学位論文

Analysis on developmental mechanisms for morphogenesis of the vertebrate dorsal trunk using the medaka mutant *Double anal fin*

（メダカ突然変異体*Double anal fin* を用いた

体幹背側形態の発生機構に関する研究）

平成 25 年 12 月博士（理学）申請

東京大学大学院理学系研究科

生物科学専攻

河西　通

Contents

Abbreviations

✧ AP	anteroposterior
✧ BAC	bacterial artificial chromosome
✧ BMP	Bone morphogenetic protein
✧ *Da*	*Double anal fin*
✧ DV	dorsoventral
✧ dpf	days post fertilization
✧ qPCR	quantitative polymerase chain reaction
✧ Tg	transgenic
✧ WISH	whole-mount in situ hybridization
✧ wpf	weeks post fertilization
✧ *wt*	*wild type*
✧ *zic*	*zinc finger protein of the cerebellum*
✧ *zic1/zic4*	*zic1* and *zic4*

Abstract

Teleost exhibits remarkable diversity in morphology such as fins and coloration particularly on the dorsal side. However, due to the late phenotypic appearance (from larva to adult) and lack of appropriate mutants, the genetic mechanisms that regulate these dorsoventrally asymmetric external patterns are largely unknown. To address this question, I analyzed the spontaneous medaka mutant, *Double anal fin* (*Da*), which exhibits a mirror-image duplication of the ventral half across the lateral midline from larva to adult. *Da* is an enhancer mutant for *zic1* and *zic4* in which their expression in dorsal somites is lost. I show that the dorsoventral polarity in *Da* somites is lost and then demonstrate by transplantation experiments that somites and their derived tissues globally determine the multiple dorsal-specific characteristics of the body (fin morphology and pigmentation). Intriguingly, the *zic1*/*zic4* expression in wild type persists throughout life in the dorsal parts of somite derivatives *i.e.* the myotome, dermis and vertebrae, forming a broad dorsal domain in the trunk. Comparative analysis in fish further implies the conserved role of *zic1/zic4* in morphological diversification of the teleost body. Finally, I find that the dorsal fin morphology can be quantitatively modified depending on the dosage of *zic1/zic4* expression. Taken together, I conclude that the teleost trunk consists of dorsal/ventral developmental modules delimited by *zic1/zic4* from late development, and propose that different expression levels of *zic1*/*zic4* in dorsal somites contribute to regulation of adaptive multiple dorsal morphologies in vertebrates.

General Introduction

Many vertebrates display conspicuous ornaments, elaborate morphologies and brilliant colors on their body. As such, the vertebrate appearance is so diverse that we are still seeking for any explanation for to what extent the fundamental properties of the body structures are shared and to what extent they are different among vertebrates. If we look at the vertebrate body from a perspective of axes, things seem to get easier to comprehend; all vertebrate species have a DV axis along its body, and every organ is similarly aligned along the DV axis. Since the ventral surface, the belly, faces the ground in most vertebrates, many surface structures or coloration on the body tend to be concentrated on the dorsal side in order to exert their functions. Indeed, many of reptiles and fish have specialized crests or fins on the midline of the back, which serve as radiators, communication tools and/or locomotives. Many vertebrates have unique pigmentation patterns usually on their back to assimilate themselves into their surrounding environment. Thus, in principle, there is an obvious DV pattern on the surface of the vertebrate trunk. Then the questions arise as to how the DV pattern is built and what properties make the DV pattern so diverse among species. Solving these problems based on developmental biology is an essential task, which will definitely provide us fundamental and critical information about the diversity of the trunk morphology.

Exploration of the developmental mechanisms for the DV pattern of the vertebrate trunk dates back to the early works of the “organizer.” Some ninety years ago, Spemann and Mangold demonstrated that a restricted group of cells of the embryo, the dorsal blastopore lip of the amphibian gastrula, is endowed with extraordinary inducing

activities (Spemann and Mangold, 1924). When grafted to an ectopic location of a host embryo, this tissue recruits neighboring cells to form a secondary body axis. After their findings, researchers came up to the concept of body axes in developmental contexts and pursued the developmental mechanisms that form the body axes. Tremendous efforts via large scale mutagenesis, biochemical experiments and other experimental manipulation of embryos have unveiled fundamental properties operating in early development. In zebrafish and *Xenopus* embryos, the initial trigger for DV axis formation is cortical rotation that occurs just after fertilization, and this transports mRNAs and proteins toward the future dorsal direction (Miller et al., 1999; Tao et al., 2005; Tran et al., 2012). By contrast, in mice, the DV axis is believed to be provided through asymmetric cell-cell interactions of blastomere cells depending on their positions (Takaoka et al., 2007). As such, the very first step of DV axis formation seems different among species. However, the subsequent determination of cell fates along the DV axis which takes place around the gastrulation stage was found to be conserved; BMP and Wnt proteins are distributed in almost the entire embryo with the highest concentration on the ventral side, and their antagonists such as Chordin and Noggin (for BMPs) and secreted Frizzled-related proteins (for Wnts) are secreted from the dorsal side (Fig. 1, left) (Piccolo et al., 1996; Gonzalez et al., 2000; Oelgeschlager et al., 2000; Schier, 2001; De Robertis, 2006). Those antagonists that interact with their target proteins attenuate the downstream signaling, and thereby a functional gradient of BMP and Wnt activity is set up across the DV axis with their highest in the ventral and little or no activity in the presumptive dorsal mesoderm. This morphogen-mediated gradient has been thought to be the most crucial step for the global DV pattern in the embryo.

DV characters of the adult body gradually become evident from larval to adult

stages. To my knowledge, however, there have been no attempts to fill a gap between the above-mentioned DV axis determination and the final DV morphology (or appearance) of the adult trunk in vertebrates. For instance, zebrafish *bmp* mutants, in which the antagonistic balance of morphogen is disrupted, show a typical "dorsalized" phenotype, leading to the loss or reduction of ventrally located structures (Schier and Talbot, 2005). This mutant has been useful in analyzing the initial DV axis formation, but it does not give us a clue to understand the late mechanism of how the subsequent DV patterning goes on, mainly due to embryonic lethality. This is also the case for other previous studies on the earlier DV axis formation, such as UV irradiation experiments onto *Xenopus* embryos soon after fertilization, in which the disruption of microtubules results in a "ventralized" phenotype (Mise and Wakahara, 1994). Thus, our knowledge on DV patterning is still limited, such as the proper organization of the three germ layers along the DV axis during gastrulation, and we have little information on how the *final* DV pattern (which is visible on the vertebrate trunk) is created afterward. Is there a particular mechanism to retain the information of the initial gradient during late DV patterning? In other words, do all tissues of the embryo just inherit the initial DV axis as the gradient information and continuously use this information until the adult stage? (Fig. 1)

In addition to the gradient mechanism in the early embryo, there is another basic principle working in organogenesis and pattern formation. When we understand the structure of an organism, the concept of modularity often helps. Modules of development are, by definition, quasi-independent developmental units, and can be recognized at various levels ranging from protein complexes and subcellular organelles to large domains in the body (Schlosser and Wagner, 2004). The primary anatomical mod-

ules of developing embryos include cell populations, organs and segments, and they behave to a certain degree independently of each other during development but will be harmoniously integrated within an organism. The modular feature of development is thought to contribute to developmental robustness and evolutionary flexibility by allowing mosaic changes in body shape and differentiation of body structures without seriously compromising the integration of the whole organism (Kirschner and Gerhart, 1998; Bolker, 2000; Kuratani, 2009). This is best manifested in segments of insect bodies (Fig. 2A); during development each segment develops in an independent manner which is dictated by a special class of transcription factors, known as selector genes (Lewis, 1978; Blair, 1995; Kim et al., 1996), and the independency of development has allowed diverse structures in each segment by reduction, loss or modification of body parts (e.g. appendages) through changes in the activity of selector genes and/or their downstream targets during evolution and speciation (Fig. 2B) (Prud'homme et al., 2011). Indeed altering the expression profile of these genes results in a wholesale redeployment of the segments, i.e. homeotic transformation, demonstrating the existence of developmental modules that constitute the animal body (Gellon and McGinnis, 1998; von Dassow and Munro, 1999). Apart from the AP axis of the animal body, however, it has been a mystery whether there is a modular process in DV pattern formation.

Here, in my doctoral thesis, I utilize a unique medaka mutant, *Da*, and discover a modular mechanism that specifies the DV pattern in late development. In Chapter 1, I demonstrate that the medaka trunk consists of two distinct developmental modules, dorsal and ventral ones, and that they are defined by persistent *zic1/zic4* expression in somites and their derivatives. In Chapter 2, I explore how the *zic*-mediated modular mechanism is related to morphological diversity in teleosts, and discuss how the variety

of the trunk morphology is created.

Chapter 1

Mechanism of dorsal pattern formation of the medaka trunk via *zic1* and *zic4*

Introduction

Vertebrates display diverse morphology and coloration especially on the dorsal side. For instance, many of reptiles and fish have crests or fins on the midline of the trunk, which serve as radiators, communication tools and/or locomotives. Moreover, many vertebrates have unique pigmentation patterns usually on their back to assimilate themselves into their surrounding environment. Developmental biologists have long sought for the mechanisms that produce such dorsoventrally non-uniform patterns, and have revealed that the molecular gradients like BMPs in early development provide the initial cue for DV pattern formation (Schier and Talbot, 2005). However, since the above DV patterns become evident in much later development and related developmental mutants are few, it is still largely unknown what genetic mechanism underlies DV surface patterning observed in late development.

To address this question, I have focused on fish, especially medaka. Medaka (*Oryzias latipes*; order Beloniformes) is a freshwater teleost fish native to mainly Japan, Taiwan, Korea and China, and has long been used as a good model for biological studies. While it is as small as approximately three centimeter long in adult, medaka shows a clear DV pattern on the trunk (Fig. 3A). It has a dorsally flattened body shape, whereas the ventral side is roundish. Moreover, the dorsal fin is tiny and positioned in the posterior region on the body, while the anal fin is wider and more anteriorly located. Also, as for the pigmentation pattern, the dorsal surface seems dark due to melanophores, but on the ventral side silver pigment cells (iridophores) are distributed so that the belly is bright. Finally, although it is not easy to see them, the sensory organs of the

lateral line (which detects the water flow) are distributed on the ventral surface but not on the back. These dorsoventrally asymmetric phenotypes are regarded to be the result of adaptation to its natural habitat as a surface dweller.

While the natural medaka displays such DV patterns, it has been reported that there is a medaka spontaneous mutant called *Da*, which exhibits a unique "ventralized" phenotype on its surface from the larval to adult stages (Fig. 3B; Fig. 4) (Tomita, 1975; Ishikawa, 1990; Tamiya et al., 1997; Ohtsuka et al., 2004). The dorsal fin of homozygous mutant adults resembles the anal fin (white arrowhead in Fig. 3B), and distribution of pigments and lateral lines in the dorsal trunk is ventralized (arrow in Fig. 3B; Fig. 4G-L). Furthermore, they exhibit a symmetric tear-drop body shape, instead of a dorsally flattened one (Fig. 3B). Hence the dorsal half of the trunk appears to be a mirror image of the ventral half across the lateral midline. Importantly, essentially no defects are observed from cleavage to early segmentation stages, and the positioning of internal organs is normal, resulting in normal viability of homozygous *Da* mutants even in adult. These facts suggest the presence of an as-yet unaddressed late patterning mechanism acting after well-studied early DV specification (Agius et al., 2000; Schier and Talbot, 2005). *Da* mutants thus provide a unique opportunity for determining novel mechanisms that control global patterning of the vertebrate trunk.

The *Da* mutant was originally discovered in a wild population of a rice field in the 1960s by Hideo Tomita (Tomita, 1969) and has been maintained at the Laboratory of Freshwater Fish Stocks of Bioscience Center at Nagoya University as a member of "Tomita Collection" (Takeda and Shimada, 2010). Just recently has it been demonstrated that *Da* is a mutant for *zic1* and *zic4* genes (Moriyama et al., 2012). These genes are arranged head-to-head in the medaka genome and expressed in a nearly identical pattern

while the *zic1* expression is stronger, implying some common regulation of the two genes. Indeed, in the *Da* mutant a transposon insertion disrupts a transcriptional regulatory region(s) shared by the two genes (Fig. 3I) (Moriyama et al., 2012). Moreover, it was briefly reported that the *zic1/zic4* expression in dorsal somites is decreased in the mutant while the expression in neural tissues is less affected (Ohtsuka et al., 2004). These facts led me to hypothesize that Zic1/Zic4 in somites participate in dorsal patterning of ectodermal derivatives (surface organs such as fins and pigment cells) through tissue interactions during late stages of development. Vertebrates usually have five genes of the *zic* gene family, *zic1-zic5*, encoding C2H2 zinc finger transcription factors (Grinberg and Millen, 2005). Members of the *zic* gene family are known to play critical roles in a variety of developmental processes (Aruga, 2004). In particular, *zic1*/*zic4* have been well investigated in the context of neural development (Aruga et al., 2002; Grinberg et al., 2004; Elsen et al., 2008). However, despite previous descriptions of skeletal and muscular defects in the mouse *Zic1* mutants (Aruga et al., 1999; Pan et al., 2011), the role of *zic1*/*zic4* in somite-derived tissues had remained largely unknown.

In this chapter, I show by transgenic and tissue-transplantation techniques that the teleost trunk consists of the two distinct developmental modules, dorsal and ventral ones, defined by persistent *zic1/zic4* expression in somites and their derivatives and that *zic1/zic4* function as selector genes in the dorsal module. I propose that *zic1/zic4* in somites regulate late-emerging characteristics in the dorsal surface, through long-term mesodermal-ectodermal interactions.

Results

_Da_ mutation in medaka causes ventralized phenotypes in the dorsal part of somites

First, I examined the effect of the *Da* mutation on somite development, since the mutation is suggested to impair the mesodermal enhancer of *zic1*/*zic4* (Moriyama et al., 2012). The expression of *zic1*/*zic4* commences in the neural plate in *Da* mutants as well as *wt* embryos [as previously described (Ohtsuka et al., 2004; Elsen et al., 2008)] during the gastrulation stage (around st. 15; data not shown). After the onset of somitogenesis, the expression in *wt* embryos is detected in the dorsal neural tube (asterisks in Fig. 3C, G) and the dorsal part of somites (arrowheads in Fig. 3C, G; Fig. 3E). However, in *Da* the expression of *zic1/zic4* is greatly reduced in dorsal somites except for the anterior-most 2-3 somites (Fig. 3D, F, H), together with a slight decrease in the hindbrain expression (asterisks in Fig. 3D, H). I thus asked if the DV pattern of *Da* mutant somites is affected. Previous reports have shown that the myotome and axial skeleton are morphologically altered in *Da* mutants in addition to various external phenotypes (Ishikawa, 1990; Tamiya et al., 1997; Ohtsuka et al., 2004). As expected, I found that somites in *Da* mutants are ventralized as indicated by the dorsal expansion of *sim1*, a ventral dermomyotome marker (Pourquie et al., 1996) (Fig. 5A, B). Furthermore, the expression of *wnt11r*, which is expressed in the dorsal part of the *wt* somites (Olivera-Martinez et al., 2002; Garriock et al., 2005; Garriock and Krieg, 2007), was reduced in *Da* mutant somites (Fig. 5C, D). These data indicate that the dorsal characteristics of *Da* mutant somites are reduced and transformed into the ventral fate. Consistent with this, tissues derived from dorsal somites in *Da* mutants seemed to have adopted the ventral fate, *i.e.* the neural arch shortens in a similar manner to the hemal arch on the ventral side of the

vertebra (Fig. 5G, H), and the dorsal myotome shape in the *Da* mutants resembles that of the ventral myotome, resulting in abnormal outgrowth without filling the gap over the neural tube (Tamiya et al., 1997) (Fig. 5E, F). This change in myotome shape could account for the tear-drop shape of the *Da* mutant body.

Wild-type somites rescue the ventralized phenotypes of *Da* mutants

The above results suggest that the trunk surface patterns are regulated by the underlying somites via the activity of the *zic1/zic4* genes. To test this idea, I established a tissue transplantation assay in medaka embryos by improving microsurgical techniques (Haines et al., 2004) (Fig. 6A). I used transgenic medaka embryos ubiquitously expressing β-*actin* promoter-driven DsRed (Tg(β-*actin*:DsRed)) as donors, so that the transplanted tissues were readily traced. In the first series of experiments, I homotopically replaced two consecutive posterior-most *Da* mutant somites with *wt* somites at st. 24 (2 dpf; 14-16 somites), and examined the effects on the external phenotypes at st. 39 (7 dpf; larval stage), when the earliest two phenotypes can be clearly observed in the *Da* mutant (Tamiya et al., 1997). At st. 39, the trunk of *wt* embryos has a single row of melanophores on the dorsal midline, whereas *Da* mutant embryos have two rows on each side of the midline (Fig. 6B, C). These two lateral alignments of melanophores are identical to those on the ventral side. Likewise, the shape of the dorsal fin fold is transformed into a ventral type in *Da* mutant larvae (Fig. 6G, H); the anterior limit of the *wt* dorsal fin fold is positioned seven somites posterior to that of the ventral fin fold, whereas in *Da* mutant embryos, it is shifted anteriorly toward the position of the ventral fin fold.

