Modular development of the teleost trunk along the dorsoventral axis and zic1/zic4 as selector genes in the dorsal module

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SUMMARY
Teleost fish exhibit remarkable diversity in morphology, such as fins and coloration, particularly on the dorsal side. These structures are evolutionary adaptive because their back is highly visible to other individuals. However, owing 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, we have 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. We show that the dorsoventral polarity in Da somites is lost and then demonstrate using transplantation techniques that somites and their derived tissues globally determine the multiple dorsal-specific characteristics of the body (fin morphology and pigmentation) from embryo to adult. Intriguingly, the zic1/zic4 expression in the 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 further implies a central role for zic1/zic4 in morphological diversification of the teleost body. Taken together, we propose that the teleost trunk consists of dorsal/ventral developmental modules and that zic1/zic4 in somites function as selector genes in the dorsal module to regulate multiple dorsal morphologies.

KEY WORDS: Dorsoventral patterning, Modularity, Somite, Zic, Oryzias latipes

INTRODUCTION
Vertebrates display diverse morphology and coloration, especially on the dorsal side. For example, 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, that allow them to assimilate themselves into their surrounding environment. Developmental biologists have long sought the mechanisms that produce such dorsoventrally asymmetric patterns, and have revealed that molecular gradients of proteins, such as BMPs, in early development provide the initial cue for dorsoventral (DV) pattern formation (Gilbert, 2010). However, as the above DV structures become evident in much later development and related developmental mutants are few, it is still largely unknown what genetic mechanism underlies the DV surface patterning observed in late development.

In general, dorsal structures in the vertebrate trunk are diverse, whereas ventral counterparts are relatively conserved. This is reminiscent of the concept of modularity. Modules of development are, by definition, quasi-independent developmental units, and can be recognized at various levels ranging from gene networks to large domains in the body (Schlosser and Wanner, 2004). The primary anatomical modules of developing embryos include cell populations, organs and segments, and they behave to some 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 (Bolker, 2000; Kirschner and Gerhart, 1998; Kuratani, 2009). This is best manifested in segments of insect bodies; during development, each segment develops in an independent manner that is dictated by a special class of transcription factors, known as selector genes (Blair, 1995; Kim et al., 1996; Lewis, 1978). The independence of development has allowed the generation of 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 (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, which demonstrates the existence of developmental modules that constitute the animal body (Gellon and McGinnis, 1998; von Dassow and Munro, 1999). Like anteroposterior (AP) specification in insect bodies, modular mechanisms could also operate along the DV axis during vertebrate development, but no clear evidence supporting this idea is available.

To examine these modular mechanisms, we have analyzed the medaka spontaneous mutant Double anal fin (Da), which exhibits a unique ventralized phenotype on its surface from the larval to adult stages (Fig. 1A,B; supplementary material Fig. S1) (Ishikawa, 1990; Ohitsu et al., 2004; Tamiya et al., 1997; Tomita, 1975). The dorsal fin of homozygous mutant adults resembles the anal fin (Fig. 1B,
transplantation techniques that the teleost trunk consists of the two cells) through tissue interactions during late stages of development. Ectodermal derivatives (external organs such as fins and pigment that Zic1/Zic4 in somites participate in dorsal patterning of is decreased in the specification (Agius et al., 2000; Schier and Talbot, 2005). A patterning mechanism that acts after well-studied early DV segmentation stages, and the positioning of internal organs is essentially no defects are observed from cleavage to early image of the ventral half across the lateral midline. Importantly, Fig. S1M). Hence, the dorsal half of the trunk appears to be a mirror shape, instead of a dorsally flattened one (supplementary material Fig. S1G-L). Furthermore, they exhibit a teardrop body shape. (Fig. 1B, arrow; supplementary material Fig. S1M). The medaka mutant exhibits a ventralized pigmentation (arrow) and median fin morphology white arrowhead), and distribution of pigments and lateral lines in the dorsal trunk is ventralized (Fig. 1B, arrow; supplementary material Fig. S1G-L). Furthermore, they exhibit a teardrop body shape, instead of a dorsally flattened one (supplementary material Fig. S1M). 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, suggesting the presence of an as yet unaddressed late patterning mechanism that acts after well-studied early DV specification (Agui 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 discovered in a wild population in the 1960s, and our recent analysis has demonstrated that Da is a mutant for zic1 and zic4 genes (zic1/zic4) (Moriyama et al., 2012), which are arranged head to head in the genome and expressed in a nearly identical pattern, although zic1 expression is stronger. In the Da mutant, a transposon insertion disrupts a transcriptional regulatory region(s) shared by the two genes (Moriyama et al., 2012). Indeed it was briefly reported that the zic1/zic4 expression in dorsal somites is decreased in the Da mutant while expression in neural tissues is less affected (Ohtsuka et al., 2004). These facts led us to hypothesize that Zic1/Zic4 in somites participate in dorsal patterning of ectodermal derivatives (external organs such as fins and pigment cells) through tissue interactions during late stages of development.

