Development 133, 2649-2659 (2006) doi:10.1242/dev.02438

# Induction and prepatterning of the zebrafish pectoral fin bud requires axial retinoic acid signaling

**Yann Gibert, Alexandra Gajewski, Axel Meyer and Gerrit Begemann***

Vertebrate forelimbs arise as bilateral appendages from the lateral plate mesoderm (LPM). Mutants in *aldh1a2* (*raldh2*), an embryonically expressed gene encoding a retinoic acid (RA)-synthesizing enzyme, have been used to show that limb development and patterning of the limb bud are crucially dependent on RA signaling. However, the timing and cellular origin of RA signaling in these processes have remained poorly resolved. We have used genetics and chemical modulators of RA signaling to resolve these issues in the zebrafish. By rescuing pectoral fin induction in the *aldh1a2/neckless* mutant with exogenous RA and by blocking RA signaling in wild-type embryos, we find that RA acts as a permissive signal that is required during the six- to eight-somite stages for pectoral fin induction. Cell-transplantation experiments show that RA production is not only crucially required from flanking somites, but is sufficient to permit fin bud initiation when the trunk mesoderm is genetically ablated. Under the latter condition, intermediate mesoderm alone cannot induce the pectoral fin field in the LPM. We further show that induction of the fin field is directly followed by a continued requirement for somite-derived RA signaling to establish a prepattern of anteroposterior fates in the condensing fin mesenchyme. This process is mediated by the maintained expression of the transcription factor *hand2*, through which the fin field is continuously posteriorized, and lasts up to several hours prior to limb-budding. Thus, RA signaling from flanking somites plays a dual early role in the condensing limb bud mesenchyme.

**KEY WORDS: Retinoic acid, Raldh2, Limb, Pectoral fin, Somites, Spadetail, No tail, Neckless, Zebrafish**

## INTRODUCTION

Vertebrate limbs arise bilaterally from the lateral plate mesoderm (LPM) of the embryo. Within the trunk LPM, interactions between the limb bud mesenchyme and the overlying ectoderm result in the initiation of local patterning and distal outgrowth that shape the fore and hindlimb buds at the appropriate levels along the anteroposterior (AP) axis (reviewed by Capdevila and Izpisua Belmonte, 2001; Logan, 2003; Tickle, 2003).

Fibroblast growth factors (FGFs) have been shown to play an important role in limb induction. When beads are soaked in a range of fibroblast growth factors and are placed in the flank of chick embryos, they induce the formation of ectopic limb buds (Cohn et al., 1995; Ohuchi et al., 1995). Competence of the LPM to respond to these signals is initially distributed along most of the AP axis of the LPM in chick and mouse embryos (Tanaka et al., 2000).

The search for the axial sources of FGF expression in the chick embryo lead to the observation that the intermediate mesoderm (IM), situated between the somites and the LPM, is required for cell proliferation in the adjoining limb bud mesenchyme (Geduspan and Solursh, 1992; Smith et al., 1996); furthermore, FGF8 is produced in the intermediate mesoderm at the time of limb induction and, when applied to the chick flank, induces the development of additional limbs (Crossley et al., 1996; Vogel et al., 1996). On the other hand, when the induction of mesonephros, a component of IM, is blocked rostral to the future limb field, limb buds nevertheless form and develop normally (Fernandez-Teran et al., 1997). In addition, elimination of FGF8 from the IM does not affect limb initiation or outgrowth (Boulet et al., 2004). In addition, genes of the Wnt family are differentially expressed in the chick IM and LPM and are capable of inducing limb outgrowth (Kawakami et al., 2001). Specifically, forelimb initiation requires *Wnt2b* expression in the IM and/or LPM of the chick, while its source in zebrafish appears to be the somites flanking the pectoral fin field (Ng et al., 2002). Thus, the identity of axial signals required for limb development as well as their source(s) remain to be determined.

Retinoic acid (RA) is required for a variety of processes during vertebrate embryonic development. Its effect is transmitted by retinoic acid receptors (RARs) at the level of regulating the expression of target genes (reviewed by Morriss-Kay and Ward, 1999). Three enzymes, Aldh1a1-3 (previously designated Raldh1-3), catalyze the final oxidative step by which vitamin A (retinol) is converted to RA, but only Aldh1a2 has been shown to be responsible for RA synthesis during early stages of embryogenesis. During gastrulation, *aldh1a2* is expressed in embryonic mesoderm and is later found in mesodermal derivatives, such as somites, IM and LPM (Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999; Begemann et al., 2001; Chen et al., 2001; Grandel et al., 2002).

Mouse and zebrafish mutants in *aldh1a2* have been used to show that the somitic mesoderm acts a source of RA that patterns adjacent axial tissues, such as the hindbrain and pancreas; importantly, *aldh1a2* mutants also lack forelimb buds (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 2002; Linville et al., 2004; Molotkov et al., 2005; Stafford et al., 2006). The absence of forelimb buds in *aldh1a2* mutants is associated with the loss of *tbx5* expression, the earliest known marker of the developing forelimb field (Gibson-Brown et al., 1996; Tamura et al., 1999; Begemann and Ingham, 2000). In the mouse, *Tbx5* first serves to establish forelimb bud outgrowth by initiating the expression of regulatory loops of Wnt and Fgf proteins (reviewed by Capdevila and Izpisua Belmonte, 2001; Logan, 2003); while in the zebrafish, *tbx5* is required for the migration of mesenchymal LPM cells to the pectoral fin bud. As a result, mutants in both vertebrates do not form forelimb buds (Ahn et al., 2002; Garrity et al., 2002; Agarwal et al., 2003;

Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Konstanz, Germany.

*Author for correspondence (e-mail: gerrit.begemann@uni-konstanz.de)

**Accepted 10 May 2006**

Rallis et al., 2003). The failure to induce *tbx5* expression in the absence of early RA signaling therefore indicates an essential early role for RA during the establishment of the limb/fin field.

