Developmental Biology 425 (2017) 152–160

Contents lists available at ScienceDirect

Developmental Biology

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

Asymmetrically reduced expression of *hand1* homeologs involving a single nucleotide substitution in a *cis*-regulatory element

Haruki Ochi[a,*], Nanoka Suzuki[a], Akane Kawaguchi[b], Hajime Ogino[b,c]

[a] Institute for Promotion of Medical Science Research, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata, Yamagata 990-9585, Japan
[b] Department of Animal Bioscience, Nagahama Institute of Bio-Science and Technology, 1266 Tamura, Nagahama, Shiga 526-0829, Japan
[c] Amphibian Research Center, Hiroshima University, 1-3-1 Kagami-yama, Higashi-hiroshima, Hiroshima 739-8526, Japan

ARTICLE INFO

Keywords:
Tetraploidization
Hand
Whole genome duplication
Cis-regulatory element
Xenopus laevis (*X. laevis*)
Xenopus tropicalis (*X. tropicalis*)

ABSTRACT

During vertebrate evolution, whole genome duplications resulted in a number of duplicated genes, some of which eventually changed their expression patterns and/or levels *via* alteration of *cis*-regulatory sequences. However, the initial process involved in such *cis*-regulatory changes remains unclear. Therefore, we investigated this process by analyzing the duplicated *hand1* genes of *Xenopus laevis* (*hand1.L* and *hand1.S*), which were generated by allotetraploidization 17–18 million years ago, and compared these with their single ortholog in the ancestral-type diploid species *X. tropicalis*. A dN/dS analysis indicated that *hand1.L* and *hand1.S* are still under purifying selection, and thus, their products appear to retain ancestral functional properties. RNA-seq and *in situ* hybridization analyses revealed that *hand1.L* and *hand1.S* have similar expression patterns to each other and to *X. tropicalis hand1*, but the *hand1.S* expression level was much lower than the *hand1.L* expression level in the primordial heart. A comparative sequence analysis, luciferase reporter analysis, ChIP-PCR analysis, and transgenic reporter analysis showed that a single nucleotide substitution in the *hand1.S* promoter was responsible for the reduced expression in the heart. These findings demonstrated that a small change in the promoter sequence can trigger diversification of duplicated gene expression prior to diversification of their encoded protein functions in a young duplicated genome.

1. Introduction

The allotetraploid frog *Xenopus laevis* and its diploid relative *X. tropicalis* provide an excellent model system for studying gene evolution in a young duplicated genome (Evans, 2006; Hellsten et al., 2010; Uno et al., 2013). Gene-by-gene estimates from transcriptomes and whole genome-sequencing projects have shown that the *X. tropicalis* and *X. laevis* lineages diverged from a common diploid ancestor 48 million years ago (Evans, 2006; Hellsten et al., 2010; Session et al., 2016). Following this, two diploid species evolved in the *X. laevis* lineage, which subsequently hybridized to form the allotetraploid genome of *X. laevis* 17–18 million years ago. This genome duplication was significantly more recent than the ancient vertebrate whole genome duplications (WGDs), which occurred 520–550 million years ago, and the teleost-specific WGD, which occurred 225–333 million years ago (Dehal et al., 2003; Putnam et al., 2008; Near et al., 2012). It has previously been demonstrated that these ancient WGDs are suitable for studying subfunctionalization and neofunctionalization of duplicated genes. However, the much younger WGD in the *X. laevis* lineage appears to be more suitable for studying earlier events following a WGD (Hellsten et al., 2007).

Immediately after a WGD, all genes and their *cis*-regulatory elements must be present as two functionally equivalent copies, with the same expression patterns at equivalent levels—otherwise, a dosage imbalance in the gene regulatory network would cause developmental disorders. However, duplicated gene pairs often exhibit distinct expression patterns in modern vertebrates (Kleinjan et al., 2008), and massive gene loss has occurred in the teleost fish lineage, with mathematical modeling indicating that this occurred in the first 60 million years after WGD (Petit et al., 2004; Berthelot et al., 2014; Inoue et al., 2015). The evolution of the *cis*-regulatory elements must have occurred prior to the diversification of duplicated gene expression and/or the loss of one of the duplicated genes. However, although many studies have found dynamic differences in the *cis*-regulatory elements by comparing evolutionarily distant species (Kleinjan et al., 2008; Ochi et al., 2012), the initial state of *cis*-regulatory elements following a WGD remains largely unclear.

Hand1 and Hand2 are basic helix-loop-helix (bHLH) transcription

* Corresponding author.
E-mail addresses: harukiochi@med.id.yamagata-u.ac.jp (H. Ochi), nanoka.s@med.id.yamagata-u.ac.jp (N. Suzuki), akane.kawaguchi@imp.ac.at (A. Kawaguchi), oginohaj@hiroshima-u.ac.jp (H. Ogino).

http://dx.doi.org/10.1016/j.ydbio.2017.03.021
Received 15 July 2016; Received in revised form 21 March 2017; Accepted 21 March 2017
Available online 28 March 2017

factors that are expressed in multiple tissues, such as the heart, limbs, neural crest, and extraembryonic mesoderm (Firulli et al., 1998; Riley et al., 1998; Srivastava et al., 1997). Biochemical analyses have shown that Hand1 and Hand2 form both homo- and heterodimers with each other and with other bHLH proteins, such as Hairy-related transcription factors (HRT1-3) (Firulli et al., 2000, 2003). It has previously been shown that overexpression of *Hand1* in the mouse heart increases the proliferation of cardiomyocytes and disrupts cardiac morphogenesis (Risebro et al., 2006), and overexpression of the Hand1 protein in the adult mouse heart causes a predisposition toward arrhythmia (Breckenridge et al., 2009). By contrast, embryos that specifically deleted *hand1* from the embryonic myocardium exhibited defects in the left ventricle and endocardial cushions (McFadden et al., 2005). Thus, it is likely that *Hand1* is critical for the proper formation and physical functioning of the heart.

Here we chose to study the *hand1* gene in *X. laevis* as the first step toward revealing how the *cis*-regulatory element has changed in this young duplicated genome. A comparative expression analysis of *X. laevis hand* homeologs and *X. tropicalis* orthologs by *in situ* hybridization revealed that although ancestral expression in the branchial arches and epidermal cells has been preserved for each homeolog pair, the S genes of *hand1* were not detected in the developing heart. In addition, a comparative sequence, luciferase reporter, chromatin immunoprecipitation (ChIP)-PCR, and transgenic reporter analysis showed that a single nucleotide substitution in the *hand1.S* promoter was responsible for this reduced expression. This result suggested that a small change in the promoter sequence occurred, which reduced the expression of *hand* homologs in the heart without gene loss.

2. Materials and methods

2.1. Syntenic comparison

We downloaded the following sequences from the *X. laevis* (v9.1) and *X. tropicalis* (v9) genome browsers (http://www.xenbase.org/entry/) and compared the genes flanking the *hand* gene loci:

X. tropicalis hand1: chr03:9,077,411...9,527,410
X. laevis hand1.L: chr3L:15,157,662...15,607,661
X. laevis hand1.S: chr3S:13,442,349...13,942,348
X. laevis hand3.S: chr4S:103,742,452...103,942,451

2.2. Phylogenetic tree and dN/dS analyses

We used gene models mapped to the following locations in the *X. laevis* genome assembly, v9.1 (annotation v1.8 and RefSeq (*X. laevis*)):

X. tropicalis hand1: chr03:9,256,025...9,265,422
X. laevis hand1.L: chr3L: chr3L:15,411,576...15,418,045
X. laevis hand1.S: chr3S:13,684,095...13,697,003
X. laevis hand3.S: chr4S:103,876,052...103,876,528

Phylogenetic trees were constructed using the neighbor-joining method (Saitou N, 1987). The non-synonymous to synonymous substitution ratios (dN/dS ratios) between *X. laevis* homeologs and their *X. tropicalis* orthologs were calculated with PAL2NAL (Suyama et al., 2006).