In our current study, we show using transgenic and tissue-transplantation techniques that the teleost trunk consists of the two distinct anatomical modules, dorsal and ventral, that are defined by persistent zic1/zic4 expression in somites and their derivatives and that zic1/zic4 function as selector genes in the dorsal module. We propose that zic1/zic4 in somites regulate late-emerging characteristics in the dorsal surface, through long-term mesodermal-ectodermal interactions.

**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 Nagoa University (Tomita, 1992). Kusu, HNI and d-RR stains were used as wild-type controls. Embryos were incubated at 28°C and staged as previously described (Iwamatsu, 2004). The common type and ‘Double-tail’ fighting fish (Betta splendens) strains were obtained from a commercial supplier in Tokyo, Japan.

**BAC modification by homologous recombination and transgenesis**

Homologous recombination of the BAC clone was performed as previously described (Moriyama et al., 2012; Nakamura et al., 2008). The generation of transgenic lines by BAC injection into embryos of the d-RR strain was used as wild-type controls. Embryos were incubated at 28°C and staged as previously described (Iwamatsu, 2004). The common type and ‘Double-tail’ fighting fish (Betta splendens) strains were obtained from a commercial supplier in Tokyo, Japan.

**Neuramist staining and whole-mount skeletal staining**

Neuramists were stained by 5-minute exposure to 0.05 mg/ml 4-(diethylaminostyryl)-N-methylpyridinium iodide (DiAsp, Sigma) dissolved
in Yamamoto’s Ringer solution. Whole-mount skeletal staining with Alizarin Red and Alcian Blue 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 and zic4 (Ohtsuka et al., 2004); myod and pax3 (Moriyama et al., 2012); twi (Yasutake et al., 2004); sim1 (primers 5′-CTGGGTTCCTTATTTACTGCAGACTCCGGGATGATGTTTTCTATAC-3′ and 5′-TTTGGGACTATATGTGCTGGGATGATAACGGTAACCTGTCTCAAAC-3′; wnt11r (primers 5′-CAATTAGGCACACTGTCCTCAACACTGC-3′ and 5′-CTATTGGCAAGCTTACGTCTTCACAC-3′); foxd3 (primers 5′-GATGACTTGGAGAGATGAAATCCG-3′ and 5′-ACACCCCGATGTTTCTTACAT-3′). For staining of Betta 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 antibodies (anti-GFP, Medical and Biological Laboratories or Clontech [JL-8]) were used at a 1:200 dilution. Biotin-conjugated anti-rabbit IgG (Sigma) was used as a secondary antibody at a 1:250 dilution.

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- to 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 anteroposterior level as the somites were isolated and kept in 10% fetal bovine serum until the dorsal surfaces of their 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 as described previously (Komura et al., 1988). Tissues were incubated at 27°C.