In zebrafish, *aldh1a2* is expressed in the posterior mesenchyme of the developing pectoral fin and in the LPM (Grandel et al., 2002; Emoto et al., 2005). RA has been detected in the limb bud mesenchyme (Thaller and Eichele, 1987; Niederreither et al., 2002); however, RA production in the cells of the polarizing region of the limb bud has not been demonstrated. Expression analyses in zebrafish and mouse embryos mutant for *Aldh1a2* have shown that RA is required to induce sonic hedgehog (Shh), the protein responsible for the polarizing activity (reviewed by Tickle, 2003), during forelimb outgrowth (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 2002), and suggest that RA signaling may be involved in the establishment of AP polarity in the forelimb. Indeed, maternal RA supplementation of *Aldh1a2* mutant mice rescues forelimb development, but does not restore normal AP patterning of the limb (Niederreither et al., 2002). Rescued limbs are characterized by a lack or abnormal distal distribution of Shh. More recent studies, however, have proposed a different role for RA in limb patterning. Using mice mutant for *Aldh1a2*, Mic et al. (Mic et al., 2004) have shown that RA signaling is required first to initiate forelimb development and later to expand the apical ectodermal ridge (AER) along the distal ectoderm. A study by Yashiro et al. (Yashiro et al., 2004) similarly suggested that a gradient of RA signaling is required to determine proximodistal identity in the developing mouse forelimb.

In this study, we determine the crucial time period for limb induction and the source of RA. By analyzing pectoral fin development in the absence of RA, we uncover that prepatterning by RA occurs several hours prior to the budding of the pectoral fin to establish a functional zone of polarizing activity. Our findings point to an essential role for somite-derived RA in fin field induction, apical fold formation and in establishing posterior cell fates within the condensing fin mesenchyme.

## MATERIALS AND METHODS

### Zebrafish husbandry

Zebrafish strains of Konstanz wild type, *nls*$^{i26}$ (Begemann et al., 2001), *ntl*$^{tc41}$ (Odenthal et al., 1996) and *spt*$^{b104}$ (Kimmel et al., 1989) were reared and staged at 28.5°C according to Kimmel et al. (Kimmel et al., 1995).

### Pharmacological treatments

Embryos were incubated in the dark at 28.5°C in $10^{-8}$ M all-trans retinoic acid (Sigma), diluted in embryo medium, from a $10^{-2}$ M stock solution in DMSO. The pan-retinoic acid receptor antagonist BMS493 (a kind gift of Bristol Myers Sqibb) was diluted to $10^{-5}$ M and $5\times10^{-6}$ M from a $10^{-2}$ M stock solution in ethanol. DEAB (4-diethylaminobenzaldehyde) (Fluka) was applied at a concentration of $10^{-5}$ M from a $10^{-2}$ M stock in DMSO, without shielding from daylight. As controls, wild-type embryos were treated with equivalent concentrations of DMSO. Genotyping to detect homozygosity of the *nls* allele was performed as previously described (Begemann et al., 2001).

### In situ hybridization

Whole-mount in situ hybridization was performed as previously described for *aldh1a2* (Begemann et al., 2001), using the following additional probes: *dlx2a* (Akimenko et al., 1994), *hand2* (Yelon et al., 2000), *hoxc6a* (Molven et al., 1990), *hoxd11a, hoxd12a* (Sordino et al., 1995), *msxc* (Ekker et al., 1992), *myod* (Weinberg et al., 1996), *shh* (Krauss et al., 1993), *tbx5* (Begemann and Ingham, 2000), *tpm1* (Zebrafish Information Network) and *pax2a* (Krauss et al., 1991).

### Mosaic analysis

Donor embryos were injected at the one-cell stage with 2.5% lysine fixable tetramethyl-rhodamin-dextran and 3.0% lysine fixable biotin-dextran ($M_r$ 100,000)(Molecular Probes) dissolved in 0.2 M KCl. At late blastula stages, groups of 10-30 donor cells were transplanted into unlabelled host embryos of the same stage, derived from pair-matings of *nls* heterozygotes, and placed along the margins of the blastoderm, which gives rise to the mesendoderm (Kimmel et al., 1990). Transplants were carried out blindly, and host genotypes determined at 24 hpf. Transplanted cells were examined at 48 hpf for fluorescence in all *nls* hosts prior to or after fixation and in situ hybridization.

## RESULTS

### Induction of the pectoral fin field requires RA signaling during early somitogenesis stages

RA signaling during gastrulation has been shown to regulate the development of the neuroectoderm, endoderm and cardiac mesoderm, respectively (Grandel et al., 2002; Kudoh et al., 2002; Stafford and Prince, 2002; Keegan et al., 2005). In order to determine in more detail the developmental stages during which RA signaling is required to induce the pectoral fin field, we employed two complementary strategies: rescuing *neckless* (*nls*) through exogenous RA; and inhibiting fin field formation by blocking RA signaling in wild-type zebrafish.

Initiation of forelimb bud formation is characterized by the expression of *tbx5* in the anterior lateral plate mesoderm and precedes the emergence of visible forelimb buds (Ruvinsky et al., 2000; Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). Pectoral fin development is characterized by the appearance of an apical fold that expresses *dlx2a* (Akimenko et al., 1994). We treated wild-type embryos with $10^{-6}$ M BMS493, which inhibits the activation of all three RA-receptor (RAR) subforms in amniotes by reinforcing co-repressor binding to RAR (Wendling et al., 2000; Dupé and Lumsden, 2001) from 10 hpf onwards, and confirmed that fin buds do develop and express both *tbx5* and *dlx2a* (Grandel et al., 2002) (Fig. 1B,F). However, when treated with $5\times10^{-6}$ M BMS493 or with $10^{-5}$ M diethylaminobenzaldehyde (DEAB), a competitive reversible inhibitor of retinaldehyde dehydrogenases (Begemann et al., 2004) from 10 hpf onwards, no fin buds emerged and none of the genes were expressed (Fig. 1C,D,G,H). We next refined these tests of RA requirement by incubations in $10^{-5}$ M DEAB or in a range of $10^{-5}$ to $5\times10^{-6}$ M BMS493. We observed that blocking RAR activation after 13 hpf, or inhibiting endogenous RA synthesis after 12 hpf, has no effect on the onset of *tbx5* expression in the pectoral fin, while treatments commencing earlier disrupt fin field induction (Table 1A). These results suggest that RA signaling is required for fin development up to 13 hpf.

