Evolution of the fish heart by sub/neofunctionalization of an elastin gene

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The evolution of phenotypic traits is a key process in diversification of life. However, the mechanisms underlying the emergence of such evolutionary novelties are largely unknown. Here we address the origin of bulbus arteriosus (BA), an organ of evolutionary novelty seen in the teleost heart outflow tract (OFT), which sophisticates their circulatory system. The BA is a unique organ that is composed of smooth muscle while the OFTs in other vertebrates are composed of cardiac muscle. Here we reveal that the teleost-specific extracellular matrix (ECM) gene, elastin b, was generated by the teleost-specific whole-genome duplication and neofunctionalized to contribute to acquisition of the BA by regulating cell fate determination of cardiac precursor cells into smooth muscle. Furthermore, we show that the mechanotransducer yap is involved in this cell fate determination. Our findings reveal a mechanism of generating evolutionary novelty through alteration of cell fate determination by the ECM.
The de novo evolution of complex phenotypic traits is a key process in the diversification of life. However, investigation of genetic changes associated with these remains one of the greatest challenges in the field of evolutionary developmental biology. Regressive losses of complex traits have received much attention, but these kinds of studies offer limited insights into the constructive evolution of complex novel traits. Indeed, the sharpest critiques of Darwin’s theory of evolution by natural selection often centered on explaining how novel body parts arose. In this context, investigations into the de novo evolution of complex phenotypic traits are open questions for evolutionary biology. Recent studies have unravelled the genetic causes for generating evolutionary novelties and suggested that neoteny, the most rare event in comparison with subfunctionalization, and the most likely fate of duplicated genes is pseudogeneization, in which one of the duplicated copies is silenced. Thus the relationship between WGDs and phenotypic evolution is still elusive.

The bulbus arteriosus (BA) is a specialized organ in the outflow tract (OFT) of teleost heart, and is regarded as an evolutionary novelty in the teleost lineage. The BA has an important role of acting as a ‘windkessel’ organ, absorbing the energy of a bolus of blood ejected by the ventricle by elastic expansion and recoil and smoothing the pressure wave down the arterial tree. Anatomical and histological characteristics of the BA are quite different from those of other vertebrate OFTs. The BA is composed of smooth muscle and is rich in elastin, while other vertebrates’ OFTs are composed of cardiac muscle lined by a thin elastin layer. The abundance of elastic fibres in the BA is considered to have a central role for its effective physiological function.

Although the morphology of OFTs has been reported in several actinopterygian species, the phylogenetic timing of emergence of the BA is still elusive. Furthermore, genetic and developmental mechanisms underlying generation of the BA are largely unknown; how did/do teleost species, in their evolution and development, convert cell types of the BA from cardiac to smooth muscle cells? Interestingly two elastin genes, designated elastin a (elna) and elastin b (elnb), were initially identified in the zebrafish (Danio rerio) genome, compared with a single gene in other vertebrate genomes. Furthermore, it was reported in both vertebrate genomes. Furthermore, it was reported in both vertebrate genomes.

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Further study of the BA is needed to understand the evolutionary path of acquisition of the BA in vertebrate evolution. We reveal that the BA is indeed an evolutionary novelty in the teleost lineage, and that elnb, which was generated by duplication of the elastin gene in the teleost-specific 3rd round WGD (3R WGD), is involved in morphogenesis of BA. We provide evidence that elnb was subsequently neofunctionalized, acquiring a function to regulate cell fate determination of BA cells into smooth muscle cells. Furthermore, we found that the mechanotransducer gene yap is involved in the process of cell fate determination in BA. These facts suggest that alteration of the extracellular environment by a novel extracellular matrix (ECM) generated evolutionary novelty by changing cell fate determination through mechanotransduction. Taken together, our results clearly illustrate that the 3R WGD and subsequent sub/neofunctionalization of elastin genes contributed to generation of the evolutionary novel BA, and to the explosive radiation under natural selection in the teleost lineage.

