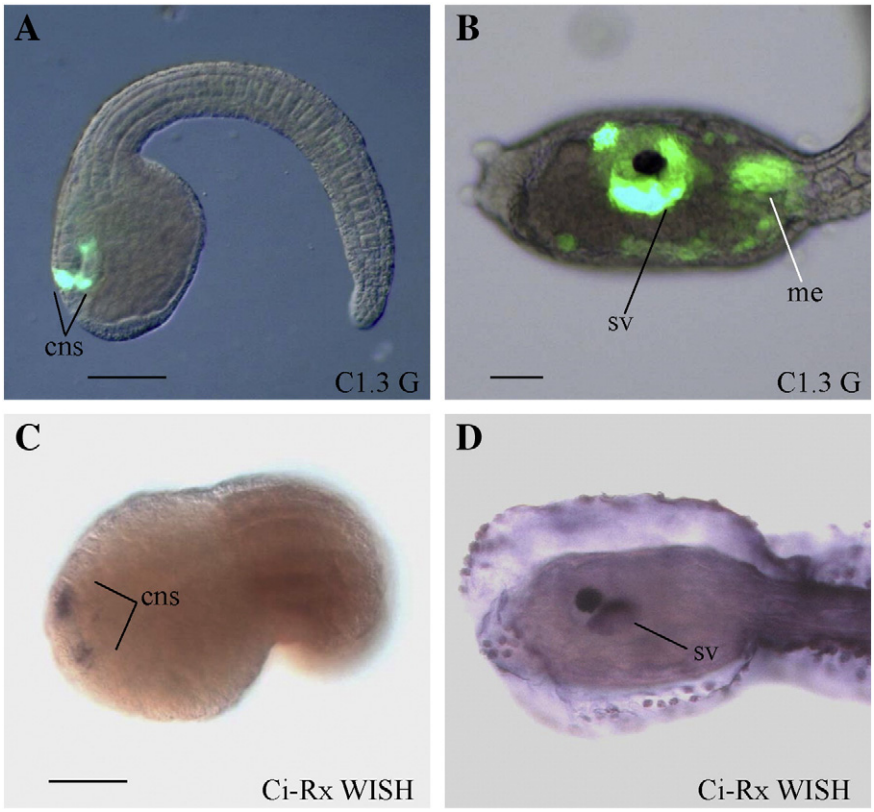



**Fig. 2.** GFP expression driven by C1.3 G reporter transgene. (A) Tailbud stage, dorsal view. Expression of the reporter gene is visible in the same three groups of cells of the endogenous transcript in the anterior brain. (B) Larva stage, lateral view. Expression of the reporter gene is visible in the sensory vesicle in the cells surrounding the ocellus in the same territory as the endogenous transcript and in some ectopic cells of the mesenchyme (white bar). (C, D) *Ci*-Rx expression pattern visualized by whole-mount *in-situ* hybridization. (C) Tailbud stage, dorsal view. Expression is in three groups of cells in the anterior brain. (D) Larva stage, lateral view. Expression is in the sensory vesicle in the cells surrounding the ocellus. Anterior is to the left in all panels. cns, central nervous system; me, mesenchyme; sv, sensory vesicle. Scale bars indicate 50 μm.

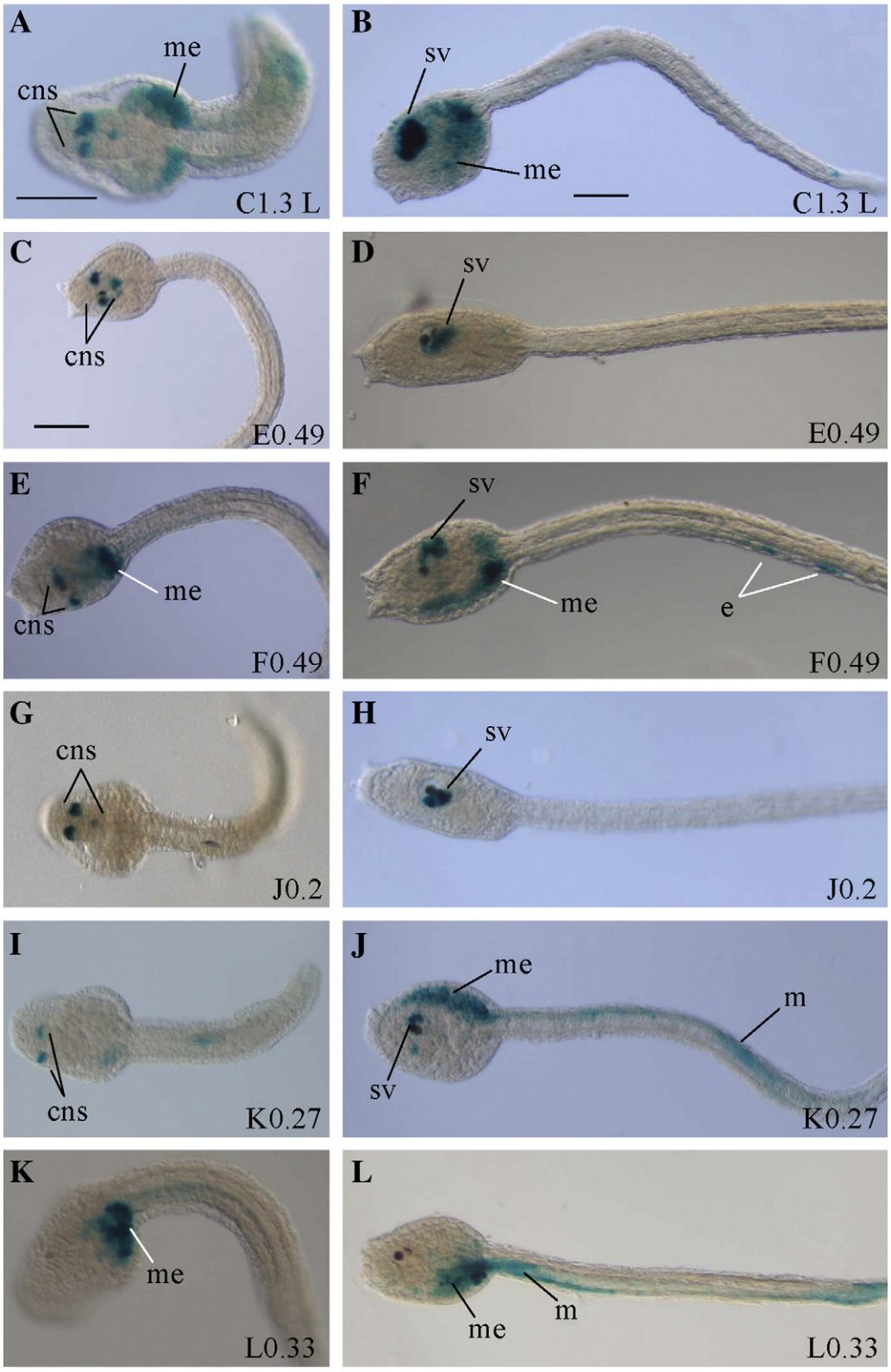



**Fig. 3.** β-galactosidase histochemical assays of LacZ expression driven by various reporter transgenes. (A, C, E, G, I) Expression of the LacZ reporter gene at tailbud stage in the anterior brain for the constructs, C1.3 L, E0.49, F0.49, J0.2 and K0.27, respectively. Lateral view of tailbud embryos. Anterior is on the left. (K) No specific expression of the reporter gene has been detected for the L0.33 construct at tailbud stage. (B, D, F, H, J) Expression of the LacZ reporter gene at larva stage in the sensory vesicle for the constructs, C1.3 L, E0.49, F0.49, J0.2 and K0.27, respectively. (L) No specific expression of the reporter gene has been detected for the L0.33 construct at larva stage. White bars indicate non-specific expression. cns, central nervous system; m, muscle; me, mesenchyme; sv, sensory vesicle. Scale bars indicate 100 μm.

