The *dlx5a/dlx6a* Genes Play Essential Roles in the Early Development of Zebrafish Median Fin and Pectoral Structures

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Abstract

The *Dlx5* and *Dlx6* genes encode homeodomain transcription factors essential for the proper development of limbs in mammalian species. However, the role of their teleost counterparts in fin development has received little attention. Here, we show that *dlx5a* is an early marker of apical ectodermal cells of the pectoral fin buds and of the median fin fold, but also of cleithrum precursor cells during pectoral girdle development. We propose that early median fin fold establishment results from the medial convergence of *dlx5a*-expressing cells at the lateral edges of the neural keel. Expression analysis also shows involvement of *dlx5a* during appendage skeletogenesis. Using morpholino-mediated knock down, we demonstrate that disrupted *dlx5a/6a* function results in pectoral fin agenesis associated with misexpression of *bmp4, fgf8a, and1* and *msx* genes. In contrast, the median fin fold presents defects in mesenchymal cell migration and actinotrichia formation, whereas the initial specification seems to occur normally. Our results demonstrate that the *dlx5a/6a* genes are essential for the induction of pectoral fin outgrowth, but are not required during median fin fold specification. The *dlx5a/6a* knock down also causes a failure of cleithrum formation associated with a drastic loss of *runx2b* and *col10a1* expression. The data indicate distinct requirements for *dlx5a/6a* during median and pectoral fin development suggesting that initiation of unpaired and paired fin formation are not directed through the same molecular mechanisms. Our results refocus arguments on the mechanistic basis of paired appendage genesis during vertebrate evolution.

Introduction

In vertebrates, appendages (limbs, wings and fins) show major structural and functional differences, but they present remarkable similarities in their developmental mechanisms [1]. Genes known to play a critical role in the initiation, growth, and patterning of tetrapod limbs (e.g. *Tbx, Hox, Fgf, Bmp* and *Shh*) are expressed in comparable spatiotemporal domain in fins [2–5] and share similar functions [5–11]. Particularly, the tetrapod *Dlx5/Dlx6* and teleost *dlx5a/dlx6a* genes are expressed in the apical ectodermal ridge (AER) of the developing limbs in mice [12–15] and in the pectoral fin fold (PFF) and median fin fold (MFF) giving rise respectively to paired and unpaired fins in zebrafish [16–19]. At early stage of fin morphogenesis, teleosts present an AER structurally homologous to the AER of tetrapods [1,20]. Later, the AER transitions into an elongated pectoral fin fold [20]. AER and fin fold structures have been demonstrated to be essential signaling centers during appendage specification and outgrowth in vertebrates [7,17,20–22]. Despite the fact that tetrapod studies have demonstrated the central role of *Dlx5/6* genes in limb formation, the implication of *dlx5a/6a* genes in teleost fin development has been little analyzed beyond examination of expression patterns.

*Dlx* genes code for an evolutionary conserved group of homeodomain transcription factors, related in sequence to the *Drosophila distalless* gene (*dll*) essential for distal appendage patterning in insects [23]. The *Dlx* genes have arisen from the ancestral *dll* gene as a result of gene duplication events [24]. In tetrapods, the *Dlx* family consists of six genes organized into the *Dlx1/2, Dlx3/4* and *Dlx5/6* bigene clusters. In zebrafish, eight *dlx* genes have been reported among which six (*dlx1a/2a, dlx3b/dlx4b, dlx5a/6a*) are arranged on chromosomes similarly to their tetrapod counterparts [24–27]. Expression and functional analyses of *Dlx5/6* have demonstrated their key roles in the development of the nervous system, of craniofacial structures, of endochondral bones and of appendages [13,14,28–38]. Simultaneous inactivation of *Dlx5* and *Dlx6* in the mouse results in a limb phenotype similar to that observed in patients affected with split-hand split-foot malformation type I (SHFM-I) [14,34,37]. Altered limb development in *Dlx5/6* null mice is associated with loss of *Bmp4*, *Fgf8* and *Msx2* expression in the medial part of the AER [14,15]. The data indicate that the *Dlx5/6* genes have a central role in vertebrate appendage formation. It was therefore of interest to examine the function of *dlx5a/6a* during the development of paired and unpaired fins.

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Here, we show that *dlx5a/6a* genes are required for the initiation of pectoral fin outgrowth and for median fin fold morphogenesis. Our results suggest distinct requirements for *dlx5a/6a* genes in paired and unpaired fin development. Moreover, the analyses demonstrate that *dlx5a/6a* are implicated in cleithrum formation and suggest that *dlx5a* is involved in fin skeletogenesis.

**Materials and Methods**

**Ethical statement**

All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee (institutional licence #BL 235). All efforts were made to minimize suffering; manipulations on adult animals were performed with the anaesthetic drug tricaine mesylate (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, Oakville, ON, Canada). Embryos were killed with an overdose of the latter drug.

**Animal maintenance**

Zebrafish and their embryos were maintained at 28.5°C according to methods described in [39]. Wild-type adult zebrafish were kept and bred in circulating water at 28.5°C with a controlled 14-h light cycle. Embryos were collected at the one-cell-stage. A Narishige IM300 microinjection was used for microinjection. Wild-type, controls, and injected embryos were raised at similar densities in embryo medium in a 28.5°C incubator. Embryos were treated with 0.0015% 1-phenyl 2-thiourea (PTU) to inhibit melanogenesis.

**Morpholino-mediated knock down and rescue experiment constructs**

For morpholino-mediated knock down, we injected or co-injected in 1 cell-stage embryos, 1 nl of *dlx5a* and/or *dlx6a* morpholinos at a concentration of either 0.4 mM or 0.8 mM. The choice of morpholino concentrations has been determined to inhibit melanogenesis.