2.3. Cloning of X. laevis hand1 homeologs

Nucleotide sequence alignments of transcript models showed that *X. laevis hand1* homeologs had a striking similarity to each other, particularly in the coding regions (Supplementary Fig 1). Thus, when developing *in situ* hybridization probe templates, we carefully chose to clone the untranslated regions and parts of the coding regions to minimize sequence similarity between the isolated DNA fragments. The resultant DNA fragments of *hand1.L* and *hand1.S* were amplified from the genomic DNA of *X. laevis J-strain* (kindly gifted by Dr. Masanori Taira, University of Tokyo) or a cDNA pool prepared from stage 26 and 35 *X. laevis J-strain* embryos (kindly gifted by Dr. Shuji Takahashi, Hiroshima University). The primer sequences used in this study are summarized in Table 1. The PCR products were cloned into either the *Bam*HI sites or *Bam*HI and *Hind*III sites of a pBluescript SKII+ plasmid and verified by sequencing analysis. The resulting plasmids were linearized for use as the templates for RNA probe synthesis.

Table 1
Primer sequences for cloning *hand* genes and promoters.
PCR primers for cloning the *hand* genes were carefully designed to include untranslated regions and parts of the coding regions to avoid cross-hybridization between homeologs, and primers for cloning the promoters were designed using the ends of the CNE. Primer linker sequences are shown in lowercase letters.

Gene	Primer sequences
X. laevis hand1.L	tagaactagtggatcTTGTGGTGCTTCCCTGAG gcagcccgggggatccGGTTCCCTTCTCCTCTT
X. laevis hand1.S	tagaactagtggatcATGAACTTGATTGGGAGCTA gcagcccgggggatccGGTTCCCTTCTCCTCTT
X. tropicalis hand1	tagaactagtggatcCAGGAATGCCACTACAGC cggtatcgataagctATGCCGCCGCTTGTATAG
X. tropicalis hand1-CNE	accgcggtggcGGCCATAGGAAATATGTAATATTGTTTT atccactagttctagAAGTTTCCACAGCTCCACT
X. laevis hand1.L-CNE	accgcggtggcGGCCAAGCATTCAGAATACTTTCC atccactagttctagGGTCTGTACAAGTTTCTGC
X. laevis hand1.S-CNE	accgcggtggcGGCCATACAACATTTTGCTCTTTT atccactagttctagGGTCTGCACAAGTTTCCAC
X. laevis hand1.L-RT-PCR	ATGAACCTGATTGGGAGCTACC TGACACCTCTCCTGGGTTC
X. laevis hand1.S-RT-PCR	ACCATGAACTTGATTGGGAGCT GTGACACCTCCCCAGGGTTG
X. laevis hand1.L-ChIP-PCR	CAGACTCTACCAATGTGAGAGACTAAACAG ATACAGCAGACCTACACAGCATGAGG
X. laevis hand1.S-ChIP-PCR	GAGAGAGGCAGGTAATTAGGTCTGTTTATTT GTTCAGTCTGTGCTGCTCTGG

2.4. *Whole-mount* in situ *hybridization*

Whole-mount *in situ* hybridization was performed using digoxigenin (DIG)-labeled antisense RNA, as described previously (Hazel et al., 2000). To eliminate cross-hybridization signals between homeologs, all *X. laevis* embryos were hybridized with probes at 60 °C and then treated with RNase A in 2× saline sodium citrate at room temperature for 30 min (Khokha et al., 2002). We omitted this RNase A treatment for *X. tropicalis* embryos with no such homeologs.

2.5. RT-PCR

Total RNA was extracted from the heart tubes of stage 36 and 40 embryos and converted to cDNA template. The primers for *hand1.L* and *hand1.S* are shown in Table 1 and Supplementary Fig 1.

2.6. Motif analysis for transcription factor-binding sites

The open-access database JASPAR v5 was used to search for potential transcription factor-binding sites in the *X. tropicalis hand1 cis*-regulatory element. The *hand1 cis*-regulatory elements for *X. tropicalis* and *X. laevis* homeologs were then aligned by ClustalW using GENETEYX, and the conserved sequences for the candidate transcription factor-binding sites were further analyzed by phylogenetic footprinting.

2.7. Construction of transgenic reporter plasmids and nucleotide substitution within the cis-regulatory elements

Candidates for the *hand1 cis*-regulatory element were amplified from the genomic DNA of the *X. tropicalis Nigerian*-strain and *X. laevis J-strain* and cloned into a firefly luciferase reporter vector (Promega, Madison, WI, USA) and a green fluorescent protein (GFP) reporter vector (Ogino et al., 2008). Nucleotide substitutions within the *hand1.S cis*-regulatory element were then performed using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio Inc., Otsu, Shiga, Japan). The primer sequences used in this study are summarized in Table 1. We used the following genomic regions in the *X. laevis* genome assembly v9.1 and *X. tropicalis* genome assembly v9:

X. laevis hand1.L: chr3L:15,410,020...15,412,700
X. laevis hand1.S: chr3S:13,696,287...13,697,746
X. tropicalis hand1: chr03:9,264,629...9,266,168

2.8. Luciferase reporter analysis

Human 293T cells were co-transfected with the firefly luciferase reporter plasmid of *hand1.L* or *hand1.S* (25 ng) and the expression vector of pCS-MT-*xt-Myod1* (200 pg), together with the *Renilla* luciferase vector (10 ng). After 24 h, the luciferase activities were measured. Each firefly luciferase activity was normalized to the *Renilla* activity using the dual luciferase assay system (Promega, Madison, WI, USA).

2.9. Chromatin immunoprecipitation (ChIP)-PCR

Myc tagged *X. tropicalis Myod1* (pCS-MT-*xt-Myod1)* plasmid was linearized with *Not*I, and mRNA was *in vitro* transcribed using the SP6 mMessage mMachine kit (Ambion Foster City, CA, USA). To note, 125 pg *Myod1* mRNA and 200 pg *mCherry* mRNA were co-injected into fertilized eggs, and 100 mCherry positive embryos were selected and fixed at stage 33/34. Sonication was performed using a Branson Sonifier 250. Chromatin samples were immunoprecipitated with anti-myc antibody (9E10, Santa Cruz) or control IgG using Dynabeads protein A immunoprecipitation kit (ThermoFisher, Waltham, MA, USA).

2.10. Transgenic reporter assay

Transgenic *X. laevis* embryos were generated using the sperm nuclear transplantation method with oocyte extracts (Kroll and Amaya, 1996). The manipulated embryos were cultured until stage 33/34, and all normally developed embryos were subjected to *in situ* hybridization to examine GFP expression with maximum sensitivity.

2.11. Ethics statement

All animal care procedures and experiments conformed to the Guidelines of Animal Experiments of Yamagata University, Japan, and the experimental protocols were approved by the Animal Research Committee of Yamagata University (26–075).