We next supplied embryos derived from parents that were heterozygous for *nls* with an exogenous supply of $10^{-8}$ M RA, commencing treatment from successively later stages onwards, and monitored expression of *tbx5* at 24 hpf, when the mesenchymal core of the pectoral fin bud has formed (Tamura et al., 1999; Begemann and Ingham, 2000) (Fig. 1Q). *nls* mutants were identified either through expansion of *krox20* (egr2b – Zebrafish Information Network) expression in the hindbrain when treated after 10 hpf (not shown), or by genotyping when treated prior to 10 hpf. In agreement with a requirement for RA after 10 hpf, treatments initiated as late as 12 hpf are sufficient to rescue *tbx5* expression to approximately wild-type levels, while treatment at 13 hpf or later is not sufficient (Table 1B).

To confirm the hypothesis that RA signaling after gastrulation is required for fin induction, RA synthesis was inhibited with DEAB in wild-type embryos starting from 5 hpf onwards, and at 11 hpf was supplanted with exogenous RA; fin bud induction in these fish proceeded indistinguishably from wild-type development (Fig. 1I,J). Together, these experiments suggest that pectoral fin field

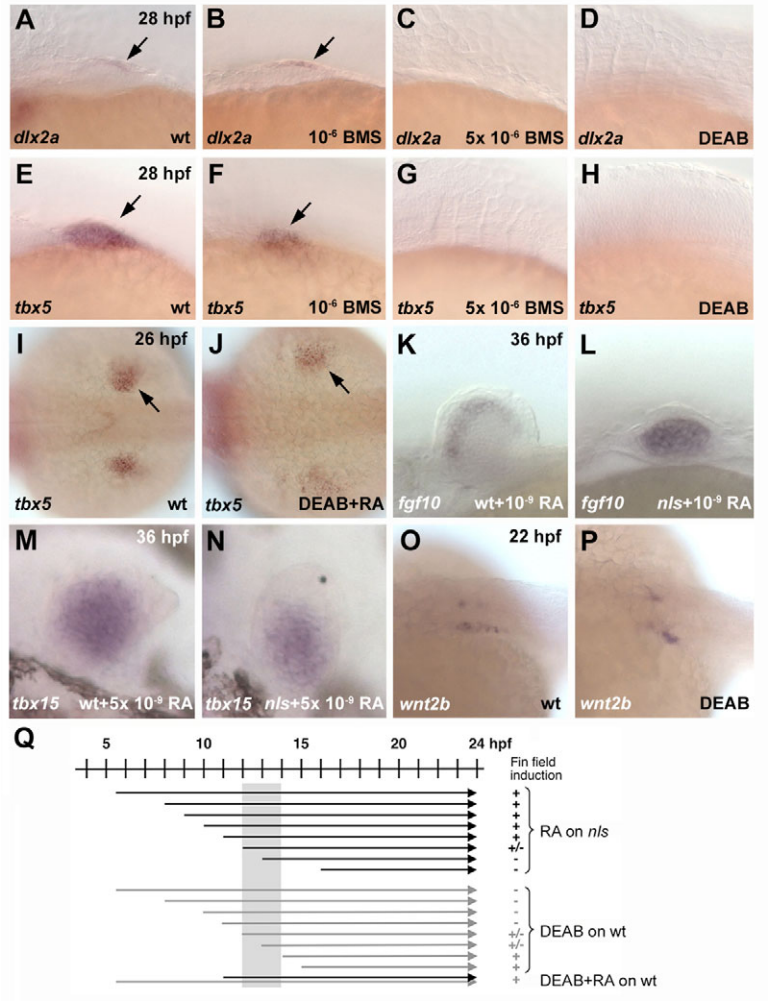


**Fig. 1. Pectoral fin field induction requires retinoic acid signaling during somitogenesis.** Anterior is towards the left: lateral (A-H,K-N), dorsal (I,J) and dorsolateral (O,P) views. Whole mount in situ hybridizations. (**A-H**) Wild-type embryos treated from 10 hpf onwards with $10^{-6}$ M and $5\times10^{-6}$ M BMS493 (B,F and C,G) and with 10 μM DEAB (D,H); untreated controls (A,E). Expression of mesenchymal (*tbx5*) and apical epidermal fold (*dlx2a*) markers (arrows) is lost at the higher antagonist concentration and in DEAB treatments. (**I**,**J**) In the absence of RA synthesis from 5 hpf onwards (10 μM DEAB), *tbx5* expression is restored to near-normal levels by treatment with $10^{-8}$ M RA from 11 hours onwards (arrows) (J). (**K-N**) $10^{-9}$ M RA (11-36 hpf) rescues *fgf10* expression (K,L) and $5\times10^{-9}$ M RA (11-36 hpf) rescues *tbx15* expression (M,N) in *nls*. (**O**,**P**) *wnt2b* expression is not abolished in wild-type animals treated with DEAB from 11 hpf onwards. (**Q**) Schematic overview of the treatment applied to wild-type embryos depicted in A-J; shaded area depicts time window in which RA signaling is required.

development requires RA signaling after the end of gastrulation and prior to the 12 hpf (six-somite stage). The sufficiency of this short period of treatment indicates that RA is required for induction, rather than maintenance of the pectoral fin field.