**In situ hybridization**

In *in situ* hybridization on whole-mount embryos and cryostat sections were performed as previously described [40,41].

For *in situ* hybridization, embryos from 12 to 96 hpf (n > 100 for each experimental groups and markers) were fixed in 4% paraformaldehyde (PFA, Millipore) in phosphate buffer saline 1× (PBS, Amresco) overnight at 4°C, dehydrated in methanol, and stored in 100% methanol at −20°C.

For *in situ* hybridization on cryostat sections, larvae (n = 16) were fixed in 4% PFA in PBS overnight at 4°C, washed in PBS and equilibrated in 30% sucrose in PBS overnight at 4°C. The samples were then embedded and frozen in O.C.T compound (Sakura Finetek) and sectioned at 10 μm. After *in situ* hybridization, sections were mounted with coverslips and Aqua Poly/Mount (Polysciences) before imaging.

The antisense mRNA probes were labeled with digoxigenin-11-UTP (Roche) and synthesized from cDNA clones: *dlx5a* [42], *dlx6a* [29], *bmp4* [43], *fgf8a* [44], *mxb* [45], *mxc* [45, and1] [46], *runx2b* [45], *coll10a1* [47]. NBT/BCIP (Roche) was used as alkaline phosphatase substrate.

**Whole-mount TUNEL assay**

The whole-mount TUNEL assay was performed on 24 hpf embryos (control embryos n = 62, *dlx5a/dlx6a* morphants n = 98) with the ApoTag peroxidase in situ apoptosis detection kit (Millipore) following the modifications described in [48].

**BrdU assay**

Decapitated embryos at 23 hpf were placed in embryo medium containing 10 mM BrdU (5-bromo-2′-deoxy-uridine, Roche) with 1% DMSO for 1 h at 28.5°C (control embryos n = 13, *dlx5a/dlx6a* morphants n = 28). Embryos were killed with an overdose of tricaine mesylate and fixed in 4% PFA in PBS overnight at 4°C, washed in PBDT (PBS DMSO 1% Tween 0.1%), dehydrated in methanol and stored in methanol 100% at −20°C. Then, the samples were rehydrated in a graded methanol-PBDT series, treated with proteinase K (10 μg/ml) for 20 minutes, post-fixed in PFA 4% for 20 minutes and incubated in 2N HCl for 1 h at room temperature.

Proliferating cells were immunodetected in wholemount embryos using a 1:100 dilution of the mouse anti-BrdU monoclonal antibody (Sigma), a 1:200 dilution of secondary anti-mouse HRP-conjugated antibody (Jackson Immuno), and revealed with DAB chromogenic substrate (Abcam).

**Histochemical picrosirius red staining**

48 hpf embryos (n = 119) were fixed overnight in 4% PFA at 4°C and washed in PBST. The embryos were incubated for 1 h in a 0.2% solution of sirius red (direct red 80, Sigma-aldrich) dissolved in saturated picric acid (Sigma-aldrich). The staining was followed by washes in distilled water. Once the water ran clear and without any red color, embryos were sequentially dehydrated into glycerol/PBS solutions and stored in 100% glycerol.
Results

dlx5a/dlx6a genes are early markers of apical ectodermal cells in developing paired and unpaired fins

Expression of the zebrafish dlx genes has been previously examined [16,19,38,49] including in the developing appendages. Even before the median fin fold becomes distinguishable, dlx5a transcripts are expressed in ectodermal cells underlying the periderm at the lateral edges of the neural keel at 15.5 hpf (Fig. 1 A, A’). From 15.5 hpf to 16 hpf, dlx5a-expressing cells follow a dynamic convergent movement toward the dorsal midline to form the presumptive median fin fold (MFF) (Fig. 1 A–D), the anterior expression limit corresponding to the MFF domain around the 8th somite [17]. Then, dlx5a expression is limited to MFF ectodermal cells at 24 hpf and 48 hpf (Fig. S1), and gradually decreases until 72 hpf when transcripts are hardly detectable (data not shown). Expression of dlx5a mirrors that of dlx6a except that transcripts seem to be present at lower levels (Fig. S2 B, D), a difference that was observed by us and others throughout the embryo using a variety of probes for this gene [18,19,29].

At the pectoral level, dlx5a transcripts are first detected at 24 hpf in apical ectodermal cells of the presumptive pectoral fin bud (Fig. 2 A). At 36 hpf, dlx5a is highly expressed in the AER of the developing pectoral fin buds (Fig. 2 B). From 36 hpf to 48 hpf, the AER develops into the pectoral fin fold (PFF) in zebrafish embryos [20]. During PFF establishment, dlx5a expression is maintained in apical ectodermal cells until 48 hpf (Fig. 2 C). Transcript levels progressively decrease in the PFF from 48 hpf to 72 hpf (Fig. 2 D). Similar observations were made for dlx6a, although transcript levels appear to be weaker (Fig. S2 A, C).

Transcripts of dlx5a are also expressed in the developing cleithrum at the base of the pectoral fin bud which extends the AER/PFF dlx5a-positive domain ventro-laterally from 36 to 72 hpf (Fig. 2 B–D). The cleithrum is one of the major bones of the pectoral girdle which supports the pectoral fins in bony fish.

Weak dlx5a expression is also detected in the endochondral disc of the pectoral fin bud at 48 hpf and 54 hpf (Fig. 2 C and data not shown). These observations show that dlx5a, and to a lesser extent...
dlx6a, are early markers of ectodermal cells giving rise to PFF and MFF structures and of precursor cells of the developing cleithrum and pectoral endochondral disc.