3. Results

3.1. Identification of X. laevis hand1 gene

A Basic Local Alignment Search Tool (BLAST) search for the previously reported *X. laevis ehand/hand1* sequence (Genbank accession number: Z95080) identified three related genes in the *X. laevis* genome sequence assembly (Fig. 1). One of these is located on chromosome 3 L and has an identical coding sequence to *ehand*, while the other two are located on the homeologous chromosome 3S and a non-homeologous chromosome 4S. A comparison of the syntenies around these loci and around the *X. tropicalis hand1* locus revealed a homeologous relationship between the putative *hand1* genes on chromosomes 3L and 3S and orthologous relationships between these and *X. tropicalis hand1* (Fig. 1A). Hence, we chose to call these *X. laevis* genes on chromosomes 3L and 3S as *hand1.L* and *hand1.S*, respectively, according to the new nomenclature method that has been used in the *X. laevis* genome sequence assembly (Session et al., 2016). The gene set that flanks the remaining gene on chromosome 4S is completely different from those flanking *hand1.L*, *hand1.S*, and *X. tropicalis hand1*. Therefore, since neither *X. tropicalis* nor other vertebrates including zebrafish, coelacanth, mouse, and human has a *hand1*-like gene in the orthologous genome region (data not shown), we inferred that this *hand1*-like gene on chromosome 4S was generated following tetraploidization rather than during the two rounds of vertebrate-specific WGDs, and thus, we named this *hand3.S*. A phylogenetic tree analysis illustrated that Hand1.L and Hand1.S are much closer to *X. tropicalis* Hand1 than Hand3 at the protein sequence level (Fig. 1B and C).

3.2. Comparative expression analysis of hand1 genes in developing X. laevis and X. tropicalis embryos

The expression of *hand1.L* was first detected in the epidermis of early neurula embryos at stage 17 using *in situ* hybridization analysis (Fig. 2B, white arrowheads). The expression of this gene then appeared in the anterior ventral portion of early tailbud embryos (Fig. 2C, white arrows), which corresponds to the presumed heart and proctodeum region (Sparrow et al., 1998). Expression also appeared in the epidermal cells at the early tailbud stage (Fig. 2C, white arrowheads). In late tailbud embryos, *hand1.L* expression was detected in the heart and branchial arches (Fig. 2D, E and K, gray and black arrowheads, respectively). Similarly, hybridization signals for the expression of its homeologous counterpart *hand1.S* were also detected in most of the *hand1.L*-expressing tissues; however, the signal intensity appeared to be lower than that of *hand1.L,* and no expression was detected in the heart (Fig. 2J, gray arrowhead, Table 2). Because *hand1* homeologs have highly similar sequences, there is a risk of these probes cross-reacting and detecting the transcripts of the other homeolog pairs (Supplementary Fig 1). To confirm whether the lower signal intensity of *hand1.S* was not due to cross-reaction with *hand1.L*, we cloned only *hand1.S*-UTR and performed *in situ* hybridization analysis (Supplementary Fig 2). Although *hand1.S*-UTR probe is more identical and shorter than probes used in the above-mentioned analysis, the signal intensity still appeared in the branchial arches and epidermal cells. Furthermore, to confirm whether the S locus transcripts are fewer than L locus transcripts in heart, we performed RT-PCR using total RNA that was extracted from the heart tubes (Fig. 2L). First, we tested the amplification efficiency of *hand1.L* and *hand1.S* primers using *X laevis* J-strain genomic DNA that contains 1 copy of *hand1.L* and *hand1.S*, respectively, and found that *hand1.L* and *hand1.S* primers have approximately equal amplification efficiency in genomic DNA (Fig. 2L). By contrast, the amplicons of *hand1.L* primer in the heart tubes were more than those of *hand1.S* primer (Fig. 2L). Thus, both *hand1.L* and *hand1.S* are expressed in the branchial arches, whereas *hand1.S* expression in the heart and epidermal cells is much lower than *hand1.L* expression. This qualitative expression divergence detected by *in situ* hybridization is consistent with the results of the RNA-seq analysis, which showed that the expression level of *hand1.L* was higher than that of *hand1.S* at stage 25, when both exhibit a peak (Fig. 3A and C) (Session et al., 2016).

In *X. tropicalis*, *hand1* showed expression in the heart, branchial arches, and epidermal cells of tailbud embryos (Fig. 4A and B, gray, black, and white arrowheads) which is very similar to the expression pattern of *hand1*.L. Therefore, assuming that *X. tropicalis hand1* represents the ancestral-type expression pattern, we infer that

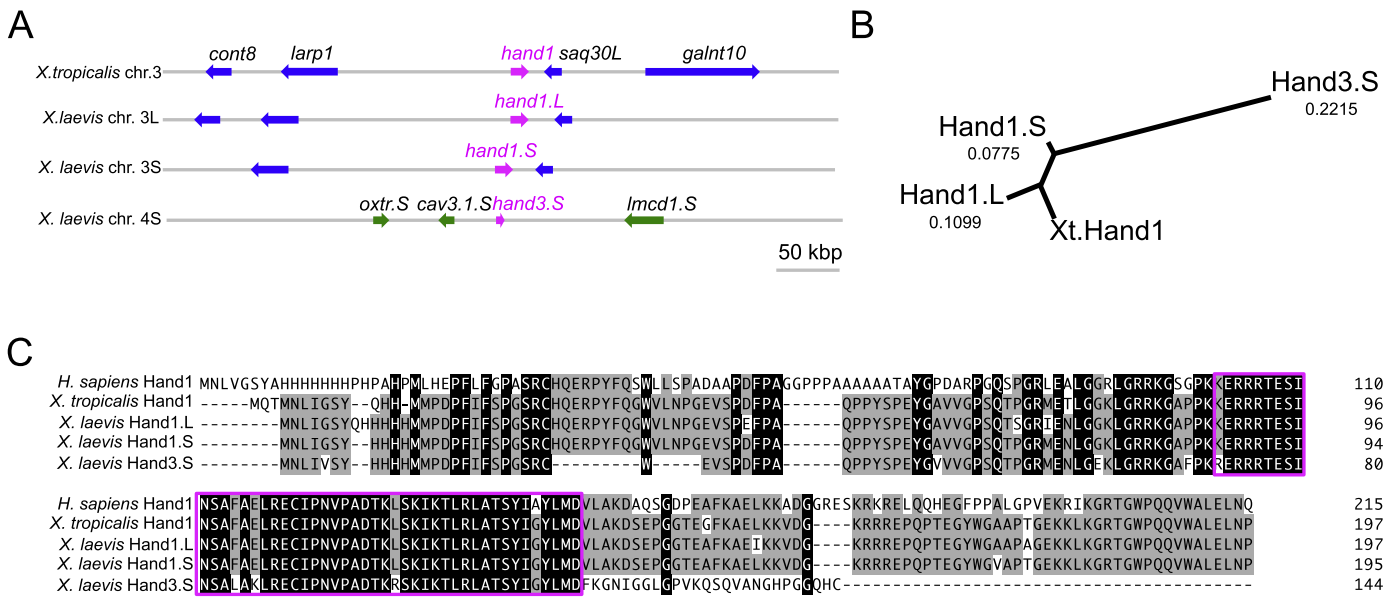

Fig. 1. (A) Synteny conservation analysis of *hand1*. These genes were identified in the *Xenopus laevis* genome assembly *X. laevis* (v9.1) and *X. tropicalis* (v9). Arrows indicate the orientation of transcription. Only part of the chromosome around the *hand* loci is shown. (B) A phylogenetic tree analysis of *hand1* gene products. This tree was constructed using the neighbor-joining method. Numbers indicate the ratios of non-synonymous to synonymous divergence (dN/dS) between *X. tropicalis* and *X. laevis* homeologs. (C) Amino acid alignments of the Hand1 family. The bHLH DNA-binding domains are indicated by the magenta boxes.

hand1.L has taken over most of the ancestral function following tetraploidization, whereas *hand1.S* has partially lost this.