Quantitative PCR

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′-AGGCCCTTTTCCGTGTCGCCCTCC-3′ and 5′-CCACGCTGTGGACGTGATGCT-3′; zic4, 5′-AGAAGCCTGTTTCATGCCCCTG-3′ and 5′-TGCTTGGGAGACGGTCTGCTG-3′; β-actin, 5′-TGCCGACCTGTTGGTAGAACAAG-3′ and 5′-CCATGAACACCTGCTGTGCTG-3′.

RESULTS

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

First, we examined the effect of the Da mutation on somite development, as 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 wild-type embryos as previously described (Elsen et al., 2008; Ohtsuka et al., 2004) during the gastrulation stage (around stage 15; data not shown). After the onset of somitogenesis, the expression in wild-type embryos is detected in the dorsal neural tube (Fig. 1C,G, arrowheads; asterisks) and the dorsal part of somites (Fig. 1D,H, arrowheads), together with a slight decrease in the hindbrain expression (Fig. 1D,H, asterisks). We thus asked whether 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; Ohtsuka et al., 2004; Tamiya et al., 1997). As expected, we found that somites in Da mutants are ventralized, as indicated by the dorsal expansion of sim1, a ventral dermomyotome marker (Pourquié et al., 1996) (Fig. 1I,J). Furthermore, the expression of wnt11r, which is expressed in the dorsal part of the wild-type somites (Garriock et al., 2005; Garriock and Krieg, 2007; Olivera-Martinez et al., 2002), was reduced in Da mutant somites (Fig. 1K,L). These data indicate that the dorsal characteristics of Da mutant somites are lost 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. 1O,P); 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. 1M,N). This change in myotome shape could account for the teardrop 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, we adopted tissue transplantation techniques (Haines et al., 2004) (Fig. 2A; see also supplementary material Movie 1). We 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, we homotopically replaced two consecutive posterior-most Da mutant somites with wild-type somites at stage 24 [2 days post-fertilization (dpf); 14- to 16-somite stage], and examined the effects on the external phenotypes at stage 39 (7 dpf; larval stage), when the earliest two phenotypes can be clearly observed in the Da mutant (Tamiya et al., 1997). At stage 39, the trunk of wild-type 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. 2B,C). These two lateral alignments of melanophores are identical to those on the ventral side. Likewise, the shape of the dorsal finfold is transformed into a ventral type in Da mutant larvae (Fig. 2G,H); the anterior limit of the wild-type dorsal finfold is positioned seven somites posterior to that of the ventral finfold, whereas in Da mutant embryos, it is shifted anteriorly towards the position of the ventral finfold.

In Da mutants transplanted with DsRed-labeled wild-type somites, the position of the melanophores was shifted towards the midline on the operated side (Fig. 2D; n=29/30). This effect was restricted to the transplanted tissues expressing DsRed. Thus, the positioning of the melanophores was locally rescued by wild-type somites. Similarly, a rescue of the dorsal finfold shape was observed in the area of the transplanted wild-type somites; the protrusion of the dorsal finfold was suppressed, resulting in a posterior shift of the dorsal finfold (Fig. 2I; n=16/21). These rescued phenotypes were never observed when the control Da mutant somites were transplanted into Da mutant hosts, 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 finfold mesenchyme in the trunk (Le Douarin and Kalcheim, 1999). However, several lines of evidence argued against their contribution to the phenotype rescue (Fig. 2F,K; supplementary material Fig. S2A-F). One is that homotopically transplanted wild-type neural tubes containing neural crest cells failed to rescue the melanophore pattern or finfold morphology in Da mutant hosts (Fig. 2F,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 (Fig. 2K, arrows and arrowheads, respectively).