**Results**

**The phylogenetic timings of BA acquisition and the 3R WGD.**

It has been reported that the OFT in some non-teleost fish consists of myocardium, and exhibits variable numbers of valves inside the lumen. This type of OFT is called a ‘conus arteriosus’ and is regarded as an ancestral characteristic of the vertebrate OFT. To determine whether the BA is indeed an evolutionary novelty exclusive to the teleost lineage, we confirmed the phylogenetic timings of emergence of the BA in representative actinopterygian fish by anatomical and histological analyses using Elastica van Gieson staining and cardiac- and smooth-muscle-specific antibodies (θ-sarcomeric actin, green; myosin light-chain kinase, magenta). We found that the OFTs of Chondrostei (Polypteriformes; Polypterus senegalus and Acipenseriformes; Acipenser gudelentaedtii) and Holostei (Semionotiformes; Lepisosteus oculatus) were composed of myocardium with a thin elastin layer inside the lumen. In contrast, OFTs of Teleostei (Osteoglossomorpha, Ostegoellus bicirrhous; Elopomorpha, Anguilla japonica; Osteiophysi, Danio rerio; Neoteleosti, Oryzias latipes) were elastin-rich and composed of smooth muscle, which are the features of the BA (Fig. 1a,b). Anatomical and histological analyses showed that the OFTs of actinopterygian fish were markedly different before and after the 3R WGD (Fig. 1). These results are consistent with previous reports and clearly show that the phylogenetic timing of BA acquisition is linked to the 3R WGD. These data suggest that BA would have been acquired as a result of 3R WGD during teleost evolution.

**Subfunctionalization of an elastin gene in teleosts.**

Next we confirmed whether two elastin paralogues were generated by the 3R WGD by molecular phylogenetic analysis. The resultant phylogenetic tree showed that elna and elnb were duplicated after the split of the spotted gar (Semionotiformes, a non-teleost fish, Fig. 2a, black diamond). This phylogenetic timing is linked to that of 3R WGD. However, elastin phylogeny is limited in its taxonomic coverage and alignment sequence length, so next we scanned flanking regions in the genome for further confirmation. This led to the identification of LIM domain kinase (limk) 1a and 1b genes as neighbours of elna and elnb, respectively, which should allow higher resolution with more unambiguously aligned sites (Fig. 2b). The phylogenetic tree of limk1a and limk1b in various vertebrates revealed that the duplication of these genes also occurred after the split of the spotted gar (Fig. 2c, black diamond). Taken together, these results indicate that genomic loci including eln and limk1 genes was duplicated in the 3R WGD.
To understand the molecular mechanism underlying BA formation, we examined the expression patterns of *elna* and *elnb* in developing teleost embryos. In zebrafish, we found that *elnb* expression was initiated at 3 days post-fertilization (dpf) in the BA in a region-specific manner, and the expression was intensified at 4 dpf (Fig. 2g–i, arrowheads). In contrast, *elna* was expressed not only in the BA but also other tissues, such as the cranial skeleton and swim bladder (Fig. 2d–f and Supplementary Fig. 2a). These results are consistent with previous reports. Next we examined the expression patterns of *elna* and *elnb* in other teleost species to investigate whether expression patterns of *elna* and *elnb* are conserved, using medaka (*Oryzias latipes*) and threespine stickleback (*Gasterosteus aculeatus*) embryos. We found that *elnb* expression patterns were restricted to the BA, while *elna* was observed in various tissues in both medaka (Fig. 2j) and stickleback (Fig. 2k), similar to zebrafish. The differential expression patterns of *elna* and *elnb* in teleosts suggest that subfunctionalization occurred in these genes, and the restricted expression of *elnb* in the BA suggests that *elnb* may be involved in organogenesis of the BA.

To clarify the relationship between the kinetics of *elnb* expression and BA morphogenesis in heart development, we examined the timing of smooth muscle cell differentiation in the OFT region. We found that smooth muscle cells appeared at 4 dpf, just after the onset of *elnb* expression (Fig. 2n,o, arrowhead). Furthermore, we studied OFT development in *Polypterus senegalus* embryos (Polypteriformes, a non-teleost fish), whose OFT is composed of cardiomycocytes even in the adult (Fig. 1). The *Polypterus* embryonic OFT was also composed of cardiomycocytes at 3 and 5 dpf, and smooth muscle cells were never observed in the OFT region (Fig. 2p,q). To examine the expression pattern of the *eln* gene in non-teleost fish possessing cardiac OFT, we isolated the *Polypterus elastin* gene and performed in situ hybridization in *Polypterus* embryos. As expected, *Polypterus* had one elastin gene, and its expression was observed in various tissues including OFT, which is similar to that of zebrafish *elna* (Supplementary Fig. 1). These results suggest that *elnb* expression is tightly related to smooth muscle differentiation in the OFT both in development and evolution.

