Tunicate Neurogenesis: The Case of the *SoxB2* Missing CNE

Evgeniya Anishchenko and Salvatore D'Aniello

Abstract The discovery of the *SoxB2*/*Sox21* regulatory element, conserved from basal metazoa to human, opened novel perspectives to study the conservation among distant related genomes. This discovery represents exceptional maintenance of an almost identical enhancer structure controlling a gene that is fundamental for nervous system development. The activity of metazoan *SoxB2* enhancers was previously demonstrated in zebrafish embryos by cross-species experiments.

Here we tested the activity of human and amphioxus orthologue *cis*-regulatory sequences in embryos of the tunicate *Ciona intestinalis* through a transgenic approach, and found out that *SoxB2* enhancers retained their activity in neuronal differentiation even in a non-vertebrate chordate.

This result was unexpected since the conserved *SoxB2* enhancer was not found in *Ciona* in previous studies. Nevertheless, we adopted a different comparative approach and performed a phylogenetic footprinting analysis using two congeneric tunicate species, *C. intestinalis* and *Ciona savignyi*, that, in fact, evidenced a conserved *SoxB2* 3′ element. The discovered element could potentially be the missing orthologous *SoxB2* enhancer previously identified in human, zebrafish, and amphioxus.

A detailed search for possible transcription factors revealed the massive presence of Sox, Pou and Fox binding sites as found in other deuterostomes. Nevertheless, whether the conserved *SoxB2* element of *Ciona* possesses a functional ability as gene transcriptional enhancer remains to be demonstrated experimentally.

Keywords Evolution • Transgenesis • Nervous system • *Ciona* • *Cis*-regulatory enhancers

E. Anishchenko • S. D'Aniello (✉)
Department of Biology and Evolution of Marine Organisms (BEOM), Stazione Zoologica Anton Dorhn, Villa Comunale, 80121 Napoli, Italy
e-mail: salvatore.daniello@szn.it

V. Zazzu et al. (eds.), *Mathematical Models in Biology*,
DOI 10.1007/978-3-319-23497-7_7

1 Introduction

One of the most intriguing mechanisms of nervous system (NS) development is related to the neuronal lineage specification, which is of great interest to science and subject of numerous and intensive studies. Nevertheless, we are still far from a complete understanding of these processes. The discovery of an evolutionary conserved non-coding element (CNE) in the animal kingdom by Royo et al. [16] started a discussion about this key aspect of neural state regulation in animal development. It was found to be an example of gene regulatory element conservation in all metazoans, from the cnidarian *Nematostella* to human. Only the exonic regions of genes were known to have such a degree of conservation among animals so different in body shape and complexity, diverging from a common ancestor around 600 My ago [14].

Royo and colleagues discovered a highly conserved CNE that regulates the *SoxB2* gene (SRY-box B), recognizable at the sequence level within metazoans, and explored its functional significance in transphyletic *cis*-regulatory DNA experiments. The invertebrate *SoxB2*, and the orthologous gene in vertebrates *Sox21*, are involved in neuronal development, differentiation and regeneration, indicating that these genes are responsible for the pluripotent features of presumptive neuronal tissues in animal [7, 9, 10, 15, 17–19, 21]. The sequence comparison of *SoxB2*/*Sox21* CNE among human (*Homo sapiens*), zebrafish (*Danio rerio*), amphioxus (*Branchistoma floridae*), acorn worm (*Saccoglossus kowalevskii*), sea urchin (*Strongylocentrotus purpuratus*) and cnidarian (*Nematostella vectensis*) showed an evolutionary conserved region of 200 bp, located at the 3′ of the gene in all analysed loci [16]. Royo and collaborators demonstrated trough transgenic experiments on zebrafish embryos that CNEs from diverse animal genomes were functional regulative elements for different stages of neurogenesis, including patterning and development of the vertebrate forebrain. Similarly, the reporter gene expression driven by human *SOX21* CNE and sea urchin *SoxB2* CNE was functional in developing the nervous system of *Drosophila*, despite absence of clear sequence orthology. This was the first study pointing to the fact that the regulatory state recognized by a conserved DNA sequence may have been redeployed at different levels of the developmental regulatory program during evolution of the complex central nervous system (CNS).

