Development 140, 1486-1496 (2013) doi:10.1242/dev.088567

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

Toru Kawanishi[1], Takuya Kaneko[1,*], Yuuta Moriyama[1,‡], Masato Kinoshita[2], Hayato Yokoi[3], Tohru Suzuki[3], Atsuko Shimada[1] and Hiroyuki Takeda[1,§]

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 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,

[1]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo, Tokyo, Japan. [2]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto, Kyoto, Japan. [3]Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan.

*Present address: Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
‡Present address: Institute of Cellular and Molecular Biosciences, University of Tokyo, Bunkyo, Tokyo, Japan
§Author for correspondence (htakeda@biol.s.u-tokyo.ac.jp)

Accepted 21 January 2013

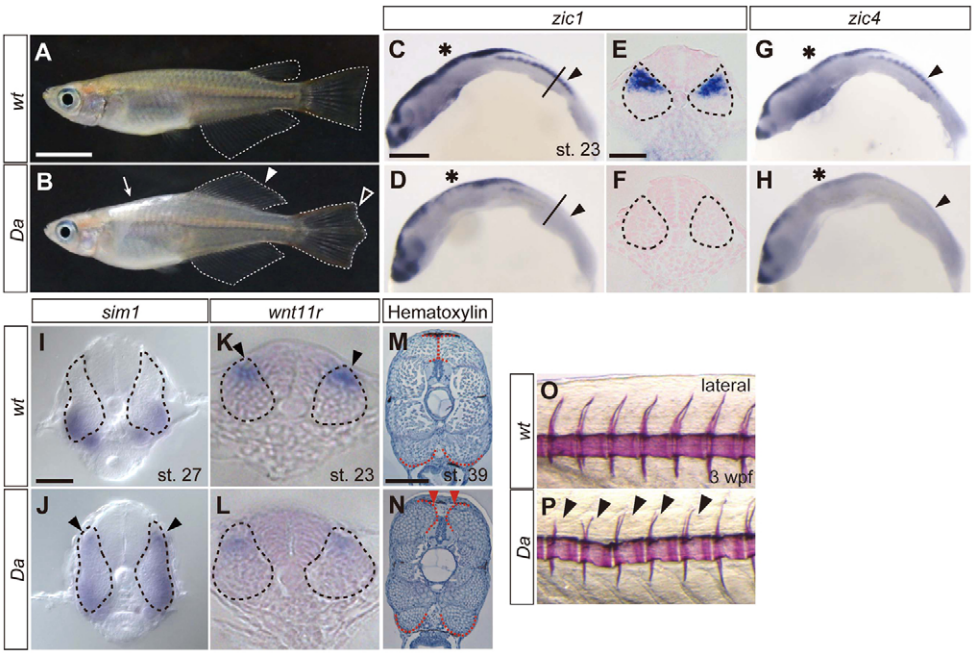

Fig. 1. Ventralized phenotypes of *Da* mutants. (**A**,**B**) The *Da* medaka mutant exhibits a ventralized pigmentation (arrow) and median fin morphology (arrowheads), as well as a teardrop body shape. (**C**,**D**,**G**,**H**) Expression patterns of *zic1* and *zic4* in the wild-type (C,G) and *Da* 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.

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 (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-*Sce*I 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

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′-CTGGGTTCTCATTTACTGCAGAC-3′ and 5′-TTGTGGACTATAGTGGCGTAACTC-3′); *wnt11r* (primers 5′-CAAATGGCTAACACTGTCTCAAAC-3′ and 5′-CTATTTGCAAACGTATCTCTCCAC-3′); *foxd3* (primers 5′-GATGACTTGGAAGATGAAATCG-3′ and 5′-ACACCCCGATGATGTTTTCTATAC-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 host embryos 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′-AGCCCTTTCCGTGTCCGTTCC-3′ and 5′-CCGACGTGTGGACGTGCATGT-3′; *zic4*, 5′-AGAAGCCGTTTCCATGCCCGT-3′ and 5′-TGCTGTTGGCGAAGCGTCTGT-3′; β-*actin*, 5′-TGCCGCACTGGTTGTTGACAACG-3′ and 5′-CCATGACACCCTGGTGCCTGG-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. 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