We then extended our analysis of the rescued phenotypes to 4 weeks post-fertilization (wpf) because some of the Da external phenotypes appear late. The distribution pattern of the iridophores (silver pigment cells), which emerges at around 2-3 wpf at the level of the 3rd to 12th somite, was also rescued when we performed somite transplantation at stage 23 (10-12 somites); the ectopic dorsal iridophores on the Da mutant trunk was suppressed at the site of transplantation at 4 wpf (Fig. 2L,M). Moreover, the medial positioning of the melanophores remained unchanged (Fig. 2N,O; supplementary material Fig. S2G-I) and the anterior limit of the dorsal fin maintained its posteriorly shifted position, even when the dorsal finfold was replaced with an adult-type dorsal fin, containing fin rays, during metamorphosis (3 wpf; Fig. 2P,Q; supplementary material Fig. S2J-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 underlie the dorsal external organs (Fig. 2R-U’); the GFP-positive mesenchymal cells were found to gradually invade into the dorsal finfold at 7 dpf.
(Fig. 2R,S) and to become elongated along the proximodistal axis in the developing dorsal fin at the larval stage (Fig. 2T,T’, arrowheads). They also distributed just beneath the dorsal melanophores at 7 dpf (Fig. 2U,U’, arrowheads). These imply that the somite derivatives continue to function in external patterning throughout late development and growth.

Taken together, we 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 wild-type somites upon the external phenotypes in the Da hosts, zic1/zic4 could act throughout early to late DV patterning. We thus traced zic1/zic4 expression in wild-type 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 region (Fig. 3A), although, as development

![Fig. 3. zic1/zic4 define the dorsal domain of the trunk throughout life.](image-url)

(A) Expression patterns of zic1, zic4, myod, twist and pax3 in wild-type embryos at stage 27 (24 somites). Each somitic compartment (myotome, sclerotome or dermomyotome), defined by the expression of myod, twist or pax3, respectively, is depicted in the schematic. 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. (B) The BAC construct used for the transgenesis of Tg(zic1:GFP/zic4:DsRed). (C-G”) Fluorescent images of Tg(zic1:GFP/zic4:DsRed) transgenic line during embryogenesis (stage 27 (C) and stage 39 (D)) and at adult stages (E-G”). Arrowheads and dashed lines in C indicate somites and their dorsal and ventral boundaries, respectively. Arrowheads in D-E” indicate the ventral boundary of GFP and DsRed expression, showing a linear boundary along the AP axis. (F-F”) Transverse sections of the adult transgenic medaka at the level indicated by a dashed line in E reveal an expression boundary shared by the myotome and the vertebrae at the almost same DV level. (G,G”) Lateral views of the adult transgenic vertebrae demonstrate the DV boundary by GFP and DsRed expression (F”,G”) Bright-field images of the same samples as in F,G, respectively. Arrowheads in F”G” indicate the ventral boundary. Asterisks in E,F,F” indicate autofluorescence of pigment cells (leucophores). (H,H”) Histological section of the transgenic medaka adult stained with an anti-GFP antibody shows that the ventral boundary of the GFP domain in the myotome (arrowheads) and the vertebrae (arrows) almost corresponds to the level of the horizontal myoseptum (arrowheads). (H,F”) Magnified view of the rectangular region in H. (I,F”) The dermis also shows a clear dorsal domain of GFP expression. Transgenic adult fish were stained with the anti-GFP antibody after removing the scales that had covered the dermis. The ventral boundary in the dermis (red arrow, the upper dermis above scales; black arrow, the lower dermis beneath scales) is placed near the horizontal myoseptum (arrowheads). In I’, the myotome was not stained because of the lack of antibody penetration when the staining was performed on an unsectioned whole sample. (J,K) Expression analysis of zic1/zic4 at the adult stage by quantitative PCR. The expression levels of zic1 (J) and zic4 (K) in the dorsal (white) or ventral (black) region of the myotome, dermis and median fin (fin) were assessed, after normalized with the basal β-actin expression. The zic1/zic4 expression at the adult stage is high only in the dorsal region (P<0.05 in all six cases), consistent with the persistent GFP expression in the transgenic line Tg(zic1:GFP/zic4:DsRed). Error bars indicate s.d. Scale bars: 50 μm for A; 200 μm for C; 500 μm for D,G; 1 cm for E,E”. }
proceeds, the expression becomes weaker in the myotome compared with other derivatives. To further track the zic1/zic4-expressing cells for a longer period of development, we have generated transgenic medaka lines (Tg[zic1:GFP/zic4:DsRed]) by introducing a bacterial artificial chromosome (BAC) construct encoding zic1- and zic4-responsive reporter genes into wild-type medaka (Fig. 3B). 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 owing to the position effect. We 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. 3C,D). 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. 3E). Transverse sections revealed that the dorsal expression domain internally expands in the entire dermis, myotome and vertebra (Fig. 3F,G). We precisely determined the expression boundary by making histological sections stained with anti-EGFP antibodies (Fig. 3H,H') and found that the expression boundary in the myotome morphologically corresponds to the horizontal myoseptum (Fig. 3H', arrowheads), which separates the myotome into the prospective epaxial and hypaxial muscles. However, no such histological landmark in the dermis and vertebra is observed (Fig. 3H') or has been reported. Intriguingly, the expression boundary lies at nearly the same DV level among the somite-derived tissues (Fig. 3H'). Quantitative PCR analyses confirmed that the GFP and DsRed expression pattern reflects that of the endogenous zic1/zic4 expression at the adult stage (Fig. 3J,K). From these, we 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