Knock down of dlx5a/6a leads to severe appendage defects

To analyze the implication of dlx5a/6a genes in appendage development, we performed dlx5a and dlx6a knock down in zebrafish embryos using morpholinos (MO). We performed micro-injections at the 1-cell stage with one morpholino or co-injection of two dlx morpholinos at two different concentrations (0.4 mM and 0.8 mM). Embryos injected with translation-blocking MOs against dlx5a and/or dlx6a exhibited characteristic and reproducible moderate to severe phenotypes compared to control embryos (Fig. 3 A). When observed at 48 hpf, a moderate phenotype is defined as presence of a "curved tail" and hypoplastic pectoral fins whereas the severe phenotype corresponds to a "curly tail" associated with agenesis of pectoral fins. Moreover, embryos with a severe phenotype are generally smaller in size when compared to controls and display craniofacial malformations (Fig. 3 C).

In single dlx5a morphants (0.8 mM), 118/236 (50%) of the embryos exhibit the normal phenotype whereas 111/236 (47%) show moderate and 7/236 (3%) display severe phenotypes (Fig. 3 B). Knock down of dlx6a (0.8 mM) leads to mostly normal phenotype (269/286) and we obtain a low proportion of moderate (13/286) and severe (4/286) phenotypes. The co-injection of dlx5a and dlx6 MOs (0.4 mM each) increases the rate of moderate (232/817; 28%) and severe (227/817; 28%) phenotypes. When we performed (n = 627) and confirmed the results obtained using translation-blocking MOs (0.8 mM each) increases the rate of moderate (232/817; 28%) and severe (227/817; 28%) phenotypes. When we increase the concentration of the injected MOs (0.8 mM each), the double knock down results mainly in severe phenotype (704/950) and low rate of moderate (137/950; 14%) and normal (109/950; 12%) phenotypes. Co-injection of splice-blocking dlx5a/dlx6a MOs was performed (n = 627) and confirmed the results obtained using translation-blocking MOs. The phenotypes of single }dlx{ morphants compared to double dlx5a/6a morphant embryos underline the potentially redundant function of the }Dlx{ paralogs in vertebrates [13,14,50–52]. Moreover, the results show that the phenotypes observed in double morphants are dose-dependent. When we doubled the concentration of the MOs from 0.4 mM to 0.8 mM each, the rate of severe phenotype also increases from 27.8% to 74.1%. Based on the above observations, we performed subsequent experiments by injecting dlx5a + dlx6a MOs at 0.8 mM and we considered the severe phenotype embryos as specimens in which the dlx5a/dlx6a knock down was more efficient. Thus, the dlx5a/dlx6a morphants analyzed in the study are embryos presenting a severe phenotype.

We performed dlx5a/dlx6a mRNA rescue experiments to test the specificity of the phenotypes obtained with the dlx5a and dlx6a MOs (Fig. 3 C). We mutated 5'-UTR sequences in the dlx mRNAs that were co-injected with the dlx5a/6a MOs to prevent MO binding. The results show that co-injection of dlx5a/6a MOs and dlx5a/6a mRNA MOs (70 ng/µl each) increases three times the proportion of normal phenotype and decrease almost twice the number of severe phenotype embryos compared to the dlx5a/6a double morphants co-injected with GFP mRNA (200 ng/µl) (Fig. 3 C). The experiments suggest that these phenotypes are specific to the dlx5a/6a knock down. The phenotypic aspects, including craniofacial malformations, pectoral fin and MFF defects, not fully rescued in the embryos with mild and severe phenotype, can be explained by the aberrant ubiquitous dlx5a/6a overexpression which leads to mild developmental defects when injected alone in the embryo (data not shown).

The dlx5a/6a genes are required for the induction of pectoral fin outgrowth and cleithrum differentiation

To better understand the effects of dlx5a/6a knock down on zebrafish fin development, we analyzed the expression of different markers known to be involved in appendage specification and morphogenesis, including some that have been shown to be affected in Dlx5/6−/− mouse embryos [14,15].

First, we examined expression of bmp4, msxB and msxC genes that are expressed in the presumptive pectoral fin bud of 24 hpf CT embryos, at a position where pectoral fin buds will appear a few hours later (Fig. 4 A, E, G). In 24 hpf dlx5a/6a morphants, we observed that the expression of the analyzed genes is lost in the presumptive pectoral fin bud during pectoral fin specification (Fig. 4 A', E', G').

We then analyzed the expression of markers that are involved during pectoral fin morphogenesis. At 48 hpf, bmp4 transcripts are found in the pectoral fin fold (PFF), the underlying mesenchyme and in the whole pectoral fin mesoderm (Fig. 4 B), whereas fgf8a expression is only detected in the PFF (Fig. 4 C). At the same stage, andl1 is expressed in epithelial cells of the pectoral fin bud, and in distal mesenchymal cells invading the fold [46] (Fig. 4 D). Moreover, Expression of msxB is limited to PFF cells and to the adjacent mesenchymal cells whereas msxC is only detected in mesenchymal cells underlying the PFF [45] (Fig. 4 F, H).

In contrast, 48 hpf dlx5a/6a morphants exhibit a severe decrease or complete loss of expression of the analyzed genes in the pectoral region (Fig. 4 F', H').

These results show that the dlx5a/6a knock down leads to severely impaired or abolished expression of genes implicated in early vertebrate appendage development, a phenotype characterized by agenesis of pectoral fin buds.