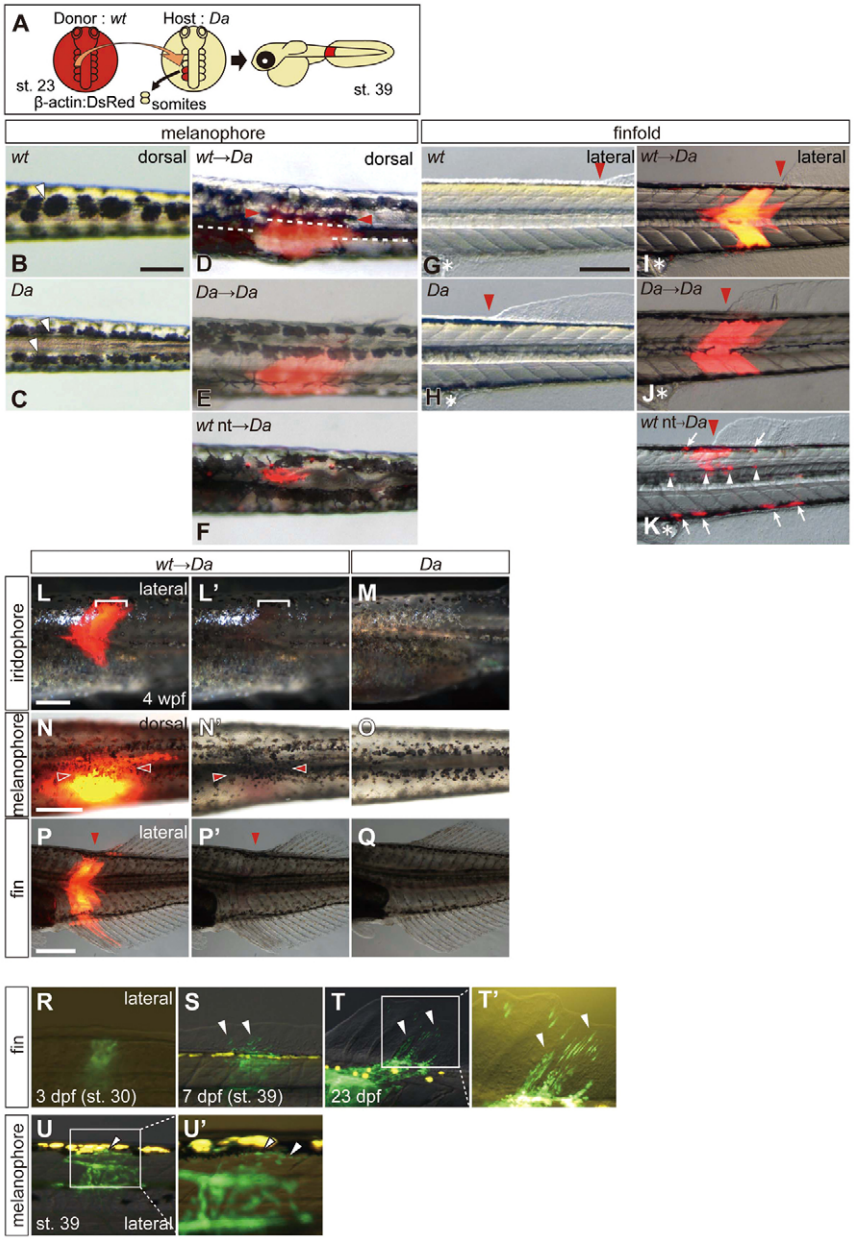

Fig. 2. Ventralized phenotypes in *Da* mutants are rescued by wild-type somites. (**A**) The somite transplantation rescue experiment. (**B-K**) Transplantation of wild-type somites (D,I) into *Da* mutant medaka embryos locally rescues the ventralized phenotypes (red arrowheads in D and I), whereas neither *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′**,**N**,**N′**,**P**,**P′**) 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.

