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 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. 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 evolutional flexibility by allowing mosaic changes in body shape and differentiation of body structures without seriously compromising the integration of the whole organism (Bolk, 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,
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 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.

**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

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**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 sim1 in wild-type (I) and Da mutant (J) embryos at stage 27 (24 somites). Arrowheads indicate ectopic expression. Dashed lines delineate the somites. (K,L) Expression pattern of wnt11r in wild-type (K) and Da mutant (L) embryos at stage 23. Arrowheads indicate strong expression in the dorsal part of wild-type somites. Dashed lines delineate the somites. (M,N) Myotomal morphology at stage 39. Dashed lines delineate the myotome. Arrowheads indicate the gap between the dorsal myotomes in the Da mutant. (O,P) Vertebral morphology of the wild-type (O) and Da mutant (P) 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: 1 cm for A,B; 200 μm for M,N; 100 μm for C,D,G,H; 50 μm for E,F,I,J.
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’-CTGGGTCTCTATTACTGCGAC-3’ and 5’-TTTGGG-ACATATGGTGGCGTAACTC-3’); wnt11r (primers 5’-CAATAGAG- TAAACACTGTTCACCAAC-3’ and 5’-CTATTTGCAAACGTATCTC- CAC-3’); foxd3 (primers 5’-GATGACTTGGAGAGTGAAATCG-3’ and 5’-ACACCCCGATGTTGTTTCTACAC-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 transplantation. Wild-type or Da mutant somites or neural tube at the same stage as the donors were mounted in 1% low melting temperature agarose with 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’-AGGCTCTTCTCCCGTCCGTTCC-3’ and 5’-CCAGCTGCTGGACGTGCAATG-3’; zic4, 5’-AGAAGGCCGTTCATCCGGCTC-3’ and 5’-CGCTTGGCAGACGCAGTCTG-3’; β-actin, 5’-TGCCCACGTGCTGGTTGGAACATC-3’ and 5’-CCATGACAACCTGCGTGCTG-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, 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 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. 1J,L). 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). Transplantation of wild-type 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 wild-type dorsal neural tubes (F,K) have this effect. Red arrowheads in G,K indicate the anterior limit of the dorsal finfold. White arrowheads in B,C indicate melanophores. Asterisks indicate the cloaca. White arrowheads and arrows in K indicate dorsal root ganglia and pigment cells, respectively, derived from the donor neural tube. (L-Q) Rescued phenotypes at the post-hatching stage (4 wpf). Wild-type somites labeled with DsRed were transplanted into Da mutant embryos in the same way as described in A. (L,L'/H11032, N,N'/H11032, P,P'/H11032) Iridophores (L,L'), emerging at 2-3 wpf, are rescued (or suppressed, brackets) on the transplantation site; the rescue of melanophores (N,N'; medial shift of melanophores) and dorsal finfold (P,P'; 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. 2. Ventralized phenotypes in Da mutants are rescued by wild-type somites.** (A) The somite transplantation rescue experiment. (B-K) Transplantation of wild-type 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 wild-type dorsal neural tubes (F,K) have this effect. Red arrowheads in G,K indicate the anterior limit of the dorsal finfold. White arrowheads in B,C indicate melanophores. Asterisks indicate the cloaca. White arrowheads and arrows in K indicate dorsal root ganglia and pigment cells, respectively, derived from the donor neural tube. (L-Q) Rescued phenotypes at the post-hatching stage (4 wpf). Wild-type somites labeled with DsRed were transplanted into Da mutant embryos in the same way as described in A. (L,L'/H11032, N,N'/H11032, P,P'/H11032) Iridophores (L,L'), emerging at 2-3 wpf, are rescued (or suppressed, brackets) on the transplantation site; the rescue of melanophores (N,N'; medial shift of melanophores) and dorsal finfold (P,P'; 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.

**Fig. 3.** zic1/zic4 define the dorsal domain of the trunk throughout life. (A) Expression patterns of zic1, zic4, myod, twi and pax3 in wild-type embryos at stage 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. The expression domain of zic1/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' 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 (arrow) almost corresponds to the level of the horizontal myoseptum (arrowheads). (H') Magnified view of the rectangular region in H. (I,J) 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,D'; 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; Pourquie 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 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, 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 the zic-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 persistent regionization 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 Betta splendens during embryogenesis (12 somites). The dashed lines in G-J delineate somites in transverse sections at the level indicated by solid lines in GJ, respectively. Arrowheads indicate the somites. zic1 expression in the somites (arrowheads in GJ) is absent in Double-tail embryos (J). Scale bars: 2 cm for A,B,D,E; 200 μm for G,H,JJ.

Modular organization of the vertebrate trunk
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; Vasilisauskas 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.
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.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Fig. 6. Model for determining the dorsal structures using the zic1/zic4 domain. Schematic illustration of dorsoventral 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 (left), and then becomes autonomously maintained for the entire life (middle and 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. S1. Ventralized phenotypes of Da mutants at larval and adult stages. (A,B,K,L) Staining with DiAsp (0.05 mg/ml) visualizes the ectopic deposition of neuromasts (sensor complexes of the lateral line) on the dorsal sites (arrowheads) in Da mutant embryos at 2 wpf (B) and in 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 wild-type and Da mutant adult males. Magnified views in (C'-F') correspond to the white boxes in C-F, respectively. 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 wild-type and Da mutant adult trunk surface, showing that while the dorsal skin of wild type has only melanophores and leucophores, that of Da also possesses iridophores (arrowheads in H), similar to the ventral side of wild type. (M) Contours of wild-type and Da mutant male adults (excluding fins), showing that the dorsal part of Da is more rounded, forming a teardrop body shape. Dashed line indicates the lateral midline. Embryos and adults in A-F',K-M are shown in lateral view with anterior towards the left. Scale bars: 500 μm for A; 1 cm for C.
Fig. S2. Further validation of transplantation experiments. (A,B) Expression pattern of foxd3 (early neural crest marker) and zic1 in wild type at stage 23 (14 somites). Bracket indicates the site from which the somites are dissected. 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 wild type, stained with DAB at 3 dpf (stage 27). The labeled cells in C formed a smoothly packed tissue whereas 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) were transplanted into d-rR hosts that lack melanophores (b/b) at stage 23 (14-16 somites). This panel shows a dorsal view of a transplant at stage 39, No melanophores appeared 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 stage 39. Dorsal view is shown with anterior towards the left. (G-L) Phenotypes of dorsal patterns after transplantation of wild-type somites (labeled with DsRed) into Da mutant embryos at stage 39 (G,J) and 2 wpf (H,K). Melanophore (G-H’) and dorsal finfold (J-K’) rescues are maintained at stage 39, and also at 2 wpf, when the dorsal finfold 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 (I,L). Scale bars: 100 μm for C; 500 μm for G,H.
**Fig. S3. Phenotype of the heterozygous Da mutant.** Lateral view of wild-type (A) and heterozygous (Da/+, B) and homozygous (C) Da mutant males. The heterozygous Da mutant exhibits the wild-type phenotypes in the pigmentation pattern and caudal fin skeleton. However, the size of the dorsal fin shows intermediate between that of wild type and Da (asterisks). Asterisks indicate the anterior limit of the dorsal fins. Scale bar: 1 cm.

**Movie 1. Experimental procedure of somite transplantation.** In the first half of the movie, somites are isolated from donor embryos. In the latter part, a hole is made in a host embryo embedded in agarose, and the donor somites are implanted through the hole.