As previously mentioned, dlx5a is highly expressed in the developing cleithrum of wild-type embryos from 36 hpf to 72 hpf (Fig. 2 B-D). The cleithrum is a dermal bone [1] located at the base of the pectoral fin buds and is the first bone that mineralizes at the axial level during early zebrafish development [1,53]. In 36 hpf control embryos, dlx5a expression in the cleithrum is associated with runx2b expression (Fig. 5 A), an early/intermediate stage marker of osteoblast differentiation. At 48 hpf, runx2b expression is maintained in the cleithrum and is also detected in the opercular and ceratobranchial-5 bones at the craniofacial level (Fig. 5 C). At the same stage, cells of the developing cleithrum highly express the col10a1 gene (Fig. 5 E), an intermediate/late stage marker of osteoblast differentiation in zebrafish [47,53,54]. Interestingly, we show that dlx5a/6a knock down leads to a drastic loss of runx2b and col10a1 expression in the cleithrum at 36 hpf and 48 hpf (Fig. 5 B-F), even in embryos presenting a moderate phenotype with hypoplastic pectoral fin buds (data not shown). At 48 hpf, loss of runx2b expression in dlx5a/6a morphants is also observed in the opercular and ceratobranchial-5 bone precursors (Fig. 5 D), structures which develop from a dlx5a-positive domains (Fig. S3) [38,49].

The present data demonstrate that dlx5a/6a genes are required, directly or indirectly, for osteoblast differentiation of the cleithrum in zebrafish.

Knock down of dlx5a/6a impacts on median fin fold morphogenesis

To study the effects of the dlx5a/6a knock down in unpaired fin development, we analyzed the expression of bmp4, fgf8a, andl1, msxB and msxC genes in the median fin fold (MFF) of dlx5a/6a morphants at 16 hpf, 24 hpf (during MFF specification) and 48 hpf (during MFF morphogenesis). At 16 hpf and 24 hpf, no
notable changes in bmp4, fgf8a and mnx expression in the median fin fold are observed in the morphants compared to controls (Table 1, data not shown). At 48 hpf, mnxB is expressed in distal mesenchymal cells of the MFF whereas mnxC shows a larger anterior expression domain [Fig. 6 A–B, black arrowheads]. Both transcripts are also detected in the spinal cord. In dlx5a/6a morphant embryos, mnxB and mnxC expression is still present in the spinal cord, but only a few mnx-positive cells are detected in mesenchymal cells in the distal part of the MFF [Fig. 6 A′–B′, black arrowheads], whereas aberrant mnxB expression is detected in MFF apical ectodermal cells [Fig. 6 A′, red arrowhead]. In contrast, the fgf8a expression initially observed in MFF ectodermal cells at 48 hpf is not affected in the morphants (Table 1, data not shown). The latter results suggested developmental defects of MFF mesenchymal cells.

The MFF of dlx morphants presents a granular aspect which seems to be associated with absence of actinotrichia. The actinotrichia are non-calcified fibrils which develop in the early zebrafish fins. They act as a scaffold for the migration of mesenchymal cells and, later, for fin ray formation during zebrafish fin skeletogenesis. The actinotrichia defects in our morphants were further investigated by examining and1 expression during posterior axis development. The and1 gene is a member of the actinodin family which encodes proteins that are essential structural components of zebrafish fin rays and fin folds and required for actinotrichia formation [46]. In control embryos, and1 expression is observed in epidermal cells of the fin fold at 24 hpf.

Figure 3. Phenotypes obtained with different dlx5a/dlx6a morpholinos and mRNA treatments. (A) Phenotypes observed in dlx5a/6a morphant embryos. Lateral view of control (CT) and dlx5a/6a morphant embryos at 48 hpf. The dlx5a/6a gene knock down results in moderate "curved tail" and severe "curly tail" phenotypes compared to controls. The moderate and severe phenotypes are associated with hypoplasia and agenesis of pectoral fin bud respectively as shown in the pectoral region magnifications. Scale bar for all panels 100 μm. (B, C) The graphics show the percentages of normal (blue bars), moderate (green bars) and severe (red bars) phenotypes obtained at 48 hpf following injection of different dlx5a/ dlx6a MOs and dlx5a/dlx6a mRNAs. For each treatment, the number (n) of specimens analyzed is indicated and each experiment was performed at least 3 times. The B graph shows the following treatments: control embryos injected with H2O; control embryos injected with a control MO (1.6 mM); single morphants injected with either dlx5a or dlx6a MOs (0.8 mM); double morphants co-injected with dlx5a and dlx6a MOs at two different concentrations (0.4 mM or 0.8 mM each). The C graph shows rescue experiments: control embryos injected with GFP mRNA (200 ng/μl); control embryos co-injected with dlx5a/6a morpholinos (0.8 mM each) and GFP mRNA (200 ng/μl) and embryos co-injected with dlx5a/6a morpholinos (0.8 mM each) and dlx5a/dlx6a mRNAs (70 ng/μl each).

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Figure 4. Impaired expression of bmp4, fgf8a, and1 and msx genes in the pectoral fin region of dlx5a/6a morphants. Whole mount in situ hybridization for bmp4 (A, A’–B, B’), fgf8a (C, C’), and1 (D, D’), msxB (E, E’–F, F’) and msxC (G, G’–H–H’) at 24 and 48 hpf in dorsal views of control (A–H) and dlx5a/6a morphant (A’–H’) embryos. At 24 hpf, bmp4, msxB and msxC genes are expressed in apical ectodermal cells of the presumptive pectoral fin bud (PPF) in control embryos (A, E, G). In dlx5a/6a morphants, bmp4 expression is lost or altered in the presumptive pectoral fin bud (A’) and the msxB and msxC transcripts are hardly detectable (E’, G’). In 48 hpf control embryos, fgf8a is detected in PPF ectodermal cells (C), and1 expression is observed in the distal mesenchyme and in epithelial cells of the PFB but not in the PFF (D). The msxB gene is expressed in the PFF and the underlying mesenchyme (E), and msxC is detected in the mesenchymal cells but not in the PFF (H). In contrast to what is observed in controls at 48 hpf, dlx5a/6a morphants show a marked decrease or loss of expression of the PFB markers associated with pectoral fin agenesis (B’–D’,...
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F’. H’). (I) Schematic representation of the pectoral fin bud at 48 hpf summarizing the expression of *dlx5a* and the analyzed PFB markers in their corresponding cellular types. Scale bars 50 μm.