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 underline 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

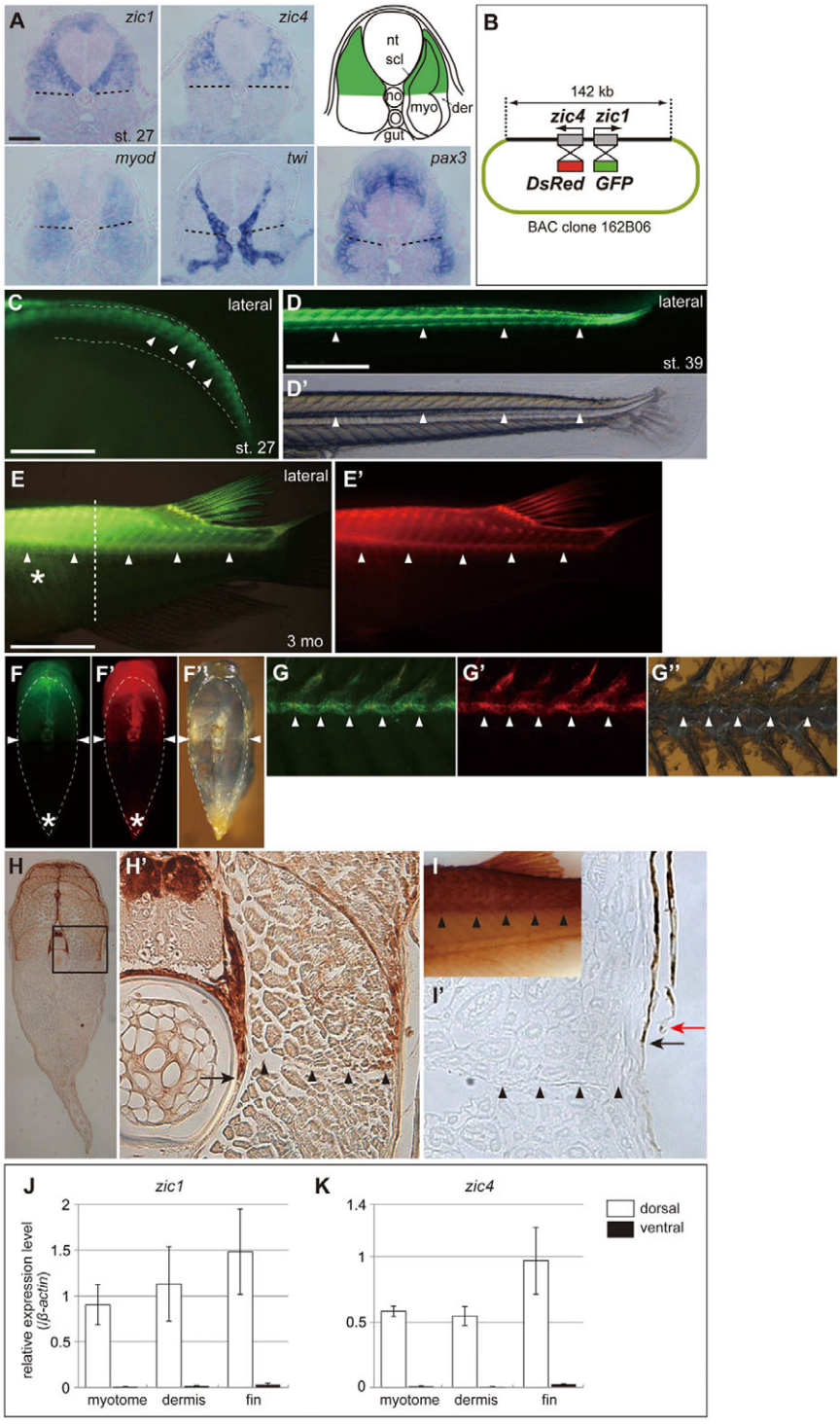

Fig. 3. *zic1/zic4* define the dorsal domain of the trunk throughout life. (**A**) Expression patterns of *zic1*, *zic4*, *myod*, *twi* and *pax3* in wild-type embryos at stage 27 (24 somites). Each somitic compartment (myotome, sclerotome or dermomyotome), defined by the expression of *myod*, *twi* or *pax3*, respectively, is depicted in the schematic. The expression domain of *zic1* 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 (arrow) almost corresponds to the level of the horizontal myoseptum (arrowheads). (H′) Magnified view of the rectangular region in H. (**I**,**I′**) 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-I′). 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 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.