To test if development in RA-rescued fins in *nls* proceeds normally, we analyzed the expression of further genes involved in pectoral fin development. *fgf10* acts genetically downstream of *fgf24* (Fischer et al., 2003) and is expressed in the fin mesenchyme at 24 hpf (Ng et al., 2002). In RA-rescued fins of *nls* mutants, *fgf10* is expressed strongly (Fig. 1K,L), suggesting that its upstream component *fgf24* must also be active. *tbx15* is strongly expressed throughout the pectoral fin mesenchyme starting from 30 hpf (Begemann et al., 2002) and is detected in RA-rescued *nls* fins (Fig. 1M,N). We conclude that genes downstream of *tbx5* in the fin mesenchyme are activated following RA-treatment in *nls*. By contrast, expression of *wnt2b*, which is required for *tbx5* expression and at 22 hpf is found in the ventral part of somites flanking the pectoral fin bud (Ng et al., 2002), is not abolished in wild type fish treated with DEAB or a pan-antagonist of all RA-receptors from 11-22 hpf (Fig. 1O,P and not shown), which includes the time of fin induction and early patterning. Thus, RA is dispensable for somitic *wnt2b* expression. Like *aldh1a2*, *wnt2b* acts upstream of *tbx5* during fin induction (Ng et al., 2002), so that both genes might operate in parallel pathways, Alternatively, earlier *wnt2b* expression outside the somites may be required to regulate *tbx5*.

## Somitic mesoderm is required for pectoral fin induction

During segmentation stages, *aldh1a2* is expressed in the somitic and intermediate mesoderm (Begemann et al., 2001; Grandel et al., 2002). As both these embryonic structures are in close proximity to the future pectoral fin field, we wanted to distinguish whether the source of RA that triggers pectoral fin induction is the intermediate or somitic *aldh1a2* expression domain. To this end, we genetically ablated the somitic mesoderm in embryos double mutant for *spadetail* (*spt*) and *no tail* (*ntl*) (Amacher et al., 2002; Goering et al., 2003), taking advantage of the fact that double-mutants for the allelic combination of $spt^{b104}$ and $ntl^{tc41}$ lack somitic mesoderm, but retain a reduced intermediate mesoderm (IM), as marked by the expression of *pax2a*, a marker of the pronephric duct and the glomerulus (Majumdar et al., 2000) (Fig. 2A-D). Similarly, somitic *aldh1a2* expression is reduced in *spt* and is absent in $spt^{b104}/ntl^{tc41}$ (Fig. 2E-H′). Because a faint *aldh1a2* staining remains in double mutants, we wanted to identify unambiguously double-mutant embryos by simultaneously monitoring both *myod* and *aldh1a2* expression. At 13 hpf, when RA signaling is required for pectoral fin field induction, *aldh1a2* is expressed in the wild-type IM, and can be clearly detected at reduced levels in the IM of $spt^{b104}/ntl^{tc41}$ (Fig. 2J,J′). These results establish that in $spt^{b104}/ntl^{tc41}$ the IM is likely to produce RA.

$spt^{b104}/ntl^{tc41}$ double mutants do not develop any morphological signs of pectoral fin development and *tbx5* expression in the pectoral fin field is indistinguishable from wild type in *ntl* (Fig. 3B). In

agreement with the reduction of *aldh1a2* expression in trunk mesoderm, *tbx5* expression is slightly reduced in *spt* (Fig. 3C). By contrast, double-mutant embryos lack *tbx5* expression (Fig. 3D), suggesting that fin field induction requires RA signaling from somites rather than from IM.

## RA is sufficient to rescue fins in the absence of somites

As the lack of *tbx5* expression in *ntl/spt* could either be due to the lack of somite-derived signals distinct from RA or because the LPM may not be competent to form fins, we asked if exogenous application of RA after gastrulation would rescue pectoral fin field induction. Interestingly, application of $10^{-8}$ M RA, beginning at 10 hpf, efficiently rescues *tbx5* expression in $spt^{b104}/ntl^{tc41}$, suggesting that the LPM is competent to respond to a somite-derived signal to form fins (Fig. 3I,K). As RA application after gastrulation does not reconstitute somite development in double mutants (Fig. 3J-M), RA is identified as the signal emanating from somites that induces the pectoral fin field. Moreover, these findings further suggest that somite-derived signals other than RA are not required to initiate *tbx5* expression.

## RA from anterior somites induces pectoral fin field formation

In order to determine more directly the source of RA, we next investigated whether somitic cells of wild-type origin were able to induce *tbx5* expression in the lateral plate mesoderm of *nls* mutants. We transplanted lineage-labeled wild-type cells at blastula stages into the lateral marginal zone of *nls* mutant hosts, a region fated to give rise to trunk somites (Melby et al., 1996). *nls* hosts were identified by their hindbrain phenotype at 22-24 hpf and scored for the number and distribution of transplanted cells (Table 2). In such an experiment, we not only identified the *nls* hosts with rescued fins, but the presence of wild-type cells in somites without rescue is equally informative. None of the *nls* hosts had donor cells in

**Table 1. The temporal requirement of RA for pectoral fin field induction**

**A Inhibition of pectoral fin development**

| Beginning of antagonist treatment* | Sample size (*n*) | Antagonist (concentration) | Number of embryos lacking *tbx5a* expression in pectoral fins (% of sample) | Loss of *tbx5a* expression |
|---|---|---|---|---|
| 5.3 hpf | 60 | BMS ($10^{-5}$ M) | 60 (100%) | + |
| | 39 | BMS ($5\times10^{-6}$ M) | 39 (100%) | + |
| 8 hpf | 64 | BMS ($10^{-5}$ M) | 64 (100%) | + |
| | 41 | BMS ($5\times10^{-6}$ M) | 41 (100%) | + |
| 10 hpf | 102 | BMS ($10^{-5}$ M) | 102 (100%) | + |
| | 41 | BMS ($5\times10^{-6}$ M) | 41 (100%) | + |
| | 30 | DEAB ($10^{-5}$ M) | 30 (100%) | + |
| 11 hpf (3 s) | 40 | BMS ($10^{-5}$ M) | 40 (100%) | + |
| | 30 | DEAB ($10^{-5}$ M) | 30 (100%) | |
| 12 hpf (6 s) | 40 | BMS ($10^{-5}$ M) | 40 (100%) | + |
| | 30 | DEAB ($10^{-5}$ M) | 26 (87%) | |
| 13 hpf (8 s) | 40 | BMS ($10^{-5}$ M) | 18 (45%) | +/– |
| | 30 | DEAB ($10^{-5}$ M) | 0 (0%) | |
| 14 hpf (10 s) | 40 | BMS ($10^{-5}$ M) | 0 (0%) | – |
| | 30 | DEAB ($10^{-5}$ M) | 0 (0%) | |
| 14.5 hpf (11 s) | 40 | BMS ($10^{-5}$ M) | 0 (0%) | – |
| 15.5 hpf (13 s) | 40 | BMS ($10^{-5}$ M) | 0 (0%) | – |