A detailed study focused on the regulation of *Sox21b* (fish ortholog of *SoxB2*) expression highlighted 19 regulatory DNA elements conserved between vertebrates (human, chicken, mouse, frog, zebrafish and fugu) [13]. Transgenic experiments using conserved fragments from the fugu genome in zebrafish showed that the majority of these CNEs were able to generate tissue-specific expression patterns in the CNS and sensory organs, in agreement with *Sox21b* expression domains. As expected, one of the enhancers analysed in this study corresponded to the evolutionary conserved element discovered by Royo and colleagues, the CNE17 in the *Sox21b* locus [13]. Nevertheless, CNE17 and CNE6 were the only enhancer elements able to drive the expression of the reporter gene in the lens, which

represents an innovation in vertebrates. A possible explanation for this could be that CNE17, orthologous to the highly conserved metazoan CNE, was co-opted in the fish lineage for the lens expression, as a consequence of the sub-functionalization of the two fish paralogs, *Sox21a* and *Sox21b* [10].

An evolutionary puzzling case remained to be solved. As mentioned above, one of *SoxB2*/*Sox21* enhancer was found to be conserved in highly distant related animals and transcriptionally active during CNS development, indicating a key role in nervous system evolution. Nevertheless, in previous studies it was not possible to detect any trace of the *SoxB*2 enhancer conservation in the lineage of tunicates, the sister group of vertebrates [5] which are considered important model systems for the study of evolution and development in chordates. Tunicates, differently from cephalochordates, are highly diverged from the common chordate ancestor, both morphologically and genetically, and this represents an additional difficulty for evolutionary biologists that take advantage of homologies between body structures and sequence conservation as main principles. Here we tried, therefore, to reveal the potentiality of tunicates in our understanding of deuterostome NS evolution.

The ascidian *C. intestinalis* represents a very useful animal model to perform in vivo transgenic assays because it possesses most of the molecular pathways and gene repertoire as the rest of chordates. Nevertheless, the *Ciona* genome shows divergent characteristics that sometimes can represent a limitation to experimental approaches and on the other hand species-specific genomic events, such as gene loss, can be considered an experimental advantage in evolutionary devoted studies.

2 Results

Two main evolutionary questions prompted us to choose *C. intestinalis* as the model organism for this study, taking into account the advantage of the easy application of transgenesis approaches that are very well established for *Ciona*.

First, is the ascidian embryonic transcription factors (TF) machinery able to recognize the transcriptional information contained in cross-species enhancers, considering the loss of the evolutionary conserved *SoxB2* CNE? Second, could the presence of the conserved *SoxB2* CNE be masked at sequence level by the highly divergent genome of ascidians?

To answer these questions, that are interesting per se from an evolutionary point of view, we performed a series of computational and transgenic experiments. To understand whether the regulation of the *SoxB2* enhancer is maintained in *Ciona*, despite the loss of the orthologous region, we carried out transgenic experiments in *C. intestinalis*, introducing exogenous DNA regulative fragments in developing embryos. More in detail, we used the technique of transgenesis by electroporation of a purified plasmid containing the putative enhancer with a GFP reporter gene into fertilized eggs. In the first series of in vivo experiments we used CNE fragments, amplified by PCR on genomic DNA, corresponding to *SoxB2*/*Sox21* CNEs from