**elnb** was neofunctionalized and involved in BA morphogenesis. To elucidate the exact function of *eln* genes for smooth muscle differentiation and BA formation, we performed knockdown experiments using antisense morpholino oligonucleotides (MOs) in zebrafish embryos. Three weeks after injection of MOs (4 ng each), sections were prepared to examine Eln synthesis and BA morphology. The *elnb* morphants exhibited severe hypoplasia of the BA and decreased elastin accumulation, while *elna* morphants exhibited no obvious defect in BA morphology (Fig. 3a). *elna* morphants exhibited a swim bladder defect, where *elna* expression level is relatively high (Supplementary Fig. 2a,b). To characterize the effect of *elna* and *elnb* knockdown on BA formation and function, we quantified the motions of each morphant BA cells by using the motion vector prediction method, which quantifies cell movement in a non-invasive manner. For motion vector prediction analysis, we used mild phenotype morphants (2 ng each) to reduce secondary effects. This small
dose of elnb MO resulted in normal morphology of the BA. As a result, the averaged contraction deformation distances were significantly reduced in elna and elnb morphant BA cells (Fig. 3b). The contraction duration of elna morphants was slightly reduced but not significant, while that of elnb morphants was greatly reduced compared with those of control embryos (Fig. 3c). These results suggest that elna and elnb are involved in the function of BA, and Elnb is likely a major contributor to BA elasticity, which contributes significantly to its function. 

The elna and elnb morphants could survive and grow to adults under mild conditions (2 ng MO injection). We performed anatomical and histological analyses to examine the developmental effects of elna and elnb knockdown. Surprisingly, we found that ectopic cardiomyocytes were induced in the BA treated with elnb MO at 6 months after injection (Fig. 3d, arrowhead). Further we confirmed that ectopic cardiomyocytes were observed in the BA in the embryonic hearts at 4 days after injection (Fig. 3e, arrowhead). Such ectopic cardiomyocyte formation in the BA was observed only in elnb morphants and not in elna morphants. Next, we confirmed the phenotypes of genetic mutants of elna and elnb, which were generated by CRISPR/Cas9 system and possessed the mutations to each coding sequences. We observed ectopic cardiomyocytes in the BA of elnb genetic mutant embryos similar to those of morpholino knockdown embryos (Supplementary Fig. 3a,b). It was reported that latent TGF-β binding protein 3 (ltbp3) marks the second heart field (SHF) in zebrafish heart development, and embryos with ltbp3 knocked down did not exhibit Elnb expression in the OFT region. We performed histological and anatomical analyses in ltbp3 knockdown embryos and found that cardiomyocytes were ectopically formed in the hypomorphic BA (Fig. 3f). However, elnb gene expression in the BA was not altered in ltbp3 morphants (Fig. 3g). We confirmed the exact expression kinetics of ltbp3 in heart development and found that ltbp3 was expressed in the BA at 3 and 4 dpf (Fig. 3h,i and Supplementary Fig. 4). It has been reported that some lbps regulate elastic fibre assembly of elastin by interacting with fibrin in mammalian skin and lung cells. These results indicate that lbtp3 does not affect proto-elnb production but is required for the maturation and function of Elnb.