**B Rescue of pectoral fin development**

| Beginning of RA treatment | Sample size (*n*) | Number of *nls* mutants† (expected number of *nls* mutants) | Number of *nls* mutants expressing *tbx5a* in pectoral fins (% of rescued *nls* mutants) | Rescue of *tbx5a* expression in *nls* |
|---|---|---|---|---|
| DMSO control 5.3 hpf | 51 | 6 (13) | 6 (100%) | – |
| 5.3 hpf | 55 | Not detectable (18) | Not detectable (100%) | + |
| 8 hpf | 80 | Not detectable (20) | Not detectable (100%) | + |
| 9 hpf | 80 | Not detectable (20) | Not detectable (100%) | + |
| 10 hpf | 33 | 6 (8) | 6 (100%) | + |
| 11 hpf (3s) | 49 | 9 (12) | 9 (100%) | + |
| 12 hpf (6s) | 117 | 35 (29) | 33 (94%) | + |
| 13 hpf (8s) | 134 | 36 (33,5) | 0 (0%) | – |
| 16 hpf (14s) | 43 | 10 (11) | 0 (0%) | – |
| 19 hpf (20s) | 50 | 10 (12,5) | 0 (0%) | – |
| 22 hpf (26s) | 38 | 12 (9,5) | 0 (0%) | – |

*Incubations lasted until 24 hpf, at which stage *tbx5* expression was assayed. s, number of somites.
†As identified by expansion of *krox20* expression in the hindbrain and reduction of *hoxb4a* expression in the anterior spinal cord, or by genotyping.

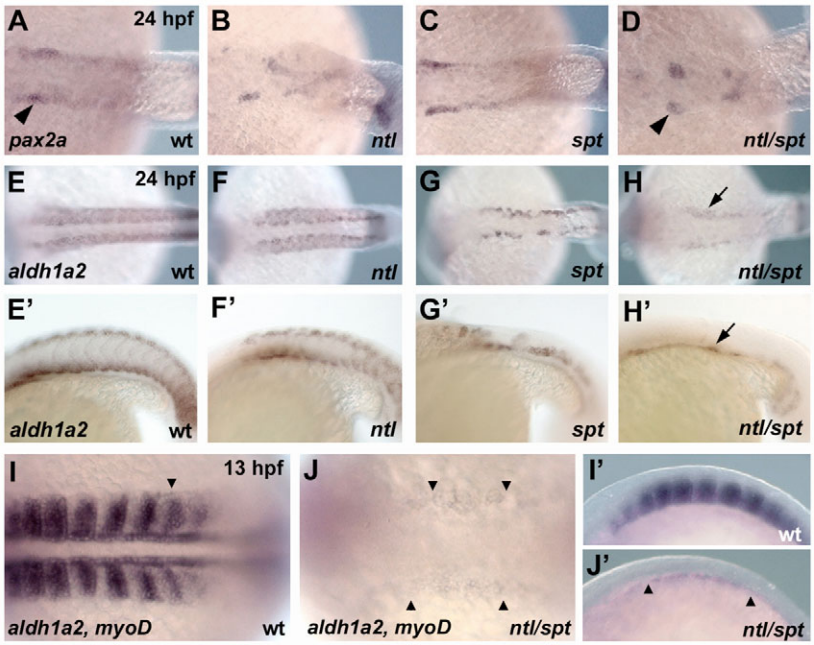


**Fig. 2. Reduction of somitic mesoderm in *no tail/spadetail* double-mutants.** Whole-mount in situ hybridizations. Anterior is towards the left, dorsal (A-J) and lateral views (E′-J′). (**A-D**) *pax2a* expression confirms the presence of intermediate mesoderm in *ntl/spt*; glomeruli are compacted in *ntl/sp* (arrowheads). (**E-H**,E′-H′) Expression of *aldh1a2* in somites is largely unchanged in *ntl* (F,F′), but reduced in *spt* (G,G′) and absent in *ntl/spt* double-mutant embryos (H,H′); expression in double-mutants is upregulated in the ventral trunk (arrows), consistent with expression in intermediate mesoderm. (**I-J′**) Double-staining reveals *aldh1a2* expression in the intermediate mesoderm (delimited by arrowheads) of wild-type and *ntl/spt* embryos; the latter lack *myod* expression (J,J′).

derivatives of the intermediate mesoderm. In one host with rescued fins ($n$=2), the majority of muscle fibers in somites 5-7 were donor derived, with only a few wild-type cells in somite 3 and 4 (Fig. 4A-C). In another successful rescue, the majority of muscle fibers in somites 3-6 were wild-type derived (Fig. 4D-F). In hosts with only a few wild-type cells occupying somites 4-7 ($n$=6), or where donor cells gave rise to part or all of the muscle fibers in somites posterior to somite 8 ($n$=6), no rescue was observed (Table 2; Fig. 4H). Interestingly, one transplanted embryo showed very strong contribution of wild-type cells to the dorsal halves of somites 1-8, yet did not lead to pectoral fin development (Fig. 4G).

These transplantation experiments show that RA signaling from somites 3-7 is sufficient for pectoral fin development; moreover, it appears that signaling originates from cells in the ventral myotome.