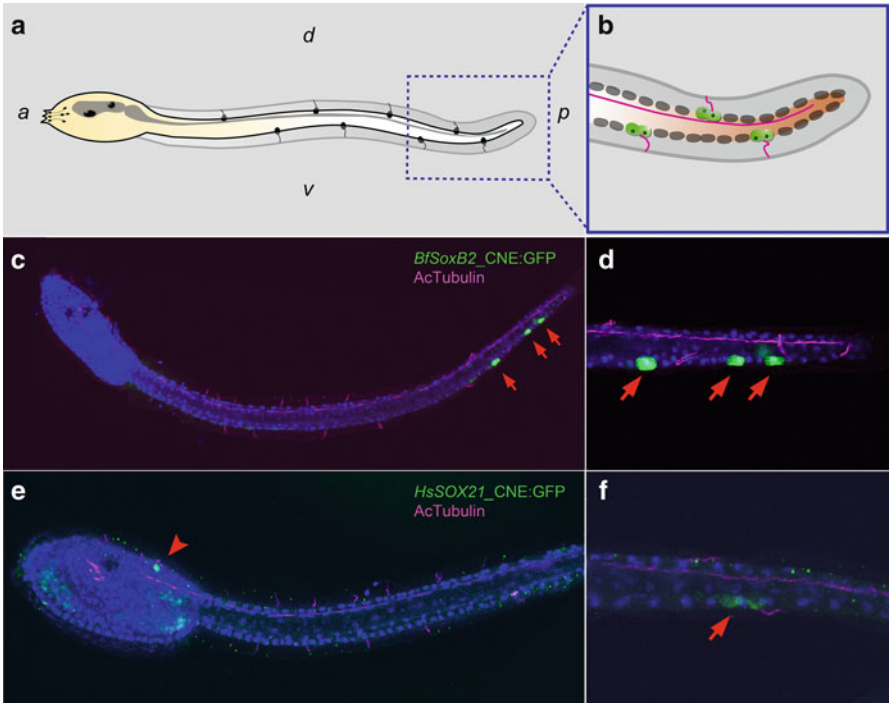

Fig. 1 Transgenic larvae using human and amphioxus cnes driving GFP expression in neural territories. (**a**) *Ciona intestinalis* 20 hpf larvae body plan in a schematic representation: *a* anterior, *p* posterior, *d* dorsal, *v* ventral. The CNS is indicated in *grey*. (**b**) Magnification of larval tail. Pairs of caudal epidermal neurons are indicated in *green*. (**c**, **d**) amphioxus *SoxB2* CNE drives GFP expression in *Ciona* ectodermal neurons in the tail (*red arrows*). (**e**, **f**) Human *Sox21* CNE resulted active in *Ciona* ectodermal neurons in the tail (*red arrows*) and in the head (*red arrowhead*)

different animal models: acorn worm *S. kovalewskii* (hemichordate), sea urchin *S. purpuratus* (echinoderm), *B. floridae* (cephalochordate) and human *H. sapiens* (vertebrate). These DNA fragments correspond to the enhancers previously used in transgenic experiments on zebrafish by Royo et al. [16].

Transgenic experiments using *B. floridae* and human *H. sapiens* CNEs gave positive results as shown in Fig. 1. The *SoxB2* enhancer from *B. floridae* was able to drive the expression of the GFP reporter gene in paired tail epidermal neurons in about 70 % of the larvae (red arrows in Fig. 1c, d). This result was confirmed by the human *SOXB2* enhancer activity in about 60 % of the larvae (red arrow in Fig. 1f), albeit that we recorded fainter signals. Interestingly, the human DNA construct was also able to drive the GFP expression in another neuronal compartment in the head region (arrowhead in Fig. 1e). In Fig. 1a we present a diagram of the *Ciona* larval body plan with the central nervous system in grey. Figure 1b is a magnification of the tail region showing in green the paired epidermal neurons, GFP positive with both the human and cephalochordate exogenous DNA constructs.

To answer the second evolutionary question we performed phylogenetic footprinting analyses that showed an extremely low degree of conservation between *C. intestinalis* and other deuterostomal orthologous regions containing the conserved *SoxB2* CNE. Nevertheless, to go deeper into this comparative analysis and trying to find the orthologous CNE in tunicates, we compared two congeneric species that diverged 180 My ago [1], with the aim to reveal conserved non-coding regions that were unrecognizable when searched between tunicates and distant related species. We therefore performed a Vista analysis that allowed to discover three main regions highly conserved between *C. intestinalis* and *C. savignyi* in the 3′ of *SoxB2* (Fig. 2a), that could correspond to the orthologous region previously found conserved in other deuterostomes. A local alignment was performed showing a very high degree of sequence conservation between the two *Ciona* species, at least 70 % identical in non-coding regions (Fig. 2b). Hence, a detailed in silico analysis was performed using Jaspar software in order to reveal potential transcription factor binding sites (TFBS), and compare them with those predicted in sea urchin, amphioxus and human *SoxB2* enhancers [16]. This allowed the identification of a cluster of several Sox and Fox binding sites in the 3′ CNE-1 peak, which was not detected in other 5′ and 3′ CNEs (Figs. 2b and 3a–c).