We next examined how the dorsal expression domain of zic1/zic4 is established and maintained. In wild-type 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 donor somites, which have begun to express zic1/zic4 at the time of transplantation, are re-specified by the surrounding tissues after transplantation. We confirmed this by examining reporter gene expression in somites transplanted from Tg(zic1:GFP/zic4:DsRed) to wild-type hosts (Fig. 4A). Five days after transplantation, they all acquired the dorsal expression of GFP (Fig. 4B; n=20/20). The dorsal expression of GFP in transplants was maintained until adulthood (Fig. 4C). 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; Hirsinger et al., 1997; Marcelle et al., 1997; Pourquié et al., 1993; 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. We speculated that zic1/zic4 expression becomes less dependent on external signals as development proceeds. We tested this idea by in vitro culture of somite-derived cells at several time points of development, and examined whether they were able to maintain zic1/zic4 expression. For this analysis, we 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. We assumed that the DsRed expression driven by the zic4 promoter can be neglected owing to the relatively weak level of transcription compared with the β-actin promoter. We found that somite cells at the segmentation stage (2 dpf) lost GFP expression within 1 day of the onset of culture (Fig. 4D-E'), 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 (stage 30, 5 dpf), the expression lasted for at least 11 days in vitro (Fig. 4F-H'). We also confirmed this autonomy in fibroblasts taken from the dermis of transgenic adults Tg(zic1:GFP/zic4:DsRed) at least for 1 week, whereas no induction of GFP signals in those from a non-expressing ventral region (Fig. 4I-J'). 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, we 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 domain of the zic1/zic4 expression with robustness.