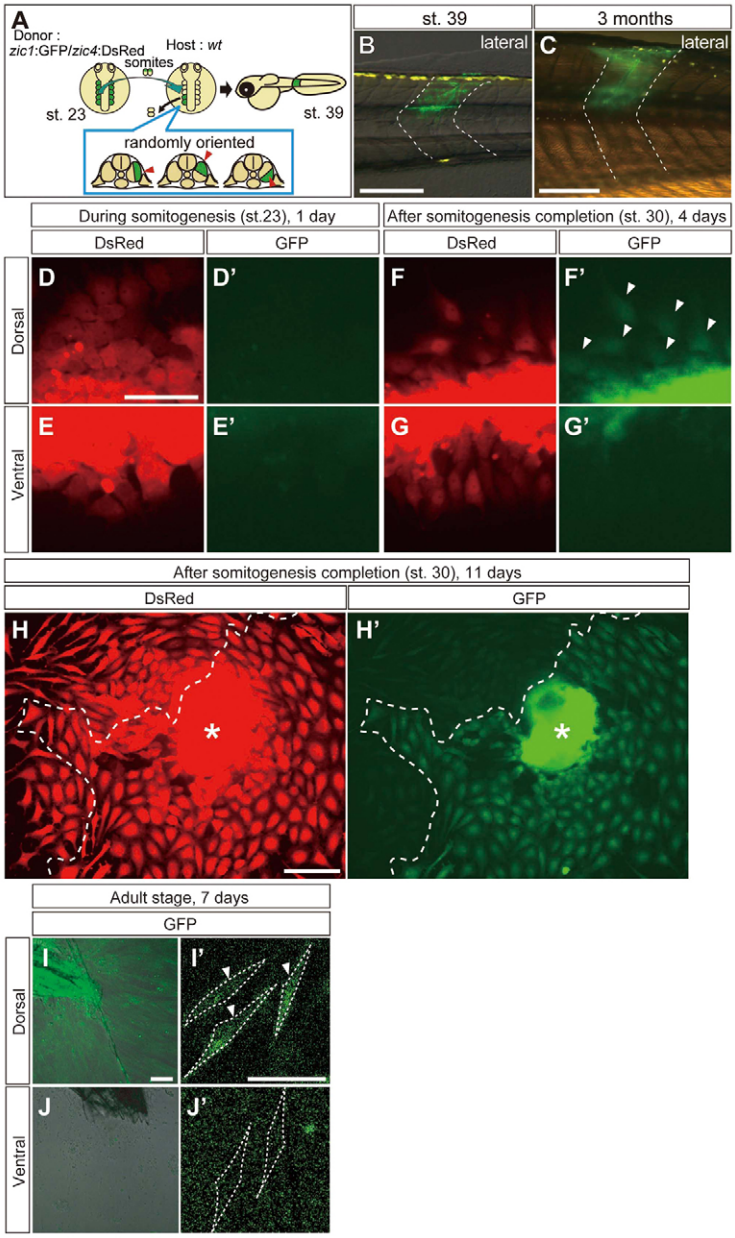

Fig. 4. Cell-non-autonomous and -autonomous expression of *zic1* in the somite lineage. (**A**) The transplantation experiments using the transgenic line Tg(*zic1*:GFP/*zic4*:DsRed) as donors as in B and C. (**B**,**C**) Somites, when transplanted randomly with respect to their orientation, acquired the dorsal expression of GFP at stage 39 (B) and 3 months post-fertilization (C). Dashed lines delineate the somites. Anterior is towards the left. The mosaicism of GFP fluorescence observed in B is due to the scattered distribution pattern of sclerotomal cells, which have stronger expression of GFP compared with myotomal cells. (**D-H′**) Explant culture of somites from Tg(*zic1*:GFP/*zic4*:DsRed/β-*actin*:DsRed) embryos at stage 24 (16 somites; D-E′) and stage 30 (the stage of somitogenesis completion; F-G′). (D-E′) Cells crawling out of the somites of stage 24 embryos do not express GFP even at the dorsal part (D′). (F-H′) Cells crawling out of stage 30 embryos maintain GFP expression in the dorsal area of the somites after 1 day (F′, arrowheads) and 11 days (H′, right of the dashed line) in culture. Asterisks in H,H′ indicate the explanted somite fragment. (**I-J′**) Primary culture of adult fibroblasts taken from dorsal and anal fins. GFP fluorescence is persistently detected in the dorsal fin fibroblasts after 1 week in culture (I), but not in the anal fin fibroblasts (J). (I′,J′) Magnified images showing individual fibroblasts (arrowheads and dashed lines). The GFP fluorescence in the dorsal fin fibroblasts is also observed after a 1-month culture (data not shown). Scale bars: 500 μm for B; 1 cm for C; 50 μm for D-G′; 100 μm for H-J′.