In *Da* mutants transplanted with DsRed-labeled *wt* somites, the position of the

melanophores was shifted toward the midline on the operated side (Fig. 6D; n = 29/30). This effect was restricted to the transplanted tissues expressing DsRed. Thus the positioning of the melanophores was locally rescued by *wt* somites. Similarly, a rescue of the dorsal fin fold shape was observed in the area of the transplanted *wt* somites; the protrusion of the dorsal fin fold was suppressed, resulting in a posterior shift of the dorsal fin fold (Fig. 6I; n = 16/21). These rescued phenotypes were never observed when the control *Da* mutant somites were transplanted into *Da* mutant hosts (Fig. 6E, J), excluding the possibility that the phenotypic change resulted from the transplantation procedure itself.

During the transplantation experiments, the transplanted somites might be contaminated with neural crest cells. Neural crest cells, a potent group of ectodermal cells, migrate out of the dorsal-most neural tube as segmentation proceeds, and give rise to diverse cell lineages including pigment cells and the median fin fold mesenchyme in the trunk (Le Douarin and Kalcheim, 1999). However, several lines of evidence argued against their contribution to the phenotype rescue (Fig. 6F, K; Fig. 7A-F). One is that homotopically transplanted *wt* neural tubes containing neural crest cells failed to rescue the melanophore pattern or fin fold morphology in *Da* mutant hosts (Fig. 6F, K; n = 11/11 for F, n = 8/8 for K), while donor-derived pigment cells or dorsal root ganglia, which are derived from the neural crest, were normally seen in the hosts (arrows and arrowheads respectively in Fig. 6K). Moreover, neural crest cells were not distributed around the transplanted somites (Fig. 7A, B) and unlikely to accompany the somites during transplantation (Fig. 7C-F).

I then extended our analysis of the rescued phenotypes to 4 wpf because some of the *Da* external phenotypes appear late. The distribution pattern of the iridophores,

which emerges at around 2-3 wpf at the level of the 3rd to 12th somite, was also rescued when I performed somite transplantation at st. 23 (10-12 somites); the ectopic dorsal iridophores on the *Da* mutant trunk was suppressed at the site of transplantation at 4 wpf (Fig. 8G-H). Moreover, the medial positioning of the melanophores remained unchanged (Fig. 8A-C, I-J) and the anterior limit of the dorsal fin maintained its posteriorly-shifted position even when the dorsal fin fold was replaced with an adult-type dorsal fin, containing fin rays, during metamorphosis (3 wpf; Fig. 8D-F, K-L). Thus the late-emerging external phenotypes are also rescued by somite transplantation. Furthermore, our lineage analysis using the transgenic fish (Tg(*zic1*:GFP/*zic4*:DsRed)) (described below) revealed that the *zic1*-expressing somite-derived cells broadly underline the dorsal external organs (Fig. 9); the GFP-positive mesenchymal cells were found to gradually invade into the dorsal fin fold at 7 dpf (Fig. 9A, B) and to become elongated along the proximodistal axis in the developing dorsal fin at the larval stage (Fig. 9C, C', arrowheads). They also distributed just beneath the dorsal melanophores at 7 dpf (Fig. 9D, D', arrowheads). These imply that the somite derivatives continue to function in external patterning throughout late development and growth.

Taken together, I concluded that the somite-derived cells function in patterning of pigment distribution and fin morphology on the dorsal side and that the lack of *zic1/zic4* activity in somites accounts for the *Da* phenotypes.

zic1/_zic4_ expression in somites delineates the dorsal domain of the trunk

Given the proposed long-term effects of *wt* somites upon the external phenotypes in the *Da* hosts, *zic1/zic4* could act throughout early to late DV patterning. I thus traced *zic1*/*zic4* expression in *wt* somites from embryo to adult. During the somitogenesis stage,

the somite differentiates into the sclerotome, dermomyotome and myotome, as indicated by *twist*, *pax3* and *myod* expression, respectively. All of these somite derivatives were found to express *zic1*/*zic4* in their dorsal portion (Fig. 10), although, as development proceeds, the expression becomes weaker in the myotome compared to other derivatives. To further track the *zic1*/*zic4*-expressing cells for a longer period of development, section in situ hybridization was tried but found to be too less sensitive to detect *zic* expression in late stages, and I have generated transgenic medaka lines (Tg(*zic1*:GFP/*zic4*:DsRed)) by introducing a BAC construct encoding *zic1*- and *zic4*-responsive reporter genes into *wt* medaka (Fig. 11A). All of the established lines (n=9) exhibited the expression of GFP and DsRed recapitulating the endogenous expression of *zic1* and *zic4*, in both neural tubes and dorsal somites, at embryonic and larval stages, indicating that the BAC construct contains *cis*-elements sufficient to drive the endogenous expression. This was further confirmed at the adult stage by quantitative PCR (see below). The fluorescence intensity varied among the individual lines, probably due to the position effect. I thus focused on one of the lines in the following analyses because of its high level of GFP expression.

Live imaging analysis of Tg(*zic1*:GFP/*zic4*:DsRed) first revealed that at larval stages, all somite derivatives maintain the dorsal *zic1*/*zic4* expression and share the ventral expression boundary even after their lineage separation (Fig. 11B-C'). Surprisingly, the domain-like expression in the somite derivatives persisted even at the adult stage, and the clear boundary between *zic*-expressing and non-expressing cells was maintained along the AP axis (Fig. 11D, D'). Transverse sections revealed that the dorsal expression domain internally expands in the entire dermis, myotome and vertebra (Fig. 11E-F''). Interestingly, the expression boundary in the myotome morphologically corresponds to

the horizontal myoseptum (arrowheads in Fig. 11E-E''), which separates the myotome into the prospective epaxial and hypaxial muscles. However, while no such histological landmark in the dermis and vertebra is observed or has been reported, the expression boundary lies at nearly the same DV level among the somite-derived tissues. Quantitative PCR analyses confirmed that the GFP and DsRed expression pattern reflects that of the endogenous *zic1*/*zic4* expression at the adult stage (Fig. 12). From these, I concluded that *zic1*/*zic4* expression delineates the dorsal domain in the trunk, which is maintained until the adulthood.

Two distinct regulations of *zic1/zic4* transcription

Next I examined how the dorsal expression domain of *zic1*/*zic4* is established and maintained. In *wt* embryos, *zic1*/*zic4* expression is initiated in newly formed somites and is maintained thereafter. In the aforementioned transplantation experiments, the orientation of the donor somites in the *Da* mutant hosts was unable to be controlled and thus was random with respect to their original DV and AP axes. In spite of this, the somites rescued the *Da* mutant phenotypes at later development stages in most cases, suggesting that *zic1/zic4* expression in donor somites, which have begun at the time of transplantation, are re-specified by the surrounding tissues after transplantation. I confirmed this by examining reporter gene expression in somites transplanted from Tg(*zic1*:GFP/*zic4*:DsRed) to *wt* hosts (Fig. 13A). Five days after transplantation they all acquired the dorsal expression of GFP (Fig. 13B; n = 20/20). The dorsal expression of GFP in transplants was maintained until adulthood (Fig. 13C). Hence, the expression domain of *zic1* in somites at its initial stage is under the influence of the surrounding tissues. This result is consistent with those of chick grafting experiments in which Wnts

and BMPs from the neural tube, lateral plate and surface ectoderm pattern the somite along the DV axis (Aoyama and Asamoto, 1987; Aoyama and Asamoto, 1988; Pourquie et al., 1993; Hirsinger et al., 1997; Marcelle et al., 1997; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998; Vasiliauskas et al., 1999).

As embryos grow rapidly, the signaling environment could change around the somite, and so could be the case for gene regulation. I speculated that *zic1*/*zic4* expression becomes less dependent on external signals as development proceeds. I tested this idea by in vitro culture of somite-derived cells at several time points of development, and examined if they were able to maintain *zic1/zic4* expression (Fig. 14A). For this analysis, I used a double transgenic line carrying both *zic1*:GFP/*zic4*:DsRed and β-*actin*:DsRed, to monitor the level of the *zic1* expression in green and the basal transcription activity in red. I assumed that the DsRed expression driven by the *zic4* promoter can be ignored due to the relatively weak level of transcription compared to the β-*actin* promoter. I found that somitic cells at the segmentation stage (2 dpf) lost GFP expression within one day after the onset of culture (Fig. 14B-C'), confirming that *zic1* expression depends on external signals from the surrounding tissues. By contrast, GFP expression tended to be maintained for longer periods in cells taken from embryos with completion of somitogenesis (st. 30; 5 dpf) (Fig. 14D-E'); the expression lasted for at least 11 days in vitro (Fig. 14F, F'). This indicates that the *zic1* expression is cell-autonomously maintained at later stages and does not require special external signaling cues for its maintenance.

Taken together, I conclude that the dorsal expression of *zic1* in somites is initially established by the signals derived from their surrounding tissues but is later maintained in a cell-autonomous manner. This mechanism could facilitate the life-long do-

main of the *zic1/zic4* expression with robustness.

Discussion

A novel late patterning mechanism centered by Zic in somites

In this study, I took advantage of the medaka *Da* mutant, an enhancer mutant for *zic1/zic4*, and have provided experimental evidence that the dorsal characteristics of the fish trunk, such as fin, body shape and pigmentation pattern, are orchestrated by Zic1/Zic4 in the somite. The body shape appears to be a manifestation of myotome outgrowth, and the other surface organs could be specified through local mesodermal-ectodermal interaction during late organogenesis. As a support of the idea of local interaction, I observed that mesenchymal cells derived from transplanted somites underlined the host epidermis and invaded into median fins. Pigment cells, localized in the interface between dermis and epidermis, is known to be influenced by the dermis in their distribution (Tosney, 2004). For fins, the present study demonstrates that the underlying mesoderm regulates the position of their outgrowth, and thereafter fin development proceeds by co-operation of the epidermis, dermis and neural crest cells. Indeed, our recent lineage analysis combined with tissue transplantation revealed that fin rays and most mesenchymal cells are derived from the somite while neural crest cells mainly contribute to the nervous system in median fins(Shimada et al., 2013).

Moreover, *zic1/zic4* expression is likely to be required for late surface morphogenesis because some of the *Da* phenotypes (such as iridophore pigmentation) only appear during puberty to sexual maturation. The continuous action of Zic1/Zic4 was also supported by my transplantation experiment that implanted *wt* somites and their derivatives exerted their rescue effects in *Da* mutants until the adult stage. Therefore,

the surface pattern of the vertebrate trunk could be established through long-term actions of the somite-derived tissues patterned by Zic1/Zic4.

Since ectodermal organs develop at specific times and in distinct regions of the trunk, the mechanism of mesodermal-ectodermal interaction could differ depending on an organ. Melanophores are known to be attracted by the chemokine Sdf1 (Svetic et al., 2007), which might be secreted from the dorsalmost and ventralmost somites to establish the dorsal and ventral melanophore alignments. The size of the dorsal fin fold in zebrafish can be modified by treating FGF (Abe et al., 2007). The FGF pathway could be involved in defining the morphology of the medaka ectodermal fin fold via interaction with somitic cells.

Dorsal fin positioning via *zic1/zic4*

Despite its variation among fish, how the dorsal fin is positioned along the AP axis in the fish trunk has been one of the mysteries in embryogenesis for a long time. To my knowledge, this study is the first identification of factors that are involved in the dorsal fin positioning. Based on my transplantation experiments between the *Da* mutant and *wt*, *zic1/zic4* expression in somites was found to regulate the position and morphology of the dorsal fin.

Among developmental and evolutionary biologists, it has been generally believed that the position of median fins including the dorsal fin is determined by the *hox* genes (Mabee et al., 2002; Freitas et al., 2006; Cole and Currie, 2007). The most plausible reason for this assumption is that the median fins including the dorsal fin are thought to be evolutionarily homologous to the paired fins, i.e. the pectoral and pelvic fins (Romer and Parsons, 1986). There is growing evidence that the paired fins and their

evolutionary derivatives, the tetrapod limbs, are positioned through the activity of *Hox* (Kuratani, 2009), and thus the more primitive, median fins seem likely to rely on the *hox* genes too. Moreover, it was reported that for shark, the position of the median fin fold is delineated by *Hoxd* and *Tbx18* genes, which specify the paired limb positions (Freitas et al., 2006). Additionally, the anal fin always begins from the position of the cloaca (trunk-tail transition) in all teleost fish (Janvier, 1996), which is reminiscent of the fact that the position of forelimbs always coincides with the position of cervical-thoracic transition which is determined by *Hoxc6* expression (Burke et al., 1995).

However, there was no difference in expression patterns of *hoxa10a, hoxa10b, hoxa11b, hoxd11a* [which are expressed in somites around the dorsal fin (Takamatsu et al., 2007)] between *wt* and *Da* embryos (Y. Koyama and T. Kawanishi, unpublished data). Thus, while there still remains a possibility that other unexamined *hox* genes are involved in the process, *zic1/zic4* could act downstream to or in parallel with *hox* genes to modulate the dorsal fin position.

Regulatory mechanism of *zic1/zic4* expression pattern in somites

I have shown that the regulatory mechanism of *zic1/zic4* expression changes from early to late development. Like dorsomedial-ventrolateral patterning of amniote somites, teleost somites are first dorsoventrally patterned at segmentation stages by the signals derived from surrounding tissues. This pattern thus reflects the initial DV pattern determined by the gradient of the Wnt and BMP activities (Pourquie et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Yusuf and Brand-Saberi, 2006). It has been reported that in the neural plate, the expression of *zic* genes are promoted by Wnt signaling (Gamse and Sive, 2001; Nyholm et al., 2007) and inhibited by BMPs (Grinblat et al., 1998;

Tropepe et al., 2006); *zic* expression in the neural tube is also regulated by these factors (Aruga et al., 2002). Thus it is possible that Wnts and BMPs regulate *zic1/zic4* expression in somites as well. Indeed, *wnt* and *bmp* genes are expressed in the dorsally located neural tube and ventrally located lateral plate mesoderm, respectively, in fish (Dick et al., 1999; Elsen et al., 2008). Thus far, I have tried chemical treatment assays in medaka embryos using KN-93 (inhibitor of non-canonical Wnt pathway; blocks CaMKII activity) (Wu and Cline, 1998) and dorsomorphin (BMP inhibitor; blocks the activity of Smad proteins) (Yu et al., 2008) in order to identify the upstream factors, but failed (data not shown). Therefore, according to my primary culture experiment, there must be some inductive signal which positively regulates *zic1/zic4* expression in somites, and this signal should mediate other pathways than these.

However, once established, the dorsal expression of *zic1/zic4* becomes no longer dependent on the external signals by the hatching stage, and is maintained for life. Given their different developmental history long after lineage separation (e.g. cell growth and turnover), special mechanisms must be required to ensure the spatially robust expression borders for over a long period of life. Epigenetic regulation of key developmental genes could be one of the mechanisms to assure such robustness. Indeed, the autonomous maintenance of *zic1/zic4* expression at later stages in my primary culture assay supports this idea. Furthermore, it was recently found that the promoter regions of both *zic1/zic4* in the genome of the dorsal somitic cells are gradually tagged with active histone marks (reduced H3K27me3 and high H3K4me2) from hatchling to adult (R. Nakamura, unpublished data).

Modular organization of the vertebrate trunk

One of the most important findings in the present study is the dorsal domain defined by the persistent *zic1/zic4* expression. Consistent with this, previous studies reported that the myotome and dermomyotome are subdivided along the dorsomedial-ventrolateral axis in amniotes (Selleck and Stern, 1991; Ordahl and Le Douarin, 1992), and that this subdivision is a result of lineage separation of somitic cells (Selleck and Stern, 1991; Ordahl and Le Douarin, 1992) and is regulated by signals emanated from surrounding tissues (Pourquie et al., 1993; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998; Vasiliauskas et al., 1999; Cheng et al., 2004). Thus the present study is the first demonstration that not only the myotome and dermomyotome but all the somite derivatives are subdivided into the dorsal and ventral parts and that they form the persistent dorsal and ventral domains. *zic1*/*zic4* are the molecular entity of the dorsal domain.

Recently, Rinn et al. reported that the embryonic *HOX* pattern is epigenetically maintained in human adult fibroblasts in foot and is required to maintain its site-specific identity (Rinn et al., 2008). Likewise, the *zic1/zic4* expression is maintained from embryo to adult. Prolonged expression of developmentally crucial transcription factors could therefore be a general feature in animal development. In this study, the somite-derived organs are found to be persistently dorsoventrally divided by almost linear borders across organ boundaries along the entire AP axis. This persistent regionalization is reminiscent of *Drosophila* body compartmentalized by *engrailed* expression in the posterior segments (Garcia-Bellido and Santamaria, 1972; DiNardo et al., 1985). In *Drosophila*, *engrailed* is considered to serve as positional information to maintain the posterior identity regardless of morphological changes during development. Among vertebrates, the present study is the first attempt to visualize robust regionalization of

the adult body by live imaging of transgenic fish.

The dorsal domain defined by the *zic1/zic4* expression represents a developmental module because the loss of *zic1/zic4* activity does not affect the ventral part of the trunk. Since *zic1/zic4* globally determine the fates of various organs on the dorsal side of the trunk, they serve as selector genes in the dorsal module. Based on the above discussion, I propose a model for late patterning of the vertebrate trunk: Somites inherit the DV axis information provided from the surrounding environment, decode it into a binary state of *zic* expression (on/off) and form the dorsal and ventral domains in the trunk to provide positional information. In the dorsal domain, *zic1/zic4* continue to act on shaping and patterning of the body through long-term mesodermal-ectodermal interaction on the dorsal side to create dorsal specific structures (Fig. 15). In the ventral domain, the ventral module functions to establish ventral specific structures. Considering the fact that when *zic1/zic4* are depleted in *Da* mutant somites the dorsal region exhibits the ventral characters, it is likely that the trunk surface organs are originally destined to show ventral characters as a default state even in the dorsal region and that they will acquire the dorsal fate according to the action of *zic1/zic4*-mediated dorsal module.