## Sources and requirement of RA in the developing pectoral fins

To resolve the role of RA in pectoral fin bud development and during the outgrowth phase, we first determined the expression pattern of *aldh1a2* in more detail and in later stages of fin development than previously shown. *aldh1a2* is first re-expressed at 28 hpf in the posterior-most part of the pectoral fin bud and in the adjacent LPM (Fig. 5A,B). Between 32 and 48 hpf, *aldh1a2* is expressed in the entire pectoral fin mesenchyme (Fig. 5C,D) and becomes restricted proximally at later stages (Fig. 5E,H). Thus, during initiation of fin outgrowth, RA is produced from a posterior source of *aldh1a2* expression, is synthesized uniformly in the entire fin at later stages, and becomes gradually restricted to the proximal anterior fin mesenchyme by day 5.

We next investigated the roles of RA signaling during later stages of pectoral fin development. Wild-type embryos treated with either BMS493 (not shown) or DEAB from 16 hpf onwards develop pectoral fin buds, although they are smaller than in wild type (Fig. 6). This suggests that the initiation of bud outgrowth is independent of RA, although normal cell proliferation within the pectoral fin bud may require full RA signaling.

To determine to what extent fin patterning is affected in the absence of RA, we examined gene expression in the fin bud mesenchyme and apical fold. The bHLH transcription factor *hand2* is required for the expansion of the pectoral fin-forming

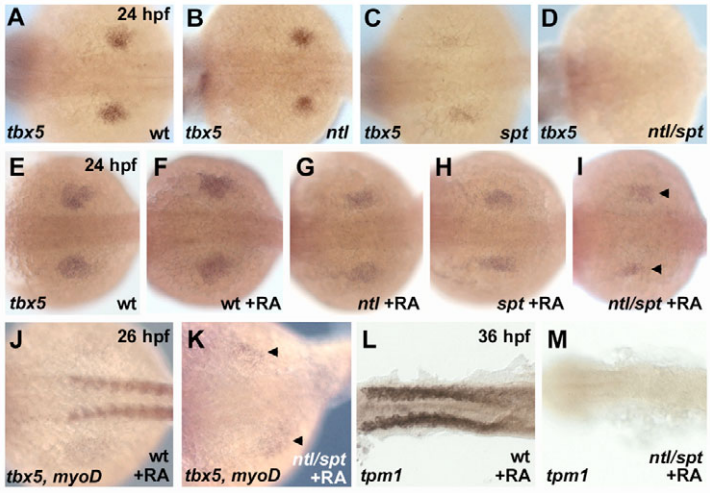


**Fig. 3. Retinoic acid rescues pectoral fin field induction in the absence of paraxial mesoderm.** Whole-mount in situ hybridizations. Anterior is towards the left, dorsal views. (**A-D**) Expression of *tbx5* in pectoral fin buds is unchanged in *ntl* (B), but reduced in *spt* (C) and absent in *ntl/spt* double-mutant embryos (D). (**E-M**) Application of $10^{-8}$ M RA, beginning at 10 hpf, rescues *tbx5* expression in *ntl/spt* pectoral fins (arrowheads in I,K); rescue occurs in the absence of myogenic differentiation, demonstrated by the absence of *myod* (J,K) and *α-tropomyosin-1* (*tpm1*) expression (L,M) in RA-treated double mutants. Four rescue experiments, each with 40 embryos, were performed (total numbers of phenotypes: 32 *spt*, 35 *ntl*, 11 *ntl/spt*, 82 siblings without mutant phenotype).

**Table 2. Transplanted wild-type cells in anterior somites rescue pectoral fin induction in *nls***

| Pectoral fin present | Somite (*n*)* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Yes | – | + | ++ | + | ++ | ++ | ++ | – | – | – | – | – | – | – | – | – |
| Yes | – | – | – | + | ++ | ++ | ++ | + | + | + | + | – | – | – | – | – |
| No† | ++ | ++ | ++ | ++ | + | + | ++ | ++ | + | + | – | – | – | – | – | – |
| No | + | + | + | – | – | – | + | – | + | + | – | – | – | – | – | – |
| No | – | – | + | + | – | + | – | – | + | + | + | + | – | – | – | – |
| No | – | – | – | + | – | – | – | – | – | – | – | – | – | – | – | – |
| No | – | – | – | – | + | – | + | + | – | – | + | + | – | + | + | ++ |
| No | – | – | – | – | – | – | + | – | – | + | + | + | + | – | – | – |
| No | – | – | – | – | – | – | – | – | ++ | ++ | ++ | ++ | – | – | – | – |
| No | – | – | – | – | – | – | – | – | – | + | + | – | – | – | – | – |
| No | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + |
| No | – | – | – | – | – | – | – | – | – | – | – | ++ | ++ | ++ | – | ++ |
| No | – | – | – | – | – | – | – | – | – | – | – | + | + | ++ | ++ | ++ |
| No | – | – | – | – | – | – | – | – | – | – | – | ++ | + | ++ | + | + |

Rescue of pectoral fin development in *nls* embryos.
*nls* hosts were identified by their hindbrain phenotype (otic placode abuts the first somite) at 22-24 hpf; *nls* hosts were scored for the number and distribution of transplanted cells, and presence of a pectoral fin bud, at 48-50 hpf.
*Shown are transplants located in the first 16 somites; donor-derived muscle fibres were counted in somites (vertical columns), indicated as absence (–), or fewer (+) or more (++) than ten fibers per somite.
†In this *nls* fish, all transplanted cells were located dorsal to the horizontal myoseptum.

region of the LPM and is expressed in the pectoral fin-bud mesenchyme, with the exception of its anteriormost aspect (Yelon et al., 2000). In the absence of RA, *hand2* is not expressed (Fig. 6A,B). Because mutants in *hand2* fail to express *sonic hedgehog* (*shh*), we next analyzed *shh* expression. In wild type, *shh* is expressed in the posterior fin bud mesenchyme at 28 hpf, co-localizing with the zone of polarizing activity (ZPA). In the absence of RA, *shh* is strongly downregulated (Fig. 6C,D). Both *shh* and *hand2* remain downregulated at least up to 48 hpf (not shown). Thus, downregulation of *shh* expression may be due to the immediate lack of RA, or may be a consequence of the loss of *hand2*.