To determine whether neofunctionalization occurred between elna and elnb genes, we designed a rescue experiment. We injected elasin MO and mRNA under the following conditions: (1) elnb MO and elnb full-length mRNA; (2) elnb MO and elna full-length mRNA; (3) elnb MO and Polypterus elnb full-length mRNA in zebrafish embryos. When we injected elnb MO, 67.9% of embryos formed ectopic cardiomyocytes in the BA. In contrast, with a combination of elnb MO and elnb full-length mRNA, the rate of ectopic cardiomyocyte formation was decreased in a dose-dependent manner. This suggests that elnb mRNA rescues the elnb morphant phenotype. In contrast to this, injection of elnb MO and elnb full-length mRNA did not rescue the elnb morphant phenotype (Fig. 3j). To examine whether the function of elnb is novel or not, next we cloned the Polypterus elna gene and
cardiomyocytes are observed in bars, 200 μm. (b) Averaged contraction deformation distances in control, elna and elnb morphant BA. Averaged contraction deformation distances in the elna and elnb BA are significantly reduced compared with that of the control. (c) Contraction durations in control, elna and elnb morphant BA. Contraction duration in elnb BA is greatly reduced compared with that of the control. (d) Anatomy and histology of BA in control, elna and elnb morphants at 6 months post-fertilization (mpf) using double immunohistochemistry: α-sarclemic actin (cardiac muscle, green) and myosin light-chain kinase (smooth muscle, magenta). Ectopic cardiomyocytes are observed in elnb morphant BA (arrowhead). Scale bars, 200 μm. This is also the case with embryonic hearts (e). Scale bars, 50 μm. (f) Anatomy and histology of BA in ltbp3 morphants at 4 dpf. Ectopic cardiomyocytes are observed in ltbp3 morphant BA with deformation of heart morphology. Scale bar, 50 μm. (g) elnb expression pattern in ltbp3 morphants at 4 dpf. Expression pattern of elnb is not altered in ltbp3 morphants. Arrowhead indicates elnb expression. Scale bar, 200 μm. (h) ltbp3 expression pattern at 4 dpf embryos. ltbp3 is expressed in the BA (arrowheads). Scale bars, 200 μm. (j) Functional divergence of elna and elnb in morphogenesis of the BA. Rescue experiment of elnb morphants by injection of elna, elnb and Polypterus eln full-length mRNAs. Percentages of ectopic cardiomyocytes in the BA are shown in each condition. elna and Polypterus eln mRNA did not rescue the elnb morphant phenotypes while elnb mRNA did. *P < 0.05, NS: P > 0.05. Dashed lines depict outlines of the BA. Values are reported as mean ± s.d. and P values were determined by the t-test. NS, not significant.

Conducted rescue experiment by injection of elnb MO and Polypterus eln mRNA. In the situation of subfunctionalization (the functions of elna and elnb in teleosts share that of ancestral eln) Polypterus eln mRNA would be able to rescue the elnb morphant phenotype. In contrast to this, in the situation of neofunctionalization (the function of elnb in teleost is different from that of ancestral eln; elnb acquired the new function), Polypterus eln mRNA would not be able to rescue the elnb morphant phenotype. We cloned two isoforms of Polypterus eln transcripts (isolorm1 and isorom2) and we found that both of these Polypterus eln mRNAs did not rescue the elnb morphant phenotype (Fig. 3j). These results indicate that the function of elnb is distinct from those of elna and ancestral eln; neofunctionalization likely occurred between these two paralogues.

elna regulates cardiac/smooth muscle differentiation. As described above, we observed ectopic cardiomyocytes in the BA of elnb morphants (Fig. 3d,e). To explain this phenotype, we designed two hypotheses; elnb regulates (1) migration pattern of cardiac precursor cell, or (2) fate determination of BA cells. To test this, we examined cell migration patterns of both BA and ventricle precursor cells. First, we conducted cell lineage tracing experiments by injection of the fluorescent tracer Dil. We injected Dil into the BA or cardiogenic (atrium or ventricle) progenitor fields at the 7-somite stage, as previously reported (Fig. 4a,b)45, and traced the contributions of Dil at 4 dpf (Fig. 4c,d). Contribution ratios of the BA field to the BA, the BA field to cardiogenic, the cardiogenic field to the BA and cardiogenic field to cardiogenic were not significantly changed in either elna or elnb morphants compared with the control (Fig. 4e, P > 0.05). We next measured the lengths of the BA and ventricle because it was previously reported that the sizes of the BA and ventricle change reciprocally when the proportionate contribution of these common precursor cells from SHF is altered45. We found that differences in the lengths of the BA and ventricles were not significant between control and elna and elnb morphants (Fig. 4f,g, P > 0.05). It was reported that phosphorylation levels of Smad1/5/8 in the arterial pole at 30 hours post-fertilization (hp) is important for regulation of the proportionate contribution of common precursors from the SHF to the BA and ventricle.45. We assumed that the phosphorylation level of Smad1/5/8 would be downregulated in elnb morphants if the migration of common precursors was affected. We examined the phosphorylation levels of Smad1/5/8 in elna and elnb morphants and found that the levels of pSmad1/5/8 signal were not changed from those of the control at 30 hpf (Supplementary Fig. 5a). Furthermore, we confirmed that elnb was not expressed in the BA at 30 hpf, suggesting that elnb is independent of phosphorylation levels of Smads (Supplementary Fig. 5b). Taken together, these results show that elnb regulates the cell fate determination of cardiac precursor cells in the BA, not their migration patterns. Elnb promotes differentiation of precursor cells into smooth muscle cells, and in the case where elnb expression is decreased or lost, the cardiac precursor cells differentiate into cardiomyocytes.
The next question was how ehnb regulates cell fate determination. From our experiments, we hypothesized that mechanotransduction might underlie BA cell fate determination because Elnb is a component of ECM that provides mechanical stress to cells. We focused on YAP, a mechanotransducer that senses mechanical signals and changes cell fate determination