the E0.49 (Figs. 3C, D) and the F0.49 (Figs. 3E, F) constructs were able to induce the expression of LacZ in the same territories as the endogenous *Ci*-Rx at tailbud and larva stages. Comparing these results with those obtained with the C1.3 L construct, it seems that the E0.49 fragment is slightly more efficient than the F0.49 with respect to induction in the CNS and sensory vesicle, but that both are less efficient

than the previously analyzed C1.3 L fragment (Figs. 3A, B). Moreover, it is evident that the F0.49 fragment also showed ectopic expression in the trunk mesenchyme and in the tail epidermis in a high percentage of transgenic embryos (Figs. 3E, F).

### *C. intestinalis vs C. savignyi: comparative sequence analysis*

Having identified two fragments capable of driving appropriate expression in the sensory vesicle, we decided to carry out a comparative sequence analysis between *C. intestinalis* and *C. savignyi* to highlight the presence of putative conserved sequences between these two closely related species. For this comparison, we used the new *C. intestinalis* genome database (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html/), which already contains a DNA sequence comparison between these two *Ciona* species performed with the VISTA bioinformatic tool (Mayor et al., 2000; http://genome.lbl.gov/vista/index.shtml); *C. savignyi* sequence was taken from the Broad Institute database (http://www.broad.mit.edu/annotation/ciona/). We examined 3 kb upstream of the exon 1 and 1.2 kb downstream of the exon 5 for a total sequence length of 11.2 kb of the *Ci*-Rx genomic locus. This analysis revealed the presence of three conserved non-coding sequences (Fig. 4). The first extends from position −2559 to −2440. The second, which is the only region with more than 75% conserved identity, is in between position −603 and −407. The third conserved sequence, which is positioned between the third and fourth exons, extends from position +3349 to +3473.

Based on these results we decided to investigate *in vivo* the ability of these three conserved regions to specifically activate reporter gene expression in the *Ci*-Rx territories. The first conserved sequence is located within the B1.6 construct, which already has been assayed by electroporation experiments and did not recapitulate endogenous expression (Fig. 1). Consistent with this observation, this reporter construct (M0.1) is unable to activate any specific expression, despite the high level of homology with *C. savignyi* (Fig. 4). In addition, the conserved intronic region located between the third and fourth exons of the *Ci*-Rx gene (INTR 0.12; Fig. 4) did not show any reporter gene activation, indicating that this sequence also lacks a positive regulatory element in this context. However, the last conserved sequence (J0.2), which is located within the E0.49 construct and is partially included in the F0.49 construct (Fig. 1A), was able to drive CNS and sensory vesicle specific expression. Therefore, sequence within J0.2 is likely at least in part responsible for the CNS and sensory vesicle specific expression.

### *Identification of a Ci-Rx specific enhancer*

While J0.2 was able to drive neural specific expression, both E0.49 and F0.49 constructs, which together cover the region from −897 to −31 of the *Ci*-Rx promoter, were also able to drive specific expression. To determine if other sequences within this region are also responsible for CNS and sensory vesicle expression, we made five constructs to cover this region. The E0.49 construct was covered by three partially overlapping constructs G0.14, H0.14 and J0.2, which was the conserved sequence from the Vista analysis analyzed above (Figs. 1A and 4). The F0.49 region, which covered from −517 to −31, was subdivided into two constructs, K0.27 and L0.33 (Fig. 1A). Of these constructs, G0.14 and H0.14 did not show any specific expression (data not shown), while L0.33 drove only ectopic expression not in the CNS or sensory vesicle (Figs. 3K, L). However, the J0.2 and K0.27 constructs drove the reporter gene expression in the nervous system of electroporated embryos at both tailbud and larva stages (Figs. 3G–J). Therefore, these results suggest that these two overlapping sequences contain the element(s) necessary to activate *Ci*-Rx transcription.

To further isolate the minimal sequence responsible for *Ci*-Rx activation, the J0.2 sequence was subdivided into a series of six smaller partially overlapping fragments, J-A through J-F (Fig. 5A). Electroporation experiments showed that the J-B, J-C, J-D and J-E constructs were unable to activate the expression of the reporter gene (Fig. 5B; data not shown). However, the J-A and J-F constructs were able to specifically activate LacZ reporter gene in the sensory vesicle (Fig. 5B). Moreover, the J-A and J-F constructs were able to closely recapitulate the endogenous *Ci*-Rx expression in the sensory vesicle at both the tailbud and larva stages (Figs. 6A–D). Of these two constructs, the J-F fragment produced stronger expression, in terms of signal intensity, with respect to the J-A construct (Figs. 6A–D). However, both fragments were less efficient than the entire J0.2 (Figs. 3G, H).

### *Putative Onecut DNA-binding sites are found in the conserved Ci-Rx promoter sequence*

In order to identify possible binding sites recognized by known transcription factors present in the J-A and J-F sequences, we used the Genomatix professional database of vertebrate TFs (http://www.genomatix.de/cgi-bin/eldorado.main.pl). The Genomatix analysis indicated the presence of multiple potential TF binding sites on both sequences, including those for HNF6 (Onecut). The Cut proteins contain a bipartite DNA-binding domain formed by the Cut domain and a homeodomain and are classified into groups containing one, two or three Cut domains.

Specifically, the J-F sequence contained the presence of a Onecut site (Fig. 6G), while the J-A fragment contained the consensus sequence of a Cut-like gene, called Clox (Fig. 6G). Vista comparison of the J0.2 enhancer between *C. intestinalis* and *C. savignyi* indicated that both the Clox and Onecut binding sites were perfectly conserved (Fig. 8A).