Chapter 2

zic1 and *zic4*-mediated organization of the dorsal trunk structures and their phenotypic variation in the teleost clade

Introduction

Fish are one of the most prosperous groups of vertebrates and encompass more than 40,000 species reported so far. When we visit an aquarium, we are often surprised at the enormous diversity of fish in their appearance. They exhibit different pigmentation, fin morphologies and body shape, depending on their habitat. Biologists have long been fascinated by these exquisite structures and tried to explain how these various characteristics are created, mainly from an evolutionary aspect such as adaptation or sexual selection.

About 100 years ago D'Arcy Thompson, a theoretical biologist, tackled this problem from another aspect. He regarded these diverse morphologies as quantitative deformation of their original forms, and tried to simplify the situations by introducing mathematical transformation (Fig. 16) (Thompson, 1917). His work has inspired biologists to speculate that various morphologies observed can be explained by a simple model with a certain original form and a few parameters defining morphological deformation. However, because of the lack of knowledge on the mechanisms of development at that time, his interpretation of animal morphological variation had little contribution to the question of how they are created through morphogenetic processes.

In Chapter 1 of my thesis, I revealed that the medaka trunk is developmentally composed of the dorsal and ventral modules, and that the morphogenesis of the dorsal trunk structures is regulated by the transcription factors Zic1/Zic4. To comprehend the morphological variation from a developmental aspect, I wondered how this modular mechanism is linked to the diverse phenotypes observed in fish. While I was searching

for a clue, I came up with the fact that the dorsal fin of the heterozygous *Da* mutant displays the intermediate size between that of the *wt* and homozygous *Da* mutant. The quantitative change of the dorsal fin, though it was not described in detail, prompted me to investigate the relationship between the variation of the dorsal trunk structures and the levels of *zic1/zic4* expression; in other words, the diverse dorsal phenotypes observed in fish could be attributed to the variation of the spatial pattern or dosage of *zic1/zic4* expression.

In this chapter, I first describe dosage-dependent effects of *zic1/zic4* expression on the various dorsal phenotypes. I then show that the *zic*-mediated dorsal patterning is conserved among fish and investigate how the various dorsal trunk morphologies among fish species are created under the control of *zic*-mediated mechanism.

Results

Quantitative change of the dorsal fin morphology and the body contour induced by the *zic1* and *zic4* loci

To investigate if the variation in dorsal trunk structures among vertebrates is linked to the *zic1/zic4* genes, I first focused on the heterozygous *Da* mutants, which apparently show intermediate phenotypes as to the dorsal fin and the body shape (Fig. 17A-C). I quantified these dorsal phenotypes as follows. The dorsal fin has a unique exoskeleton, lepidotrichia (or soft fin rays), and the number of the lepidotrichia is thought to represent the size of the dorsal fin (Fig. 17D). In *wt* adult males, the number of lepidotrichia is 6.0 on average (n = 5), while that for the heterozygous and homozygous *Da* mutant males are 7.4 (n = 5) and 15.0 (n = 3), respectively (Fig. 17E). As a control, the anal fin, a ventral counterpart, was also measured, where the zic1/zic4 genes are not expressed. There was no significant difference among the three lines in the number of the anal fin rays (*wt*: 18.2; *Da*: 17.0; *Da*/+: 17.2; Fig. 17F). Next, the dorsal body shape was quantified by extracting three characteristic points to approximate the body contour by an arc (Fig. 17G). Again, the curvature of the dorsal contour was intermediate in heterozygous *Da* mutants when compared to wt and homozygous *Da* mutants (*wt*: 0.36; *Da*: 0.74; *Da*/+: 0.55 in arbitrary units; Fig. 17H). On the other hand, I did not observe any difference in the pattern of pigmentation and neuromast distribution between heterozygous *Da* mutants and *wt* (data not shown). Taken together, these results suggest that the dorsal fin and body shape can be quantitatively changed according to the number of active *zic1/zic4* alleles.

To further assess the effect of *zic* gene number on the dorsal structures, a BAC construct harboring the intact *zic1* gene was introduced to *wt* medaka to create a *zic1* overexpressing line Tg(*zic1/zic4*:GFP) (Fig. 18A-D). Interestingly, the dorsal fin of the transgenic line was smaller than that of *wt*, (Fig. 18B) and in some cases the dorsal fin was completely lost (Fig. 18C). Quantitative analysis confirmed that the number of dorsal fin rays was significantly decreased in the transgenic line, while the number of the anal fin rays was not altered (Tg dorsal fin: 4.2; Tg anal fin: 18.4; Fig. 18G, H). Alizarin Red staining revealed that the bony component of the lepidotrichia and supporting endoskeleton called radials were normal in the *zic1*-overexpressing line (Fig. 18E, F), indicating that the reduction in the number of the lepidotrichia was not caused by malformed ossification in the dorsal fin. Collectively, I concluded that the *zic* genes regulate the size of the dorsal fin in a dosage-dependent manner.

Double-tail variant of betta points out conservation of *zic1*/*zic4* function among teleosts

In order to explore the possibility of *zic1/zic4* involvement in dorsal diversification, I needed to first know if the *zic1/zic4* function in somites is conserved among species. For this reason, I focused on one variant of betta (*Betta splendens*; Perciformes), which had been established during domestication (Fig. 19A-F). This variant, known as 'Double tail,' exhibits typical *Da* phenotypes in terms of fin morphology when compared with the common-type betta; the shape and position of the dorsal fin is transformed into those of the anal fin (Fig. 19D, E, arrowheads), and the caudal-most vertebrae do not bend dorsally similar to what is observed in medaka *Da* (Ishikawa, 1990; Moriyama et al., 2012), leading to duplicated caudal fin lobes in this variant (Fig. 19D, E, brackets).

The distribution of the lateral line is also ventralized (Fig. 19C, F, red arrowheads). I compared the expression of *zic1*/*zic4* in the common type and Double-tail betta embryos. In common-type betta, *zic1*/*zic4* are expressed in the dorsal part of somites and neural tissues, whereas *zic1*/*zic4* expression is specifically lost in Double-tail somites (Fig. 19G-J), suggesting the conserved function of Zic1/Zic4 in somites in betta surface patterning. Although which genetic change causes the mesodermal loss of *zic1/zic4* expression in Double tail has not been addressed, the Double-tail gene is known to be recessive as is the case of the medaka *Da* mutant, and thus it is reasonable to think that the regulatory region shared by both *zic1* and *zic4* is mutated in Double tail as well. Collectively, I revealed that the function of the *zic1/zic4* genes in somites is conserved among teleosts.

Conserved spatial pattern of *zic1/zic4* expression in various fish

Given the conserved function of *zic1/zic4*, I hypothesized that alteration of *zic1/zic4* expression is the cause of various dorsal trunk structures among fish. I first searched for teleosts which exhibit reduced dorsal characteristics in the entire of a species. Angelfish (*Pterophyllum* sp.; Perciformes) and discus (*Symphysodon* sp.; Perciformes) matched this criterion and display apparently symmetric trunk surface structures including the position and size of the dorsal fins (Fig. 20A, B). It could be expected that the expression level of *zic1/zic4* in somites is diminished in these species. I successfully obtained fertilized eggs during breeding. I conducted WISH staining on these embryos and found that both species expressed *zic1/zic4* in the dorsal part of every somite (Fig. 20D-G). Next I picked up a fish species which has instead enormously different dorsal and ventral trunk structures. Catfish (*Corydoras aeneus*; Siluriformes) has enhanced dorsal

body curvature with its extremely flat ventral surface (Fig. 20C). However, the embryos again exhibited the dorsally restricted expression of *zic1/zic4* in somites (Fig. 20H, I). Therefore, it is further confirmed that the dorsal expression of *zic1/zic4* in somites is widely conserved among teleost species irrespective of their body shape. Thus such a drastic variation of the dorsal trunk structures among species is most likely independent of the spatial change of *zic1/zic4* expression.

Discussion

WISH analysis in a variety of fish embryos revealed that the *zic1/zic4* expression is conserved among teleosts. Furthermore, the betta Double tail variant exhibited loss of *zic1/zic4* expression in somites, associated with the altered dorsal trunk morphologies. These results indicate that the dorsal specific structures observed in a wide variety of teleosts are based on the *zic1/zic4*-mediated modular mechanism acting in the dorsal domain of the trunk.

In the subsequent analyses, I showed that by changing the dosage of *zic1/zic4* expression, some of the medaka dorsal trunk structures, i.e. the dorsal fin morphology and the dorsal contour, could be modified quantitatively. Considering the fact that these dorsal structures become identical to ventral ones when *zic1/zic4* expression in the somites is gone, it is reasonable to say that the small dorsal fin and dorsally flattened shape seen in *wt* medaka are created by quantitative alterations added to already existing ventral structures, but not by adopting distinct mechanisms (Fig. 21). To my knowledge, this is the first report in vertebrates that transcriptional factors regulate the variety of morphologies along the DV axis in a dosage-dependent manner.

Mechanism of quantitative change in the dorsal fin size depending on the *zic1/zic4* expression levels

zic1/zic4 are expressed in the dorsal part of all somites, and there is no biased expression observed along the AP axis. However, these facts are apparently contradictory to the ability of determining the dorsal fin position along the AP axis. There are two possi-

ble mechanisms to solve this paradox. One is the gradient-and-threshold model. In this model, certain AP axis information outside the *zic1/zic4*-mediated module forms a posterior (high) to anterior (low) gradient of the activity for dorsal fin production and is interpreted by *zic1/zic4* in a quantitative manner via a threshold. If *zic1/zic4* are in high dose, the threshold increases and only the posterior region where the activity level is above the threshold will have a small dorsal fin; if *zic1/zic4* are in low dose, the threshold lowers and the fin expands toward the anterior side, just like the *Da* mutant or the betta Double tail. The other possible explanation is based on protein maturation; while the mRNA expression is uniformly distributed along the AP axis, the more anterior somites have more abundant Zic proteins since somites are formed from the anterior side. In this way, the dorsal Zic module obtains an anterior (high) to posterior (low) gradient of Zic activity. However, in my preliminary experiment in which the anterior somites from the region that does not protrude the dorsal fin were replaced with the posterior somites from the region that protrudes the fin, or vice versa, the dorsal fin did not show any phenotype (data not shown). This suggests that the anteroposteriorly graded factors for determining the dorsal fin position is not maintained within the somites. This favors the "gradient-and-threshold" model. Taken together, *zic1/zic4* genes not only determine the dorsal module but also participate in the AP patterning of the dorsal fin.

Making variations in the dorsal trunk structures

Given the conserved expression pattern and function of *zic1/zic4*, how do teleosts create different types of dorsal trunk structures using the developmental module of *zic1/zic4*? Here I raise two possibilities: to change the dosage of *zic1/zic4* expression in somites and to change the gene network downstream of *zic1/zic4*. When the *zic* expression level

is altered genetically, the medaka exhibited various size of the dorsal fin with a negative correlation, and it did not impair the ossification processes of dorsal fin structures. Thus the variation of the expression level of *zic1/zic4* derives the dorsal characteristics including the dorsal fin and dorsal body shape among fish, as if *zic1/zic4* behaved as a parameter of D'Arcy Thompson's spatial transformation (Fig. 21). According to my WISH results for various fish, however, all embryos had relatively high levels of *zic1/zic4* expression in dorsal somites, suggesting that this dosage-dependent mechanism is unlikely to have been adopted among these fish species. Considering the conserved expression pattern and function of *zic1/zic4*, such drastic diversity of body appearance across species is probably caused by modifications in their downstream genes. Thus, it is reasonable to think that the quantitative changes of *zic1/zic4* expression drives minor changes in dorsal trunk structures often observed within species like two different strains of medaka or betta, whereas the quantitative or qualitative differences in the downstream genes yield diversity in dorsal structures among distinct teleost species or genera.

General Discussion

Dorsal domain: novel developmental module starting in somites

In my doctoral study, I attempted to reveal the developmental mechanism underlying the late DV patterning of the vertebrate trunk. In Chapter 1, I demonstrated that the trunk consists of two distinct developmental modules, dorsal and ventral ones, and that they are defined by lifelong *zic1/zic4* expression. The concept of "dorsal" had been utilized only as a direction toward the back, and the dorsal region had not been anatomically defined (Romer and Parsons, 1986). Here, one of my achievements is to describe for the first time the existence of the dorsal domain in our body with a clear boundary and to clearly define the "dorsal" and "ventral" in the context of developmental biology from late developmental stages to the end of life. The DV pattern of the trunk does not simply utilize the initial gradient information inherited from the early embryo, but is built by the binary information of Zic1/Zic4 in somites. *zic1/zic4* expression in somites thus represents a regionalization process that fills a gap between the initial DV axis information and the adult DV pattern.

Furthermore, the *zic1/zic4*-mediated developmental module works in somites. Somites were long regarded as a mere source of precursors that give rise to axial and appendage muscle, vertebrae and ribs, and dermis (Wolpert and Tickle, 2011) with a few exceptions (Tosney, 2004; Gammill et al., 2006). In my study, somites were found to have unexpectedly major patterning activities on the ectoderm and neural crest, e.g. to regulate the dorsal fin morphology and pigmentation pattern, probably through local cell-cell interaction. Therefore, somites and their derivatives should be considered as

crucial elements to drive the variation of the trunk morphology in vertebrate anatomy and their evolutionary process.

In Chapter 2, I investigated and affirmed the generality of this *zic*-mediated mechanism among teleosts. To what extent does it work among vertebrates other than teleosts? Compared to fish, which usually has a relatively long tail region, amniotes have a large portion of thorax and abdomen in the body, and most somite-mediated patterning, if any, should be largely masked by or overlapped with signals emanated from the viscera. Moreover, based on the expression pattern of Zic1 in amniotes (Nagai et al., 1997; Sun Rhodes and Merzdorf, 2006) and the position of the myoseptum that likely divides the dorsal and ventral domain in amniotes (thoracolumbar fascia in human; septum laterale in other amniotes) (Ahmed et al., 2006), the dorsal domain seems relatively small. Nevertheless, the *zic1/zic4*-mediated mechanism should also work in other vertebrates since the fundamental feature of dorsoventrally asymmetric patterns on the trunk is shared among vertebrates. Further genetic and developmental analyses, especially in amniotes, are required to confirm this idea.

Downstream mechanism of *zic1/zic4*-mediated dorsal patterning

I have identified *zic1/zic4* as an important clue to understanding the relationship between the initial DV information and the final DV pattern on the trunk. In order to reveal the developmental mechanism of the dorsal patterning in more detail, the downstream genes need to be identified as well. However, the downstream genes of *zic* genes in the neural tube or neural plate identified to date, such as *ApoE* and *Math1* (Aruga, 2004), are either not expressed in the somites or uniformly expressed in both the dorsal and ventral part of somites. Thus far I attempted to find the candidate genes by compar-

ing the expression profiles of somitic cells isolated from the *wt* and *Da* mutant embryos using RNA microarrays. Although I had backcrossed *Da* with *d-rR* (*wt*) for three times to make the genetic backgrounds closer to each other, most of the differently expressed genes were related to the housekeeping functions, implying that the downstream genes of *zic1/zic4* are activated in response to *zic1/zic4* in a rather small fashion. Further molecular analysis will be required.

Significance of developmental modularity in DV patterning

In general, the modular construction of the animal body could promote diversification in form and size during evolution; one module can adopt a novel phenotype without affecting the others (Wagner et al., 2007). It has been argued that making a modular mechanism to dissociate from others is a critical step for morphological evolution (Raff, 1996). Here, the dorsal module in late development enables diversification of dorsal specific characteristics without any alteration of the ventral structures. Since the dorsal domain is generally faced with the environment and other external stimuli, this modular system should provide fast environmental adaptation. Indeed, the dorsal trunk structures seem to be much more various than the ventral among vertebrates.

In addition, according to Chapter 2 the *zic1/zic4*-mediated module has the ability to quantitatively regulate the morphology of dorsal structures including the dorsal fin size and body shape. In natural conditions, ever-changing environments sometimes yield quick alterations of multiple body structures at the same time; for instance, change of water flow conditions eventually results in simultaneous quantitative changes of fish body shape and also the size and position of fins (McGuigan et al., 2003; Haas et al., 2010). Hence, the *zic*-mediated module, by which

multiple structures can be changed simultaneously in a quantitative manner, could be the implementation of adaptive functions required under such natural conditions.

Materials and Methods

Fish strains

The medaka (*Oryzias latipes*) *Da* mutant used here was originally isolated from a wild population in Aichi prefecture, Japan (Tomita, 1969), and has been maintained as a closed colony in the Laboratory of Fish Stocks at Nagoya University (Tomita, 1992). *Kusu*, *HNI* and *d-rR* stains were used as wild type controls. Embryos were staged according to the previous paper (Iwamatsu, 2004). The common-type and 'Double-tail' *B. splendens* strains, *Pterophyllum* sp. were *Symphysodon* sp. were obtained from a commercial supplier in Tokyo, Japan. Embryos of *C. aeneus* were a kind gift from R. Toyoizumi.

BAC modification by homologous recombination and transgenesis

The BAC construct harboring *zic1/zic4*:DsRed made by Y. Moriyama was modified to encode *zic1/zic4*:GFP by homologous recombination as previously described (Nakamura et al., 2008; Moriyama et al., 2012). Primers used are as follows: TCATCATTATTATTTTGCCTGCTGTCAAGTTATTCTAAAATTAGGAAGTACCAC CGGTCGCCACCATGGT (zic4_5'UTR-EGFP5'); ACTTTGCACATAAAATATGCAT GATGCACACAGATATGATCTCCACTGACGTCGACCAGTTGGTGATTTT (zic4_ 3'UTR-EGFP3'). The BAC construct encoding *zic1*/*zic4*:GFP was made with the following primers: The modified BAC construct encoding *zic1*:GFP/*zic4*:DsRed was provided by Y. Moriyama (Moriyama et al., 2012). Generation of transgenic lines by BAC injection into embryos of the *d-rR* strain was performed as previously described

(Nakamura et al., 2008). We utilized the I-*Sce*I meganuclease method to increase the probability of successful germ line transmission as previously described (Rembold et al., 2006).