To test this, we knocked down and genetically mutated yap and found ectopic cardiomyocytes in the BA of yap morphants and mutants similar to ehnb morphants (Fig. 4h,i and Supplementary Fig. 3c). Furthermore, we found that ehnb expression in the BA was not altered in yap morphants (Fig. 4j). These results suggest the possibility that yap is involved in cell fate determination of BA cells through mechanotransduction.

Discussion

In this study, we revealed the genetic and developmental basis underlying morphogenesis of the BA, an evolutionary novelty in the teleost lineage that sophisticates their single circulatory system and contributes to their explosive radiation in aquatic environment

We first confirmed the phylogenetic timings of emergence of the BA in actinopterygian evolution and found that only teleost
species have BA (Fig. 1). This result indicates that the BA is a synapomorphy of teleost lineage. The OFT in fish heart, which is located between the ventricle and the ventral aorta, has received different names in the history of zoology and this persists today. It has variously been called ‘conus arteriosus’, ‘bulbus arteriosus’, ‘bulbus aortae’, ‘truncus arteriosus’ and even ‘bulbus cordis’. It has been generally assumed that in most teleosts the conus arteriosus is vestigial or even absent coincident with the remarkable development of the BA in this zoological group. It has been suggested that in some non-teleost fishes the conus arteriosus is vestigial or even absent coincident with the remarkable development of the BA in this zoological group. Recent studies reported the existence of BA in the teleost, with BA elastin showing a decreased elastic properties of polymeric elastin from BA and ventral aorta differ significantly from those of non-teleost fishes (Fig. 1). Thus, it is not plausible to regard these as ‘BA’ or a homologous organ to BA. These caused further confusion in this field. Given this situation, here we would like to redefine the BA as a teleost-specific organ with a large amount of elastic fibres and composed of smooth muscle cells, which may be endowed by Elnb. Our findings strongly indicate that BA is a synapomorphy in the teleost lineage (Figs 1 and 2).

We revealed that neofunctionalization had occurred among eln genes in teleost evolution (Fig. 3). Gene duplication and subsequent subfunctionalization and neofunctionalization have been often addressed by focusing on the phenomena of teleost-specific 3R WGD. However, the consequences of the 3R WGD, especially its impact on the massive radiation of teleosts in the aquatic environment, have been matter of controversial debate. Although the WGDs are regarded as a potent force providing novel genes for evolutionary adaptation and innovation, it is less clear whether the 3R WGD was directly linked to the acquisition of evolutionary novelty in infraclasse Teleosti. Our results demonstrated that the 3R WGD and subsequent neofunctionalization of eln genes contributed to acquisition of BA, which is believed to be one of the most important evolutionary novelties for adaptive radiation in the aquatic environment. Our study sheds light again on the importance of gene duplication for evolution, as proposed by Susumu Ohno in his book Evolution by Gene Duplication. In addition to the previous study that revealed convergent evolution of electric organ in Mormyridae and Gymnotiformes driven by neofunctionalization of Scn4a gene, our study clearly illustrates that WGD and subsequent neofunctionalization contributes to generation of phenotypic evolutionary novelty under natural selection and the teleost-specific 3R WGD demarcates infraclass Teleostei.

Our results are highly suggestive that elastogenesis of Elnb is regulated by ltbp3, which is known to be a SHF marker in zebrafish. It is reported that some ltbps regulate elastic fibre assembly of elastin, and these facts raise the possibility that assembly of Elnb in the BA would be regulated by ltbp3. However, not only teleosts but also other vertebrates have ltbp3 in their genomes. In this context, it is likely that ltbp3 is expressed in the OFT in basal actinopterygians, followed by ehnb expression in the OFT region after the 3R WGD, and then elastogenesis would be promoted by ltbp3. The expression patterns of ltbp3 in non-teleost fish await further study, and these results will contribute to further understanding of the origin of the SHF in vertebrate evolution.