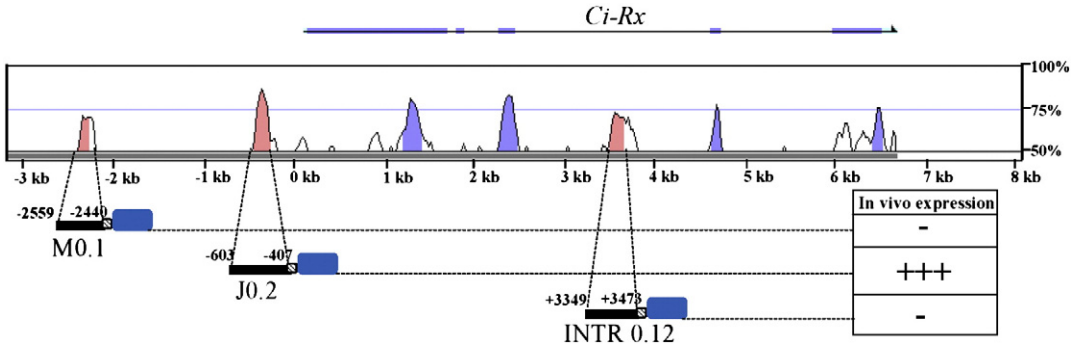
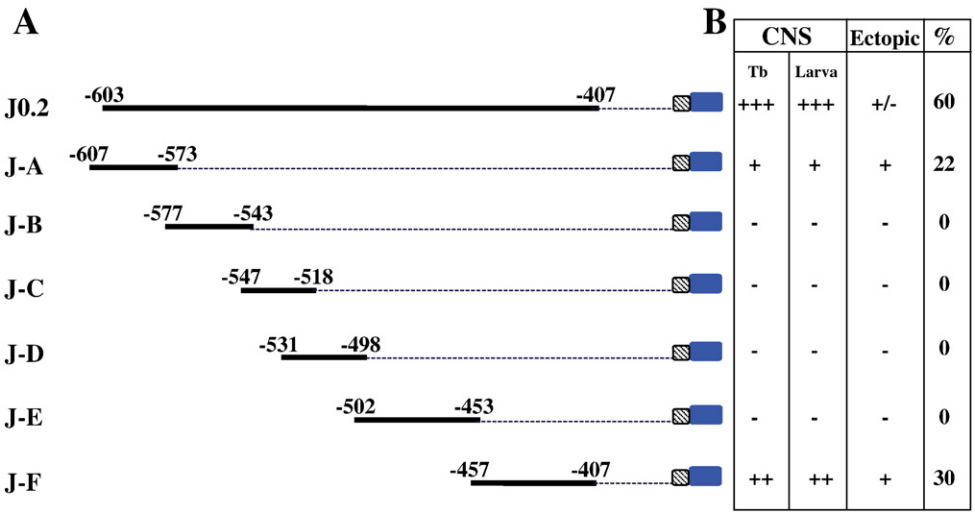



**Fig. 4.** mVista sequence alignment plot between *C. intestinalis* and *C. savignyi*, with *Ci*-Rx exons shown as blue boxes. Curve represents levels of sequence identity in a 50 bp window. Blue peaks are exons, while pink peaks are non-coding sequences. Below the alignment are the constructs used in this study that have conserved non-coding sequences. The scoring chart indicates expression driven by each transgene in *C. intestinalis* embryos at tailbud and larva stages. "+" symbols denote relative intensity and penetrance of LacZ.




| | CNS | | Ectopic | % |
|---|---|---|---|---|
| | Tb | Larva | | |
| J0.2 | +++ | +++ | +/- | 60 |
| J-A | + | + | + | 22 |
| J-B | - | - | - | 0 |
| J-C | - | - | - | 0 |
| J-D | - | - | - | 0 |
| J-E | - | - | - | 0 |
| J-F | ++ | ++ | + | 30 |

**Fig. 5.** (A) Diagrams of deleted transgene constructs of the J0.2 non-coding sequence. Black bars indicate non-coding sequences. Blue box represents the LacZ reporter gene. Dashed box indicates human β-globin basal promoter. (B) Scoring chart for expression driven by each transgene in *C. intestinalis* embryos at tailbud and larva stages. Number of "+" symbols denotes relative intensity and penetrance of LacZ expression. % indicates number of embryos showing expression of the reporter gene. CNS, central nervous system; tb, tailbud stage.

*Comparison of Ci-Rx and Ci-Onecut expression*

In order to assess if *Ci*-Onecut is a viable candidate to regulate *Ci*-Rx expression, we carried out whole mount *in situ* hybridization to determine if their expression patterns overlap. At the tailbud stage *Ci*-Onecut expression was detected along the antero-posterior axis of the central nervous system in the precursors of the sensory vesicle of the visceral ganglion and in some cells of the spinal cord (Figs. 7A, B). Later at larva stage, *Ci*-Onecut expression in the nervous system is restricted to the sensory vesicle and the visceral ganglion (Fig. 7C). This pattern of *Ci*-Onecut expression is consistent with what has been reported previously in *Halocynthia roretzi* and *C. intestinalis* (Moret et al., 2005; Sasakura and Makabe, 2001). Importantly, comparing the expression of *Ci*-Rx and *Ci*-Onecut at tailbud and larva stages suggested that some of the cells marked by *Ci*-Onecut could overlap with cells in the anterior nervous system that express *Ci*-Rx. Therefore, we then investigated the colocalization of these two genes using double fluorescent *in situ* hybridization. Using confocal microscopy, we found that the most anterior cells expressing *Ci*-Rx consistently also co-express *Ci*-Onecut (Figs. 7E–G). Furthermore, we observed *Ci*-Onecut co-localization in the two more anterior groups of cells that express *Ci*-Rx. Unfortunately, it was not been possible to establish if *Ci*-Onecut is expressed in the more posterior cell marked by *Ci*-Rx, because *Ci*-Rx expression in this cell is too faint and transient to be detected with this technique.

*Ci-Onecut binding is necessary for anterior neural expression of Ci-Rx*

To study the role of *Ci*-Onecut in the activation of the *Ci*-Rx enhancer, we used the J0.2 fragment for mutational analyses. To test its dependence on the putative Onecut DNA binding elements, we made point mutations in five nucleotides of the core Onecut binding sequence (Fig. 8B). Electroporation experiments of the J0.2 MUT construct, carrying the 5 point mutations, ($n>150$ embryos analyzed in at least three independent experiments) showed only 29% of the embryos with reporter gene expression in contrast to the 65% of positive embryos electroporated with the wild type J0.2 construct (Fig. 8C). This result supports the hypothesis that the Onecut binding site is involved in the activation of *Ci*-Rx expression. To further verify this hypothesis, we created a new synthetic construct, called OC2, which contains 60 nucleotides with a tandem repetition of the Onecut binding site (Fig. 6G). The OC2 construct was also able to direct expression of the reporter gene in the specific territories of *Ci*-Rx when electroporated in *Ciona* eggs (Figs. 6E, F). To determine if the Clox binding site found in the J-A fragments is also involved in the activation of the *Ci*-Rx enhancer, the Clox binding site in the J-A construct was mutated with 5 nucleotides changes (J-A MUT; Fig. 8B). When electroporated into *Ciona* eggs, the J-A MUT construct did not show any expression of the reporter gene, while the J-A construct was able to drive expression of the LacZ in the 25% of the electroporated embryos (Fig. 8C). Together, these results reveal that at least two Onecut binding sites are involved in Rx transcriptional activation.

*Ci-Onecut binds and activates the Ci-Rx enhancers*

To address the ability of the Onecut protein to directly bind the *Ci*-Rx enhancer, we next carried out electrophoretic mobility shift assays (EMSA). *In vitro Ci*-Onecut protein was synthesized and combined with either the oligonucleotides encoding the Onecut binding site (Onecut wt) or the Clox binding site (Clox wt), respectively (Table S3). Both the oligonucleotides were able to form specific complexes with *Ci*-Onecut (Fig. 9 lanes 1, 7). In both cases, the retarded band disappeared in the competition with 100–200-fold excess of specific unlabeled oligonucleotide (Fig. 9, lanes 2–3, 8–9), but was not affected by 200-fold excess of the random (R) oligonucleotide (Fig. 9, lanes 4, 10). To further confirm the retarded bands were due to the specific binding of *Ci*-Onecut to these oligonucleotides, the competition assay was performed using mutated oligonucleotides (called Onecut MUT and Clox MUT), which were made by changing the same five nucleotides as used in the analysis above (Fig. 8B). The mutated oligonucleotides were not able to compete with WT oligonucleotides for *Ci*-Onecut (Fig. 9, lanes 5–6, 11–12), nor were the mutated oligonucleotides able to be retarded by *Ci*-Onecut alone (data not shown). Therefore, these results suggest that *Ci*-Onecut is directly involved in the *Ci*-Rx enhancer activation and is able to recognize the Onecut binding sites.