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and in all MFF cells at 48 hpf (Fig. 7 A–B). As previously reported [46], *and1* transcripts are not co-expressed with *dlx5a* in the apical ectodermal cells at 24 hpf, but rather in epithelial cells directly adjacent to the apical ectodermal tissue (Fig. S4 A). In 24 hpf *dlx5a/6a* morphants, *and1* is detected in the MFF but transcripts show a shorter antero-posterior expression domain (Fig. 7 A’, Fig. S4 B). At 48 hpf, transcripts are also observed in a less extended MFF domain (Fig. 7 B’).

We then performed picrosirius red staining to analyze the cellular organization of the median fin fold and to reveal the presence of actinotrichia. In CT embryos at 48 hpf, actinotrichia are clearly visible (Fig. 7 C, the white lines indicate their orientations and lengths). In *dlx5a/6a* morphants, actinotrichia do not form and the fin fold is smaller and granular.

We used the zebrafish ET-37 enhancer-trap line [55], expressing GFP in migrating mesenchymal cells of the fin fold, to examine MFF cell migration following *dlx5a/6a* knock down. In 54 hpf control embryos, migrating mesenchymal cells in the MFF are aligned and show filopodia orientated in the direction of migration (Fig. 7 D, the white line indicates the direction of migration). In *dlx5a/6a* morphants, mesenchymal cells are disorganized and do not show filopodia (Fig. 7 D–D’). Moreover, the median fin fold phenotype observed in the double morphants is associated with increased cell death and decreased proliferation at 24 hpf (Fig. S5).

### dlx5a is expressed during fin skeleton formation

Several studies in tetrapod species have demonstrated the role of *Dlx5/6* genes in endochondral bone development [14,36,57]. The expression of *dlx5a/6a* has also been reported in some craniofacial bones that develop by either endochondral or intramembranous ossification [38,49]. To further examine the potential implication of *dlx5a* in appendage skeletogenesis, we completed *dlx5a* expression analysis at later stages during fin bone formation. We performed *in situ* hybridization on sections of wild-type larvae from around 6 days (4.2 mm) to one month post fertilization (8.7 mm).

Expression of *dlx5a* was first detected in the perichondrium of the hyurals in 6.2 mm larvae (data not shown) whereas no expression was observed in the forming radials. Slightly later, in 6.6 mm larvae, *dlx5a* expression is maintained in the parahypural and hypural perichondrium (Fig. 8 B, black arrowheads) and transcripts are also observed in maturing chondrocytes (Fig. 8 B, blue arrowheads). In the same specimen, *dlx5a* is expressed in cells surrounding the distal radials during radial segmentation (Fig. 8 C, black arrowheads), including in the zone of segmentation (ZS) (Fig. 8 C, green arrowheads), and in developing lepidotrichia of the anal fin (Fig. 8 C, orange arrowheads), but not in the proximal radials (Fig. 8 C). Expression of *dlx5a* in the ZS is still detected in 7.3 mm larvae while radial segmentation is almost completed (Fig. S6 B, blue arrowheads) but not in 8.7 mm larvae after radial segmentation (Fig. S6 C, black asterisks), whereas expression is maintained but decreased in cells surrounding the distal radials (Fig. S6 C). Moreover, *dlx5a* transcripts are observed in maturing chondrocytes (Fig. 8 D, blue arrowheads) and in the flanking perichondrium of anal proximal radials in 7.3 mm larvae (Fig. 8 D, black arrowheads). Later, *dlx5a* expression is detected in well-developed lepidotrichia of the dorsal fin in 8.7 mm fish (Fig. 8 E, orange arrowheads). The results suggest that *dlx5a* is implicated in the formation of skeletal components of the developing zebrafish fins.

### Discussion

*dlx5a/6a* genes are essential for the initiation of pectoral fin development

During zebrafish development, the three *dlx* bigene clusters, *dlx1a/2a, dlx3b/4b* and *dlx5a/6a*, are expressed in fin primordia [16]. However, no fin phenotypes have been observed in *dlx1a/2a* and *dlx3b/4b* morphants (E. Heude unpublished observations, personal communication from A. Fritz, [30,58]). In mouse, combinatorial mutations of several *Dlx* genes have shown that only *Dlx5/6* and *Dlx2/5* mutants display distal limb malformations [14,36,59]. Thus, the *Dlx5/Dlx6* genes seem to play a critical role in the formation of appendages in vertebrates.

In zebrafish, the *dlx5a/6a* genes are early markers of apical ectodermal cells of the presumptive and developing pectoral fin buds and median fin fold (Figs. 1, 2, Figs. S1, S2) suggesting their role in early specification and morphogenesis of these structures.