Our results are also suggestive of an important role of Elnb in the fate determination of BA cells. From our cell lineage tracing experiments, we provide strong evidence that Elnb regulates cell fate determination of BA cells into smooth muscle cells (Fig. 4). Organogenesis and morphogenetic movements involve dynamic remodelling of ECM scaffolds in the embryo. In addition to physical connections of cells within tissues, ECMS act as the three-dimensional elastic scaffolds that resist cell-traction forces and thereby regulate tissue development by altering physical force distributions, changing the cellular force balance and modulating cell shape. Previous studies have indicated that material properties of polymeric elastin from BA and ventral aorta differ significantly in the teleost, with BA elastin showing a decreased elastic modulus and increased viscoelasticity. The different functions between Elna and Elnb, reported here, are consistent with these facts. Further we found that the mechanotransducer yap was involved in the process of cell fate determination of BA cells. These facts suggest that the different elasticity and stiffness of Elnb in BA affects organogenesis of BA by regulating the cell fate determination coordinating with mechanotransduction. This is the first report that elastin, a type of ECM, regulates the cell fate of cardiac progenitor cells.

Taken together, our study permits us to propose the following scenario for the evolution and development of BA in the teleost; in the evolution of teleosts, the elastin gene was duplicated by the 3R WGD and one of the two paralogues, elnb, was relaxed from constraints of transcriptional regulation and changed its expression pattern to be restricted to BA (subfunctionalization). This relaxation was applied to not only transcriptional regulations but...
and staged according to Iwamatsu58. Adult stickleback (\(14/10\)-h-light/dark cycle. d-rR strains were used as wild type. Collected embryos using the primary antibodies anti-

**Methods**

**Collection of fish embryos and adult fish.** Zebrafish (\(D.\) rerio) were maintained at 28.5 °C on a 14/10-h-light/dark cycle. Embryos were obtained from natural crosses of wild-type fish of the Tubingen AB background. Embryos were cultured in plastic plates (140 mm diameter) filled with 10% seawater at 16°C. Eggs were obtained from natural crosses of wild-type of the Tubingen AB background. Collected embryos were maintained at 28.5 °C, sorted into 1/3 Ringer media (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl\(_2\), 1.7 mM HEPES at pH 7.2) and staged according to hfpg at 28.5 °C and morphological criteria56,57. Medaka (\(O.\) latipes) were maintained at 28 °C on a 14/10-h-light/dark cycle. d-R strains were used as wild type. Collected embryos were maintained at the same conditions as described above. Embryos were cultured and staged according to Iwamatsu58. Adult stickleback (\(G.\) aculeatus) were maintained in 3421 tanks with 10% seawater at 16 °C in the fish facility of the National Institute of Genetics (Mishima, Japan). Artificial insemination was conducted to obtain fertilized eggs, and the embryos were cultured in plastic plates (140 mm diameter) filled with 10% seawater at 16 °C. Staging was conducted according to a previous study59. Polypterus (\(P.\) senegalus) embryos were obtained from natural crosses of wild-type fish. Collected embryos were maintained at 28.5 °C, sorted into 1/3 Ringer media (39 mM NaCl, 0.97 mM KCl, 1.8 mM CaCl\(_2\), 1.7 mM HEPES at pH 7.2) and staged according to Takeuchi et al60. Adult fish of Polypterus (\(P.\) senegalus), sturgeon (\(A.\) gueldenstaedti), spotted gar (\(P.\) senegalus) and stickleback (\(P.\) elna) were anesthetized with 0.016% tricine (MS-222, Sigma Chemical Co.) and fixed with 4% paraformaldehyde in PBS at the desired stages. All experimental reagents and chemicals were purchased according to the animal ethics committee of the University of Tokyo.

**Frozen sections.** For cryosections, embryos and fish hearts were washed with PBS and embedded in 10% (w/v) sucrose/PBS solution at 4°C overnight. Embryos were then placed in 30% (w/v) sucrose/PBS solution overnight at 4°C. Embryos and fish hearts were placed in a 1:1 solution of 30% sucrose and optimal cutting temperature compound and frozen at –80 °C. Using a Leica CM 3050 S cryostat, we obtained 7 μm sections, which were mounted on glass slides and allowed to dry at room temperature.