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

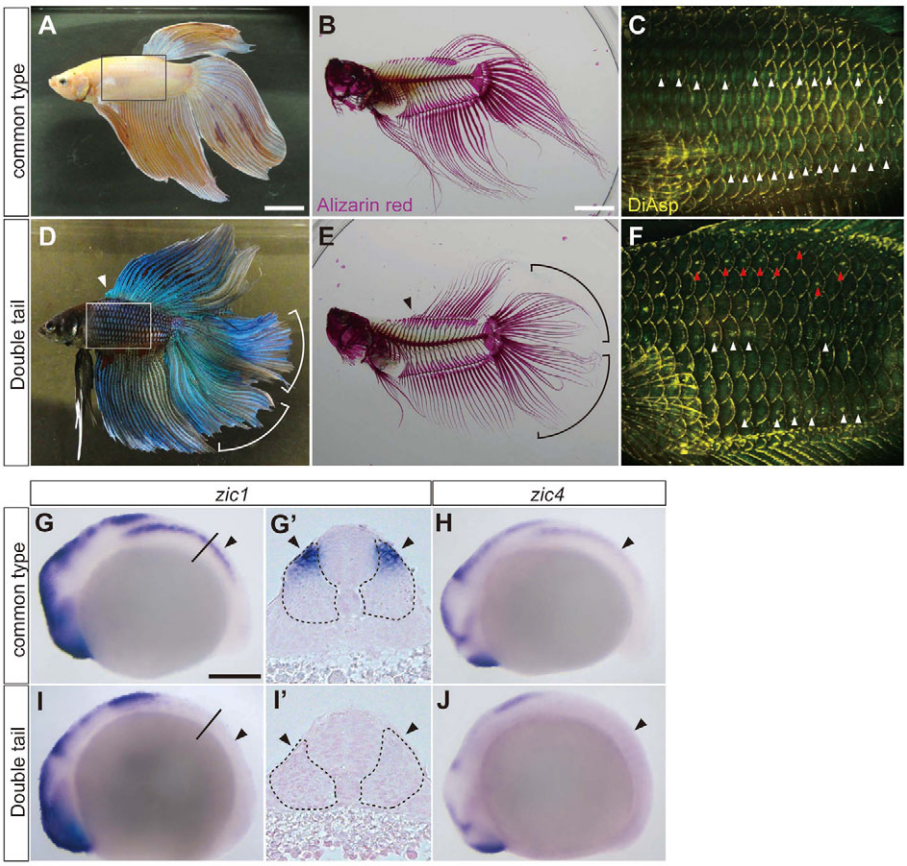

Fig. 5. *Betta splendens* and its *zic1* and *zic4* expression pattern. (**A-F**) Common type (A-C) and Double-tail (D-F) adult male *Betta splendens*. Skeletal staining with Alizarin Red (B,E) shows the fin positioning and morphology. The Double-tail variant has an anteriorly expanded dorsal fin (arrowheads 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 (I,J). Scale bars: 2 cm for A,B,D,E; 200 μm for G,H,I,J.

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 gradient of the Wnt and BMP activities (Hirsinger et al., 1997; Marcelle et al., 1997; Pourquié et al., 1996; Yusuf and Brand-Saberi, 2006). However, once established, their dorsal expression stops being dependent on the external signals by the hatching stage and is maintained for life. The sustained expression of *zic1/zic4* is likely to be required for late surface morphogenesis because some of the *Da* phenotypes (such as iridophore pigmentation) appear only between puberty and sexual maturation. The continuous action of Zic1/Zic4 was also supported by the transplantation experiment that implanted wild-type somites and their derivatives exerted their rescue effects in *Da* mutants until the adult stage. Based on these results, we propose a model for late surface patterning of the vertebrate trunk that somite-derived tissues inherit the DV positional information derived from the surrounding environment, decode it into a binary state of *zic* expression (on/off), and continue to act on shaping and patterning of the body through long-term mesodermal-ectodermal interaction on the dorsal side (Fig. 6).