Neuromast staining and whole-mount skeletal staining

Neuromasts were stained by 5-minute exposure to 0.05 mg/ml 4-(4-diethylamino-styryl)-N-methylpyridinium iodide (DiAsp, Sigma) dissolved in Yamamoto's Ringer solution. Whole-mount skeletal staining with Alizarin Red was performed as previously described (Ohtsuka et al., 2004).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described previously (Takashima et al., 2007). Signals were visualized with NBT/BCIP tablets (Roche), BM Purple (Roche) or Fast Red tablets (Roche). The probes used for medaka staining are as follows: *zic1*, *zic4* (Ohtsuka et al., 2004); *myod*, *pax3* (Moriyama et al., 2012); *twi* (Yasutake et al., 2004); *sim1* (primers: 5'-CTGGGTTCTCATTTACTGCAGAC-3', 5'-TTGTGGACTATAGTGGCGTAACTC-3'); *wnt11r* (primers: 5'-CAAATGGCTA ACACTGTCTCAAAC-3', 5'-CTATTTGCAAACGTATCTCTCCAC-3'); *foxd3* (primers: 5'-GATGACTTGGAAGATGAAATCG-3', 5'-ACACCCCGATGATGTTTTCTA TAC-3'). For the staining of *B. splendens*, we used *zic1* and *zic4* probes synthesized from cDNA cloned from Japanese pufferfish (*Takifugu rubripes*).

Immunohistochemistry

Whole-mount immunostaining was processed as previously described (Koshida et al.,

2005). The primary antibody, anti-DsRed (Clontech) was used at a 1:200 dilution. Biotin-conjugated anti-rabbit IgG (Sigma) was used as a secondary antibody at 1:250 dilution. Signals were visualized with 3,3'-diaminobenzidine (DAB; Roche).

Tissue transplantation

Tissue transplantation in medaka was performed in accordance with the protocol used previously in zebrafish (Haines et al., 2004), with some modifications. The trunk regions of the donor embryos (Tg(β-*actin*:DsRed) or Tg(*zic1*:GFP/*zic4*:DsRed)) at the 14-16-somite stage were treated with 20 mg/ml pancreatin (Wako) for several minutes. The most caudal two successive somites or the dorsal neural tube at the same AP level as the somites were isolated and kept in 10% fetal bovine serum until transplantation. Wild type or *Da* mutant host embryos at the same stage as the donors were mounted in 1% low melting temperature agarose with their dorsal surfaces of the posterior trunk exposed. The somites or neural tube of the host embryos were extirpated from the same region as the donor tissues. The host embryos into which the donor somites or neural tube were transplanted were incubated to the hatching stage.

Primary tissue culture

Somite culture was performed based on the previously established medaka cell culture method (Komura et al., 1988). Medaka embryos with chorion were soaked in Dakin's solution (0.4% sodium hypochlorite) for 40 seconds, washed with sterilized PBS, soaked in 70% ethanol for 5 seconds, washed with sterilized PBS and dechorionated with sterilized forceps. Somites were excised after digestion with 20 mg/ml sterilized pancreatin, placed in the culture medium [10 ml FBS (Life Technologies), 1.5 ml Pen

Strep (Life Technologies), 38.5 ml Leibovitz's L-15 medium (Life Technologies)], covered with a cover slip and incubated at 27°C.

qPCR

Total RNA of adult fish was extracted with ISOGEN (Nippon Gene) from the ventral or dorsal trunk tissues i.e. the myotome, dermis and fins. SuperScript III (Invitrogen) was used for subsequent cDNA synthesis. The transcription levels were quantified with THUNDERBIRD SYBR (TOYOBO) and Stratagene Mx3000P (Agilent Technologies). The primers used for PCR were as follows: *zic1*: 5'-AGCCCTTTCCGTGTCCGTTCC-3', 5'-CCGACGTGTGGACGTGCATGT-3'; *zic4*: 5'-AGAAGCCGTTTCCATGCCCGT-3', 5'-TGCTGTTGGCGAAGCGTCTGT-3'; β-*actin*: 5'-TGCCGCACTGGTTGTTGACAACG-3', 5'-CCATGACACCCTGGTGCCTGG-3'.

Figures

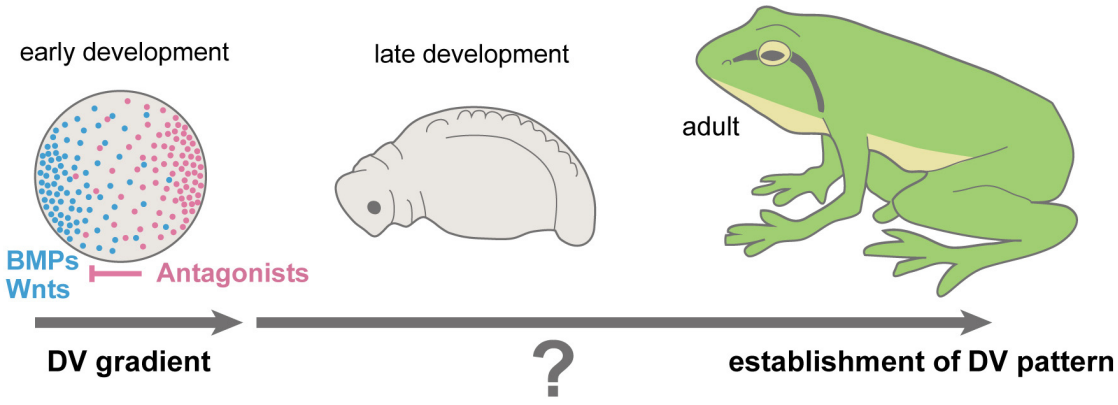

Fig. 1. DV pattern formation during development.

In early development, the antagonistic action of morphogens such as BMPs and Wnts determines the initial DV axis (left). However in late development after gastrulation, it is not clear what mechanisms undergo to establish the final DV pattern.

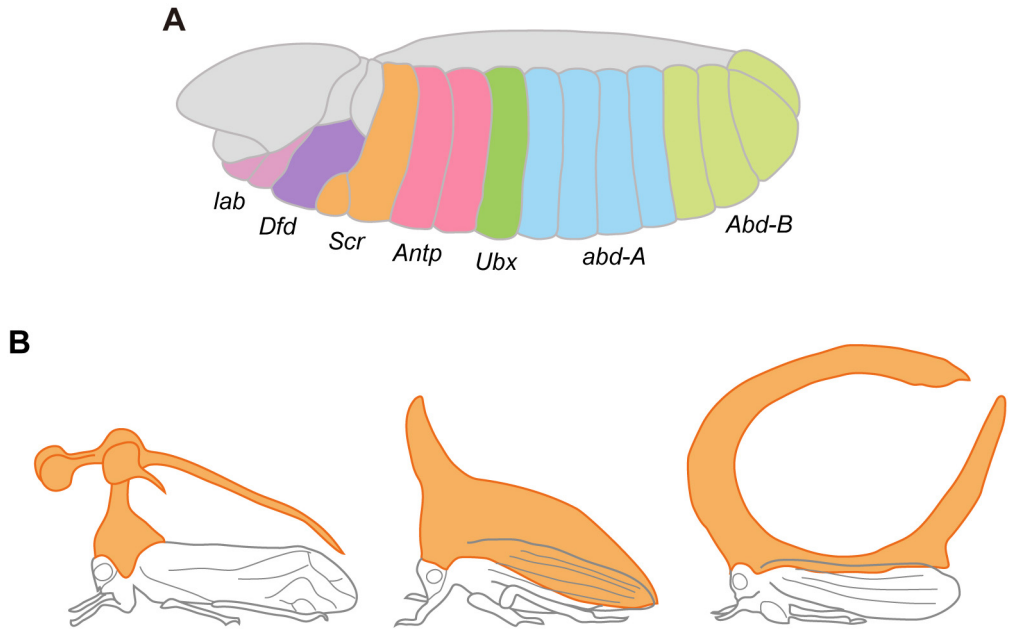

Fig. 2. Developmental modularity.

(A) Example of developmental modularity in *Drosophila.* Embryonic segments are delineated by segmentation genes and subject to the developmental modules driven by these genes to make specific structures.

(B) According to Prud'homme et al. (2011), downstream genes of *Scr* in treehoppers are diversified so that they have various types of "helmets," derivatives of the first thoratic segment (orange).

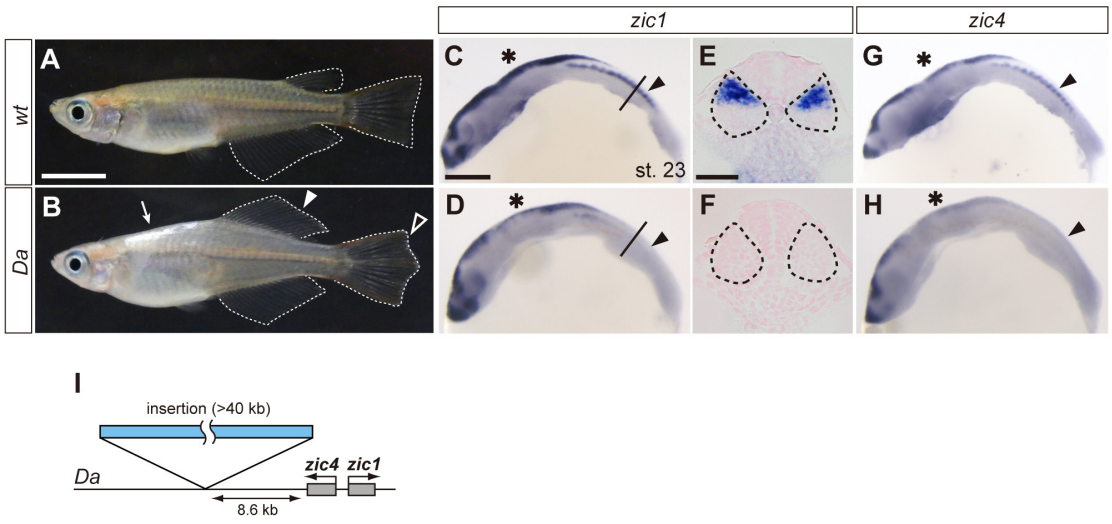

Fig. 3. *Da* mutant and the causal genes.

(A, B) The *Da* medaka mutant exhibits a ventralized pigmentation (arrow) and median fin morphology (arrowheads) as well as a tear-drop body shape.

(C-H) Expression patterns of *zic1* and *zic4* in the *wt* (C and G) and *Da* mutant (D and H) medaka at st. 23 (12 somites). Arrowheads and asterisks indicate the somite and neural tube, respectively. (E, F) *zic1* expression in transverse sections of *wt* and *Da* mutant embryos at st. 23 at the level of the solid lines in (C) and (D). Dashed lines delineate the somites.

(I) In the *Da* genome, a DNA transposon (blue) is inserted into the vicinity of *zic1/zic4* loci, resulting in perturbation of the expression. Scale bars equal 1 cm for (A); 100 µm for (C); 50 µm for (E).

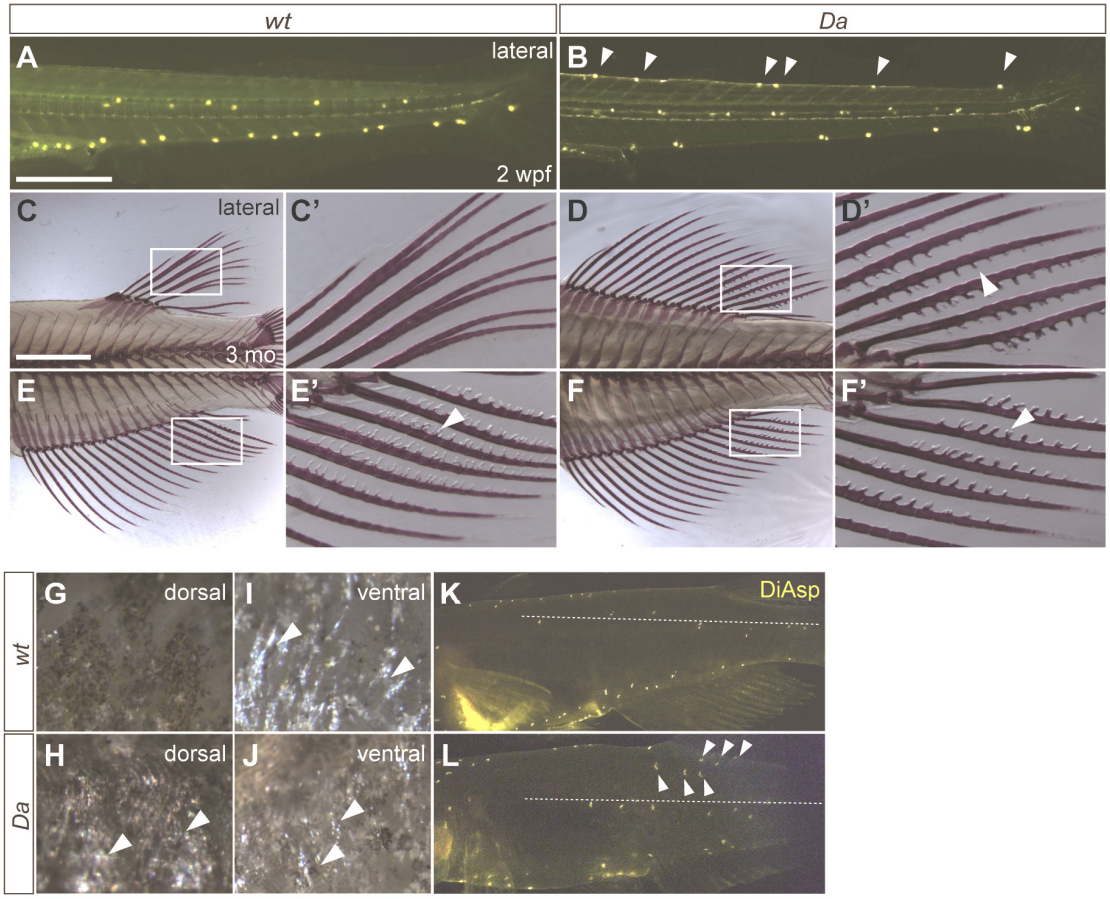

Fig. 4. Ventralized phenotypes of *Da* mutants at larval and adult stages.

(A, B, K, L) Ectopic deposition of neuromasts (sensor complexes of the lateral line) on the dorsal sites (arrowheads) in *Da* mutant embryos at 2 wpf (B) and adult (L). Dashed lines indicate the lateral midline of the trunk.

(C-F') Skeletal patterns of the dorsal (C, D) and anal fins (E, F) in *wt* and *Da* mutant adult males. (C'-F') are magnified views corresponding to the white boxes in (C-F), respectively. Note that the papillary processes (arrowheads), which normally develop only on the anal fin in response to testosterone, are also observed on the dorsal fin in the *Da* mutant.

(G-J) Pigmentation patterns of the *wt* and *Da* mutant adult trunk surface, showing that while the dorsal skin of *wt* has only melanophores and leucophores, that of *Da* possesses iridophores (arrowheads in H) in addition, similarly to the ventral side of *wt*.

Embryos and adults in (A-F') and (K-L) are shown in lateral view with anterior to left. Scale bars equal 500 μm for (A); 1 cm for (C).

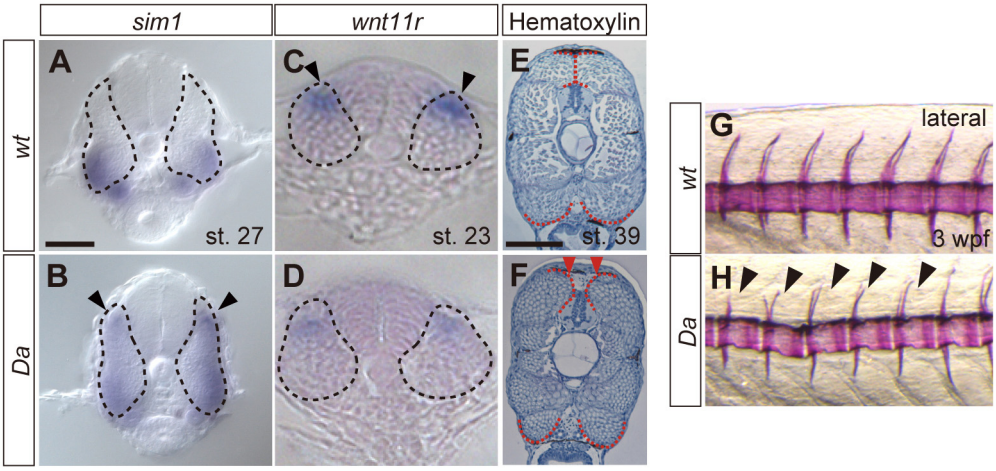

Fig. 5. Ventralized phenotypes of *Da* mutant somites.

(A, B) Expression pattern of *sim1* in *wt* (A) and *Da* mutant (B) embryos at st.27 (24 somites). Arrowheads indicate ectopic expression. Dashed lines delineate the somites.

(C, D) Expression pattern of *wnt11r* in *wt* (C) and *Da* mutant (D) embryos at st. 23. Arrowheads indicate strong expression in the dorsal part of *wt* somites. Dashed lines delineate the somites.