3.3. Comparative transgenic cis-regulatory reporter analysis of hand1 homeologs

A comparative expression analysis using *in situ* hybridization showed that *hand1.L* has taken over most of the ancestral function following tetraploidization, whereas *hand1.S* is no longer expressed in the heart. These findings suggest that the *cis*-regulatory elements of the *hand1.S* locus have changed since tetraploidization.

We initially compared the genomic sequence of a 13.3-kb segment encompassing *X. tropicalis hand1* with the orthologus region in *X. laevis* L and S using the VISTA alignment tool (Frazer et al., 2004). This analysis showed that the sequences surrounding the proximal promoter were highly conserved between *X. tropicalis hand1*, *hand1.L*,

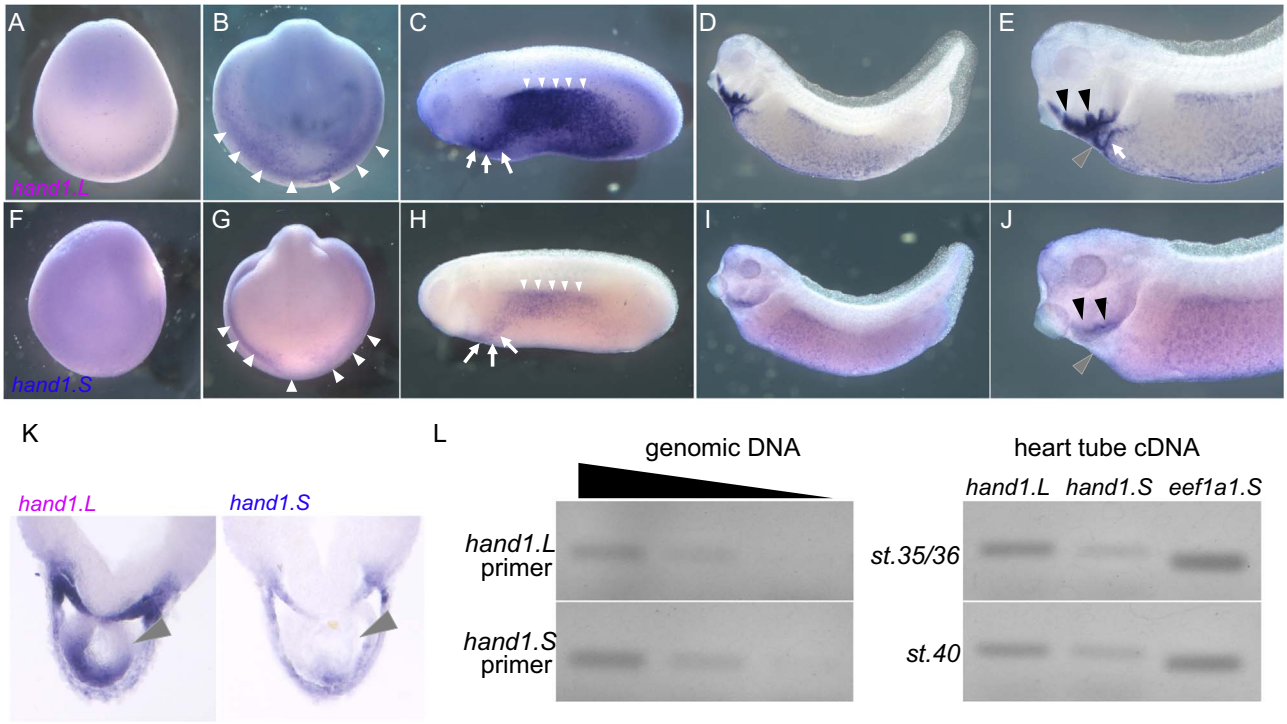

Fig. 2. Developmental expression of *hand1.L* and *hand1.S* in *Xenopus laevis*. (A–K) The whole embryos showing expression at stages 15 (A and F), 17 (B and G), 19 (C and H), and 35/36 (D and I), and their high-magnification images (E and J). Cross-section showing heart at stage 35/36 (K). White arrowhead: epidermal cells; white arrow: anterior ventral portion; gray arrowhead: heart; black arrowhead: branchial arches. (L) RT-PCR analysis of *hand1.L* and *hand1.S*. The amplification efficiency of each primer pair was confirmed using genomic DNA from J-strain (5 ng, 1 ng, and 0.2 ng, respectively). RT-PCR was performed with cDNA from the heart tubes. *Eukaryotic translation elongation factor 1 alpha 1/EF-1 alpha* (*eef1a.S*) was used as internal control.

Table 2
Scoring results from the *hand1.L* and *hand1.S is situ* hybridization. *hand1.L* and *hand1.S* expression patterns were examined at stage 33/34. The percentage indicates the number of embryos that showed the expression pattern in the left column among the examined embryos.

Expression pattern of embryos	*hand1.L* (% of embryos that showed the expression pattern in the left column)	*hand1.S*
heart, branchial arches, epidermal cells	96.2	3.8
branchial arches, epidermal cells	3.8	96.2
	n = 26	n = 26

and *hand1.S*, making these conserved noncoding elements (CNEs) candidates for the *hand1* enhancer (Fig. 5A). We then analyzed the putative transcription factor-binding motifs to find the responsible transcription input that changed *hand1.S* expression following tetraploidization. The open-access database JASPAR ver. 5 was used to define potential transcription factor-binding motifs using *X. tropical hand1 cis*-regulatory elements (Mathelier et al., 2014). The resultant candidate transcription factors were then narrowed down on the basis of their heart expression using the Expression Atlas (Petryszak et al., 2014), followed by phylogenetic footprinting (Fig. 5). This resulted in the identification of the putative Myod1-binding motif, which is upstream of the TATA-binding protein (TBP)-binding motif and is separated from it by Myog, Elf5, Nfic, and Nkx3-2 (Fig. 5A). A detailed sequence comparison of the core DNA-binding motifs indicated that a single nucleotide substitution had occurred in the enhancer box (E-box) of the Myod1 putative binding site in *hand1.S* promoter (Fig. 5B). Previous studies showed that mutations in E-box, such as CAnnTG to TGnnTG and CAnnTG to GAnnTG, effectively abolished the binding of bHLH transcription factors (Blackwell and Weintraub, 1990; Czernik et al., 1996; Ohno et al., 2003). Next, we examined whether the single nucleotide substitution in the E-box of *hand1.S* promoter show the differential enhancer activities to Myod1 input. We first cloned the proximal promoter elements shown in Fig. 5A into the firefly luciferase reporter plasmid and then performed luciferase reporter analysis. The luciferase activity of *X. tropicalis hand1* and *hand1.L* promoters was enhanced by *X. tropicalis* Myod1 expression (Fig. 5C). By contrast, Myod1 did not increase the *hand1.S* promoter activity. Subsequently, we performed Chromatin immunoprecipitation analysis to know whether the Myod1 directly binds to *hand1.L* promoter in *X. laevis* embryo. The ChIP-PCR analysis showed that Myod1 enriched on the *hand1.L* promoter, while not on the *hand1.S* promoter (Fig. 5D). Thus, Myod1 seems to preferentially up-regulate the expression of *hand1.L* thought the its promoter region.