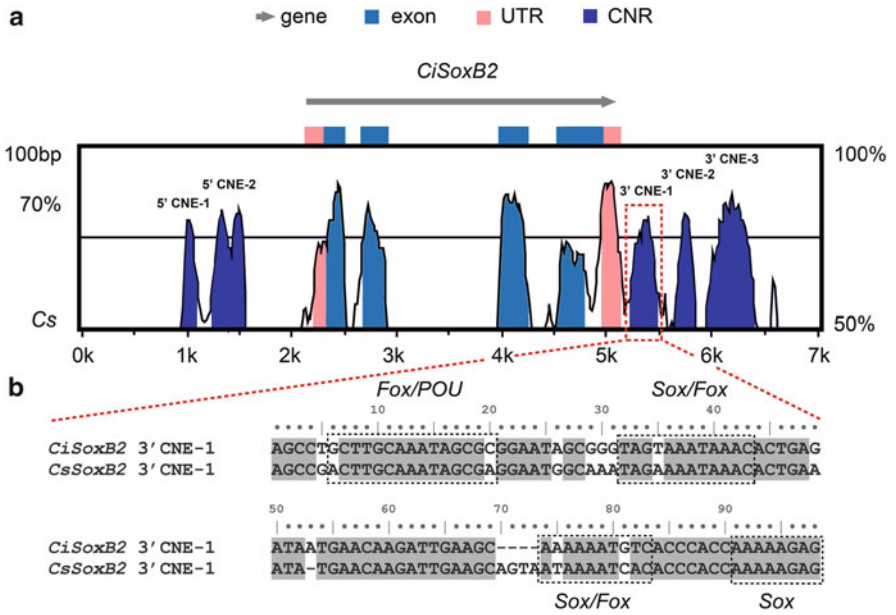

Fig. 2 Phylogenetic footprinting and *Ciona*'*s* CNE alignment. (**a**) Vista analysis between *SoxB2* loci of *Ciona intestinalis* and *Ciona savignyi. Dark blue peaks* represent conserved non-coding elements between the two species, *pink* indicates the *SoxB2* 5′ and 3′ UTRs and *blue* the exons. (**b**) Alignment of *Ciona*'s *SoxB2* 3′CNE-1. Potential binding sites for Pou, Sox and Fox are highlighted by frames

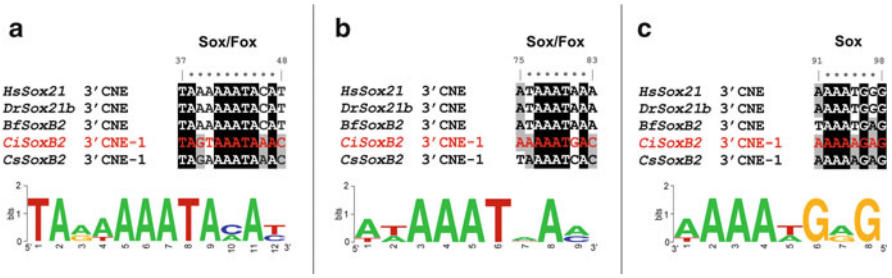

Fig. 3 Diagrams of TF binging sequences conserved in deuterostomes. *Ciona*'s *SoxB2* 3′CNE-1 contains Sox and Fox (**a**, **b**) and Sox (**c**) binding sites that are found in orthologous sequences from *Ciona savignyi*, amphioxus, zebrafish and human. *Black* background indicates a 100 % match of identity between all species considered

Three short sequence fragments were found to be highly conserved in 3′ CNE-1, which could be the potential binding targets for Sox and Fox (Fig. 3a–c). The level of conservation of the multiple sequence alignment using other chordates (amphioxus, zebrafish and human) was 67 % (Fig. 3a), 56 % (Fig. 3b) and 63 % (Fig. 3c).