(E, F) Myotomal morphology at st. 39. Dashed lines delineate the myotome. Arrowheads indicate the gap between the dorsal myotomes in the *Da* mutant.

(G, H) Vertebral morphology of the *wt* (G) and *Da* mutant (H) larvae, anterior to the cloaca, at 3 wpf. In *Da* mutants, the neural spines (prospective neural arches; arrowheads) are shortened to almost the same length as the hemal spines (prospective hemal arches).

Scale bars equal 50 μm for (A); 200 μm for (E).

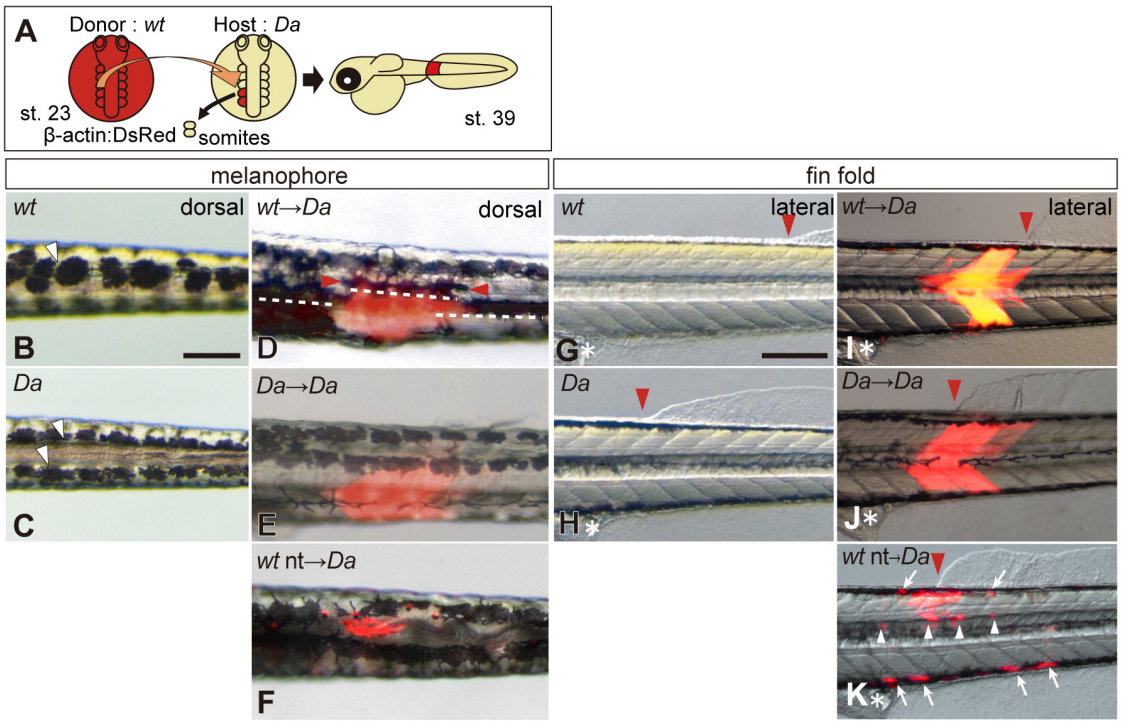

Fig. 6. Ventralized phenotypes in *Da* mutants are rescued by wild-type somites.
(A) Schematic diagram of the rescue experiment by somite transplantation as in (B)-(K). (B-K) Transplantation of *wt* somites (D, I) into *Da* mutant medaka embryos locally rescues the ventralized phenotypes [red arrowheads in (D) and (I)], whereas neither *Da* mutant somites (E, J) nor *wt* dorsal neural tubes (F, K) have this effect. Arrowheads in (D) indicate the medially shifted melanophores. Red arrowheads in (G)-(K) indicate the anterior limit of the dorsal fin fold. White arrowheads in (B) and (C) indicate melanophores. Asterisks indicate the cloaca. White arrowheads and arrows indicate dorsal root ganglia and pigment cells, respectively, derived from the donor neural tube.
Scale bars equal 200 μm.

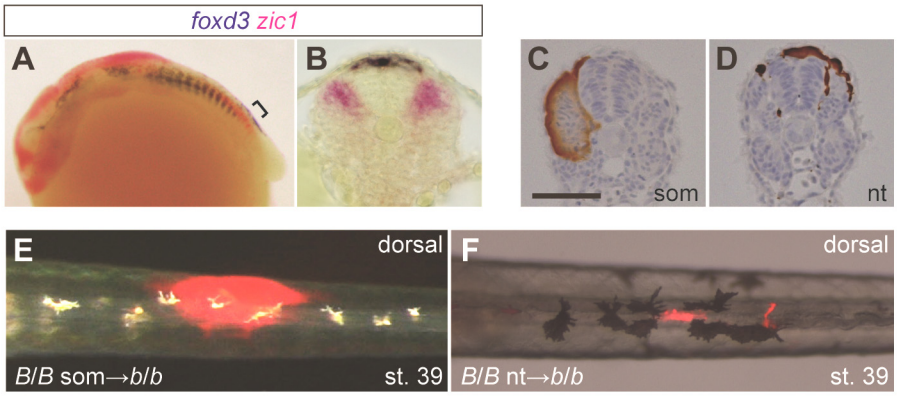

Fig. 7. Further validation of somite transplantation experiments.

(A, B) Expression pattern of *foxd3* (early neural crest marker) and *zic1* in *wt* at st. 23 (14 somites). Bracket indicates the site from which the somites are dissected. Note that *foxd3*-expressing dorsal neural tube and neural crest cells do not express *zic1*.

(C, D) Transverse sections of somite (C) and dorsal neural tube (D) transplants from Tg(β-*actin*:DsRed) to *wt*, stained with DAB at 3 dpf (st. 27). Note that the labeled cells in (C) form a smoothly packed tissue while in (D) they showed mesenchymal shape, located at both the medial and lateral side of the somite.

(E, F) Additional evidence for lack of neural crest cell contribution during the somite transplantation experiments. (E) Somites derived from a melanophore-containing strain '*Kusu*' (*B*/*B*, labeled with DsRed by having crossed with a β-*actin* promoter-driven DsRed transgenic line) are transplanted into *d-rR* hosts which lack melanophores (*b*/*b*) at st. 23 (14-16 somites). This panel shows a dorsal view of a transplant at st. 39, No melanophores appears in 19 out of 19 transplants. (F) Control experiments by transplanting the dorsal neural tube of a *B*/*B* embryo into a *b*/*b* host. Melanophores appear around the transplantation site at st. 39. Dorsal view is shown with anterior to left.

Scale bar equals 100 μm.

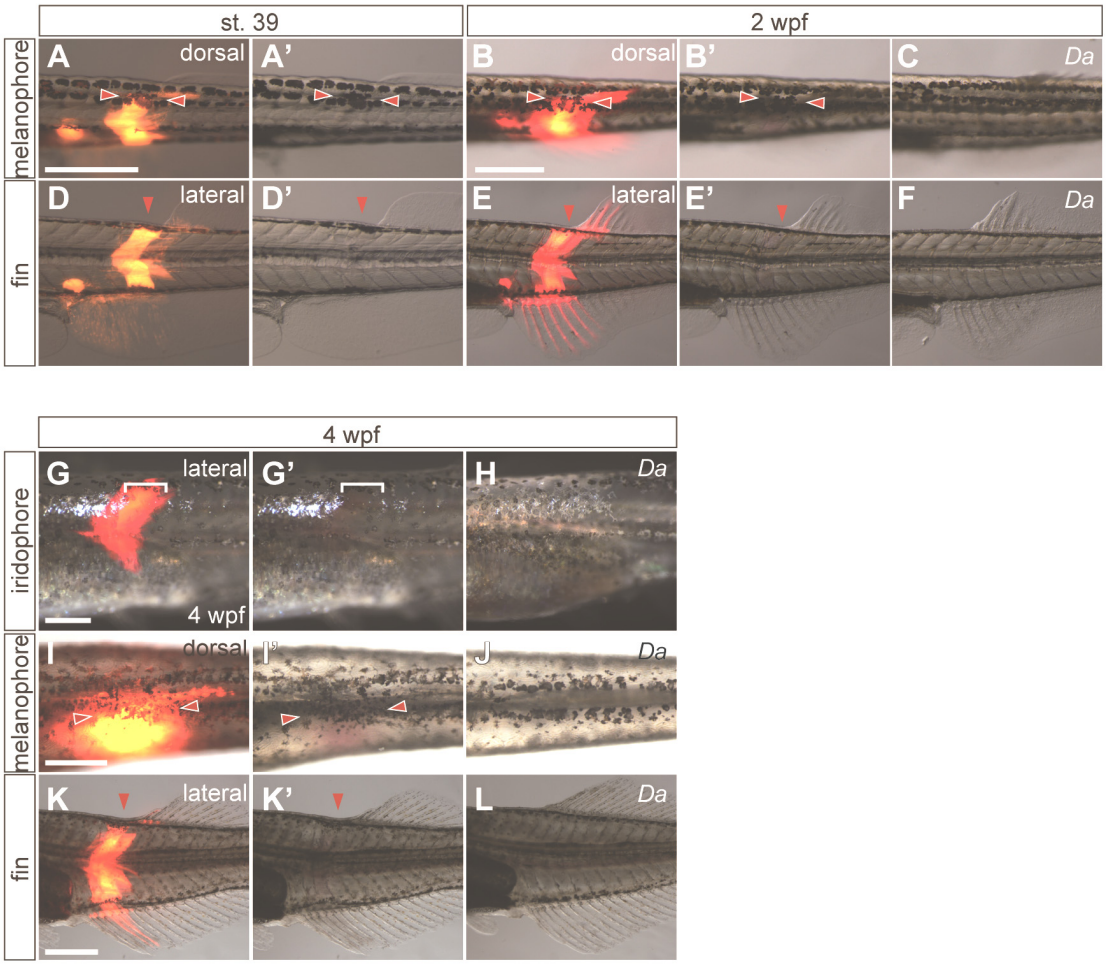

Fig. 8. Phenotype rescue of *Da* by somite transplantation at the larval stage.

(A-F) Phenotypes of dorsal patterns after transplantation of *wt* somites (labeled with DsRed) into *Da* mutant embryos at st. 39 (A, D) and 2 wpf (B, E). (A'), (B'), (D') and (E') are the same images as (A), (B), (D) and (E), respectively, except that DsRed fluorescence is not merged. Melanophore (A, A', B, B') and dorsal fin fold (D, D', E, E') rescues are maintained at st. 39, and also at 2 wpf, when the dorsal fin fold starts to be replaced with the dorsal fin with fin rays. Arrowheads indicate the rescued sites. Homotopic regions of the *Da* mutants at 2 wpf are also shown (C, F).

(G-L) Rescued phenotypes at the post-hatching stage (4 wpf). (G'), (I') and (K') are the same images as (G), (I) and (K), respectively, except that DsRed fluorescence is not merged. Iridophores (G, G'), emerging at 2-3 wpf, are rescued (or suppressed, brackets) on the transplantation site; the rescue of melanophores (I, I'; medial shift of melanophores) and dorsal fin fold (K, K'; posterior shift of dorsal fin fold) are maintained after hatching (4 wpf; arrowheads). The equivalent regions of the *Da* mutants are also shown (H, J and L).

Scale bars equal 500 μm for (A), (B), (G) and (I); 1 mm for (K).

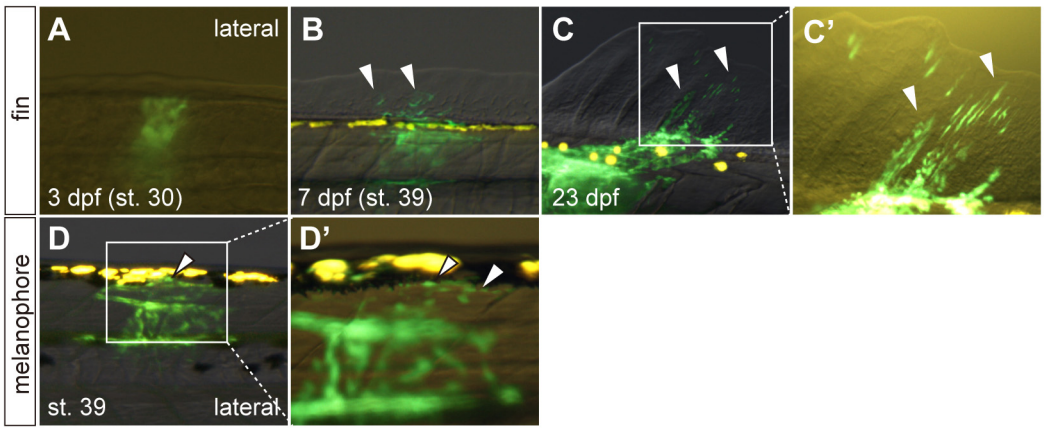

Fig. 9. Distribution of *wt* somitic cells after somite implantation.

Lineage analysis of the GFP-positive somitic cells. Somites dissected from transgenic fish Tg (*zic1*:GFP/*zic4*:DsRed) are transplanted into *wt* embryos at the somitogenesis stage.

(A-C') Somitic cells expressing GFP gradually invade the dorsal fin fold (arrowheads) and become elongated along the proximodistal axis. (C') is a magnified view corresponding to the white box in (C). Yellow fluorescence is the autofluorescence derived from leucophores (white pigment cells).

(D, D') Somitic cells expressing GFP are present underneath melanophores at st. 39 (arrowheads). (D') is a magnified view corresponding to the white box in (D). Yellow fluorescence is the autofluorescence derived from leucophores.

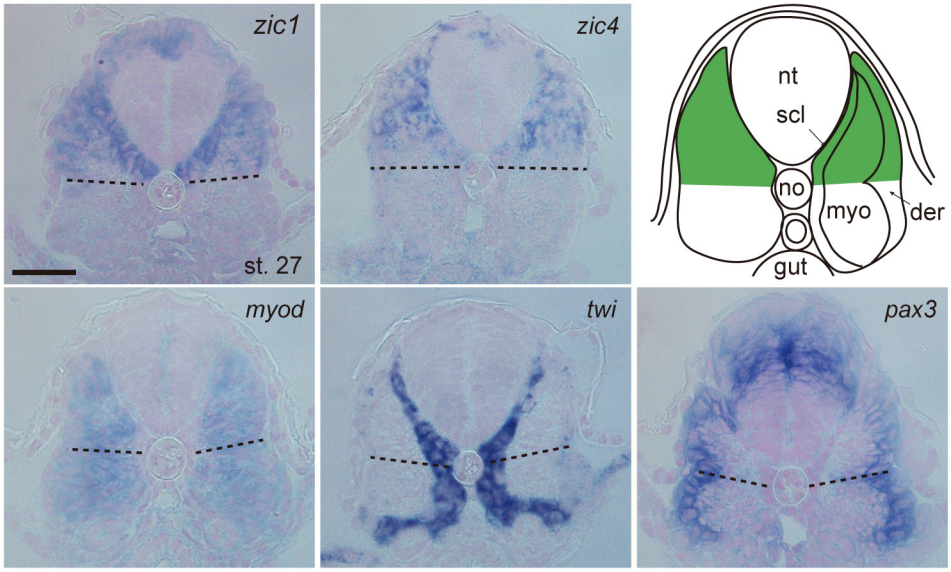

Fig. 10. *zic1/zic4* expression covers all somitic components.

Expression patterns of *zic1*, *zic4*, *myod*, *twi* and *pax3* in *wt* embryos at st. 27 (24 somites). Each somitic compartment, myotome, sclerotome or dermomyotome defined by the expression of *myod*, *twi* or *pax3* respectively, is depicted in the schematic diagram (upper right). The expression domain of *zic1* and *zic4* is represented in green. Dashed lines indicate the horizontal myoseptum. nt, neural tube; no, notochord; gut, gut tube; scl, sclerotome; myo, myotome; der, dermomyotome.

Scale bar equals 50 µm.

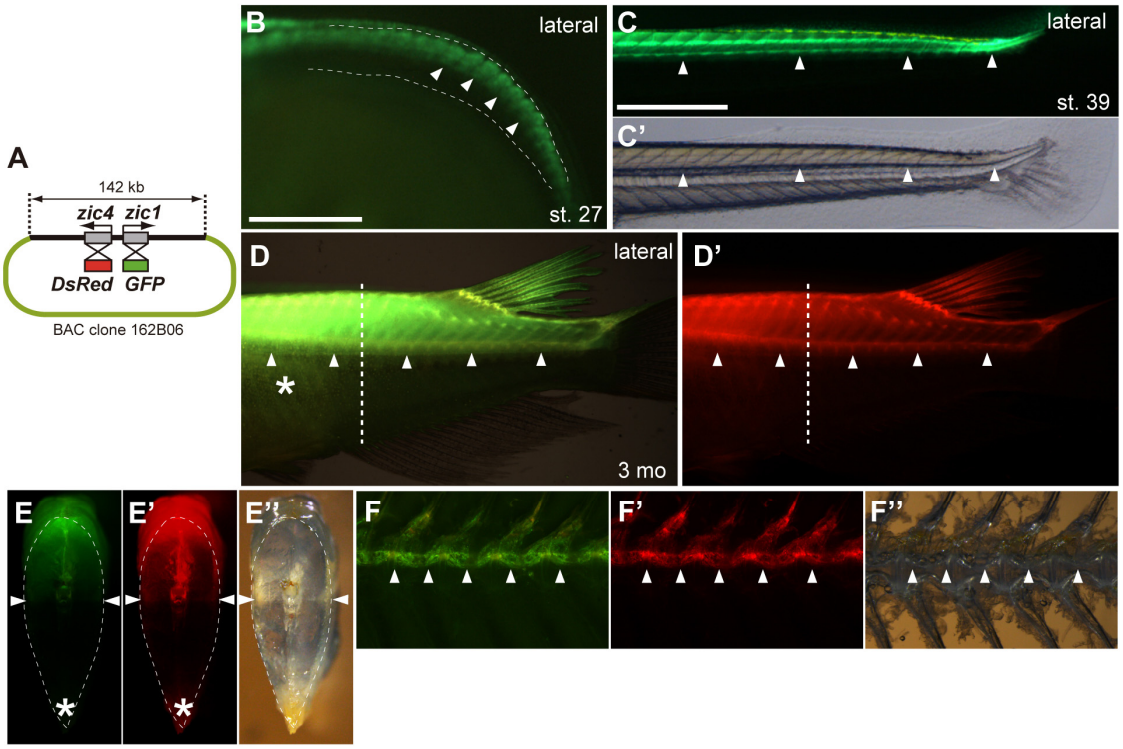

Fig. 11. *zic1/zic4* define the dorsal domain of the trunk throughout life.