**Elastica van gieson staining.** For Elastica van Gieson staining, sectioned samples were washed in PBS and stained with Weigert’s iron hematoxylin solution (Muto Pure Chemicals Co., Ltd) for 5 min. Then, the samples were washed with running water for 10 min. Finally, the samples were stained with Elastica van Gieson solution (Muto Pure Chemicals Co., Ltd) for 5 min. After staining, samples were dehydrated and mounted.

**Immunohistochemistry.** Immunohistochemistry was performed as described41 using the primary antibodies anti-\(\alpha\)-sarcomeric actinium (mouse monoclonal (1:200, Sigma, EA-53), anti-myosin light-chain (mouse monoclonal (1:1200, Sigma, K36), muscle-specific MF20 (1:100, Developmental Studies Hybridoma Bank, University of Iowa, IA), and anti-phospho-Smad1/5/8 mouse monoclonal (1:200, Cell Signaling, #9511).

**Molecular phylogenetics.** Peptide sequences with high similarities to coding sequences of zebrafish elastin and LIM domain kinase (immk) 1A/1B were retrieved from public databases using alnem2. The retrieved sequences were aligned with Xced, which was the multiple sequence alignment algorithm employed48. Using unambiguously aligned amino-acid sites, molecular phylogenetic trees were inferred preliminarily with the neighbour-joining method using Xced and finally with the maximum-likelihood method using PhyML version 3.0 (ref. 63). Among-site rate heterogeneity was taken into account by assuming a gamma distribution. The G model (\(G = 4 + 1\)). In both the neighbour-joining and maximum-likelihood methods, bootstrap resampling was performed with 100 replicates.

**Whole-mount in situ hybridization.** Whole-mount in situ hybridization of zebrasfish, medaka, stickleback and Polypterus embryos was performed as previously64, except that the signals were visualized with BM Purple (Roche). Primer sequences for cloning genes of interest were as follows: zebrasfish elna (primers: 5′-AGTCTTTCGCTGAGGGTGTC-3′, 5′-AAACAGTCCACGCACCTG-3′), zebrafish elnb (5′-CAGCGGAGCTTGAGAGATGGGG-3′, 5′-GCCAACCCTGGAGGTGTCGTC-3′), medaka elna (5′-GGCCAGCTTGAGGGTGTC-3′, 5′-CTCCCTCACTTGAGAGC-3′), medaka elnb (5′-TGTTGCTTCCTGGAGAGC-3′), and Polypterus elnb (5′-TCTCCTCGGCGTGAAACTC-3′, 5′-GGTGAATCCAGCGGAC-3′).

**Morpholino and mRNA injections.** Antisense MOs were designed to block translation and were purchased from Gene Tools, LLC (Philomath, OR, USA). The sequences of MOs used in this study were as follows: elna (5′-TGTTGATCGTA CATTGGTCTTGTC-3′, 5′-CCGGCGCATCCGTGATGAATAAC-3′) and control (5′-CCTCTATTCCATGTTAACATTTATA-3′, supplied as standard control morpholino oligo by Gene Tools, LLC). Primer sequences for cloning genes of interest were as follows: isoform1 elna (5′-ATGGCAGAAGCAGAAGGCAGGGTC-3′, 5′-ATTTCCTTTTCTTCCACAGATTTGTCGTTAGGAC-3′), isoform2 (5′-ATGGCGACAAGAAGGCAGGGTC-3′, 5′-CTAGACCGTTAGTACCCATATATAGTGC-3′).

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**Microscopy.** Whole-mounted embryos were imaged on a Leica M205 FA automated fluorescence stereomicroscope with the imaging system AF6000 (Leica Microsystems, Wetzlar, Germany). Section samples were imaged on a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

**Video microscopy.** Movie images of beating zebrafish hearts were recorded as sequential phase-contrast images with a \(2 \times 20\) objective lens and \(0.7\) conversion lens at a frame rate of \(580\) fps, a resolution of \(1,024 \times 512\) pixels, and a depth of \(8\) bits using Sony imaging system (Sony Corporation, Tokyo, Japan).