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; Vasiliauskas 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

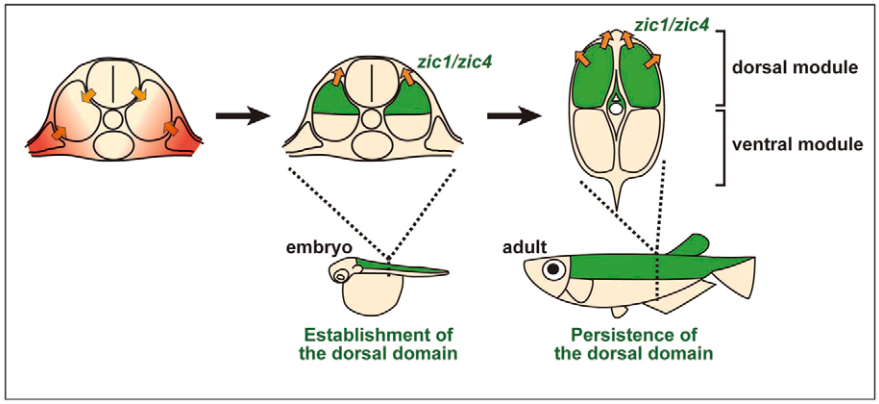

Fig. 6. Model for determining the dorsal structures using the *zic1/zic4* domain. Schematic illustration of dorsoventral patterning in fish during early to late development. The dorsal expression domain of *zic1/zic4* in the somite is established during embryogenesis by signals from their surrounding 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.

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. Toyoizumi 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 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

References

Abe, G., Ide, H. and Tamura, K. (2007). Function of FGF signaling in the developmental process of the median fin fold in zebrafish. *Dev. Biol.* **304**, 355-366.

Abzhanov, A., Protas, M., Grant, B. R., Grant, P. R. and Tabin, C. J. (2004). Bmp4 and morphological variation of beaks in Darwin's finches. *Science* **305**, 1462-1465.

Abzhanov, A., Kuo, W. P., Hartmann, C., Grant, B. R., Grant, P. R. and Tabin, C. J. (2006). The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature* **442**, 563-567.

Agius, E., Oelgeschläger, M., Wessely, O., Kemp, C. and De Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development* **127**, 1173-1183.

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

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

Aruga, J. (2004). The role of Zic genes in neural development. *Mol. Cell. Neurosci.* **26**, 205-221.

Aruga, J., Mizugishi, K., Koseki, H., Imai, K., Balling, R., Noda, T. and Mikoshiba, K. (1999). Zic1 regulates the patterning of vertebral arches in cooperation with Gli3. *Mech. Dev.* **89**, 141-150.

Aruga, J., Tohmonda, T., Homma, S. and Mikoshiba, K. (2002). Zic1 promotes the expansion of dorsal neural progenitors in spinal cord by inhibiting neuronal differentiation. *Dev. Biol.* **244**, 329-341.

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

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

Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134**, 25-36.

Cheng, L., Alvares, L. E., Ahmed, M. U., El-Hanfy, A. S. and Dietrich, S. (2004). The epaxial-hypaxial subdivision of the avian somite. *Dev. Biol.* **274**, 348-369.

Elsen, G. E., Choi, L. Y., Millen, K. J., Grinblat, Y. and Prince, V. E. (2008). Zic1 and Zic4 regulate zebrafish roof plate specification and hindbrain ventricle morphogenesis. *Dev. Biol.* **314**, 376-392.

Garriock, R. J. and Krieg, P. A. (2007). Wnt11-R signaling regulates a calcium sensitive EMT event essential for dorsal fin development of Xenopus. *Dev. Biol.* **304**, 127-140.

Garriock, R. J., D'Agostino, S. L., Pilcher, K. C. and Krieg, P. A. (2005). Wnt11-R, a protein closely related to mammalian Wnt11, is required for heart morphogenesis in Xenopus. *Dev. Biol.* **279**, 179-192.