To investigate *in vivo* the ability of the *Ci*-Onecut protein to bind and active the two *Ci*-Rx enhancers, we next determined if *Ci*-Onecut is able to induce the ectopic expression of the reporter constructs in the notochord, a territory where it is normally not expressed. For this purpose, we prepared a construct in which the coding sequence of *Ci*-Onecut has been cloned downstream of the *Ci*-Brachyury promoter sequence (Bra-Onecut). Brachyury is specifically expressed in the notochord and its promoter has already been characterized in *Ciona* (Corbo et al., 1997; Yamada et al., 2003). To verify its ability to induce *Ci*-Onecut ectopic expression in the notochord cells, Bra-Onecut electroporated embryos were analyzed by *in situ* hybridization

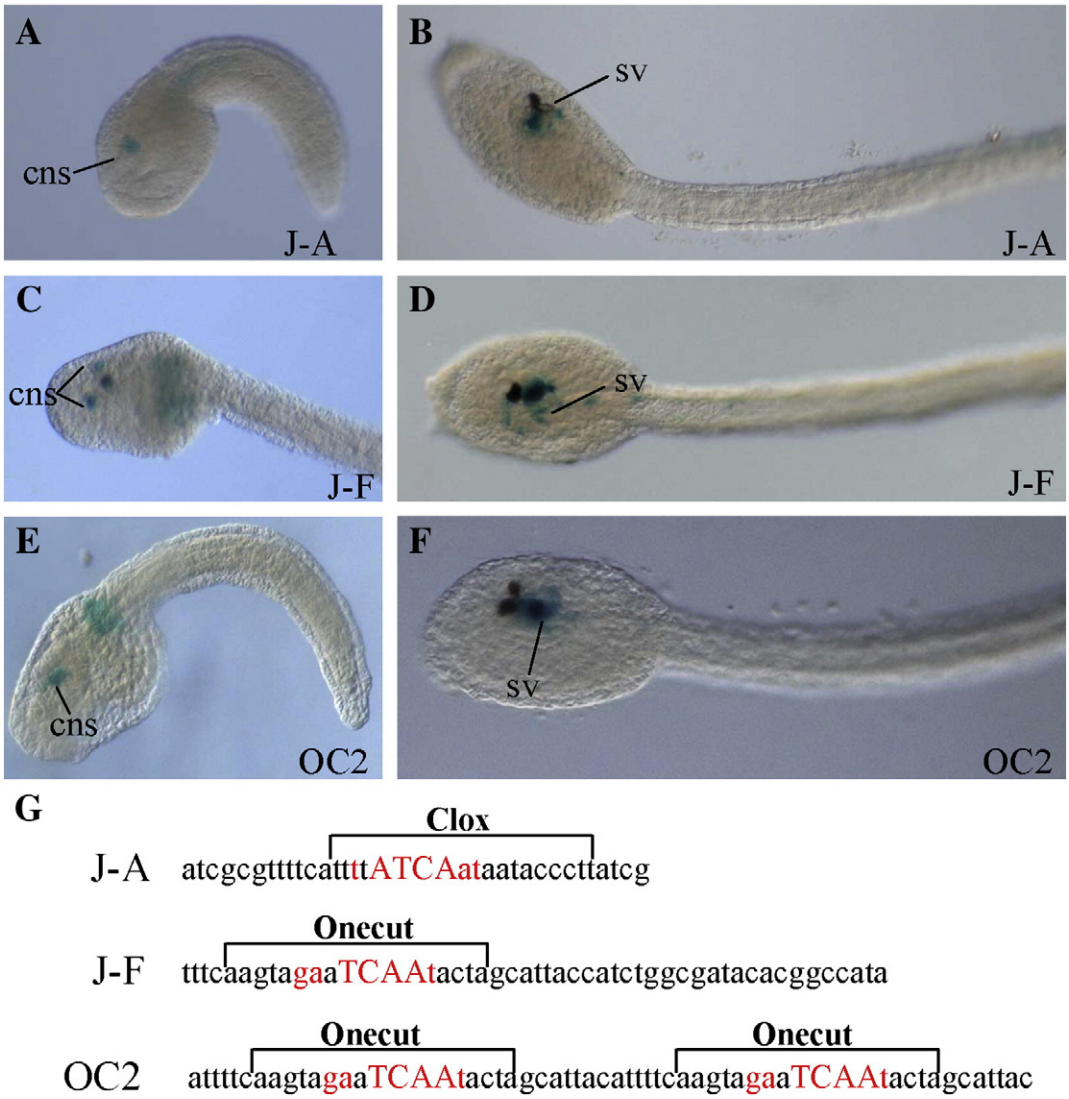



**Fig. 6.** β-galactosidase histochemical assays of LacZ expression driven by various reporter transgenes. Embryos are in lateral view with anterior to the left. (A, C, E) Expression of the LacZ reporter gene at tailbud stage in the anterior brain for the constructs, J-A, J-F and OC2 respectively. (B, D, F) Expression of the LacZ reporter gene at larva stage in the sensory vesicle for the constructs J-A, J-F and OC2, respectively. (G) Schematization of the J-A fragment containing the Clox (Cut-like) binding site, the J-F fragment containing the Onecut binding site, and the OC2 fragment containing a tandem repetition of the Onecut binding site. cns, central nervous system; m, muscle; me, mesenchyme; sv, sensory vesicle.

experiments using *Ci*-Onecut probe. *Ci*-Onecut transcript was detected in the sensory vesicle and visceral ganglion where it is normally expressed and in various notochord cells (Fig. 10A). *Ci*-Onecut is not expressed in all the notochord cells due to the mosaic incorporation of the transgene. In 40% of the electroporated embryos, we observed an alteration in the normal development of the notochord, likely due to the misexpression of *Ci*-Onecut in this tissue. Having established that Bra-Onecut drives *Ci*-Onecut expression in the notochord, we then electroporated the *Ciona* eggs with Bra-Onecut together with the wild type or mutated J0.2 and J-A constructs. 40% of the embryos coelectroporated with the J0.2 or the J-A constructs express the LacZ reporter genes in the notochord cells as well as the endogenous territories of *Ci*-Rx (Figs. 10B, C, E). However, the J0.2 MUT construct showed strongly reduced expression in the notochord cells compared to the J0.2 construct, likely because of the presence of a mutated Onecut site but a still active Clox site (Fig. 10D). The J-A MUT construct, which only contains the mutated Clox site, did not show any expression in the notochord cells (Fig. 10F). We also coelectroporated the OC2 construct together with Bra-Onecut, which produce ectopic expression in the notochord cells with very high efficiency (Fig. 10G). Therefore, the ability of *Ci*-Onecut to induce ectopic expression of the *Ci*-Rx LacZ reporters in the notochord cells, where none of the constructs of the *Ci*-Rx promoter has never been active, strongly suggests that *in vivo Ci*-Onecut is able to bind and activate *Ci*-Rx expression through both Onecut and Clox sites.

