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. Intriguingly, the zic1/zic4 expression 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. Moreover, 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. Intense analysis further implies a central role for zic1/zic4 in morphological diversification of the teleost trunk. 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 Wagner, 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; Ohtsuka 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 ectodermal derivatives (external organs such as fins and pigment that Zic1/Zic4 in somites participate in dorsal patterning of less affected (Ohtsuka et al., 2004). These facts led us to hypothesize is decreased in the patterning mechanism that acts after well-studied early DV normal, suggesting the presence of an as yet unaddressed late 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, the dorsal half of the trunk appears to be a mirror shape, instead of a dorsally flattened one (supplementary material Fig. S1M). Hence, the dorsal half of the trunk is ventralized (Fig. 1B, arrow; supplementary material Fig. S1G-L). Furthermore, they exhibit a teardrop body shape. Scale bars: 1 cm for A,B; 200 μm for M,N; 100 μm for C,D,G,H; 50 μm for E,F,J.

**Fig. 1. Ventralized phenotypes of Da mutants. (A,B) The Da medaka mutant exhibits a ventralized pigmentation (arrow) and median fin morphology (arrowheads), as well as a teardrop body shape. (C,D,G,H) Expression patterns of zic1 and zic4 in the wild-type (C,G) and Da mutant (D,H) medaka at stage 23 (12 somites). Arrowheads and asterisks indicate somites and neural tubes, respectively. (E,F) zic1 expression in transverse sections of wild-type (E) and Da mutant (F) embryos at stage 23 at the level of the solid lines in C,D. Dashed lines delineate the somites. (I,J) Expression pattern of sm1 in wild-type (I) and Da mutant (J) embryos at stage 27 (24 somites). Arrowheads indicate strong expression in the dorsal part of the trunk (arrow; 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 (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 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 Nagoya 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 performed as previously described (Nakamura et al., 2008). We used the I-SceI meganuclease method to increase the probability of successful germline 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-diethylaminostyryl)-N-methylpyridinium iodide (DiAsp, Sigma) dissolved...
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 (Morita et al., 2012); twi (Yasutake et al., 2004); sim1 (primers 5'-CTGGGTCTTCTATTACTGAGAC-3' and 5'-TTTGGGACATATAGTGGCGTAACTC-3'); wnt11r (primers 5'-CAATAGGACATAACAGTCTCGAATTCC-3' and 5'-CTATTGGCGACGAGTACAAGCTTCACC-3'); foxd3 (primers 5'-GATGACTTGGGAGATGAAATCG-3' and 5'-ACACCCCGATGTGTTCTTATAC-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 material Movie 1). We used transgenic medaka embryos ([beta-actin:DsRed] Tg([beta-actin:DsRed]) transplanted into wild-type embryos as previously described (Ohtsuka et al., 2004). The expression of zic1/zic4 is greatly reduced in dorsal somites except for the anteriormost two or three 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 and dermomyotome marker (Pourquié et al., 1996) (Fig. 1M,N). This change in myotome shape could account for the teardrop shape of the Da mutant body.

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, asterisks) and the dorsal part of somites (Fig. 1C,G, arrowheads; Fig. 1E). However, in Da, the expression of zic1/zic4 is greatly reduced in dorsal somites except for the anterior most two or three 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 and dermomyotome marker (Pourquié et al., 1996) (Fig. 1M,N). This change in myotome shape could account for the teardrop shape of the Da mutant body.
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 (or suppressed, brackets) on the transplantation site; the rescue of melanophores (N,N; medial shift of melanophores) and dorsal finfold (PP'; posterior shift of dorsal finfold) are maintained after hatching (4 wpf; arrowheads). (M,O,Q) The equivalent regions of the Da mutants are also shown.

(R-U*) Lineage analysis of the GFP-positive somitic cells. Somites dissected from transgenic fish Tg (zic1:GFP/zic4:DsRed) were transplanted into wild-type embryos at the somitogenesis stage. (R-T) Somitic cells expressing GFP gradually invade the dorsal finfold (arrowheads) and become elongated along the proximodistal axis. (U,U') Somitic cells expressing GFP are present underneath melanophores at stage 39 (arrowheads). Scale bars: 200 μm for B,C,G-K; 500 μm for L,N; 1 mm for P.
(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...
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-H′). 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; Vasilievskas 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 somitic 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, arrowheads). 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 (Svetić 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 the \( zic1/zic4 \) regulatory 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). 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
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. Terashima 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 10107483 to T. Kawani; 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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