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**Figure 5. Knock down of *dlx5a/6a* leads to a defect of cleithrum differentiation.** Dorsal views of whole mount *in situ* hybridization for *runx2b* and *col10a1* on control (A, C, E) and *dlx5a/6a* morphant (B, D, F) embryos at 36 hpf (A–B) and 48 hpf (C–F). In controls at 36 hpf, *runx2b* is expressed in precursors cells of the cleithrum (c) (A) whereas expression is absent in the pectoral region of *dlx5a/6a* morphants (B, black asterisks). At 48 hpf, expression of *runx2b* and *col10a1* is detected in differentiating osteoblasts of the cleithrum which supports the pectoral fin bud (C, E). The *runx2b* transcripts are also observed at the craniofacial level in the opercular (op) and ceratobranchial 5 (cb5) bone precursors (C). In contrast, *dlx5a/6a* morphants show a drastic loss of *runx2b* and *col10a1* expression in the pectoral region (D, F, black asterisks) and of *runx2b* expression at the craniofacial level (D, blue asterisks). Scale bar shown in F for all panels 100 μm.

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At the pectoral level, \( dlx5a \) is expressed during pectoral fin bud initiation at 24 hpf and the \( dlx5a/6a \) knock down is associated with agenesis of pectoral fin buds (Figs. 3, 4). The data indicate that \( dlx5a/6a \) genes are required for pectoral fin bud induction and outgrowth in zebrafish. We show that \( dlx5a \) expression in the pectoral fin fold progressively decreases from 36 hpf to 48 hpf to become hardly detectable at 72 hpf (Fig. 2). Interestingly, this period corresponds to the AER/PFF transition followed by PFF elongation [20]. It has been shown that the unilateral surgical ablation of the PFF induces an activation of \( dlx5a \) expression in the reformed AER, whereas \( dlx5a \) is no longer detected in the control PFF in zebrafish [20]. The data suggest that \( dlx5a \) is essential for the first step of pectoral fin specification and morphogenesis until AER/PFF transition. In mouse, the \( Dlx5/6 \) genes are expressed in the AER of limb buds from E9.5 to E12.5 [13–15]. However, the genes are not expressed in the limb field during limb bud induction at E8.5 (E. Heude unpublished observations, [60]). The data indicate that \( Dlx5/6 \) are not involved in the early specification of limb buds in mouse. Indeed, in contrast to what is observed in \( dlx5a/6a \) morphants, the \( Dlx5/6 \) null mutant mice do not show limb bud agenesis, the \( Dlx5/6 \) inactivation only alters limb morphogenesis.

The \( dlx5a/6a \) knock down leads to severe decreases or loss of expression of \( bmp4, fgf8a, and1 \) and \( msx \) genes. The \( Dlx5/6 \) null mutant mice show a loss of \( Bmp4, Fgf8 \) and \( Msx2 \) expression in the medial part of the limb buds [14,15]. A recent study demonstrated that the Dlx5 protein binds to conserved sequences in the proximity of the \( Bmp2 \) and \( Bmp4 \) loci in vitro, suggesting a direct regulation of \( Bmp \) genes [15]. Moreover, BMPs appear to act as signaling relays between \( Dlx \) and \( Msx \) genes during ectoderm-mesoderm communication for proper limb development in the mouse [15]. Dlx5 seems to have a key role in the initiation of an ectoderm-mesoderm dialog during tetrapod limb morphogenesis.

Our results suggest that \( dlx5a/6a \) expression in ectodermal cells acts upstream of \( bmp4, fgf8a \) and \( msx \) genes in the pectoral fin bud primordia to induce pectoral fin outgrowth in zebrafish. The \( dlx5a/6a \) genes seem to be crucial to induce the ectoderm-mesoderm communication at the basis of appendage outgrowth in zebrafish. However, it appears that the role of \( Dlx5/6 \) in the early development of paired appendages diverged between teleosts and tetrapods during vertebrate evolution.

| Table 1. Markers affected by the \( dlx5a/6a \) knock down during the early development of pectoral and median fins. |
|-------------------------------------------------|-------------------------------------------------|
| **Pectoral fin** | **Median fin** |
| 24 hpf | 48 hpf | 16 hpf | 24 hpf | 48 hpf |
| \( bmp4 \) | A | A | U | U | X |
| \( fgf8a \) | X | A | X | U | U |
| \( msxB \) | A | A | U | U | A |
| \( msxC \) | A | A | U | U | A |
| \( and1 \) | X | A | X | A | A |

A, affected; U, unaffected; X, not expressed.
Distinct requirement for dlx5a/6a gene function during paired and unpaired fin development

It has been shown that the early dorsal MFF apical tissue consists of epidermal cells covered by periderm [17,61]. Our results show that, at the posterior level, dlx5a is first detected in ectodermal cells underlying the periderm at the lateral edges of the neural keel, with expressing cells later converging towards the midline to cover the posterior neural rod (Fig. 1) [62,63]. From 15.5 hpf to 16 hpf, we show that ectodermal cells expressing dlx5a are laterally connected to the neural ectoderm. Thus, we propose that the presumptive dorsal MFF territory expressing dlx5a results from the medial fusion of the lateral edges of the neural keel during neural rod formation (Fig. 1 D).

We show that the dlx5a/6a knock down leads to impaired median fin fold development associated with msx and and1 misexpression in MFF mesenchymal cells, increased apoptosis and decreased proliferation in the MFF at 24 hpf (Figs. 6, 7 A, A′–B, B′, Figs. S4, S5). Moreover, similar to what was observed in and1/and2 double morphants [46], the median fin fold anomalies of dlx5a/6a morphants are characterized by defective mesenchymal migration and absence of actinotrichia (Fig. 7 C, C′–D, D′). As already mentioned, and1 is expressed in cells adjacent to the dlx5a-expressing apical ectodermal tissue in the MFF at 24 hpf. Our results suggest that the dlx5a/6a genes may be required to...
maintain proper andl expression leading to actinotrichia formation during early MFF development.