3 Discussion

The regulatory landscape of genes involved in developmental processes is constrained by enhancers that remained conserved during evolution. Therefore, *cis*-regulatory elements conserved between orthologous genes in vertebrates have been readily recognized in comparative genomic studies as soon as multiple genomes sequencing projects become available. The exceptional case of the discovery of an ancient enhancer retained in metazoans has opened new perspectives in the research field of *cis*-regulatory elements.

The direct comparison between distantly related animals can be inconclusive when the degree of nucleotide conservation is low, while on the contrary the choice of congeneric species is often fruitless because the high homology becomes uninformative in the search for non-coding active elements. In this perspective, the availability of genomes from numerous metazoan species help greatly in reconstruction of metazoan evolution. We applied a transgenic approach using human *SOX21* and amphioxus *SoxB2* enhancers exogenously in *C. intestinalis* embryos and more important we demonstrated that they were functional in pro-neural tissues, as previously demonstrated in a related study on zebrafish and *Drosophila* embryos. Here we found the putative ancestral enhancer of the *SoxB2* gene by comparing two tunicates, which was thought to be lost in such fast evolving genomes. A detailed bioinformatics search in the conserved non-coding regions on

Ciona'*s SoxB2* loci revealed a cluster of four TFBS of the Sox and Fox class in the 3′ CNE-1 (Figs. 2b and 3a–c), who correspond to the *SoxB2* CNEs reported by Royo et al. [16].

However recent studies demonstrated that the ancestral regulatory function of *SoxB2* CNE is still conserved, despite the lack of sequence similarity among different phyla [6, 11]. Furthermore, similar to our results of *Ciona* transgenic experiments, the human *SOX21* and sea urchin *SoxB2* CNEs were demonstrated to be functional in the neuroblasts of the presumptive brain and ventral nerve cord of *D. melanogaster* embryos [16]. These transgenic approaches highlighted the deep functional conservation of metazoan *SoxB2* CNEs in neurogenesis, not only in species possessing high sequence similarity but also in animals showing significantly divergent *SoxB2* regulatory elements. Recently a finding was reported of so called FCNEs (Functional Conserved Non-coding Elements) concerning those *cis*-regulatory elements that, despite a low sequence similarity across distant related species, still keep the ancestral function during developmental processes [20].

The potential transcriptional activity of the *SoxB2* CNEs identified in the present study in two *Ciona* species, despite missing a high degree of sequence similarity with other deuterostomes, remains to be experimentally confirmed in future studies.

4 Materials and Methods

4.1 Animals and Embryos

Adult individuals of *C. intestinalis* used in this study were collected from the Gulf of Naples (Italy) and kept in tanks at 18 °C until further use. To prevent spontaneous spawning in captivity, ripe animals were exposed to continuous light. Gametes were collected from the gonoducts of several animals and used for in vitro fertilization.

4.2 Comparative Genomics

To obtain DNA sequences for *SoxB2* loci, a series of databases was used: ANISEED database (www.aniseed.cnrs.fr/) for *C. intestinalis* and *C. savignyi* sequences; SpBase (www.spbase.org) for sea urchin *S. purpuratus*; JGI (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html) for amphioxus *B. floridae*, and NCBI (http://www.ncbi.nlm.nih.gov/) for human and acorn worm *S. kowalevskii* sequences.

4.3 Phylogenetic Footprinting and in Silico Analyses

Genomic sequences from the two congeneric *Ciona* species, including *SoxB2* locus plus 5 kb upstream and 5 kb downstream, were aligned using the AVID software [2]. Sequences were compared using mVISTA ([8]; http://genome.lbl.gov/vista/mvista/submit.shtml), with the following parameters: 100 bp of fragment length with 70 % of sequence identity.

In order to reveal TFBSs in *SoxB2* CNEs, dna sequences were analysed using Jaspar (http://jaspar.genereg.net/), a TF binding profile database [12]. Diagrams of POU, Sox and Fox binding sequences conserved between two *Cionas*, human and amphioxus were generated using WebLogo software [3].