Finally, we examined whether the *hand1.L* promoter has an enhancer activity in the heart using *X. laevis* reporter transgenic system. We cloned the proximal promoter elements shown in Fig. 5A into a GFP reporter vector. Transgenic embryos of the *X. tropicalis hand1* promoter with GFP showed enhancer activities in the heart, branchial arches, and epidermal cells of tailbud embryos (Fig. 6, gray arrowhead and white arrowheads), although reporter expression in the branchial arches was broader than endogenous *hand1* expression. We also observed the reporter gene expression in the pronephric duct, where endogenous *hand1* was not expressed (Fig. 6, white arrow). We have previously shown that some of enhancer activates the reporter gene expression in multiple tissues where endogenous gene is not expressed (Ochi et al., 2012; Suzuki et al., 2015). Such pleiotropic enhancers require the tissue-specific silencer to recapitulate the endogenous gene expression pattern. Indeed, we previously have shown that tissue-specific gene expression is produced through a combination of an enhancer and silencer (Ochi et al., 2012). Therefore, although the promoter used in this reporter analysis can drive reporter gene expression in heart, it may lack the tissue-specific silencers. Previous studies have shown that multiple enhancers are often retained in one gene locus for its expression (Ochi et al., 2012; Suzuki et al., 2015). Since we only examined the promoter activity, it is still possible that strong heart-specific enhancers are retained in upstream and/or downstream of *hand1* gene. Regarding the promoters of *hand1.L* and *hand1.S*, their transgenic reporter embryos showed enhancer activities in most of the *X. tropicalis hand1* promoter activating tissues. However, the *hand1.L* CNE showed promoter activity in the heart, whereas the *hand1.S* CNE failed to activate reporter expression here (Fig. 6, gray arrowheads; Table 3). Thus, we inferred that the *hand1.L* promoter has taken over most of the ancestral function following tetraploidization, whereas *hand1.S* has partially lost this function.

To confirm whether the single nucleotide substitution in the Myod1 E-box has contributed to the differential expression of *hand1.L* and *hand1.S*, we substituted a thymidine in the *mydo1* E-box of *hand1.S* with cytosine (Fig. 6). These transgenic reporter embryos carrying the single nucleotide replaced promoter exhibited expression in the hearts of tailbud embryos (Fig. 6, gray arrow; Table 3). Thus, the single

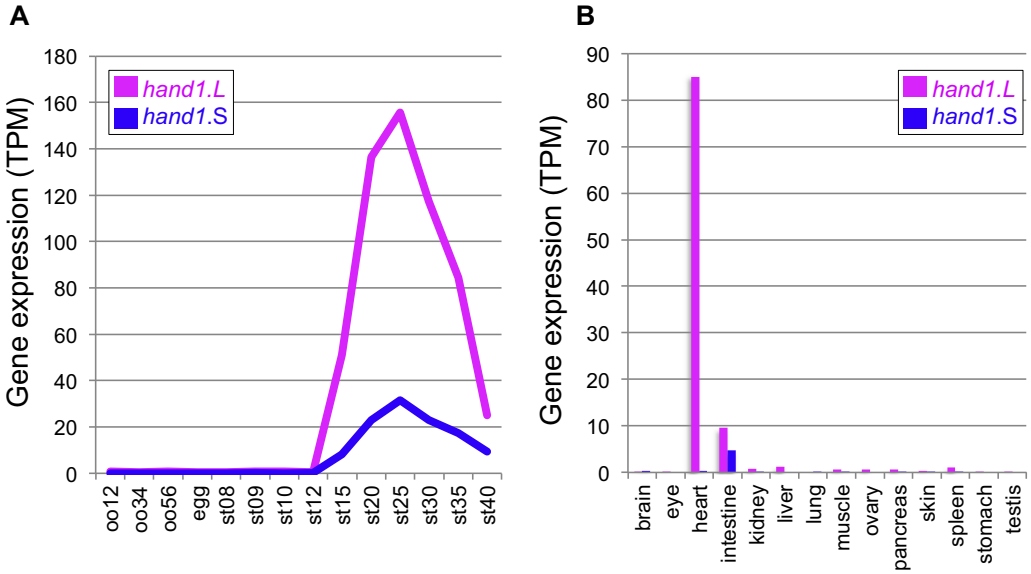

Fig. 3. RNA-seq analysis of developing embryos and adult tissues. Expression of *hand1* homeologs in developing embryos (A) and in adult tissues (B).

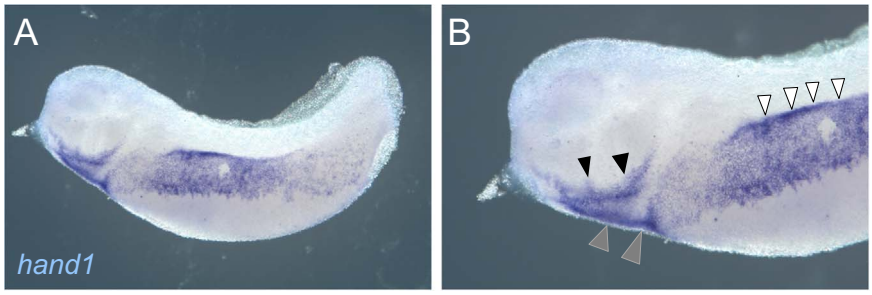

Fig. 4. Expression of *hand1* in *Xenopus tropicalis*. Expression at stage 28 at low (A) and high (B) magnification. White arrowhead: epidermal cells; gray arrowhead: heart; black arrowhead: branchial arches.