(A) Schematic of the BAC construct used for the transgenesis of Tg(*zic1*:GFP/*zic4*:DsRed). (B-F') Fluorescent images of Tg(*zic1*:GFP/*zic4*:DsRed) transgenic line during embryogenesis [st. 27 (B) and st. 39 (C)] and at adult stages (D-F''). Arrowhead and dashed lines in (B) indicate somites and their dorsal and ventral boundaries, respectively. Arrowheads in (C-D') indicate the ventral boundary of GFP (C, D) and DsRed (D') expression, showing a linear boundary along the AP axis. (C') is the bright field image of the same sample as in (C). (E, E') Transverse sections of the adult transgenic medaka at the level indicated by dashed lines in (D, D') reveal an expression boundary of GFP (E) and DsRed (E') shared by the myotome and the vertebrae at the almost same DV level. (F, F') Lateral views of the adult transgenic vertebrae demonstrate the DV boundary by GFP (F) and DsRed (F') expression. (E'') and (F'') is bright field images of the same samples as in (E) and (F), respectively. Arrowheads in (E-F'') indicate the ventral boundary. Asterisks in (D), (E) and (E') indicate autofluorescence of leucophores.
Scale bars equal 200 μm for (B); 500 μm for (C).

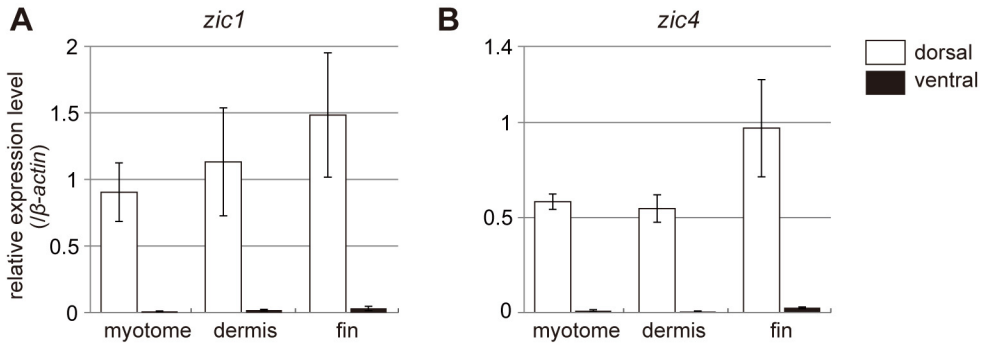

Fig. 12. *zic1/zic4* expression level at the adult stage quantified by qPCR.

Expression analysis of *zic1*/*zic4* at the adult stage by quantitative PCR. The expression levels of *zic1* (A) and *zic4* (B) in the dorsal (white) or ventral (black) region of the myotome, dermis and median fin (fin) are assessed, after normalized with the basal β-*actin* expression. Note that the *zic1/zic4* expression at the adult stage is high only in the dorsal region ($p < 0.05$ in all 6 cases), consistent with the persistent GFP and DsRed expression in the transgenic line Tg(*zic1*:GFP/*zic4*:DsRed). Error bars indicate standard deviation.

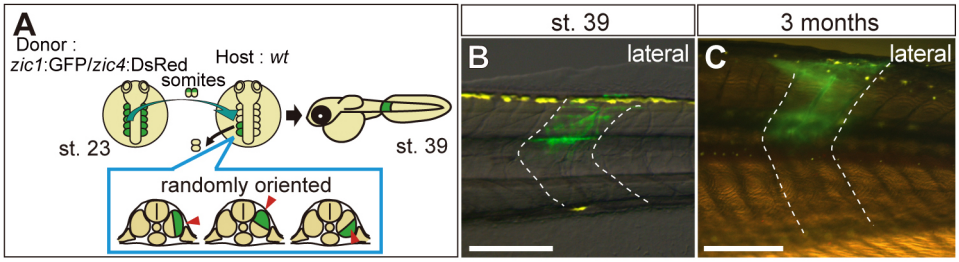

Fig. 13. Dorsal expression of *zic1*:GFP after randomized somite implantation.

(A) Schematic diagram of the transplantation experiments using the transgenic line Tg(*zic1*:GFP/*zic4*:DsRed) as donors as in (B) and (C). The orientation of transplanted somites is randomized and cannot be controlled (arrowheads).

(B, C) Somites, when transplanted randomly with respect to their orientation, acquires the dorsal expression of GFP at st. 39 (B) and 3 months post fertilization (C). Dashed lines delineate the somites. Anterior is to the left. The mosaicism of GFP fluorescence observed in (B) is due to the scattered distribution pattern of sclerotomal cells, which have stronger expression of GFP compared to myotomal cells. Yellow signals are autofluorescence of leucophores.

Scale bars equal 500 μm for (B); 1 cm for (C).

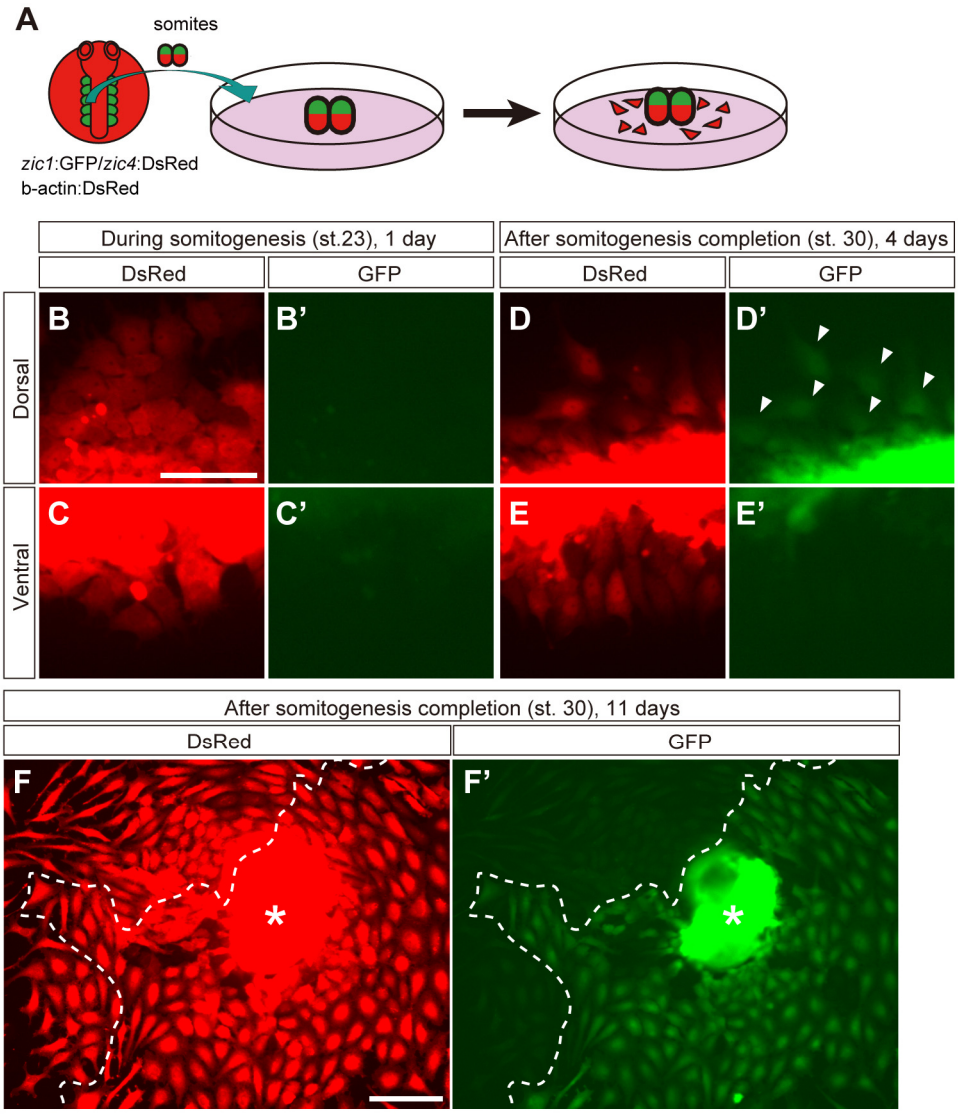

Fig. 14. Cell-non-autonomous and autonomous expression of *zic1* in the primary culture experiment.

(A) Schematic diagram of the somite culture experiments using Tg(*zic1*:GFP/*zic4*:DsRed/ β-*actin*:DsRed).

(B-F') Explant culture of somites from Tg(*zic1*:GFP/*zic4*:DsRed/β-*actin*:DsRed) embryos at st. 23 (14 somites; B-C') and st. 30 (the stage of somitogenesis completion; D-E'). (B-C') Cells crawling out of the somites from st. 23 embryos do not express GFP even at the dorsal part (B'). (D-F') Cells crawling out of st. 30 embryos maintain GFP expression in the dorsal area of the somites after 1 day (D', arrowheads) and 11 days (F', right to the dashed line) in culture. Asterisks in (F, F') indicate the explanted somite fragment.

Scale bars equal 50 μm for (B);100 μm for (F).

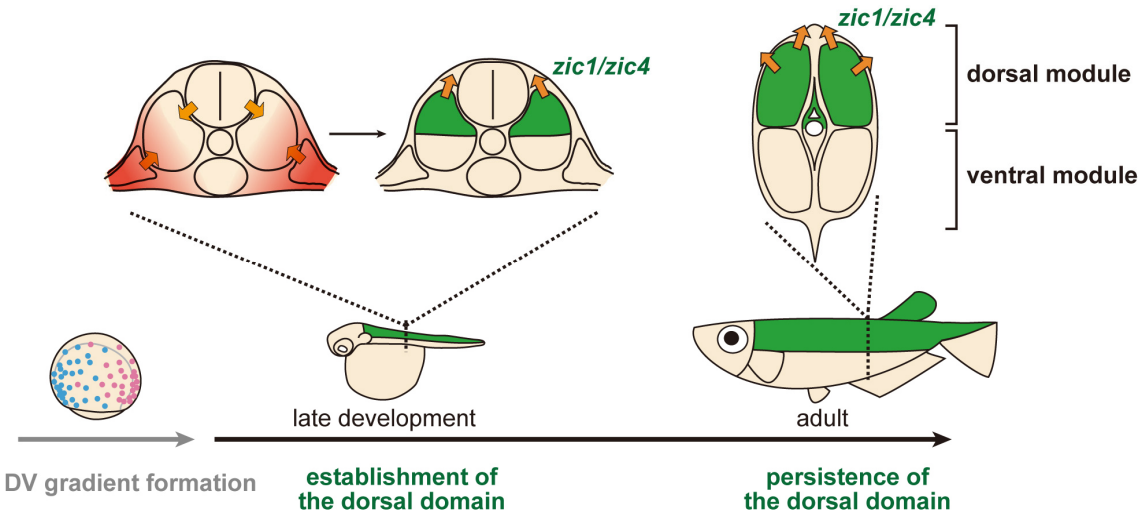

Fig. 15. Model for determining the dorsal structures using the *zic1*/*zic4* domain.
Schematic illustration of DV patterning in fish during early to late development. The dorsal expression domain of *zic1/zic4* in the somite is established during embryogenesis by signals from their surrounding tissues such as the neural tube and lateral plate (upper left), and then becomes autonomously maintained for the entire life (upper middle and upper right). During this process, the somite decodes the surrounding DV information to ON and OFF states of the *zic1/zic4* expression, leading to the formation of dorsal and ventral modules in the trunk. In the dorsal module, *zic1/zic4*-positive somite-derived cells continue to exert their inductive effects on the dorsal-specific surface structures and pigmentation pattern during late development.

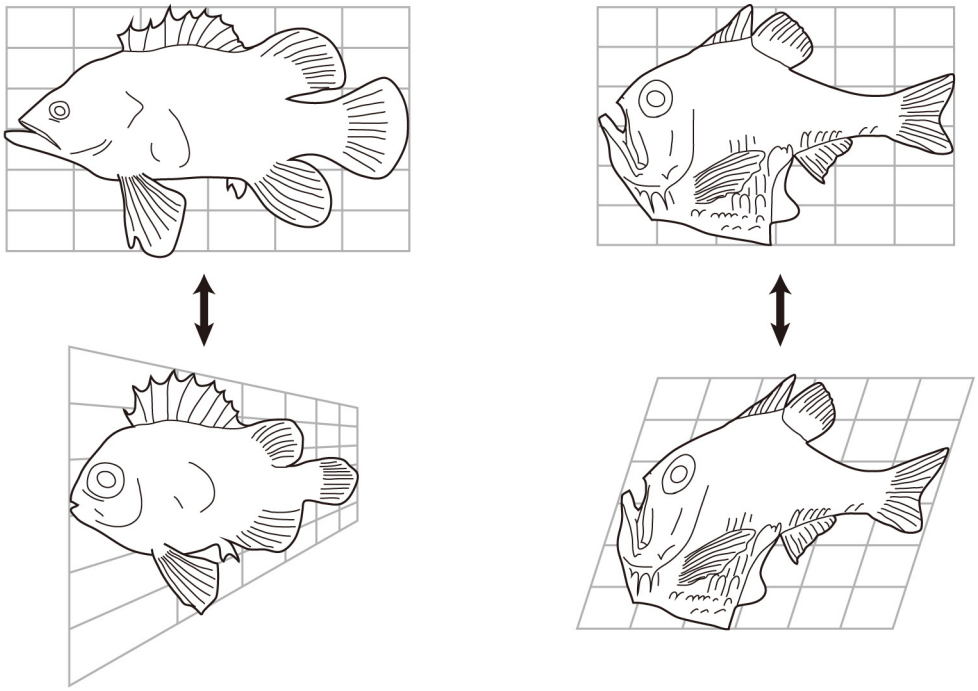

Fig. 16. Mathematical transformation of trunk morphologies proposed by D'Arcy Thompson.

Thompson regarded various types of morphologies as a result of coordinate transformation. He placed a Cartesian coordinate to the original form and distorted it with a simple transformation with some parameters to deduce various morphologies. Clockwise from upper right: freshwater hatchetfish (*Argyropelecus olfersi*), marine hatchetfish (*Sternoptyx diaphana*), short bigeye (*Pseudopriacanthus altus*), wreckfish (*Polyprion* sp.).

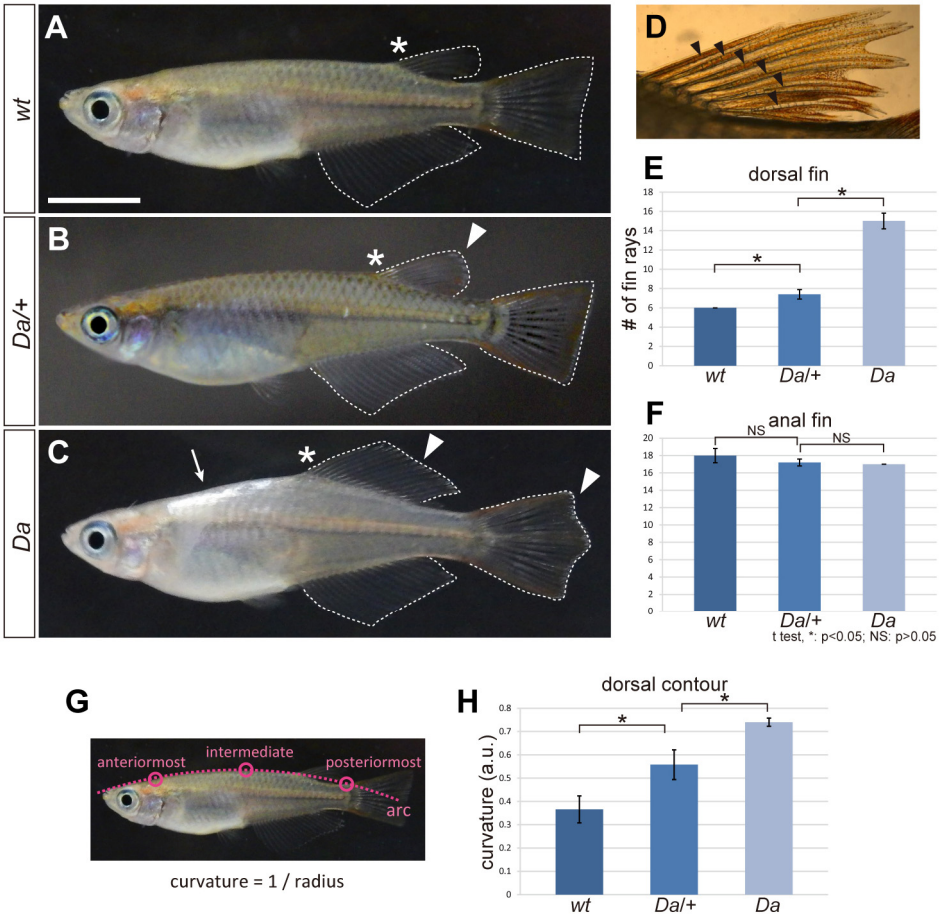

Fig. 17. Quantification of the phenotypes of wt and heterozygous and homozygous *Da* mutants.