4.4 Transgenic Experiments

Four CNEs from *S. kovalewskii*, *S. purpuratus*, *B. floridae* and *H. sapiens*, were amplified by PCR and cloned in the pSP72:CNE:2XGFP:SV40 vector, containing the GFP reporter gene and SV40 polyadenylation sequence. *C. intestinalis* transgenic embryos were obtained via electroporation experiments, as previously described [4], and observed with confocal microscopy after immunohistochemical detection. Each experiment was performed in triplicate, comparing at least 100 embryos for each single construct. Briefly, eggs were dechorionated, before fertilization to be ready to incorporate the exogenous DNA using a solution containing: 1 % sodium thioglycolate, 0.05 % proteinase E and 1N sodium hydroxide (NaOH), and afterwards washed in filtered sea water (FSW). The 200 μl of dechorionated and fertilized eggs were transferred into Bio-Rad Gene Pulser 0.4 cm cuvettes containing a 0.77 M mannitol solution and 100 μg of the exogenous DNA plasmids, and subsequently electroporated using a Bio-Rad Gene Pulser II™ with the following settings: constant 50 V and 800 μF. Electroporated eggs were transferred into petri dishes with 1 % agarose bottom with FSW and let develop at 18 °C until the desired developmental stage.

4.5 Whole Mount Immunohistochemistry

Embryos were fixed in 4 % formaldehyde during 30 min at room temperature and washed with PBT (PBS 1x, 0.1 % Tween20). Embryos were dehydrated gradually in 70 % ethanol, followed by rehydration in PBS 1x four times. To permeabilize the embryos, they were incubated in PBS containing 0.01 % Triton-100 for 30 min. Embryos were incubated in blocking buffer (PBS 1x, 0.01 % Triton-100, 30 % goat serum) over night. Next, embryos were kept in blocking buffer with 1:300 polyclonal anti-GFP Ab from rabbit (TP401; Torrey Pines Bionabs) and 1:300

monoclonal anti-Acetylated Tubulin (AcTubulin) Ab from mice (T7451; Sigma) for 2 days at 4 °C and subsequently washed with PBT changing the solution every 15 min for 4 h. Then, embryos were incubated with the secondary anti-mouse Alexa 488 Ab or anti-rabbit Alexa 633 Ab in PBT (1:500), over night at 4 °C, then washed in PBT and incubated with DAPI (D9542; Sigma) $1{:}10^4$ in PBT for 10 min. Laser scanning confocal images were obtained with a Zeiss LSM 510 META confocal microscope.

Acknowledgments The authors are grateful to Maria Ina Arnone, Margherita Branno, Annamaria Locascio, Filomena Ristoratore and Antonietta Spagnuolo for their suggestions about in vivo experiments and for sharing with us DNA plasmids for transgenesis. We thank Mara Francone for technical assistance with DNA maxi-preparation, the Marine Resources for Research Unit of Stazione Zoologica Anton Dohrn for animal fishing and maintenance. Heartfelt gratitude to Rita Marino for her excellent suggestions concerning immunohistochemical experiments. Evgeniya Anishchenko has been supported by a SZN PhD fellowship (2011–2014). This work was supported by a Marie Curie Career Integration Grant (FP7-PEOPLE-2011-CIG, PCIG09-GA-2011-293871) to Salvatore D'Aniello.