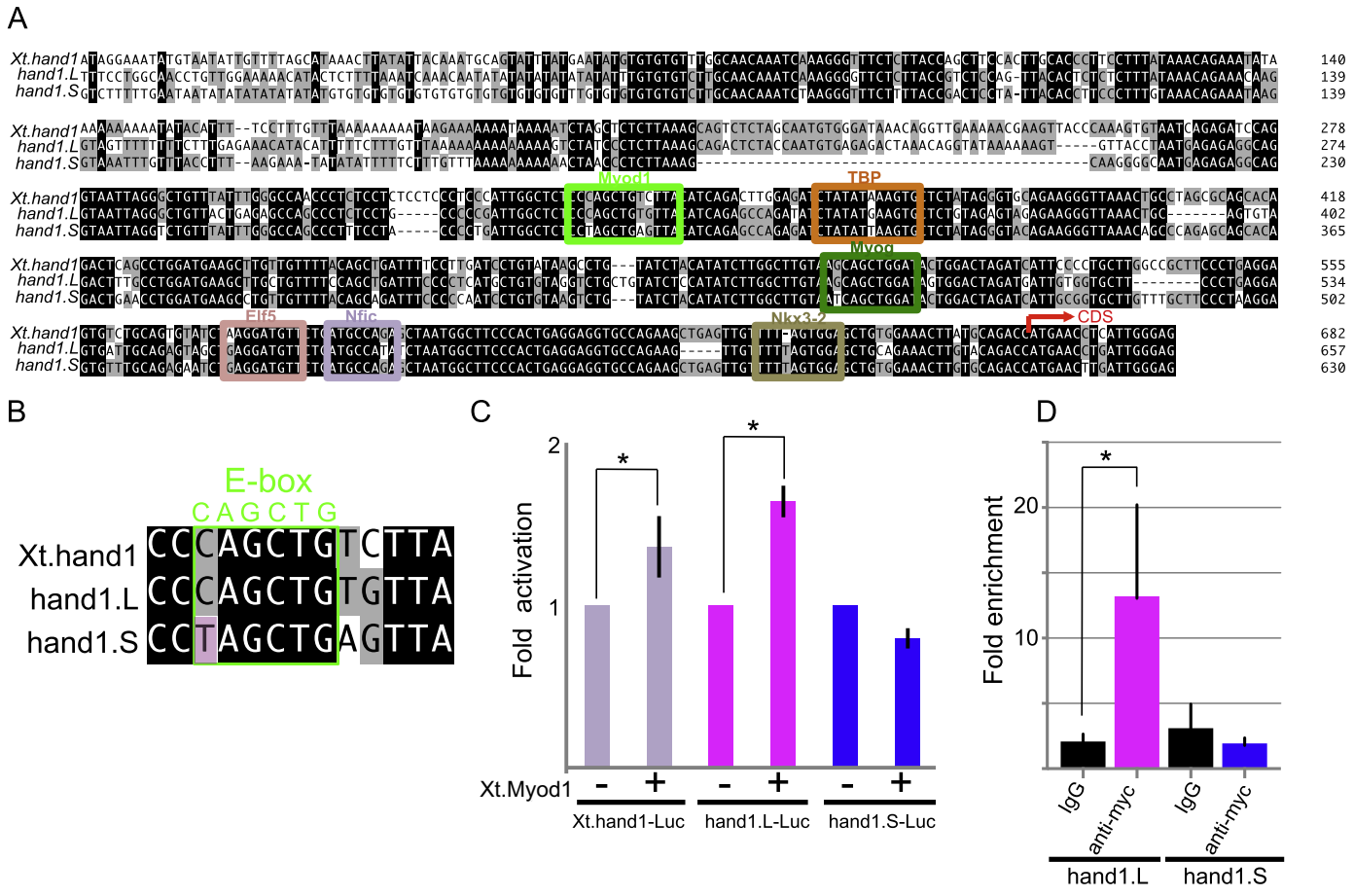

Fig. 5. Divergence of enhancer activity between *hand1.L* and *hand.S* promoters. (A-B) Alignment of the *hand1* promoter element (CNE) and potential transcription factor-binding motifs (A) and higher magnification image of potential Myod1-binding motifs. (C) Transcriptional activation of the *hand1.L* promoter by Myod1. Firefly luciferase activity was measured and normalized to Renilla luciferase using the dual luciferase assay kit. Luciferase data are expressed as fold activation relative to the empty expression vector. Error bars indicate ± SD, and an asterisk indicates p-value ($P < 0.05$, n=3, two-tailed Student's T-test). (C) Myod1 protein enriched on *hand1.L* promoter compared with *hand1.S* promoter. Error bars indicate ± SD, and an asterisk indicates p-value ($P < 0.05$, determined by one-way ANOVA with Tukey's multiple comparison test).

nucleotide substitution in the *cis*-regulatory element following allotetraploidization has produced the differential tissue expression between *hand1.L* and *hand1.S*.

4. Discussion

Here, we demonstrate that the *hand1.L* and *hand1.S* genes that were generated by allotetraploidization in *X. laevis* exhibit distinct expression patterns in the heart and that a single nucleotide substitution in the *hand1.S* promoter has contributed to this differential expression.

WGD produces two functionally equivalent *cis*-regulatory elements and gene copies, meaning that all genes must be expressed at equivalent levels immediately after this event. It is particularly important that the gene expression level of physically interacting dosage-sensitive transcription factors is maintained to prevent a dosage imbalance in the gene regulatory network, which would result in developmental disorders. The gene balance hypothesis explains the evolutionary mechanisms by which genes whose products physically interact with each other are over-retained in young duplicated genomes (Edger and Pires, 2009). However, it is well known that in modern vertebrates, many duplicated genes are expressed in distinct tissues and/or one of the duplicated genes has often been lost (Petit et al., 2004; Kleinjan et al., 2008; Ochi et al., 2012; Berthelot et al., 2014). It has been estimated that this gene loss occurred in the first 60 million years after the WGD event (Inoue et al., 2015) and so the evolution of

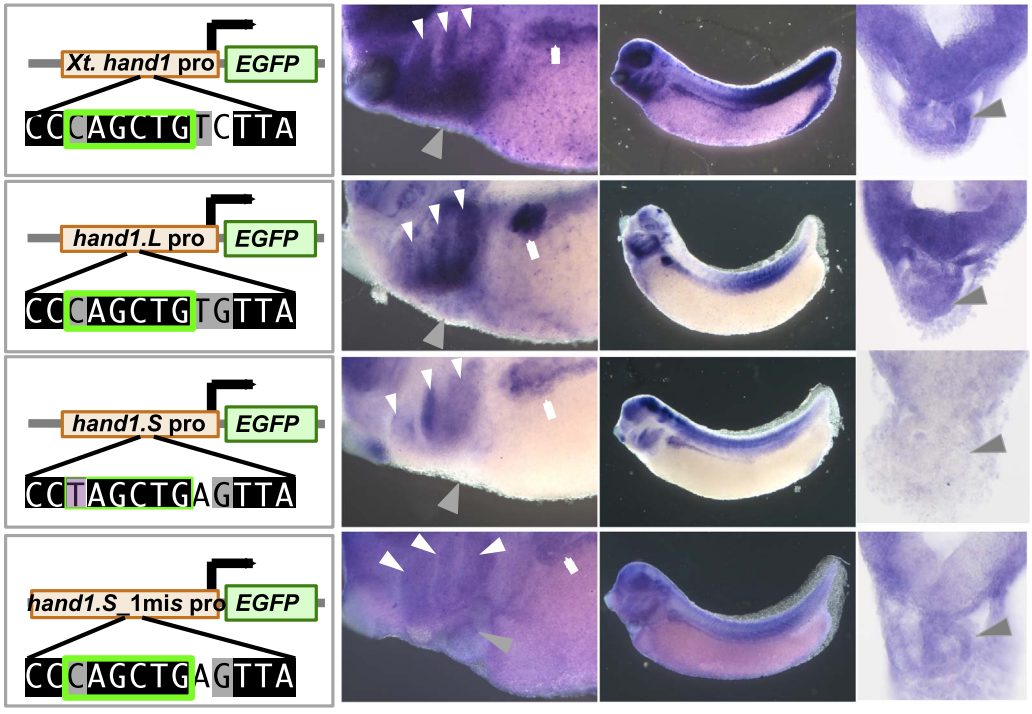

Fig. 6. Transgenic reporter analysis of the *hand1.L* and *hand1.S* promoters. Green fluorescent protein (GFP) expression pattern driven by *hand1* promoters. The reporter constructs are shown on the left, and higher magnification images and cross-section of the heart are shown in the middle. A thymidine in *hand1.S* putative Myod1-binding site was substituted into cytosine. E-box was indicated by the green boxes. Gray arrowhead: heart; white arrowhead: branchial arches; white arrows: pronephric duct.

cis-regulatory elements that cause the expression loss should have occurred more rapidly. RNA-seq analysis has indicated that not only *hand1.S* but also the Hand1 interacting protein homeologs *hand2.S* and *hairy-related transcription factor1.S* (*hey1.S*) exhibit the much lower expression than *hand2.L* and *hey1.L* (Session et al., 2016). These simultaneous reductions in *hand2.S* and *hey1.S* expression with *hand1.S* expression must have been accompanied by the simultaneous evolution of *cis*-regulatory elements for each locus. This will have involved both mutations in the enhancers/promoters and the acquisition of silencers, although the simultaneous acquisition of novel heart-specific silencers in the *hand1*, *hand2*, and *hey1* loci seems unlikely. Therefore, we believe that a simultaneous single nucleotide substitution in the *cis*-regulatory element is one of the evolutionary processes that have maintained the optimal expression balance between physically interacting dosage-sensitive transcription factors and that this genomic status may reflect the early stage of diploidization following WGD.