Gaston-Massuet, C., Henderson, D. J., Greene, N. D. and Copp, A. J. (2005). Zic4, a zinc-finger transcription factor, is expressed in the developing mouse nervous system. *Dev. Dyn.* **233**, 1110-1115.

Gellon, G. and McGinnis, W. (1998). Shaping animal body plans in development and evolution by modulation of Hox expression patterns. *Bioessays* **20**, 116-125.

Gilbert, S. F. (2010). *Developmental Biology*. Sunderland: Sinauer Associates.

Grinberg, I., Northrup, H., Ardinger, H., Prasad, C., Dobyns, W. B. and Millen, K. J. (2004). Heterozygous deletion of the linked genes ZIC1 and ZIC4 is involved in Dandy-Walker malformation. *Nat. Genet.* **36**, 1053-1055.

Haines, L., Neyt, C., Gautier, P., Keenan, D. G., Bryson-Richardson, R. J., Hollway, G. E., Cole, N. J. and Currie, P. D. (2004). Met and Hgf signaling controls hypaxial muscle and lateral line development in the zebrafish. *Development* **131**, 4857-4869.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquié, O. (1997). Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124**, 4605-4614.

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

Iwamatsu, T. (2004). Stages of normal development in the medaka Oryzias latipes. *Mech. Dev.* **121**, 605-618.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature* **382**, 133-138.

Kirschner, M. and Gerhart, J. (1998). Evolvability. *Proc. Natl. Acad. Sci. USA* **95**, 8420-8427.

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

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H. and Takada, S. (2005). Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. *Dev. Cell* **8**, 587-598.

Kuratani, S. (2009). Modularity, comparative embryology and evo-devo: developmental dissection of evolving body plans. *Dev. Biol.* **332**, 61-69.

Kusakabe, R., Kuraku, S. and Kuratani, S. (2011). Expression and interaction of muscle-related genes in the lamprey imply the evolutionary scenario for vertebrate skeletal muscle, in association with the acquisition of the neck and fins. *Dev. Biol.* **350**, 217-227.

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

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

Marcelle, C., Stark, M. R. and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. *Development* **124**, 3955-3963.

Moriyama, Y., Kawanishi, T., Nakamura, R., Tsukahara, T., Sumiyama, K., Suster, M. L., Kawakami, K., Toyoda, A., Fujiyama, A., Yasuoka, Y. et al. (2012). The medaka zic1/zic4 mutant provides molecular insights into teleost caudal fin evolution. *Curr. Biol.* **22**, 601-607.

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

Nakamura, S., Saito, D. and Tanaka, M. (2008). Generation of transgenic medaka using modified bacterial artificial chromosome. *Dev. Growth Differ.* **50**, 415-419.

Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (1998). Xenopus Zic family and its role in neural and neural crest development. *Mech. Dev.* **75**, 43-51.

Ohtsuka, M., Kikuchi, N., Yokoi, H., Kinoshita, M., Wakamatsu, Y., Ozato, K., Takeda, H., Inoko, H. and Kimura, M. (2004). Possible roles of zic1 and zic4, identified within the medaka Double anal fin (Da) locus, in dorsoventral patterning of the trunk-tail region (related to phenotypes of the Da mutant). *Mech. Dev.* **121**, 873-882.

Olivera-Martinez, I., Missier, S., Fraboulet, S., Thélu, J. and Dhouailly, D. (2002). Differential regulation of the chick dorsal thoracic dermal progenitors from the medial dermomyotome. *Development* **129**, 4763-4772.

Ordahl, C. P. and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114**, 339-353.

Pan, H., Gustafsson, M. K., Aruga, J., Tiedken, J. J., Chen, J. C. and Emerson, C. P., Jr (2011). A role for Zic1 and Zic2 in Myf5 regulation and somite myogenesis. *Dev. Biol.* **351**, 120-127.

Pourquié, O., Coltey, M., Teillet, M. A., Ordahl, C. and Le Douarin, N. M. (1993). Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc. Natl. Acad. Sci. USA* **90**, 5242-5246.

Pourquié, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.

Prud'homme, B., Gompel, N. and Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proc. Natl. Acad. Sci. USA* **104 Suppl. 1**, 8605-8612.