The function of zic1/zic4 is conserved among teleosts

Finally, we examined whether the zic-mediated dorsal patterning is a general mechanism across species. To achieve this, we searched for other species that have altered fin morphology similar to the Da mutant, and found that one variant of Betta (Betta splendens; order Perciformes, native to Thailand), which has been established during domestication, met this criterion (Fig. 5A-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 are transformed into those of the anal fin (Fig. 5A,B,D,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), which leads to duplicated caudal fin lobes in this variant (Fig. 5A,B,D,E, brackets). The distribution of the lateral line is also ventralized (Fig. 5C,F, red arrowheads). We 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; however, zic1/zic4 expression is specifically lost in Double-tail somites (Fig. 5G-J), suggesting the conserved function of Zic1/Zic4 in somites in Betta surface patterning. We have not addressed further what causes the mesodermal loss of zic1/zic4 expression in ‘Double tail’, since genomic resources of the Betta genome, such as a draft genome and BAC library, are not available at the present. Collectively, we revealed that the function of the zic1/zic4 genes in somites is conserved among teleosts.
DISCUSSION
A novel late patterning mechanism centered by Zic in somites
Members of the Zic gene family are known to play crucial 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; Elsen et al., 2008; Grinberg et al., 2004). 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 study, we 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. Supporting the idea of local interaction, we 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, are 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 cooperation of the epidermis, dermis and neural crest cells. Indeed, our recent lineage analysis combined with tissue transplantation reveals that fin rays and most mesenchymal cells are derived from the somite, whereas neural crest cells mainly contribute to the nervous system in median fins (A.S., T.K., T.K., H. Yoshihara, T. Yano, K. Inohaya, M.K., Y. Kamei, K. Tamura and H.K., unpublished). Therefore, the surface pattern of the vertebrate trunk could be established through long-term actions of the somite-derived tissues patterned by Zic1/Zic4. As 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 finfold
in zebrafish can be modiﬁed by perturbing FGF signaling (Abe et
al., 2007). The FGF pathway could also be involved in deﬁning
the morphology of the ectodermal finfold via interaction with
somitic cells. This patterning mechanism could be conserved in
part in the vertebrate lineage, as the dorsal expression of theZIP
related genes is observed from lamprey to mouse (Gaston-Massuet
et al., 2005; Kusakabe et al., 2011; Nagai et al., 1997; Nakata et al.,
1998; Rohr et al., 1999).

The regulatory mechanism of zic1/zic4 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 (Hirsinger et al.,
1998; Rohr et al., 1999).

One of the important ﬁndings in the present study is the dorsal
domain deﬁned by the persistent zic1/zic4 expression. Previous
studies reported that the somite derivatives, including the myotome
and dermomyotome are subdivided along the dorsomedial-
ventrolateral axis in amniotes (Ordahl and Le Douarin, 1992;
Selleck and Stern, 1991), and that this subdivision is a result of
lineage separation of somitic cells (Ordahl and Le Douarin, 1992;
Selleck and Stern, 1991) and is regulated by signals emanating from
surrounding tissues (Cheng et al., 2004; Pourquié et al., 1993;
Tonegawa et al., 1997; Tonegawa and Takahashi, 1998; Vassiliauskas
et al., 1999). It is thus likely that the zic1/zic4-expressing domain
corresponds to the previously proposed dorsomedial (dorsal in ﬁsh)
domain. The present study demonstrated that zic1/zic4 are the
molecular entity of the dorsal domain and that the dorsal domain
further deﬁnes the patterns of ectodermal organs.

Recently, Rinn et al. reported that the embryonic Hox gene
pattern is epigenetically maintained in ﬁbroblasts of the human adult
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. However, the present study is the ﬁrst to visualize
persistent regionalization of the vertebrate adult body by live
imaging of transgenic ﬁsh; the somite-derived organs are found to
be dorsoventrally divided by almost linear borders across organ

![Fig. 5. Betta splendens and its zic1 and zic4 expression pattern. (A–F) Common type
(A–C) and Double-tail (D–F) adult male Betta splendens. Skeletal staining with Alizarin Red
(B,E) shows the ﬁn positioning and morphology. The Double-tail variant has an
anteriorly expanded dorsal ﬁn (arrowheads in D,E) resembling an anal ﬁn and dual caudal ﬁn
lobes (brackets in D,E). Staining with DiAsp (C,F; corresponding to the boxes in A,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,F). Anterior is towards
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
solid lines in G,I, respectively. Arrowheads
indicate the somites. zic1 expression in the
somites (arrowheads in G,H) is absent in
Double-tail embryos (IJ). Scale bars: 2 cm for
A,B,D,E; 200 μm for G,H,I, J.](image)
boundaries along the entire anteroposterior axis. 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 over a long period of life. Like Hox genes, epigenetic regulation of key developmental genes could be one of the mechanisms that assures such robustness. The autonomous maintenance of zic1/zic4 expression at later stages supports this idea.