The pectoral fin bud and median fin fold share similar embryonic components including apical ectodermal tissue and mesenchymal cells migrating along the actinotrichia to invade the folds (Fig. 4 I, Fig 6. C). Both structures express a similar set of genes in the corresponding embryonic tissues. Indeed, early dlx5a expression is detected in apical ectodermal cells of the presumptive MFF at 13.5 hpf and in the PFF at 24 hpf suggesting a similar role for dlx5a in the specification of both structures. However, whereas the dlx5a/6a knock down leads to the early loss of expression of markers associated with fin bud agenesis at the pectoral level, we did not observe obvious differences in the expression of bmp4, fgf8a and msx genes in the MFF of the double morphants compared to controls at 16 hpf and 24 hpf (Table 1). Moreover, the median fin fold begins to develop in dlx5a/6a morphants and later shows developmental defects at 48 hpf. In contrast to what we described at the pectoral level, dlx5a/6a genes seem to be required for proper morphogenesis of the MFF but not for its specification.

In pectoral and median fin buds, dlx5a expression is associated, directly or indirectly, with activation of bmp4, fgf8a, msx and andl genes. Fgf8 and Dlx proteins are also detected in the MFF apical tissue of sharks suggesting that ancestral molecular mechanisms implicated in unpaired fin development might have been established during early vertebrate evolution [22]. It is now well accepted that paired appendages evolved after unpaired appendages [22,64,65]. Despite their different embryonic origins [66,67], it has been suggested that paired and unpaired fins use a common suite of developmental mechanisms, a hypothesis mainly based on expression analyses [17,22,68,69]. The latter studies support that ancestral mechanisms of median fin development have been co-opted for the development of paired appendages. In contrast, our observations show that dlx5a/6a knock down has a different impact on zebrafish pectoral and median fin development (Table 1). The early communication between MFF apical ectodermal cells and underlying structures seems to take place in dlx5a/6a morphants. However, dlx5a/6a expression in apical ectodermal cells may be required for the specification of the pectoral fin bud. Therefore, although development of paired and unpaired fins may use similar molecular mechanisms, when it comes to those involving dlx5a/6a, differences in timing, expression territory or usage of target genes may underlie the profound phenotypic differences that we observed. Alternatively, paired and unpaired fins may use different dlx5a/6a-associated molecular mechanisms for their development. The differential effects of zebrafish mutations affecting other genes on pectoral and median fin development also support differences in mechanisms (for review, [70]).

Cleithrum formation requires dlx5a/6a expression

We show that dlx5a is highly expressed in the differentiating cleithrum from 36 hpf to 72 hpf in control embryos (Fig. 2 C-D). The cleithrum extends from the base of the pectoral fin and forms the posterior edge of the gill chamber. The cleithrum is an ancestral component of the pectoral girdle not homologous to any bones in mammals. It is present in all bony fish ancestors (Osteichthyes) and stem-group tetrapods but is absent in living tetrapods [71], except frogs [72]. Apparently, loss of the cleithrum during vertebrate evolution corresponds to the appearance of neck structures for head mobility. In zebrafish, the cleithrum is a dermal bone from mesodermal origin [1,73] and is the first bone to mineralize at the axial level during early zebrafish development. Expression of dlx5a in the cleithrum is associated with expression of runx2b at 36 hpf and runx2b and col10a1 at 48 hpf, early/intermediate and intermediate/late markers of osteoblast differentiation respectively (Fig. 5 A, C, E) [47,53,54]. The expression analysis indicates that dlx5a is an early/intermediate marker of differentiating cleithrum osteoblasts.

In dlx5a/6a morphants, expression of runx2b and col10a1 is lost suggesting impaired cleithrum development (Fig. 5 B, D, F). Absence of cleithrum is not an indirect effect of the loss of pectoral fins in dlx5a/6a morphants. Many studies reported experiments that led to the absence of pectoral fins while the cleithrum was still present, often without any signs of dysmorphologies [10,11,74-77]. Moreover, dlx5a/6a knock down also leads to a loss of runx2b expression in the opercular and ceratobranchial bones (Fig. 5 C, D), skeletal structures which both develop in a dlx5a/6a-positive context at the craniofacial level (Fig. S3) [38,49]. Our results show that dlx5a/6a genes are required for cleithrum formation in zebrafish.

dl5a is involved in fin skeletogenesis

To further study the potential implication of dlx5a during zebrafish fin skeletogenesis, we extended dlx5a expression analysis to later stages of unpaired fin bone formation. The fin skeleton is a mix of endochondral and dermal bones; the radial and hypural bones which support and articulate the fins rays originate from cartilaginous structures (Fig. 8 A, blue structures), whereas the lepidotrichia (fin rays) are dermal bones [65]. We show that dlx5a is expressed in the developing endochondral and dermal structures (Fig. 8, Fig. S6). During endochondral fin skeletogenesis, dlx5a is expressed in maturing chondrocytes and in the perichondrium of hypurals and proximal radials (Fig. 8 B, D). We never detected transcripts in proliferating or resting chondrocytes. The results are consistent with what is observed during long bone formation in tetrapods and suggest a conserved Dlx5 implication in chondrocyte differentiation among vertebrate species.