### *Ci-Onecut activates Ci-Rx gene expression*

In order to assess the ability of *Ci*-Onecut protein to influence Rx endogenous expression, we overexpressed a constitutive activator (OC-VP16) or a constitutive repressor (OC-WRPW) form of *Ci*-Onecut. To induce ectopic OC-VP16 or OC-WRPW, we used an enhancer of *Ci*-Etr, which is specifically expressed in the nervous system up to the larval stage and starts to be expressed in the CNS lineage from the 110 cells stage (aniseedV3_2090 on the ANISEED web site; http://aniseed-ibdm.univ-mrs.fr/). The *Ci*-Etr enhancer is able to recapitulate its endogenous expression pattern throughout the CNS of *Ciona* embryos (Fig. 11A). Whole mount *in situ* hybridization experiments for *Ci*-Rx on embryos electroporated with the constitutive activation construct (Etr-OC-VP16) revealed the presence of a strong *Ci*-Rx signal in the sensory vesicle (Fig. 11C), compared to electroporation of control Etr-GFP construct that did not

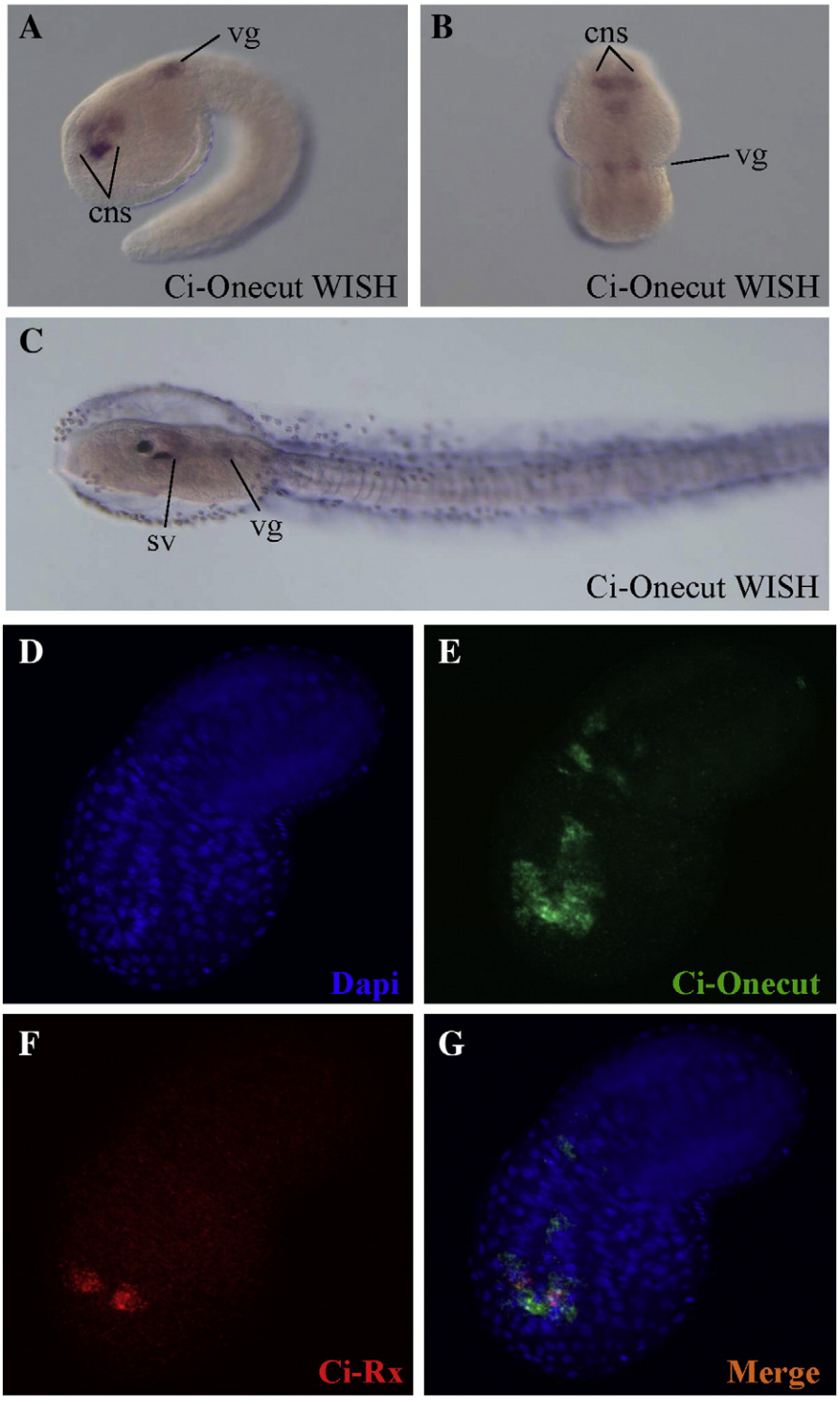



**Fig. 7.** Whole-mount *in situ* hybridization of *Ci*-Onecut and *Ci*-Rx. Anterior is to the left in all panels. (A, B) Tailbud stage lateral and dorsal view. Expression of *Ci*-Onecut is detected along the antero-posterior axis of the CNS. (C) Larva stage, lateral view. *Ci*-Onecut expression is detected in the sensory vesicle and in the visceral ganglion. (D–G) Positional relationship of *Ci*-Rx and *Ci*-Onecut gene expression domains. (D) DAPI image. (E) *Ci*-Onecut expression at tailbud stage (dorsal view). (F) *Ci*-Rx expression at the same stage. (G) Merge image of D, E, and F. Orange color in G indicates co-expression territory of *Ci*-Rx and *Ci*-Onecut. cns, central nervous system; sv, sensory vesicle; vg, visceral ganglion.

affect *Ci*-Rx expression embryo (Fig. 11B). Furthermore, 40% of embryos electroporated with the constitutive repressor construct (Etr-OC-WRPW) did not show any *Ci*-Rx endogenous expression (Fig. 11D), while 45% of the embryos showed a reduced signal (data not shown).

Therefore, these results demonstrate a functional connection between *Ci*-Onecut and *Ci*-Rx expression and that *Ci*-Onecut is not only able to activate *Ci*-Rx enhancer, but its ability to act as a transcriptional activator is also necessary for *Ci*-Rx endogenous expression in the anterior part of the nervous system. Because ectopic expression is