Prud'homme, B., Minervino, C., Hocine, M., Cande, J. D., Aouane, A., Dufour, H. D., Kassner, V. A. and Gompel, N. (2011). Body plan innovation in treehoppers through the evolution of an extra wing-like appendage. *Nature* **473**, 83-86.

Rembold, M., Lahiri, K., Foulkes, N. S. and Wittbrodt, J. (2006). Transgenesis in fish: efficient selection of transgenic fish by co-injection with a fluorescent reporter construct. *Nat. Protoc.* **1**, 1133-1139.

Rinn, J. L., Wang, J. K., Allen, N., Brugmann, S. A., Mikels, A. J., Liu, H., Ridky, T. W., Stadler, H. S., Nusse, R., Helms, J. A. et al. (2008). A dermal HOX transcriptional program regulates site-specific epidermal fate. *Genes Dev.* **22**, 303-307.

Rohr, K. B., Schulte-Merker, S. and Tautz, D. (1999). Zebrafish zic1 expression in brain and somites is affected by BMP and hedgehog signalling. *Mech. Dev.* **85**, 147-159.

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

Schlosser, G. and Wagner, G. P. (2004). *Modularity in Development and Evolution*. Chicago; London: University of Chicago Press.

Selleck, M. A. and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112**, 615-626.

Svetic, V., Hollway, G. E., Elworthy, S., Chipperfield, T. R., Davison, C., Adams, R. J., Eisen, J. S., Ingham, P. W., Currie, P. D. and Kelsh, R. N. (2007). Sdf1a patterns zebrafish melanophores and links the somite and melanophore pattern defects in choker mutants. *Development* **134**, 1011-1022.

Takashima, S., Shimada, A., Kobayashi, D., Yokoi, H., Narita, T., Jindo, T., Kage, T., Kitagawa, T., Kimura, T., Sekimizu, K. et al. (2007). Phenotypic analysis of a novel chordin mutant in medaka. *Dev. Dyn.* **236**, 2298-2310.

Tamiya, G., Wakamatsu, Y. and Ozato, K. (1997). An embryological study of ventralization of dorsal structures in the tail of medaka (Oryzias latipes) Da mutants. *Dev. Growth Differ.* **39**, 531-538.

Tomita, H. (1969). On the new mutants in body color and fins of the medaka. *Zool. Mag.* **78**, 58.
Tomita, H. (1975). Mutant genes in the medaka. In *Medaka (Killifish) Biology and Strains* (ed. T. Yamamoto), pp. 251-272. Tokyo, Japan: Keigaku.
Tomita, H. (1992). The lists of the mutants and strains of the medaka, common gambusia, silver crucian carp, goldfish, and golden venus fish maintained in the Laboratory of Freshwater Fish Stocks, Nagoya University. *The Fish Biology Journal MEDAKA* **4**, 45-47.
Tonegawa, A. and Takahashi, Y. (1998). Somitogenesis controlled by Noggin. *Dev. Biol.* **202**, 172-182.
Tonegawa, A., Funayama, N., Ueno, N. and Takahashi, Y. (1997). Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4. *Development* **124**, 1975-1984.
Tosney, K. W. (2004). Long-distance cue from emerging dermis stimulates neural crest melanoblast migration. *Dev. Dyn.* **229**, 99-108.
Vasiliauskas, D., Hancock, S. and Stern, C. D. (1999). SWiP-1: novel SOCS box containing WD-protein regulated by signalling centres and by Shh during development. *Mech. Dev.* **82**, 79-94.
von Dassow, G. and Munro, E. (1999). Modularity in animal development and evolution: elements of a conceptual framework for EvoDevo. *J. Exp. Zool.* **285**, 307-325.
Wagner, G. P., Pavlicev, M. and Cheverud, J. M. (2007). The road to modularity. *Nat. Rev. Genet.* **8**, 921-931.
Wray, G. A. (2007). The evolutionary significance of cis-regulatory mutations. *Nat. Rev. Genet.* **8**, 206-216.
Yasutake, J., Inohaya, K. and Kudo, A. (2004). Twist functions in vertebral column formation in medaka, Oryzias latipes. *Mech. Dev.* **121**, 883-894.
Yusuf, F. and Brand-Saberi, B. (2006). The eventful somite: patterning, fate determination and cell division in the somite. *Anat. Embryol. (Berl.)* **211 Suppl. 1**, 21-30.