In teleosts, the radials arise from a common mesenchymal condensation which later segments into proximal and distal components. It has been suggested that fish radial segmentation corresponds to tetrapod joint interzone formation and that both processes share similar spatio-temporal expression of genes between zebrafish, chick and mouse [78]. Our results show that dlx5a expression is detected in cells surrounding the distal radials, notably in the zone of segmentation (ZS) during radial segmentation suggesting its role in the latter process (Fig. 8 C, Fig. S6 B). It has been shown that Dlx5/6 genes are early markers of the presumptive elbow joint in chick developing limbs [79]. The study reveals that co-expression of Dlx5 and Gdf5, a gene known to regulate joint formation, corresponds to the initiation of elbow joint formation. Interestingly, gdf5 is also observed in the ZS in 6.6 mm zebrafish larvae [80] at the same stage as dlx5a transcripts (Fig. 8 C). Our data further support the potential implication of Dlx5 in appendage segmentation/joint formation in vertebrates. In parallel, we observed that dlx5a is expressed in the developing lepidotrichia of median fins (Fig. 8 C, E). Altogether, the results associated with what is observed in the cleithrum (Fig. 5) indicate that dlx5a is involved in zebrafish osteoblast differentiation. However, the role of dlx5a in zebrafish skeletogenesis requires further investigations.

Conclusion

Our results demonstrate that dlx5a/6a genes are necessary for the specification and outgrowth of the zebrafish pectoral fin buds. However, the dlx5a/6a genes do not seem to carry out the same role during the development of pectoral and median fins suggesting differences in the molecular mechanisms controlling
the early development of paired and unpaired fins. The origin of paired fins during vertebrate evolution is still controversial [22,66,67,69]. Our results can refocus arguments and may open new evolutionary perspectives on the mechanistic basis of paired appendage genesis in vertebrate species.

Supporting Information

Figure S1  Expression patterns of dlx5a during zebrafish median fin development. Whole mount in situ hybridization for dlx5a on lateral view of the posterior axis of 24 hpf (A) and 48 hpf (B) zebrafish embryos and on 10 μm parasagittal frozen sections (A’, B’) at the level indicated in (A, B). At 24 and 48 hpf, dlx5a expression is limited to apical ectodermal cells of the median fin fold (MFF). nc, notochord; nt, neural tube; sc, spinal cord. Scale bars 50 μm. (PDF)

Figure S2  Comparison of dlx5a and dlx6a expression in the developing zebrafish fins. Whole mount in situ hybridization for dlx5a (A, B) and dlx6a (C, D) in the pectoral fin bud (PFB) (A, C) and in the median fin fold (MFF) (B, D) of 30 hpf embryos. Expression of dlx5a is detected in apical ectodermal cells of both pectoral and median developing fins (A, B, black arrowheads). Expression of dlx6a mirrors dlx5a expression, however dlx6a transcripts seem to be present at lower level (C, D, black arrowheads). Scale bars 50 μm. (PDF)

Figure S3  The opercle, the ceratobranchial-5 bone and the cleithrum all develop in a dlx5a-positive context. Dorsal and lateral views of whole mount in situ hybridization for runx2b (A, A’) and dlx5a (B, B’) in the anterior region of 40 hpf zebrafish embryos. Expression of runx2b reveals the opercle (op), the ceratobranchial-5 bone (cb5) and the cleithrum (cl) (A, A’), structures which differentiate at early stage of zebrafish development. The dlx5a expression analysis at equivalent stage shows that the three bones develop in dlx5a-positive domains, explaining the loss of runx2b expression in dlx5a/6a morphants at the pectoral and craniofacial levels shown in Fig. 5 D (black and blue asterisks), ba, branchial arches. Scale bars 100 μm. (PDF)

Figure S4  Expression of andl1 in the median fin fold of control and dlx5a/6a morphant embryos. Ventral view at the posterior level of whole mount in situ hybridization for andl1 in 24 hpf controls (A) and dlx5a/6a morphants (B). The ventral view reveals that andl1 transcripts are not expressed in the apical ectodermal cells of the median fin fold (medial line, AP), but in ectodermal cells adjacent to the AP. Scale bar shown in B for the two panels 50 μm. (PDF)

Figure S5  The median fin fold defects in dlx5a/6a morphants are associated with altered cell proliferation and apoptosis. Lateral view at the posterior axis of BrdU (A, A’) and TUNEL (B, B’) assays on control (A-B) and dlx5a/6a morphant (A’,B’) embryos at 24 hpf. The BrdU assay shows that controls present proliferating cells in the median fin fold whereas no BrdU-positive cells are observed in the morphants. In parallel, the morphants show a high increase of apoptotic cells in the MFF compared to controls (B–B’). Scale bar shown in B’ for all panels 20 μm. (PDF)

Figure S6  Expression of dlx5a in fin skeletal components of late-stage zebrafish larvae. (A) Overview of the posterior axial skeleton of a one-month-old zebrafish. Endoskeletal fins supports are colored blue and red square indicates the structures analyzed in (B-C). (B, C) Whole mount in situ hybridization for dlx5a on 10 μm parasagittal frozen sections of 7.3 mm and 8.7 mm late-stage zebrafish. As seen in Fig. 8 C in 6.6 mm larvae, dlx5a expression is still detected in cells surrounding the distal radials (dr) in 7.3 mm larvae, including in the zone of segmentation (ZS) (B, blue arrowheads) when the radial segmentation is almost completed. Later, after segmentation (8.7 mm), dlx5a expression is maintained but decreases in cells surrounding the distal radials and is no longer detected in the ZS (black asterisks) (C), dr, distal radials; lp, lepidopterichia; pr, proximal radials. Scale bars B–C 10 μm. (PDF)

Checklist S1  ARRIVE Checklist. (DOC)

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Author Contributions

Conceived and designed the experiments: EH ME. Performed the experiments: EH SS. Analyzed the data: EH ME. Contributed reagents/materials/analysis tools: ME. Wrote the paper: EH ME.

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