Funding

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (Grant Nos. 16K07362, 25124704, 25125716, and 16H04828 to H. Ochi and 15K07082 to H. Ogino), as well as grants from CREST (JST to H.

Table 3
Scoring results from the *Xenopus laevis* transgenic reporter assay. Green fluorescent protein (GFP) expression patterns were examined at stage 33/34. Each construct showed reproducible multi-expression patterns, as indicated in the left column. The percentage of transgenic embryos indicates the number of embryos that showed the expression pattern in the left column among the total number of GFP-expressing embryos.

Expression pattern of transgenic embryos	*Xt.hand1-EGFP* (% of embryos that showed the expression pattern in the left column/GFP positive embryos)	*Xl.hand1.L-EGFP*	*Xl.hand1.S-EGFP*	*Xl.hand1.S-1 mis-EGFP*
heart, branchial arches, eye, somite, pronephric duct	33.3	21.9	4.3	18.5
heart, somite	0	6.3	4.3	0
branchial arches, eye, somite, epidermal cells	20	21.9	39.1	0
branchial arches, eye, somite,	13.3	9.4	0	18.5
branchial arches, somite	6.7	0	0	3.7
branchial arches, somite, pronephric duct, blood vessel	0	0	0	3.7
branchial arches, eye, somite, pronephric duct	0	0	0	3.7
branchial arches, pronephric duct	0	0	0	3.7
somite	26.7	18.8	0	3.7
somite, pronephric duct	0	12.5	0	0
eye, somite	0	6.3	39.1	40.7
neural tube	0	3.1	0	0
somite, blood vessel	0	0	8.7	3.7
somite, pronephric duct, blood vessel	0	0	4.3	0
	Number of embryos (N)			
Number of examined embryos	60	88	96	60
Number of GFP positive embryos	15	32	23	27

Ogino), the Takeda Science Foundation (to H. Ochi and H. Ogino), and Senri Foundation and YU-COE (C) (H. Ochi).

Acknowledgments

We gratefully acknowledge Drs. Shuji Takahashi, Atsushi Toyoda, Yutaka Suzuki, Taejoon Kwon, Naoto Ueno, and Atsushi Suzuki for preparing the RNA-seq TPM data for transcriptomic analyses. *X. tropicalis* was provided by the Institute for Amphibian Biology (Hiroshima University, Japan) with support in part by the National Bio-Resource Project of the AMED, Japan.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.03.021.

References

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.-M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M., Quillet, E., Guyomard, R., Galiana, D., Bobe, J., Volff, J.-N., Genêt, C., Wincker, P., Jaillon, O., Crollius, H.R., Guiguen, Y., 2014. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. Nat. Commun. 5. http://dx.doi.org/10.1038/ncomms4657.

Blackwell, T.K., Weintraub, H., 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250, 1104–1110. http://dx.doi.org/10.1126/science.2174572.

Breckenridge, R.A., Zuberi, Z., Gomes, J., Orford, R., Dupays, L., Felkin, L.E., Clark, J.E., Magee, A.I., Ehler, E., Birks, E.J., Barton, P.J.R., Tinker, A., Mohun, T.J., 2009. Overexpression of the transcription factor Hand1 causes predisposition towards arrhythmia in mice. J. Mol. Cell. Cardiol. 47, 133–141. http://dx.doi.org/10.1016/j.yjmcc.2009.04.007.

Czernik, P.J., Peterson, C.A., Hurlburt, B.K., 1996. Preferential binding of myoD-E12 versus myogenin-E12 to the murine sarcoma virus enhancer in vitro. J. Biol. Chem. 271, 9141–9149. http://dx.doi.org/10.1074/jbc.271.15.9141.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., Tomaso, A., De, Davidson, B., Gregorio, A., Di, Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E.M., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-bow, S., Desantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., 2003. The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298, 2157–2168.

Edger, P.P., Pires, J.C., 2009. Gene and genome duplications: the impact of dosage-sensitivity on the fate of nuclear genes. Chromosom. Res. 17, 699–717. http://dx.doi.org/10.1007/s10577-009-9055-9.

Evans, B.J., 2006. Multiple mechanisms promote the retained expression of gene duplicates in the tetraploid frog Xenopus laevis. PLoS Genet. 2, e56. http://dx.doi.org/10.1371/journal.pgen.0020056.

Firulli, B.A., Hadzic, D.B., McDaid, J.R., Firulli, A.B., 2000. The basic helix-loop-helix transcription factors dHAND and eHAND exhibit dimerization characteristics that suggest complex regulation of function. J. Biol. Chem. 275, 33567–33573. http://dx.doi.org/10.1074/jbc.M005888200.

Firulli, B., Howard, M.J., McDaid, J.R., McIlreavey, L., Dionne, K.M., Centonze, V.E., Cserjesi, P., Virshup, D.M., Firulli, A.B., 2003. PKA, PKC, and the protein phosphatase 2A influence HAND factor function: a mechanism for tissue-specific transcriptional regulation. Mol. Cell 12, 1225–1237. http://dx.doi.org/10.1016/S1097-2765(03)00425-8.

Firulli, A.B., McFadden, D.G., Lin, Q., Srivastava, D., Olson, E.N., 1998. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat. Genet. 18 (3), 266–270.

Frazer, K.A., Pachter, L., Poliakov, A., Rubin, E.M., Dubchak, I., 2004. VISTA: computational tools for comparative genomics. Nucleic Acids Res. 32, W273–W279. http://dx.doi.org/10.1093/nar/gkh458.

Hazel, L., Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of Xenopus laevis. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Hellsten, U., Harland, R.M., Gilchrist, M.J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N.H., Shu, S., Taher, L., Blitz, I.L., Blumberg, B., Dichmann, D.S., Dubchak, I., Amaya, E., Detter, J.C., Fletcher, R., Gerhard, D.S., Goodstein, D., Graves, T., Grigoriev, I.V., Grimwood, J., Kawashima, T., Lindquist, E., Lucas, S.M., Mead, P.E., Mitros, T., Ogino, H., Ohta, Y., Poliakov, A.V., Pollet, N., Robert, J., Salamov, A., Sater, A.K., Schmutz, J., Terry, A., Vize, P.D., Warren, W.C., Wells, D., Wills, A., Wilson, R.K., Zimmerman, L.B., Zorn, A.M., Grainger, R., Grammer, T., Khokha, M.K., Richardson, P.M., Rokhsar, D.S., 2010. The genome of the Western clawed frog Xenopus tropicalis. Science 328, 633–636. http://dx.doi.org/10.1126/science.1183670.