References

1. Bernà, L., Alvarez-Valin, F., D'Onofrio, G.: How fast is the sessile *Ciona*? Comp. Funct. Genomics (2009). 875901
2. Bray, N., Dubchak, I., Pachter, L.: AVID: a global alignment program. Genome Res. **13**, 97–102 (2003)
3. Crooks, G.E., Hon, G., Chandonia, J.M., Brenner, S.E.: WebLogo: a sequence logo generator. Genome Res. **14**, 1188–1190 (2004)
4. D'Aniello, S., D'Aniello, E., Locascio, A., Memoli, A., Corrado, M., Russo, M.T., Aniello, F., Fucci, L., Brown, E.R., Branno, M.: The ascidian homologue of the vertebrate homeobox gene *Rx* is essential for ocellus development and function. Differentiation **74**, 222–234 (2006)
5. Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H.: Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature **439**, 965–968 (2006)
6. Doglio, L., Goode, D.K., Pelleri, M.C., Pauls, S., Frabetti, F., Shimeld, S.M., Vavouri, T., Elgar, G.: Parallel evolution of chordate cis-regulatory code for development. PLoS Genet. **9**, e1003904 (2013)
7. Ferrero, E., Fischer, B., Russell, S.: *SoxNeuro* orchestrates central nervous system specification and differentiation in Drosophila and is only partially redundant with Dichaete. Genome Biol. **15**, R74 (2014)
8. Frazer, K.A., Pachter, L., Poliakov, A., Rubin, E.M., Dubchak, I.: VISTA: computational tools for comparative genomics. Nucleic Acids Res. **32**, W273–W279 (2004)
9. Kamachi, Y., Kondoh, H.: Sox proteins: regulators of cell fate specification and differentiation. Development **140**, 4129–4144 (2013)
10. Lan, X., Wen, L., Li, K., Liu, X., Luo, B., Chen, F., Xie, D., Kung, H.F.: Comparative analysis of duplicated *Sox21* genes in zebrafish. Dev. Growth Differ. **53**, 347–356 (2011)
11. Maeso, I., Irimia, M., Tena, J.J., Casares, F., Gómez-Skarmeta, J.L.: Deep conservation of *cis*-regulatory elements in metazoans. Philos. Trans. R. Soc. Lond. B Biol. Sci. **368**, 20130020 (2013)

12. Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D.J., Buchman, S., Chen, C.Y., Chou, A., Ienasescu, H., Lim, J., Shyr, C., Tan, G., Zhou, M., Lenhard, B., Sandelin, A., Wasserman, W.W.: JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic Acids Res. **42**, D142–D147 (2013)
13. Pauls, S., Smith, S.F., Elgar, G.: Lens development depends on a pair of highly conserved *Sox21* regulatory elements. Dev. Biol. **3665**, 310–318 (2012)
14. Putnam, N.H., Srivastava, M., Hellsten, U., Dirks, B., Chapman, J., Salamov, A., Terry, A., Shapiro, H., Lindquist, E., Kapitonov, V.V., Jurka, J., Genikhovich, G., Grigoriev, I.V., Lucas, S.M., Steele, R.E., Finnerty, J.R., Technau, U., Martindale, M.Q., Rokhsar, D.S.: Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science **317**, 86–94 (2007)
15. Reiprich, S., Wegner, M.: From CNS stem cells to neurons and glia: *Sox* for everyone. Cell Tissue Res. **359**, 111–124 (2014)
16. Royo, J.L., Maeso, I., Irimia, M., Gao, F., Peter, I.S., Lopes, C.S., D'Aniello, S., Casares, F., Davidson, E.H., Garcia-Fernández, J., Gómez-Skarmeta, J.L.: Transphyletic conservation of developmental regulatory state in animal evolution. Proc. Natl. Acad. Sci. U. S. A. **108**, 14186–14191 (2011)
17. Sandberg, M., Kallstrom, M., Muhr, J.: *Sox21* promotes the progression of vertebrate neurogenesis. Nat. Neurosci. **8**, 955–1001 (2005)
18. Taguchi, S., Tagawa, K., Humphreys, T., Satoh, N.: Group B *Sox* genes that contribute to specification of the vertebrate brain are expressed in the apical organ and ciliary bands of hemichordate larvae. Zoolog. Sci. **19**, 57–66 (2002)
19. Uchikawa, M., Yoshida, M., Iwafuchi-Doi, M., Matsuda, K., Ishida, Y., Takemoto, T., Kondoh, H.: B1 and B2 *Sox* gene expression during neural plate development in chicken and mouse embryos: universal versus species-dependent features. Dev. Growth. Differ. **53**, 761–771 (2011)
20. Vassalli, Q.A., Anishchenko, E., Caputi, L., Sordino, P., D'Aniello, S., Locascio, A.: Regulatory elements retained during chordate evolution: coming across tunicates. Genesis **53**, 66–81 (2015)
21. Whittington, N., Cunningham, D., Le, T.K., De Maria, D., Silva, E.M.: *Sox21* regulates the progression of neuronal differentiation in a dose-dependent manner. Dev. Biol. **397**, 237–247 (2015)