(A-C) Lateral view of *wt* (A) and heterozygous (*Da*/+, B) and homozygous (C) *Da* mutant males. The heterozygous *Da* mutant exhibits the *wt* phenotypes in the pigmentation pattern and caudal fin skeleton. However, the size of the dorsal fin shows intermediate between that of *wt* and *Da* (asterisks). Asterisks indicate the anterior limit of the dorsal fins.

(D) Fin rays can be observed in the dorsal fin (arrowheads).

(E, F) Numbers of the dorsal (E) and anal (F) fins in the three lines.

(G) Dorsal body contour is measured by approximating with an arc by using the three landmark points (pink), i.e. the posterior end of the head, the anterior limit of the caudal fin, and the intermediate point between the two points along the AP axis.

(H) Dorsal curvatures of the three lines.

Asterisks means $p < 0.05$ in Student's t test. Scale bar equals 1 cm.

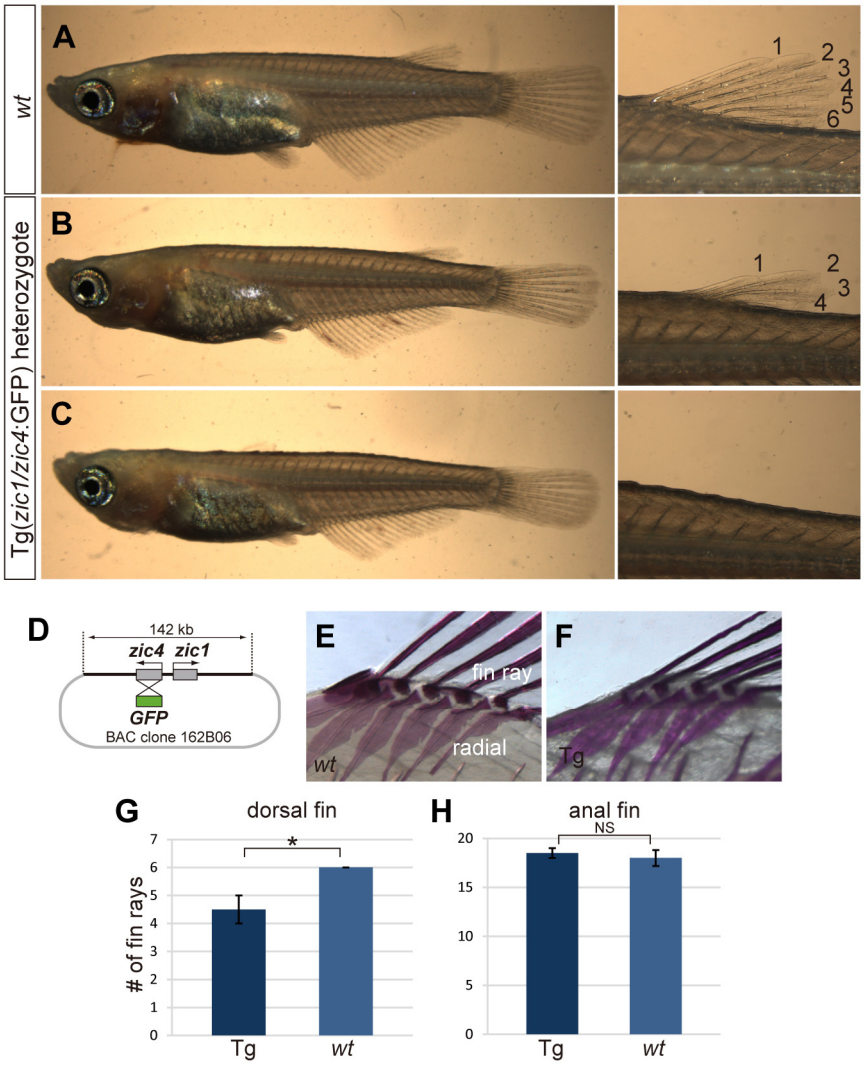

Fig. 18. Phenotypes of the *zic1*-overexpressing lines.

(A-C) Tg(*zic1/zic4*:GFP) exhibit reduction of the dorsal fin size. The degree is varied among individuals (B, C). The numbers of fin rays are counted on the right panels.

(D) BAC construct used for making Tg(*zic1/zic4*:GFP).

(E, F) Alizarin Red staining of *wt* and Tg(*zic1/zic4*:GFP) male adults.

(G, H) Numbers of the dorsal (G) and anal (H) fin rays in the two lines.

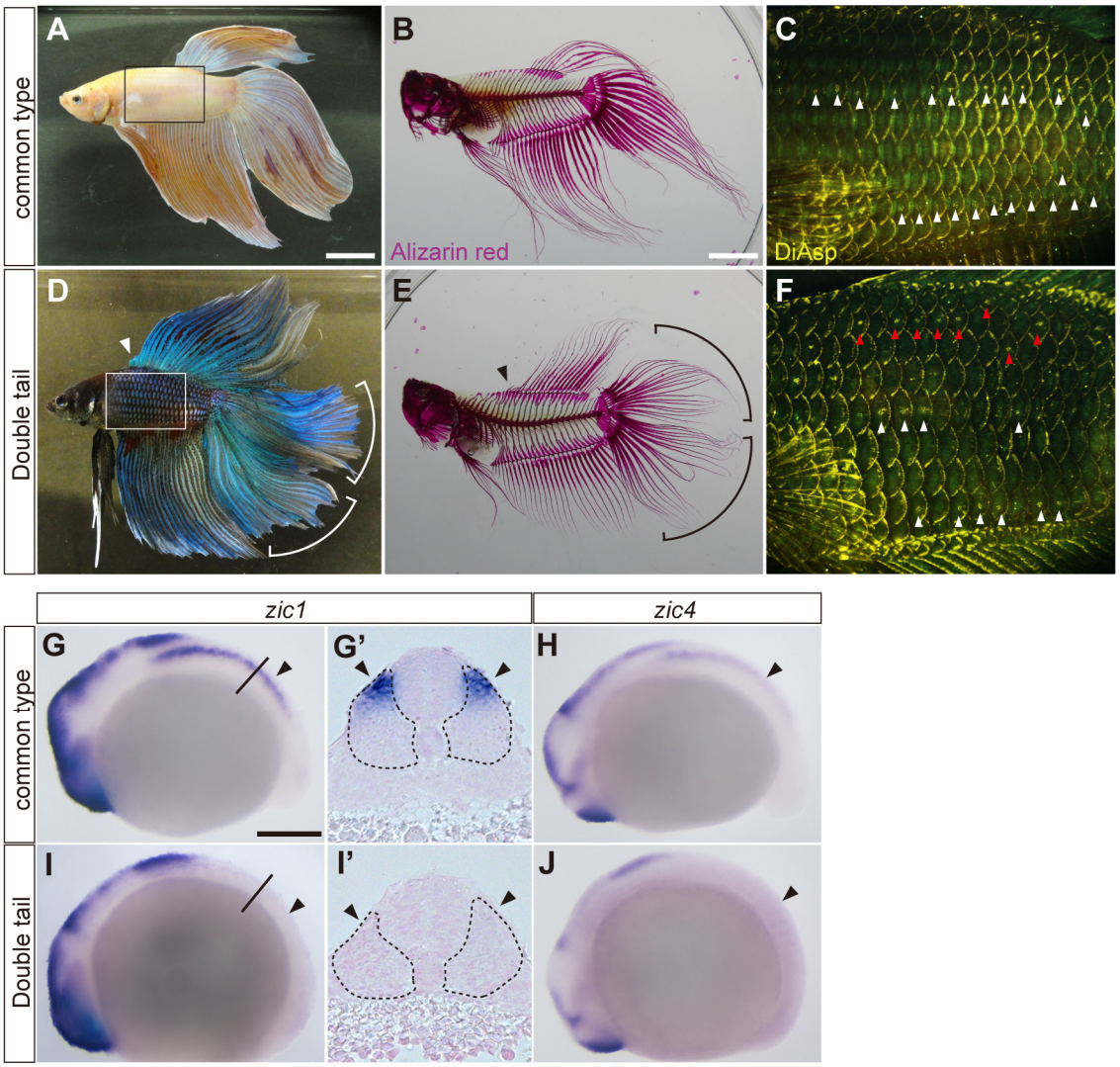

Fig. 19. *Betta splendens* and its *zic1* and *zic4* expression pattern.

(A-F) Common-type (A-C) and Double-tail (D-F) adult male *B. splendens*. Skeletal staining with Alizarin red (B and E) shows the fin positioning and morphology. The Double-tail variant has an anteriorly expanded dorsal fin [arrowheads in (D) and (E)] resembling an anal fin and dual caudal fin lobes [brackets in (D) and (E)]. Staining with 0.05 mg/ml DiAsp [(C) and (F); corresponding to the boxes in (A) and (D), respectively] reveals the ectopic deposition of neuromasts (red arrowheads) on the dorsal side in addition to the lateral and ventral sides [white arrowheads in (C) and (F)]. Anterior is to the left.

(G-J) Expression pattern of *zic1* and *zic4* in common-type and Double-tail *B. splendens* during embryogenesis (12 somites). The dashed lines in (G', I') delineate somites in transverse sections at the level indicated by black lines in (G, I), respectively. Arrowheads indicate the somites. *zic1* and *zic4* expression in the somites [arrowheads in (G) and (H)] are absent in Double-tail embryos (I and J). Scale bars equal 2 cm for (A), (B); 200 µm for (G).

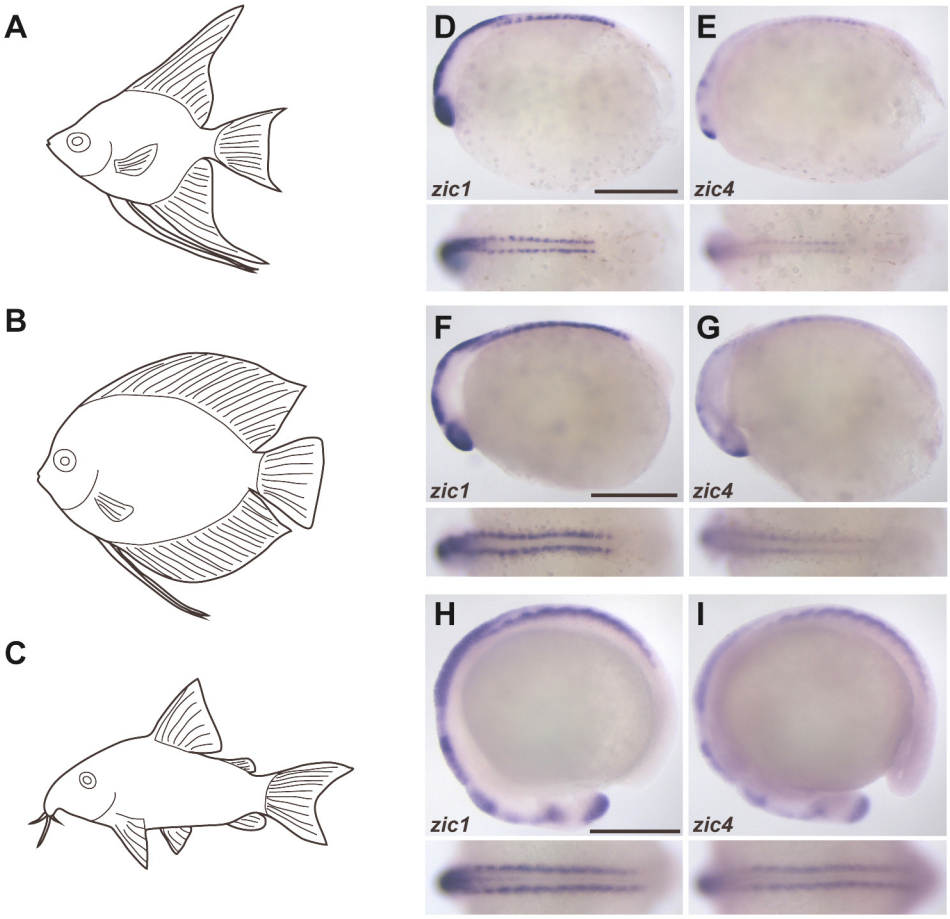

Fig. 20. Expression patterns of *zic1/zic4* in angelfish, discus and catfish.
(A-C) Adult fish appearance. Angelfish (A), discus (B) and catfish (C) are shown.
(D-I) WISH results of angelfish (D, E), discus (F, G) and catfish (H, I) embryos at somitogenesis stage (12-20 somites). In all embryos, the dorsal expression of *zic1/zic4* is observed. Scale bars equal 500 μm.

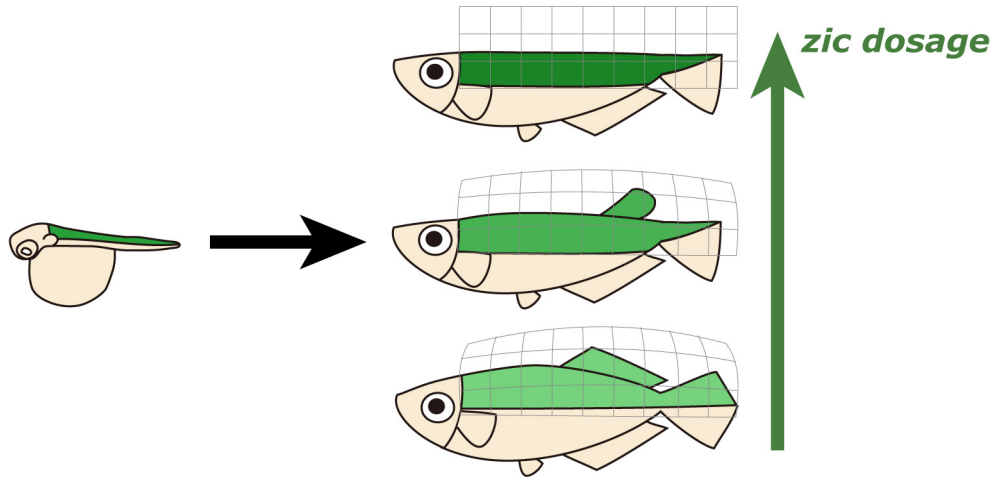

Fig. 21. Model for variation of dorsal trunk structures mediated by *zic1/zic4*.
The expression level of *zic1/zic4* in somites in late development affects the multiple dorsal trunk structures in a dosage-dependent manner, possibly contributing to diversification of trunk morphologies within species. The *zic1/zic4* expression level could be a parameter of D'Arcy Thompson's spatial transformation for explaining the variety of gross dorsal trunk morphology within species (gray meshwork).

References

Abe, G., Ide, H. and Tamura, K. (2007) Function of FGF signaling in the developmental process of the median fin fold in zebrafish, *Dev Biol* 304(1): 355-66.

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C. and De Robertis, E. M. (2000) Endodermal Nodal-related signals and mesoderm induction in *Xenopus*, *Development* 127(6): 1173-83.

Ahmed, M. U., Cheng, L. and Dietrich, S. (2006) Establishment of the epaxial-hypaxial boundary in the avian myotome, *Dev Dyn* 235(7): 1884-94.

Aoyama, H. and Asamoto, K. (1987) Do the fate of somite cells change when their axes are rotated?, *Dev Growth Differ* 29: 411.

Aoyama, H. and Asamoto, K. (1988) Determination of somite cells: independence of cell differentiation and morphogenesis, *Development* 104(1): 15-28.

Aruga, J. (2004) The role of *Zic* genes in neural development, *Mol Cell Neurosci* 26(2): 205-21.

Aruga, J., Mizugishi, K., Koseki, H., Imai, K., Balling, R., Noda, T. and Mikoshiba, K. (1999) *Zic1* regulates the patterning of vertebral arches in cooperation with *Gli3*, *Mech Dev* 89(1-2): 141-50.

Aruga, J., Tohmonda, T., Homma, S. and Mikoshiba, K. (2002) *Zic1* promotes the expansion of dorsal neural progenitors in spinal cord by inhibiting neuronal differentiation, *Dev Biol* 244(2): 329-41.

Blair, S. S. (1995) Compartments and appendage development in *Drosophila*, *Bioessays* 17(4): 299-309.

Bolker, J. A. (2000) Modularity in development and why it matters to evo-devo, *Am Zool* 40: 770-776.

Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C. (1995) *Hox* genes and the evolution of vertebrate axial morphology, *Development* 121(2): 333-46.

Cheng, L., Alvares, L. E., Ahmed, M. U., El-Hanfy, A. S. and Dietrich, S. (2004) The epaxial-hypaxial subdivision of the avian somite, *Dev Biol* 274(2): 348-69.

Cole, N. J. and Currie, P. D. (2007) Insights from sharks: evolutionary and developmental models of fin development, *Dev Dyn* 236(9): 2421-31.

De Robertis, E. M. (2006) Spemann's organizer and self-regulation in amphibian embryos, *Nat Rev Mol Cell Biol* 7(4): 296-302.

Dick, A., Meier, A. and Hammerschmidt, M. (1999) Smad1 and Smad5 have distinct roles during dorsoventral patterning of the zebrafish embryo, *Dev Dyn* 216(3): 285-98.