The dorsal domain defined by the zic1/zic4 expression could be a developmental module because the loss of zic1/zic4 activity does not affect the ventral part of the trunk. As zic1/zic4 globally determine the fates of various organs on the dorsal side, they serve as selector genes in the dorsal module. Moreover, the module in the trunk is unique in that it consists of mesodermal and ectodermal components, and the former dictates the latter. At the moment, we do not know whether the trunk module forms a truly lineage-restricted compartment, especially for the dermis and vertebra, and the answer to this awaits long-term lineage tracing, which is still technically difficult in fish.

The modular construction of the animal body could promote diversification in forms and size during evolution; one module can adopt a novel phenotype without affecting the others (for a review, see Wagner et al., 2007). In general, vertebrates exhibit a variety of color patterns and structures on the dorsal side, whereas those on the ventral side are relatively conserved. This could be achieved through modular organization and recruitment of selector genes during adaptation to ever-changing environmental conditions. Changes in the activity of one or a few selector genes in each module could thus produce local morphological specification. The Da-type mutants in fish provide a good example or this.

The unique external phenotype of Da highlights the role of zic1/zic4 in adaptive speciation of teleosts. Da mutants exhibit a large dorsal fin and teardrop body shape, which are characteristic for fast-swimming middle-layer fish (such as tuna) rather than dorsally flattened fish with a small dorsal fin, slowly swimming near the surface (such as medaka). This drastic change in external shape is caused by a spontaneous transposon insertion in the cis-regulatory region of the zic1/zic4 genes (Moriyama et al., 2012). Furthermore, the Betta variant ‘Double tail’, which was found to lose zic1/zic4 expression in dorsal somites, exhibits a similar phenotype to the medaka Da mutant. These facts imply that zic1/zic4 are broadly involved in morphological diversification within and between species. In particular, unlike in amniotes, the somite derivatives in fish underlie the larger part of the body and thus have a greater impact on body morphology. In this context, the phenotype of heterozygous Da mutants with intermediate fin morphology is particularly interesting (supplementary material Fig. S3) as it suggests a dosage-dependent action of Zic1/Zic4 [like BMP and calmodulin signaling in the beaks of Darwin’s finches (Abzhanov et al., 2006; Abzhanov et al., 2004)]. Indeed, there is emerging evidence in other model organisms that morphological diversification and evolution proceed through mutations in the cis-regulatory sequences of developmental regulatory genes (Carroll, 2008; Prud’homme et al., 2007; Wray, 2007).

In summary, we propose a Zic-mediated late patterning mechanism and modular organization of the vertebrate trunk: the DV pattern of the trunk does not simply use the initial gradient information inherited from the early embryo, but is built by the binary information of Zic1/Zic4 in somites. This modularity may contribute to a great variety of dorsal structures seen among vertebrates. Elucidation of the gene network centering around zic1/zic4 and mechanisms underlying the maintenance of the zic expression boundary will definitely help understand this complicated process.

Acknowledgements

We thank D. Kurokawa and T. Urano for assistance with Betta splendens; R. Toyozumi for providing eggs; K. Inohaya for providing a plasmid; A. V. Terashiba and R. Behringer for critical reading of the manuscript. We are grateful to Y. Ozawa for fish care.

Funding

This work was supported by the Japan Society for the Promotion of Science (JSPS) [Grant-in-Aid for JSPS fellows grant 10J07483 to T. Kawanishi; KAKENHI grants 20247030 to H.T. and 21570219 to A.S.].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088567/-/DC1
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