Hellsten, U., Khokha, M.K., Grammer, T.C., Harland, R.M., Richardson, P., Rokhsar, D.S., 2007. Accelerated gene evolution and subfunctionalization in the pseudotetraploid frog Xenopus laevis. BMC Biol. 5, 31. http://dx.doi.org/10.1186/1741-7007-5-31.

Inoue, J., Sato, Y., Sinclair, R., Tsukamoto, K., Nishida, M., 2015. Rapid genome reshaping by multiple-gene loss after whole-genome duplication in teleost fish suggested by mathematical modeling. Proc. Natl. Acad. Sci. 112, 14918–14923. http://dx.doi.org/10.1073/pnas.1507669112.

Khokha, M.K., Chung, C., Bustamante, E.L., Gaw, L.W.K., Trott, K. a., Yeh, J., Grammer, T.C., 2002. Techniques and probes for the study of Xenopus tropicalis development. Dev. Dyn. 225 (4), 499–510. http://dx.doi.org/10.1002/dvdy.10184.

Kleinjan, D.A., Bancewicz, R.M., Gautier, P., Dahm, R., Schonthaler, H.B., 2008. Subfunctionalization of duplicated zebrafish pax6 genes by cis-regulatory divergence. PLoS Genet. 4, e29. http://dx.doi.org/10.1371/journal.pgen.0040029.

Kroll, K.L., Amaya, E., 1996. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122, 3173–3183.

Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D.J., Buchman, S., Chen, C., Chou, A., Ienasescu, H., Lim, J., Shyr, C., Tan, G., Zhou, M., Lenhard, B., Sandelin, A., Wasserman, W.W., 2014. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic Acids Res. 42, D142–D147. http://dx.doi.org/10.1093/nar/gkt997.

McFadden, D.G., Barbosa, A.C., Richardson, J.A., Schneider, M.D., Srivastava, D., Olson, E.N., 2005. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development 132, 189–201. http://dx.doi.org/10.1242/dev.01562.

Near, T.J., Eytan, R.I., Dornburg, A., Kuhn, K.L., Moore, J.A., Davis, M.P., Wainwright, P.C., Friedman, M., Smith, W.L., 2012. Resolution of ray-finned fish phylogeny and timing of diversification. Proc. Natl. Acad. Sci. 109, 13698–13703. http://dx.doi.org/10.1073/pnas.1206625109.

Ochi, H., Tamai, T., Nagano, H., Kawaguchi, A., Sudou, N., Ogino, H., 2012. Evolution of a tissue-specific silencer underlies divergence in the expression of pax2 and pax8 paralogues. Nat. Commun. 3, 848. http://dx.doi.org/10.1038/ncomms1851.

Ogino, H., Fisher, M., Grainger, R.M., 2008. Convergence of a head-field selector Otx2 and Notch signaling: a mechanism for lens specification. Development 135, 249–258. http://dx.doi.org/10.1242/dev.009548.

Ohno, K., Sadeh, M., Blatt, I., Brengman, J.M., Engel, A.G., 2003. E-box mutations in the RAPSN promoter regions in eight cases with congenital myasthenic syndrome. Hum. Mol. Genet. 12, 739–748. http://dx.doi.org/10.1093/hmg/ddg089.

Petit, J., Stange-thomann, N., Mauceli, E., Bouneau, L., Jaillon, O., Aury, J., Ozouf-costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Bie, C., Jubin, C., Castelli, V., Katinka, M., Zody, M.C., Mesirov, J., Lindblad-toh, K., Birren, B., Nusbaum, C., Kahn, D., Saurin, W., Scarpelli, C., Wincker, P., 2004. Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. Nature 431, 946–957.

Petryszak, R., Burdett, T., Fiorelli, B., Fonseca, N.A., Gonzalez-Porta, M., Hastings, E., Huber, W., Jupp, S., Keays, M., Kryvych, N., McMurry, J., Marioni, J.C., Malone, J., Megy, K., Rustici, G., Tang, A.Y., Taubert, J., Williams, E., Mannion, O., Parkinson, H.E., Brazma, A., 2014. Expression Atlas update-a database of gene and transcript expression from microarray- and sequencing-based functional genomics experiments. Nucleic Acids Res. 42, D926–D932. http://dx.doi.org/10.1093/nar/gkt1270.

Putnam, N.H., Butts, T., Ferrier, D.E.K., Furlong, R.F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.-K., Benito-Gutiérrez, E., Dubchak, I., Garcia-Fernàndez, J., Gibson-Brown, J.J., Grigoriev, I.V., Horton, A.C., de Jong, P.J., Jurka, J., Kapitonov, V.V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L.A., Salamov, A.A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin-I, T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L.Z., Holland, P.W.H., Satoh, N., Rokhsar, D.S., 2008. The amphioxus genome and the evolution of the chordate karyotype. Nature 453, 1064–1071. http://dx.doi.org/10.1038/nature06967.

Risebro, C.A., Smart, N., Dupays, L., Breckenridge, R., Mohun, T.J., Riley, P.R., 2006. Hand1 regulates cardiomyocyte proliferation versus differentiation in the developing heart. Development 133, 4595–4606. http://dx.doi.org/10.1242/dev.02625.

Saitou N, N.M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees'. Mol. Biol. Evol. 4, 406–425, (doi:citeulike-article-id:93683).

Session, A.M., Uno, Y., Kwon, T., Chapman, J.A., Toyoda, A., Takahashi, S., Fukui, A., Hikosaka, A., Suzuki, A., Kondo, M., Heeringen, S.J., Van, Quigley, I., Heinz, S., Ogino, H., Ochi, H., Hellsten, U., Lyons, J.B., Simakov, O., Putnam, N., Stites, J., Wallingford, J.B., Ito, Y., Asashima, M., Ueno, N., Matsuda, Y., Veenstra, G.J.C., 2016. Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538, 1–15. http://dx.doi.org/10.1038/nature19840.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M.L., Brown, D., Olson, E.N., 1997. Regulation

of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat. Genet. 16 (2), 154–160. http://dx.doi.org/10.1038/ng0697-154.

Suyama, M., Torrents, D., Bork, P., 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 34, W609–W612. http://dx.doi.org/10.1093/nar/gkl315.

Suzuki, N., Hirano, K., Ogino, H., Ochi, H., 2015. Identification of distal enhancers for Six2 expression in pronephros. Int. J. Dev. Biol. 59, 241–246. http://dx.doi.org/10.1387/ijdb.140263ho.

Sparrow, D.B., Kotecha, S., Towers, N., Mohun, T.J., 1998. Xenopus eHAND: a marker for the developing cardiovascular system of the embryo that is regulated by bone morphogenetic proteins. Mech. Dev. 71 (1–2), 151–163. http://dx.doi.org/10.1016/S0925-4773(98)00010-0.

Riley, P., Anson-Cartwright, L., Cross, J.C., 1998. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat. Genet. 18 (3), 271–275. http://dx.doi.org/10.1038/ng0398-271.

Uno, Y., Nishida, C., Takagi, C., Ueno, N., Matsuda, Y., 2013. Homoeologous chromosomes of Xenopus laevis are highly conserved after whole-genome duplication. Heredity 111. http://dx.doi.org/10.1038/hdy.2013.65.