DiNardo, S., Kuner, J. M., Theis, J. and O'Farrell, P. H. (1985) Development of embryonic

pattern in *D. melanogaster* as revealed by accumulation of the nuclear engrailed protein, *Cell* 43(1): 59-69.

Elsen, G. E., Choi, L. Y., Millen, K. J., Grinblat, Y. and Prince, V. E. (2008) Zic1 and Zic4 regulate zebrafish roof plate specification and hindbrain ventricle morphogenesis, *Dev Biol* 314(2): 376-92.

Freitas, R., Zhang, G. and Cohn, M. J. (2006) Evidence that mechanisms of fin development evolved in the midline of early vertebrates., *Nature* 442: 1033-7.

Gammill, L. S., Gonzalez, C., Gu, C. and Bronner-Fraser, M. (2006) Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling, *Development* 133(1): 99-106.

Gamse, J. T. and Sive, H. (2001) Early anteroposterior division of the presumptive neurectoderm in *Xenopus*, *Mech Dev* 104(1-2): 21-36.

Garcia-Bellido, A. and Santamaria, P. (1972) Developmental analysis of the wing disc in the mutant engrailed of Drosophila melanogaster, *Genetics* 72(1): 87-104.

Garriock, R. J., D'Agostino, S. L., Pilcher, K. C. and Krieg, P. A. (2005) Wnt11-R, a protein closely related to mammalian Wnt11, is required for heart morphogenesis in *Xenopus*, *Dev Biol* 279(1): 179-92.

Garriock, R. J. and Krieg, P. A. (2007) Wnt11-R signaling regulates a calcium sensitive EMT event essential for dorsal fin development of *Xenopus*, *Dev Biol* 304(1): 127-40.

Gellon, G. and McGinnis, W. (1998) Shaping animal body plans in development and evolution by modulation of *Hox* expression patterns, *Bioessays* 20(2): 116-25.

Gonzalez, E. M., Fekany-Lee, K., Carmany-Rampey, A., Erter, C., Topczewski, J., Wright, C. V. and Solnica-Krezel, L. (2000) Head and trunk in zebrafish arise via coinhibition of BMP signaling by *bozozok* and *chordino*, *Genes Dev* 14(24): 3087-92.

Grinberg, I. and Millen, K. J. (2005) The ZIC gene family in development and disease, *Clinical genetics* 67(4): 290-6.

Grinberg, I., Northrup, H., Ardinger, H., Prasad, C., Dobyns, W. B. and Millen, K. J. (2004) Heterozygous deletion of the linked genes *ZIC1* and *ZIC4* is involved in Dandy-Walker malformation, *Nature Genetics* 36(10): 1053-5.

Grinblat, Y., Gamse, J., Patel, M. and Sive, H. (1998) Determination of the zebrafish forebrain: induction and patterning, *Development* 125(22): 4403-16.

Haas, T. C., Blum, M. J. and Heins, D. C. (2010) Morphological responses of a stream fish to water impoundment, *Biol Lett* 6(6): 803-6.

Haines, L., Neyt, C., Gautier, P., Keenan, D. G., Bryson-Richardson, R. J., Hollway, G. E., Cole, N. J. and Currie, P. D. (2004) Met and Hgf signaling controls hypaxial muscle and lateral line development in the zebrafish, *Development* 131(19): 4857-69.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquie, O. (1997) Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning, *Development* 124(22): 4605-14.

Ishikawa, Y. (1990) Development of caudal structures of a morphogenetic mutant (*Da*) in the teleost fish, medaka (*Oryzias latipes*), *J Morphol* 205: 219-232.

Iwamatsu, T. (2004) Stages of normal development in the medaka *Oryzias latipes*, *Mech Dev* 121(7-8): 605-18.

Janvier, P. (1996) *Early vertebrates*, Oxford: Clarendon Press.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996) Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene, *Nature* 382(6587): 133-8.

Kirschner, M. and Gerhart, J. (1998) Evolvability, *Proc Natl Acad Sci U S A* 95(15): 8420-7.

Komura, J., Mitani, H. and Shima, A. (1988) Fish cell culture: Establishment of two fibroblast-like cell lines (OL-17 and OL-32) from fins of the medaka, *Oryzias latipes*, *In Vitro Cellular & Developmental Biology* 24(4): 294-298.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H. and Takada, S. (2005) Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos, *Developmental Cell* 8(4): 587-98.

Kuratani, S. (2009) Modularity, comparative embryology and evo-devo: developmental dissection of evolving body plans, *Dev Biol* 332(1): 61-9.

Le Douarin, N. and Kalcheim, C. (1999) *The Neural Crest*, Cambridge: Cambridge University Press.

Lewis, E. B. (1978) A gene complex controlling segmentation in *Drosophila*, *Nature* 276: 565-570.

Mabee, P. M., Crotwell, P. L., Bird, N. C. and Burke, A. C. (2002) Evolution of median fin modules in the axial skeleton of fishes, *J Exp Zool* 294(2): 77-90.

Marcelle, C., Stark, M. R. and Bronner-Fraser, M. (1997) Coordinate actions of BMPs, Wnts, Shh and Noggin mediate patterning of the dorsal somite, *Development* 124(20): 3955-63.

McGuigan, K., Franklin, C. E., Moritz, C. and Blows, M. W. (2003) Adaptation of rainbow fish to lake and stream habitats, *Evolution* 57(1): 104-18.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L. and Moon, R. T. (1999) Establishment of the dorsal-ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of Dishevelled that is dependent on cortical rotation, *The Journal of cell biology* 146(2): 427-37.

Mise, N. and Wakahara, M. (1994) Dorsoventral polarization and formation of dorsal axial structures in *Xenopus laevis*: analyses using UV irradiation of the full-grown oocyte and after

fertilization, *Int J Dev Biol* 38(3): 447-53.

Moriyama, Y., Kawanishi, T., Nakamura, R., Tsukahara, T., Sumiyama, K., Suster, M. L., Kawakami, K., Toyoda, A., Fujiyama, A., Yasuoka, Y., Nagao, Y., Sawatari, E., Shimizu, A., Wakamatsu, Y., Hibi, M., Taira, M., Okabe, M., Naruse, K., Hashimoto, H., Shimada, A. and Takeda, H. (2012) The medaka *zic1/zic4* mutant provides molecular insights into teleost caudal fin evolution, *Curr Biol* 22(7): 601-7.

Nagai, T., Aruga, J., Takada, S., Gunther, T., Sporle, R., Schughart, K. and Mikoshiba, K. (1997) The expression of the mouse Zic1, Zic2, and Zic3 gene suggests an essential role for Zic genes in body pattern formation, *Dev Biol* 182(2): 299-313.

Nakamura, S., Saito, D. and Tanaka, M. (2008) Generation of transgenic medaka using modified bacterial artificial chromosome, *Dev Growth Differ* 50(6): 415-9.

Nyholm, M. K., Wu, S. F., Dorsky, R. I. and Grinblat, Y. (2007) The zebrafish *zic2a-zic5* gene pair acts downstream of canonical Wnt signaling to control cell proliferation in the developing tectum, *Development* 134(4): 735-46.

Oelgeschlager, M., Larrain, J., Geissert, D. and De Robertis, E. M. (2000) The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling, *Nature* 405(6788): 757-63.

Ohtsuka, M., Kikuchi, N., Yokoi, H., Kinoshita, M., Wakamatsu, Y., Ozato, K., Takeda, H., Inoko, H. and Kimura, M. (2004) Possible roles of *zic1* and *zic4*, identified within the medaka *Double anal fin* (*Da*) locus, in dorsoventral patterning of the trunk-tail region (related to phenotypes of the *Da* mutant), *Mech Dev* 121(7-8): 873-82.

Olivera-Martinez, I., Missier, S., Fraboulet, S., Thelu, J. and Dhouailly, D. (2002) Differential regulation of the chick dorsal thoracic dermal progenitors from the medial dermomyotome, *Development* 129(20): 4763-72.

Ordahl, C. P. and Le Douarin, N. M. (1992) Two myogenic lineages within the developing somite, *Development* 114(2): 339-53.

Pan, H., Gustafsson, M. K., Aruga, J., Tiedken, J. J., Chen, J. C. and Emerson, C. P., Jr. (2011) A role for Zic1 and Zic2 in *Myf5* regulation and somite myogenesis, *Dev Biol* 351(1): 120-7.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996) Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4, *Cell* 86(4): 589-98.

Pourquie, O., Coltey, M., Teillet, M. A., Ordahl, C. and Le Douarin, N. M. (1993) Control of dorsoventral patterning of somitic derivatives by notochord and floor plate, *Proc Natl Acad Sci U S A* 90(11): 5242-6.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West,

P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996) Lateral and axial signals involved in avian somite patterning: a role for BMP4, *Cell* 84(3): 461-71.

Prud'homme, B., Minervino, C., Hocine, M., Cande, J. D., Aouane, A., Dufour, H. D., Kassner, V. A. and Gompel, N. (2011) Body plan innovation in treehoppers through the evolution of an extra wing-like appendage, *Nature* 473(7345): 83-6.

Raff, R. A. (1996) *The shape of life*, Chicago: Univ. of Chicago Pr.

Rembold, M., Lahiri, K., Foulkes, N. S. and Wittbrodt, J. (2006) Transgenesis in fish: efficient selection of transgenic fish by co-injection with a fluorescent reporter construct, *Nat Protoc* 1(3): 1133-9.

Rinn, J. L., Wang, J. K., Allen, N., Brugmann, S. A., Mikels, A. J., Liu, H., Ridky, T. W., Stadler, H. S., Nusse, R., Helms, J. A. and Chang, H. Y. (2008) A dermal *HOX* transcriptional program regulates site-specific epidermal fate, *Genes Dev* 22(3): 303-7.

Romer, A. S. and Parsons, T. (1986) *The vertebrate body*: Saunders Company.

Schier, A. F. (2001) Axis formation and patterning in zebrafish, *Curr Opin Genet Dev* 11(4): 393-404.

Schier, A. F. and Talbot, W. S. (2005) Molecular genetics of axis formation in zebrafish, *Annu Rev Genet* 39: 561-613.

Schlosser, G. and Wagner, G. P. (2004) *Modularity in development and evolution*, Chicago and London: The University of Chicago Press.

Selleck, M. A. and Stern, C. D. (1991) Fate mapping and cell lineage analysis of Hensen's node in the chick embryo, *Development* 112(2): 615-26.

Shimada, A., Kawanishi, T., Kaneko, T., Yoshihara, H., Yano, T., Inohaya, K., Kinoshita, M., Kamei, Y., Tamura, K. and Takeda, H. (2013) Trunk exoskeleton in teleosts is mesodermal in origin, *Nat Commun* 4: 1639.

Spemann, H. and Mangold, H. (1924) Ueber Inducktion von Embryonalanlagen durch Implantation artfremder Organisatoren, *Roux' Arch. Entwicklungsmech.* 100: 599-638.

Sun Rhodes, L. S. and Merzdorf, C. S. (2006) The *zic1* gene is expressed in chick somites but not in migratory neural crest., *Gene Expr Patterns* 6: 539-45.

Svetic, V., Hollway, G. E., Elworthy, S., Chipperfield, T. R., Davison, C., Adams, R. J., Eisen, J. S., Ingham, P. W., Currie, P. D. and Kelsh, R. N. (2007) Sdf1a patterns zebrafish melanophores and links the somite and melanophore pattern defects in *choker* mutants, *Development* 134(5): 1011-22.

Takamatsu, N., Kurosawa, G., Takahashi, M., Inokuma, R., Tanaka, M., Kanamori, A. and Hori, H. (2007) Duplicated *Abd-B* class genes in medaka *hoxAa* and *hoxAb* clusters exhibit differential expression patterns in pectoral fin buds, *Dev Genes Evol* 217(4): 263-73.

Takaoka, K., Yamamoto, M. and Hamada, H. (2007) Origin of body axes in the mouse

embryo, *Curr Opin Genet Dev* 17(4): 344-50.

Takashima, S., Shimada, A., Kobayashi, D., Yokoi, H., Narita, T., Jindo, T., Kage, T., Kitagawa, T., Kimura, T., Sekimizu, K., Miyake, A., Setiamarga, D. H., Murakami, R., Tsuda, S., Ooki, S., Kakihara, K., Hojo, M., Naruse, K., Mitani, H., Shima, A., Ishikawa, Y., Araki, K., Saga, Y. and Takeda, H. (2007) Phenotypic analysis of a novel *chordin* mutant in medaka, *Dev Dyn* 236(8): 2298-310.

Takeda, H. and Shimada, A. (2010) The art of medaka genetics and genomics: what makes them so unique?, *Annu Rev Genet* 44: 217-41.

Tamiya, G., Wakamatsu, Y. and Ozato, K. (1997) An embryological study of ventralization of dorsal structures in the tail of medaka (*Oryzias latipes*) *Da* mutants, *Dev Growth Differ* 39(4): 531-8.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005) Maternal *wnt11* activates the canonical wnt signaling pathway required for axis formation in *Xenopus* embryos, *Cell* 120(6): 857-71.

Thompson, D. W. (1917) *On Growth and Form*, Cambridge: Cambridge Univ. Press.

Tomita, H. (1969) On the new mutants in body color and fins of the medaka, *Zool Mag* 78: 58.

Tomita, H. (1975) Mutant genes in the medaka. in T. Yamamoto (ed.) *Medaka (Killifish) Biology and Strains*. Tokyo, Japan: Keigaku.

Tomita, H. (1992) The lists of the mutants and strains of the medaka, common gambusia, silver crucian carp, goldfish, and golden venus fish maintained in the Laboratory of Freshwater Fish Stocks, Nagoya University, *The Fish Biology Journal MEDAKA* 4: 45-47.

Tonegawa, A., Funayama, N., Ueno, N. and Takahashi, Y. (1997) Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4, *Development* 124(10): 1975-84.

Tonegawa, A. and Takahashi, Y. (1998) Somitogenesis controlled by Noggin, *Dev Biol* 202(2): 172-82.

Tosney, K. W. (2004) Long-distance cue from emerging dermis stimulates neural crest melanoblast migration, *Dev Dyn* 229(1): 99-108.

Tran, L. D., Hino, H., Quach, H., Lim, S., Shindo, A., Mimori-Kiyosue, Y., Mione, M., Ueno, N., Winkler, C., Hibi, M. and Sampath, K. (2012) Dynamic microtubules at the vegetal cortex predict the embryonic axis in zebrafish., *Development* 139: 3644-52.

Tropepe, V., Li, S., Dickinson, A., Gamse, J. T. and Sive, H. L. (2006) Identification of a BMP inhibitor-responsive promoter module required for expression of the early neural gene *zic1*, *Dev Biol* 289(2): 517-29.

Vasiliauskas, D., Hancock, S. and Stern, C. D. (1999) SWiP-1: novel SOCS box containing WD-protein regulated by signalling centres and by Shh during development, *Mech Dev* 82(1-2):

79-94.

von Dassow, G. and Munro, E. (1999) Modularity in animal development and evolution: elements of a conceptual framework for EvoDevo, *J Exp Zool* 285(4): 307-25.

Wagner, G. P., Pavlicev, M. and Cheverud, J. M. (2007) The road to modularity, *Nat Rev Genet* 8(12): 921-31.

Wolpert, L. and Tickle, C. (2011) *Principles of Development*, Oxford: Oxford Univ. Press.

Wu, G. Y. and Cline, H. T. (1998) Stabilization of dendritic arbor structure in vivo by CaMKII, *Science* 279(5348): 222-6.

Yasutake, J., Inohaya, K. and Kudo, A. (2004) Twist functions in vertebral column formation in medaka, *Oryzias latipes*, *Mech Dev* 121(7-8): 883-94.

Yu, P. B., Hong, C. C., Sachidanandan, C., Babitt, J. L., Deng, D. Y., Hoyng, S. A., Lin, H. Y., Bloch, K. D. and Peterson, R. T. (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism, *Nat Chem Biol* 4(1): 33-41.

Yusuf, F. and Brand-Saberi, B. (2006) The eventful somite: patterning, fate determination and cell division in the somite, *Anat Embryol (Berl)* 211 Suppl 1: 21-30.

Acknowledgments

Here I would like to thank all the people who supported my research. First of all, I would like to express my deepest appreciation to my supervisor, Dr. Hiroyuki Takeda. I definitely owe my completion of the Ph.D. course to his substantial support and great patience in my research progress. His sincere attitude toward biology boosted my willing to do science more.

Besides my supervisor, I extend my gratitude to the other members of the committee, Drs. Hirokazu Tsukaya, Hideki Endo, Koji Akasaka and Masanori Taira, for constructive advice and encouraging comments to my doctoral thesis.

I would also like to thank Dr. Masato Kinoshita for providing a wonderful Tg line Tg(β-*actin*:DsRed), without which I could not have deepened my research to this extent. I thank Drs. Tohru Suzuki, Hayato Yokoi and Keiji Inohaya for providing plasmids for RNA probes. I also appreciate their help of obtaining fish embryos to Drs. Daisuke Kurokawa and Ryuji Toyoizumi.

Throughout my graduate period, I have been deeply indebted to Dr. Atsuko Shimada, who always assisted me with in-depth discussions and encouragements. Without her persistent help, this thesis would not have been materialized. Also, my thankful acknowledgment goes to Dr. Tatsuya Tsukahara and other members of Takeda Lab for providing opportunities for exciting daily discussions and helpful feedback to my research. I am especially grateful to Ms. Yasuko Ozawa for her exquisite fish care.

Finally, I dedicate my doctoral thesis to my parents, who gave me a chance to attend the Ph.D. course and have supported me with great patience all the time.