*hoxd11a* and *hoxd12a* are markers of the posterior mesenchyme of wild-type fin buds at 40 hpf (Sordino et al., 1995) (Fig. 6E,G), the expression of which does not depend on *shh* (Neumann et al., 1999). When RA synthesis is suppressed, expression of *hoxd11a* and *hoxd12a* is downregulated (Fig. 6E-H). *hoxc6a* is marker of the anterior half of the fin mesenchyme at 28 hpf and is restricted more anteriorly as the fin bud grows (Molven et al., 1990) (Fig. 6I). When RA signaling is abolished, *hoxc6a* is expanded posteriorly to cover the anterior half of the mesenchyme at 38 hpf (Fig. 6J). These findings indicate that early AP patterning of the developing fin bud is compromised in the absence of RA, leading to an expansion of the anterior marker *hoxc6a* and the downregulation of markers of the posterior fin bud mesenchyme.

To test whether loss of RA signaling affects the development of the apical fold, a structure required for fin outgrowth, we examined *dlx2a* expression. As in wild type, *dlx2a* is expressed along the AP axis of the apical fold during fin budding in the absence of RA synthesis (Akimenko et al., 1994) (Fig. 6K,L). In a subset of embryos treated with DEAB from 14 hpf, rather than 16 hpf, onwards, slight variations in the AP-extent of *dlx2a* expression was observed, such as downregulation in the anterior and posterior apical fold (not shown). This suggests that full RA signaling is required to establish a complete apical fold.

## Axial RA signaling patterns the fin field prior to budding

*aldh1a2* is widely expressed in the pectoral fin bud (Fig. 5), whereas in the mouse it is restricted to the lateral plate mesoderm (Mic and Duester, 2003; Mic et al., 2004; Yashiro et al., 2004). We therefore determined if pectoral fin patterning is mediated by RA synthesized within the fin bud mesenchyme, by blocking signaling after initiation, but prior to *aldh1a2* expression in the fin bud. Wild type were

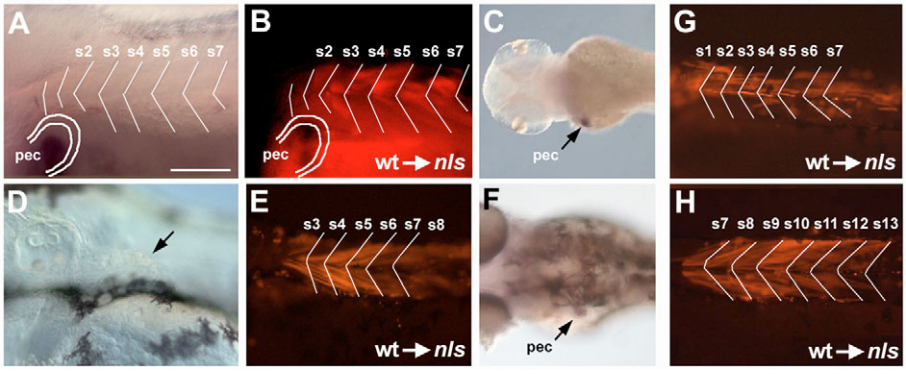


**Fig. 4. Retinoic acid signaling from anterior somites rescues pectoral fin development in *nls*.** Anterior is towards the left: lateral (A,B,D,E,G,H) and dorsal views (C,F). Transmitted light (A,C,D,F) and fluorescent (B,E,G,H) images taken at 48 hpf. (**A-C**) Wild-type donor cells (red fluorescence in B) occupying somites (s) 2-7 in a *nls* host are able to rescue pectoral fin (pec) development and *tbx5* expression on the transplanted side (dark area in pectoral fin in A,B; arrow in C); chevron-shaped lines mark somite boundaries. The embryo in B was processed for in situ hybridization prior to photography, resulting in apparent enhancement of unspecific autofluorescence within the yolk. (**D-F**) Wild-type donor cells occupying most of somites 3-6 in a *nls* host rescue pectoral fin development (arrow in D) and *tbx5* expression (arrow in F). (**G**,**H**) Donor cells transplanted to the dorsal aspect of somites 1-7 (G) or to somites posterior to somite 6 (H) are unable to rescue pectoral fin development (not shown). Scale bar: 100 μm.

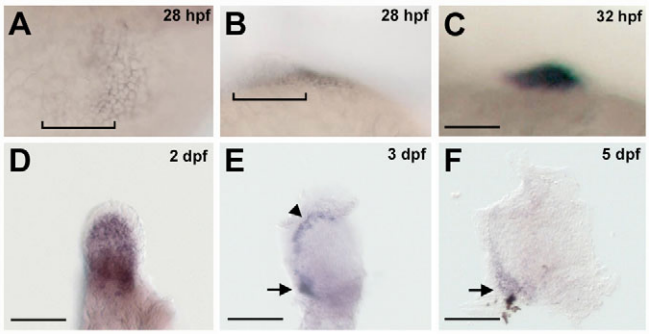


**Fig. 5. Expression of *aldh1a2* in wild-type pectoral fins.** Whole-mount in situ hybridizations. Anterior is towards the left: dorsal (A) and lateral views (B-F). Pectoral fin buds at 28 hpf (A,B), 32 hpf (C), and 2, 3 and 5 dpf (D-F). (**A**,**B**) *aldh1a2* is expressed in the posterior part of developing pectoral fins and adjoining lateral plate mesoderm; bracket demarcates the fin proper. (**C**,**D**) *aldh1a2* expression is restricted to the fin mesenchyme. (**E**,**F**) From 3 dpf onwards, expression is restricted to the proximal, anterior and distal margins of the mesenchyme, and is downregulated in the distal margin by 5 dpf. Scale bars: 100 μm.

incubated in 10 μM DEAB from 14, 18 and 22 hpf onwards, and assayed for *shh* expression at 32 hpf. Inhibiting RA signaling from 14 hpf results in reduced fin buds devoid of detectable *shh* expression and inhibition from 18 hpf onwards lead to a reduction of most *shh* expression (Fig. 6O,P). By contrast, blocking RA signaling after 22 hpf had little effect on *shh* expression (Fig. 6M,N). This suggests that AP patterning within the pectoral fin field occurs throughout early to mid-somitogenesis and prior to the formation of the limb bud. As *aldh1a2* is expressed in the fin bud after its AP pattern has been established, somite-derived RA is required to pattern the developing field along with the condensation of the fin mesenchyme.