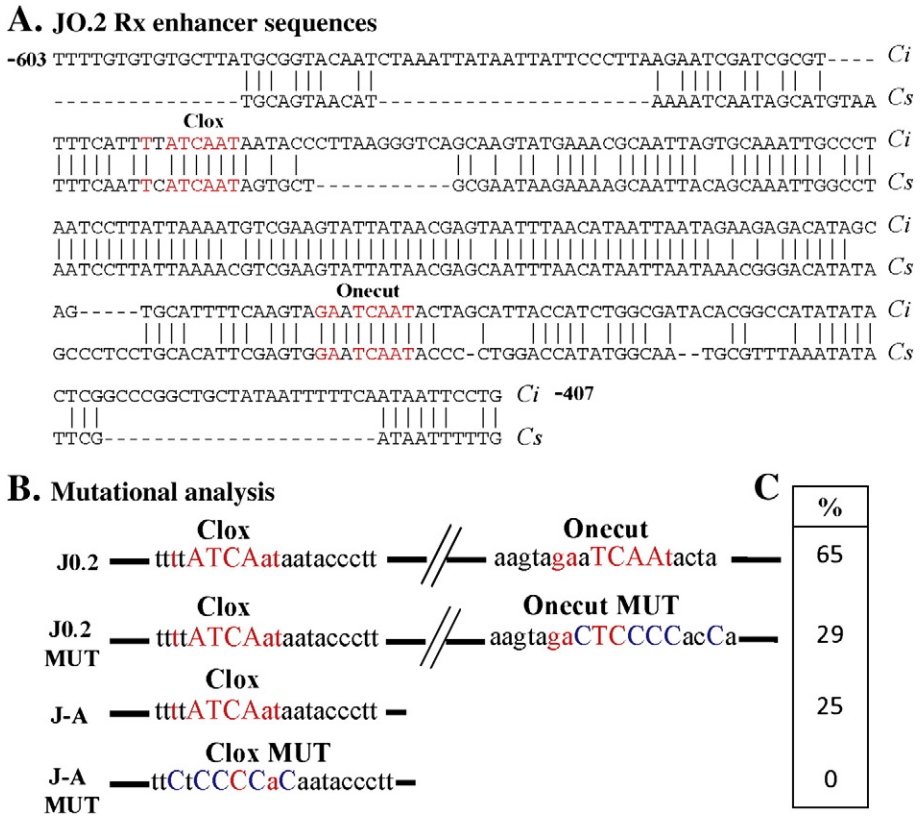



**Fig. 8.** (A) Phylogenetic footprinting of the Onecut elements (red letters) in the J0.2 sequence between *C. intestinalis* (*Ci*) and *C. savignyi* (*Cs*). Vertical bars indicate conserved nucleotides. (B) Schematization of the constructs used for mutational analysis. Blue indicates mutated nucleotides in the Onecut and Clox binding sites. (C) Scoring chart for expression driven by each transgene in *C. intestinalis* embryos at tailbud and larva stages.

restricted to the sensory vesicle, this suggests other limiting factors are also necessary to promote ectopic *Ci*-Rx expression in the CNS.

## Discussion

In all vertebrate species in which Rx genes have been studied, the expression pattern is highly conserved and it has been demonstrated that they are involved, not only in the patterning of the nervous system but also in the development of the eye fields. In invertebrate species, although Rx genes are expressed in the nervous system, they do not share any expression in the eye structures (Arendt et al., 2004; Eggert et al., 1998; Mannini et al., 2008). Therefore, it seems that *Rx* gene function was likely restricted to the brain in the common ancestor of vertebrate and invertebrate, while during Deuterostome evolution Rx genes acquired a new role in the development of the eye structures.

In *C. intestinalis*, we previously demonstrated that the Rx gene has a key function in the development of photosensitive organ, since knockdown of this gene resulted in a larva that lacks the ocellus and the photoreceptor cells and has completely lost the ability to respond to the light stimuli variations (D'Aniello et al., 2006). These data clearly show that the new function of Rx gene in the development of the photosensitive structure has been acquired during chordate evolution before tunicate divergence. In this context, studying Rx gene in an experimental model such as *C. intestinalis* represents a great opportunity to clarify the genetic cascade of Rx genes and to shed light on the evolutionary mechanisms that led to the acquisition of a new function in eye development. In the attempt to answer these intriguing questions, we characterized the regulatory mechanism underlying *Ci*-Rx specific activation during *C. intestinalis* embryogenesis.

Previously, we identified a 2.9 kb genomic region located upstream of the *Ci*-Rx coding sequence responsible for *Ci*-Rx tissue specific activation (D'Aniello et al., 2006). In the present study, we build on this initial observation and perform analysis of this promoter. First, we performed a detailed deletion analysis of the 2.9 kb sequence and tested the ability of these fragments to reproduce *Ci*-Rx expression profile. Computational analysis of the Rx gene locus between two ascidian species, *C. intestinalis* and *C. savignyi*, revealed the presence of three conserved non-coding sequences. Among them, only one (J0.2) was functionally active and able to activate reporter gene expression in the *Ci*-Rx territories consisting of three groups of cells of the developing brain and at the larva stage in the cells surrounding the ocellus (Figs. 3G, H). Interestingly, this region of the promoter was the most conserved and the only one showing higher than 75% conservation. Recently, it has been shown that Nodal controls *Ci*-Rx negative regulation on the left side of the embryo and gives rise to the proper right sided localization of the ocellus and photoreceptor cells (Yoshida and Saiga, 2011). Unfortunately, *Ciona* rapid development and LacZ stability do not permit to observe the switch on and off of the enhancer at the different stages. This hitch does not allow to establish if the Rx enhancers that we have identified are involved in the response to signaling.

Subsequently, to better narrow the *Ci*-Rx enhancer(s), this J0.2 positive fragment has been subdivided and allowed the positive identification of two fragments, called J-A and J-F, able to direct the expression of the reporter gene in the same territories of the endogenous transcript (Figs. 6A–D). Computational search for binding sites revealed that both sequences contain binding sites for the Onecut TF, also called HNF6 in vertebrates. Interestingly, the expression profile of this transcription factor in *C. intestinalis* (Moret et al., 2005) and in *H. roretzi* (Sasakura and Makabe, 2001) appeared similar to that of *Ci*-Rx in the CNS at tailbud stage and in the sensory vesicle at larva stage.

In literature the role of these genes in the development of the nervous system is reported. In *D. melanogaster* a direct role of Onecut

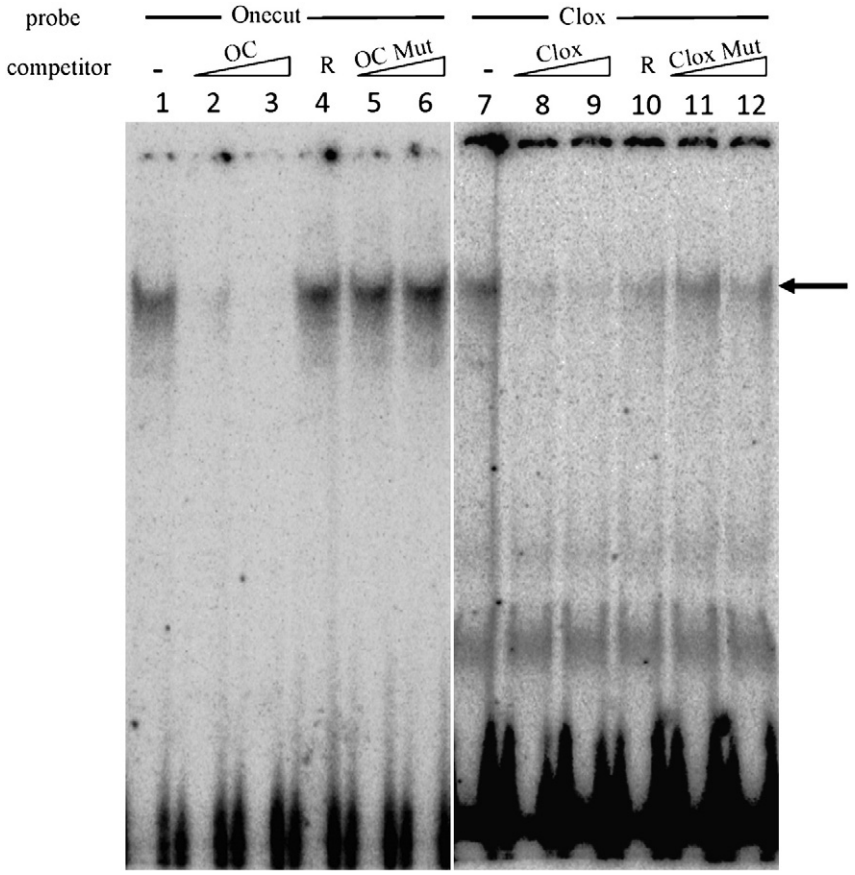



**Fig. 9.** Gel-shift analysis with *Ci*-Onecut protein and wild-type and mutated oligonucleotides. One shifted band is observed in lanes 1 and 7 where the *in vitro* translated protein was incubated with the OC (Onecut wt, Table S3) and Clox (Clox wt, Table S3) labeled oligonucleotides, respectively. The specific band disappears in the presence of the unlabeled oligonucleotides (lanes 2–3, 8–9), while is not affected by the random one (lanes 4, 10) or by the mutated ones (lanes 5–6, 11–12). Arrow indicates specific retarded band. Increasing amount of cold probe and mutated oligo are represented by open triangles. OC Mut and Clox Mut indicate Onecut MUT and Clox MUT oligonucleotides (Table S3).