**Motion vector analysis.** Motion vectors of beating zebrafish hearts were obtained using a block-matching algorithm, as described previously39. Briefly, each frame was divided into square blocks of \(N \times N\) pixels. For a maximum motion displacement of \(w\) pixels per frame, the current block of pixels was matched to the corresponding block at the same coordinates in the previous frame within a square window of \(4w + 2w\). Here we set the parameters to \(N = 16\) and \(w = 8\) as determined empirically based on the throughput speed of calculation and accuracy of the block-matching criterion. The best match on the basis of a matching error criterion yielded the displacement of each block. The mean absolute error was used as the matching criterion. The matching function is given by

\[
M(i,j) = \frac{1}{N^2} \sum_{m,n} \sum_{i,j} |f(m,n) - f_{m,n} - i,j - m,n) + n + j| \leq w
\]

where \(f(m,n)\) represents the intensity at coordinates \((m,n)\) in the current block of \(N \times N\) pixels, and \(f_{m,n} - i,j - m,n) + n + j\) represents the intensity at new coordinates \((m + i,n + j)\) in the corresponding block in the previous frame. We performed the above calculation for every \(4 \times 4\) pixels in the frame with \(1,024 \times 512\) pixels, and we obtained \(32,768\) motion vectors (\((1,024 \times 512) / (4 \times 4)\) pixels).
The spatial average of the motion vector magnitude was defined by the following equation:

$$\overline{|V|} = \frac{1}{N_{roi}} \sum_{i=1}^{N_{roi}} |V_i|$$

(2)

where $|V|$ = absolute value of motion vector, $N_{roi}$ = number of valid motion vectors in ROI, and $x_i$ and $y_i$ represent the components of the $i$th vector. By plotting the special average $|V|$ against time, we can obtain information regarding the deformation speed of zebrafish hearts, that is, contraction and relaxation. Because we averaged the magnitude of the motion vectors as shown in the formula above, contraction and relaxation motion both gave positive values. In the present study, we simply termed ‘average speed’ for the special average $|V|$.

**Analysis of the BA motion.** We placed the ROI on the BA area of zebrafish hearts in each group, that is, control, eha n MO and eha b MO. From the result of motion vector analysis, we distinguished the BA contraction and analysed the contraction duration and the averaged contraction deformation distance of the contraction ($N = 4$).

**Dye labelling.** Dil (2.5 mg ml$^{-1}$ in dimethylsulphoxide) was injected into the anterior lateral plate modesmer of zebrafish embryos at the 7-somite stage. The embryos were anaesthetized with 0.016% tricaine and embedded in 3% methylcellulose for stability during injection. After incubation, the embryos were again anaesthetized and placed in 3% methylcellulose during imaging at 4 dpf.

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**Acknowledgements**

We would like to thank A. Shimada for providing technical tips of DiI labelling experiments; K. Takahashi and T. Kunihiro for assistance with motion analysing experiments; and S. Tomita, J. Kitano and B. Bruneau for critical reading of the manuscript. This study was supported by Grants-in-Aid for Scientific Research from the MEXT (Ministry of Education, Culture, Sports, Science and Technology of Japan; K.K.-T.), by the Japan Society for the Promotion of Science (Y.M., JSPS research fellow) and by JSPS through the ‘Funding Program for Next Generation World-Leading Researchers (Next Program)’, initiated by the Council for Science and Technology Policy (CSTP) (K.K.-T.).

**Author contributions**

Y.M. designed the study, performed the majority of the experiments and analysed the data; F.I. collected the stickleback embryos; H.T. provided the medaka embryos; T.Y. and M.O. collected the *Polypterus* embryos; S.K. performed the phylogenetic analysis; F.W.K. searched for elna and elnb homologues in the medaka and stickleback genomes and the eln homologue in the *Polypterus* genome and identified candidate sequences of these genes; Y.M. wrote the paper; K.K.-T. supervised the study and helped to write the manuscript with input from all authors.

**Additional information**

**Accession codes:** All nucleotide sequences reported in this study have been deposited in GenBank/EMBL/DDBJ under the accession codes LC099968 (*Oryzias latipes elna*), LC099969 (*Oryzias latipes elnb*), LC099970 (*Gasterosteus aculeatus elna*), LC099971 (*Gasterosteus aculeatus elnb*), LC099966 (*Polypterus senegalus eln isoform1*) and LC099967 (*Polypterus senegalus eln isoform2*).

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Moriyama, Y. et al. Evolution of the fish heart by sub/neo-functionalization of an elastin gene. *Nat. Commun.* **7**:10397 doi: 10.1038/ncomms10397 (2016).

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