as a transcriptional activator in the central and peripheral nervous system and, in particular, in the formation of photoreceptors has been demonstrated (Nguyen et al., 2000). In zebrafish and in mammals these genes are expressed in various parts of the nervous system and in particular in the retina and pineal gland where Rx genes are also expressed (Hong et al., 2002; Landry et al., 1997). We verified by double *in situ* hybridization that Onecut and Rx genes are expressed in the same territories in the anterior developing brain of *C. intestinalis* tailbud embryos (Fig. 7). We then used *in vivo* and *in vitro* approaches to demonstrate that *Ci*-Onecut binds and activates the two predicted *Ci*-Rx enhancer elements. First, we demonstrated *in vivo* that once J-F and J-A are mutated in the Onecut binding site, the expression of the reporter gene is dramatically reduced (Fig. 8). Analysis of these two sequences by EMSA assay revealed that, they are able to form a specific retarded complex with the *Ci*-Onecut protein *in vitro* synthesized (Fig. 9). Furthermore, to demonstrate that Onecut is not only necessary but also sufficient to activate *Ci*-Rx enhancers we overexpressed *Ci*-Onecut in the notochord cells (Fig. 10). This experiment highlighted that when *Ci*-Onecut is translated in an ectopic tissue (the notochord) it is able to recognize the binding sites present in the J-A, J-F and OC2 sequences and induce LacZ expression in the notochord cells. Furthermore, mutations of the Onecut sites present in these sequences abolish ectopic activation of the J-A and J-F Rx enhancer in the notochord cells (Figs. 10D, F). Finally, we provided evidence that Onecut transcriptional activation is necessary and sufficient to activate *Ci*-Rx endogenous expression in the sensory vesicle (Fig. 11).

Similar to Rx genes, the expression pattern and function of Onecut is highly conserved in analyzed invertebrates and vertebrates. *Ci*-Onecut (presented here and Moret et al., 2005) appears conserved within tunicates as *H. roretzi* (Sasakura and Makabe, 2001). In *D. melanogaster*, the Onecut homolog has been demonstrated to have a direct role in the central and peripheral nervous system and it has been described to have a role in the formation of photoreceptors (Nguyen et al., 2000). In zebrafish and mammals, Onecut genes are expressed in the nervous system, including the retina and pineal gland where Rx genes are also expressed (Hong et al., 2002; Landry et al., 1997). Furthermore, by morpholino experiments, it has been shown that *Ci*-Onecut controls Chox10 and Irx genes (Imai et al., 2009). These genes seem to be implicated in retina and photoreceptors development in zebrafish and mouse embryos (Katoh et al., 2010; Leung et al., 2008). These results suggest that Onecut could have a conserved genetic pathway involved in the formation of photosensitive structures.

It is interesting that although it has been widely reported in multiple species that Onecut expression and function is in the same territories of Rx, such as the nervous system, pineal gland and retina, it had never been implicated in being a direct regulator of the Rx genes. To date the only detailed study on Rx regulatory elements was performed in *Xenopus*. Although conserved binding sites for *Otx2* and *Sox2* have been identified in *Xenopus* Rx enhancer elements (Danno et al., 2008), we did not find Otx2 or Sox2 binding sites in the J0.2 sequence or any of the consensus sequences. *C. intestinalis* diverged from modern vertebrates more than 500 Ma ago. However, the function of Rx gene seems to be conserved in the development of photosensitive structures (D'Aniello et al., 2006). Our data suggest that Onecut directly regulates Rx enhancer elements. Therefore, it will be interesting to investigate if there are conserved Onecut enhancers that also regulate Rx gene expression in vertebrates. However, it is also possible that the Onecut gene was an

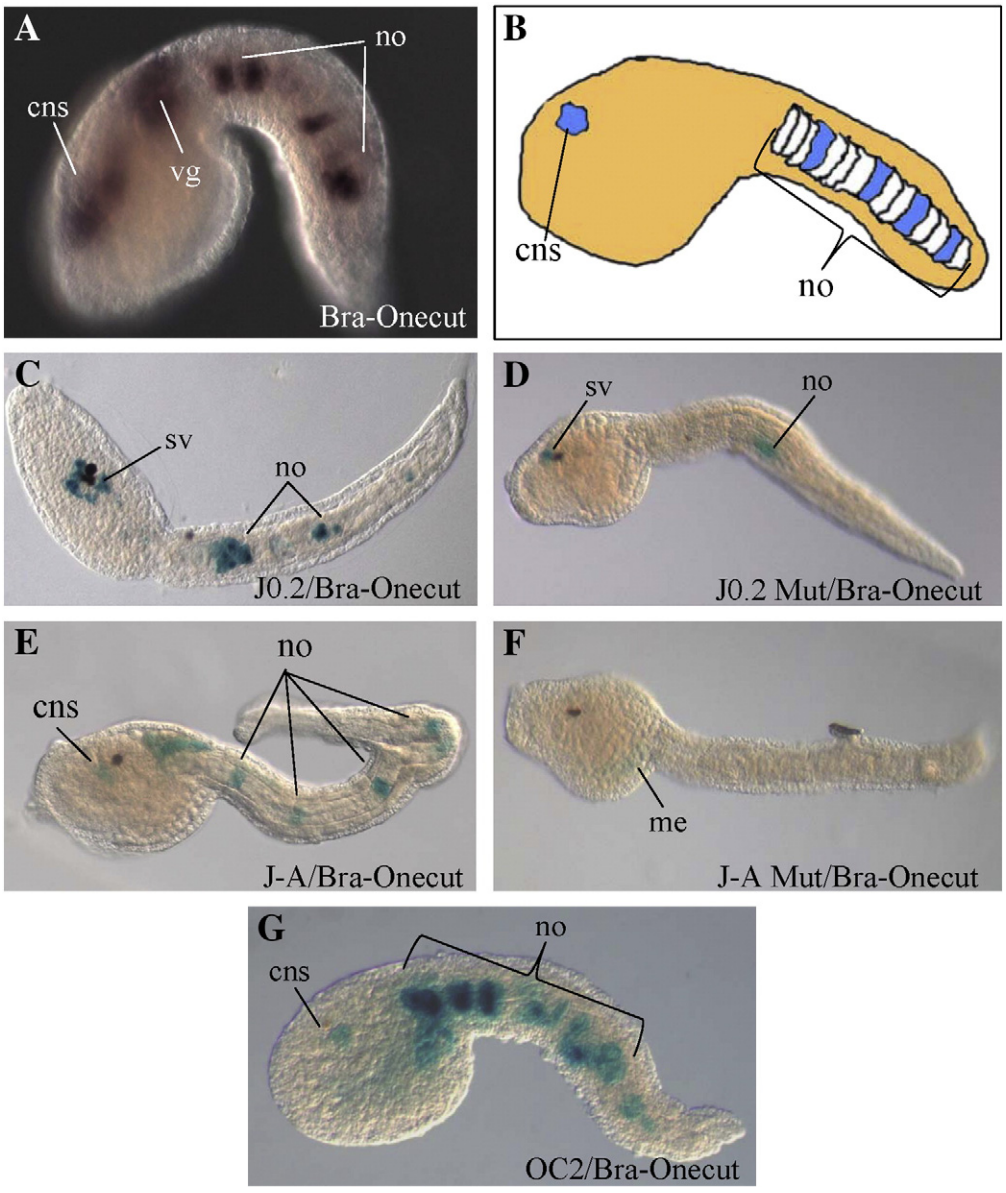



**Fig. 10.** (A) Spatial expression of *Ci*-Onecut in embryos electroporated with the Bra-Onecut construct at tailbud stage. The expression is visible not only in the *Ci*-Onecut endogenous territories but also in the notochord cells. (B) Predicted result of co-electroporation of the Bra-Onecut construct together with the constructs containing *Ci*-Rx non-coding sequence upstream of the LacZ reporter gene. (C, D) Larva embryos co-electroporated with Bra-Onecut together with J0.2 construct (C) and J0.2 Mut construct (D), respectively. (E, F) Larva embryos co-electroporated with Bra-Onecut together with J-A construct (E) and J-A Mut construct (F), respectively. Expression of the reporter gene has been detected not only in the *Ci*-Rx endogenous tissue in the anterior brain, but also in the notochord cells when the wild type constructs are used, while no signals are detected with the mutated constructs. (G) Embryo at late tailbud stage co-electroporated with Bra-Onecut construct together with the OC2 construct. LacZ expression is visible in the central nervous system and in the notochord. All images are lateral view with anterior to the left. cns, central nervous system; no, notochord; sv, sensory vesicle.

ancestral regulator of Rx expression, but that due to the accumulation of mutational events vertebrate Rx genes acquired new regulatory mechanisms and the dependence on Onecut has been lost.

While considering the origin of Onecut and Rx regulation in the sensory vesicle, it is also important to understand the possible homology of the ascidian ocellus, to photosensitive structures in vertebrates (D'Aniello et al., 2006; Horie et al., 2008; Sakurai et al., 2004; Tsuda et al., 2003). Until recently, the prevailing hypothesis was that the ocellus was ancestral to the vertebrate eye. However, in recent years, some authors have proposed that the ascidian ocellus could be homologous to the vertebrate median eye, also called epiphysis or pineal gland. The photoreceptive function of the pineal gland is less conserved and is still present only in some non-mammal vertebrate species. In support of this hypothesis, similar to the ascidian ocellus, the epiphysis of amphibian and teleost larvae triggers the shadow response, when the developing lateral eyes are still not competent to respond to light stimuli (Foster and Roberts, 1982). In addition, the ascidian ocellus and vertebrate epiphysis are both derived from cells located in the lateral part of the embryonic neural plate (Eagleson and Harris, 1990; Nishida, 1987). Together, these similarities between the ocellus and vertebrate epiphysis suggested a possible homology between these two structures.

Despite the similarities of the ocellus and the epiphysis, other characteristics do not permit us to distinguish whether or not the ocellus is truly homologous to the eyes or epiphysis. For instance, as in vertebrates, ascidian photoreceptors are ciliary in origin (Eakin and Kuda, 1971), hyperpolarize to light (Gorman et al., 1971), and express Opsin1. Furthermore, the sequence of *Ci*-Opsin1 is highly homologous to both vertebrate retinal and pineal opsins (Kusakabe et al., 2001). With respect to Rx genes, vertebrate Rx genes are expressed in both the pineal organ and the retina (Hong et al., 2002; Mathers and Jamrich, 2000) and

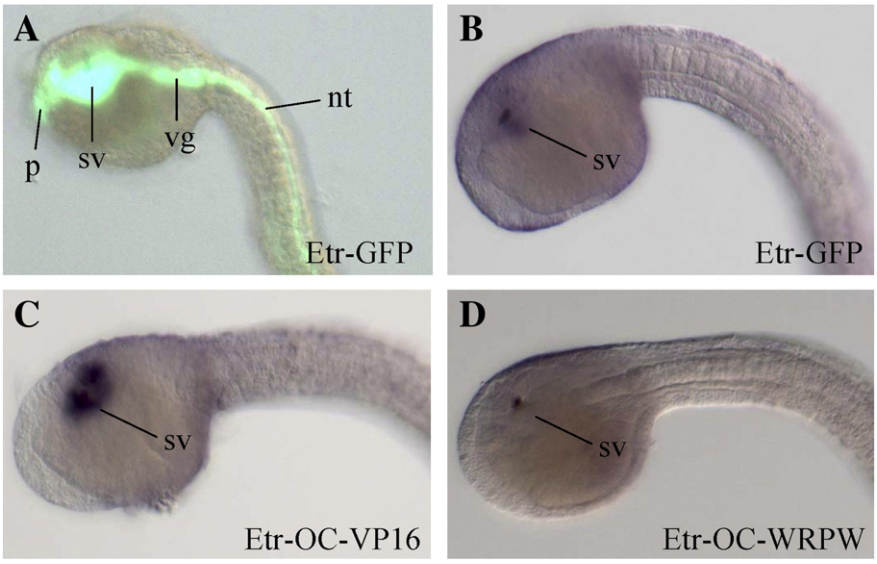



**Fig. 11.** (A) Merged bright-field/fluorescent image of GFP expression driven by the Etr promoter at tailbud stage. Transgene expression occurs throughout the nervous system. (B–D) *Ci*-Rx expression pattern visualized by whole-mount *in-situ* hybridization on embryos electroporated with Etr-GFP (B), Etr-OC-VP16 (C) and Etr-OC-WRPW (D) constructs. (C) The Etr-OC-VP16 construct promotes *Ci*-Rx expression in the sensory vesicle, while (D) the Etr-OC-WRPW construct inhibits *Ci*-Rx expression. nt, neural tube; p, palps; sv, sensory vesicle; vg, visceral ganglion.

play a specific role in the formation of projection neurons of zebrafish pineal gland (Cau and Wilson, 2003). Taking into account a parsimonious theory of evolution, a possible scenario could be that the ocellus represents the ancestral structure of both light sensing organs, which diversified into the vertebrate pinealocyte and retinal photoreceptors (Klein, 2006). In this context, it would explain why Onecut and Rx genes are both expressed in the pineal gland and the retina of many vertebrate species. Further studies in *Ciona* as well as in various vertebrate species will help to elucidate the evolutionary mechanisms that led to the formation of the ascidian ocellus and vertebrate pineal gland and eye.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.05.584.

## Acknowledgments

We are deeply grateful to Joshua Waxman (Cincinnati Children's Hospital Medical Center, Ohio, USA) for a critical reading of the manuscript and for the final English revision of the paper. Special thanks to Nunzia Pastore, Laura Gaveglia and Claudia Racioppi for their help in preparing constructs and performing electroporation during their undergraduate thesis. We thank Alessandro Amoroso for technical assistanceand the SZN Molecular Biology Service members for sequencing and technical assistance. We acknowledge Giovanna Benvenuto of the SZN Confocal Microscopy Service for confocal microscopy assistance.

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