### Effects of RA depletion during pectoral fin outgrowth

To examine the function of *aldh1a2* expression within the developing fin bud, we compared the effects of blocking RA production by incubation in DEAB in wild type between 11 and 17 hpf, and during budding and outgrowth of the fin after 28 hpf. Larvae ($n$=80) resulting from the former treatment lack pectoral fins. At 6 dpf, only the exoskeletal component of the shoulder girdle, the cleithrum, is present, while all endoskeletal derivatives, which develop from within the fin bud mesenchyme, are absent. Only in a few cases ($n$=5/80) a much reduced scapulocoracoid had formed (Fig. 7B). Larvae ($n$=80) incubated in DEAB from 28 hpf onwards exhibit defects in the development of proximal skeletal elements of the shoulder girdle ($n$=80) (Fig. 7C). More distal regions, including the endoskeletal disc, do not show any defects that are detectable at the molecular or morphological level (Fig. 7D-G). We conclude that RA is required for the proper differentiation of the cartilaginous elements of the shoulder girdle along their dorsoventral extent (relative to the animal's axis).

## DISCUSSION

### Somites provide RA for pectoral fin field induction

The contribution of RA signaling to the establishment of the forelimb field is poorly understood and the source for RA signaling establishing the fin/limb field has remained elusive. We have investigated this RA requirement in more detail and show that RA induces pectoral fin field formation after gastrulation and prior to the formation of somites 6-8. Thus, RA signaling between 12 and 13 hpf is sufficient for pectoral fin field induction as marked by *tbx5* expression. We show that RA signaling originates from expression of *aldh1a2* in somites 3-7, which appear during this time (Fig. 4). RA synthesis from anterior somites thus fulfils several important developmental roles: induction of the fin field, as well as neural patterning and motoneuron differentiation in the anterior spinal cord (Begemann et al., 2004; Linville et al., 2004). Our findings also explain the results of a previous study, in which treatment of zebrafish embryos with citral, an unspecific inhibitor of retinaldehyde dehydrogenases, from 80% epiboly to the two-somite stage leads to morphologically normal fish that lack the pectoral fins (Vandersea et al., 1998).

We observed a notable correspondence of temporal RA requirement with the development of the somites flanking the prospective forelimb field. In zebrafish, the pectoral fin develops

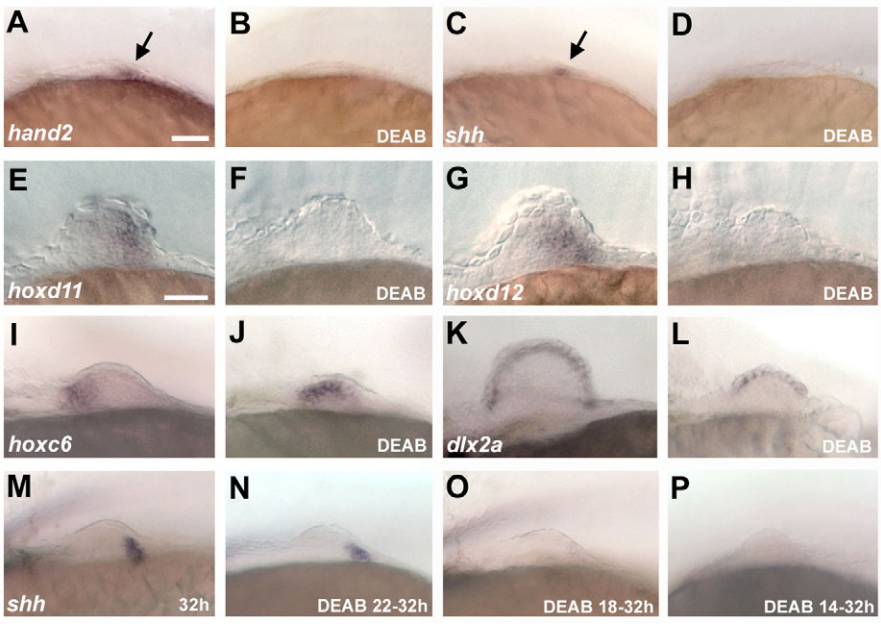


**Fig. 6. Expression of marker genes in the mesenchyme and ectoderm of wild-type and pectoral fins.** Treatment with 10 μM DEAB, starting from 16 hpf to 28 hpf (A-D), 38 hpf (I,J) and 40 hpf (E-H,K,L); and from differing starting times to 32 hpf (M-P). Anterior is towards the left. (**A**,**B**) *hand2* is expressed in the medial and posterior mesenchyme of wild type (arrow), but is not expressed in the absence of RA. (**C**,**D**) *shh* is expressed in the posterior mesenchyme (arrow), but is not expressed in the absence of RA. (**E-H**) *hoxd11* and *hoxd12* are expressed in the posterior mesenchyme and fail to be induced in the absence of RA. (**I**,**J**) *hoxc6*, which is normally restricted to the anterior mesenchyme, is expanded posteriorly in DEAB-treated embryos. (**K**,**L**) *dlx2a*, a marker of the apical fold, is normally expressed in the absence of RA. (**M-P**) *shh* induction is lost upon early inhibition of RA signaling. Scale bars: 50 μm.

lateral to the second and third somite (Grandel and Schulte-Merker, 1998), and RA is required while these somites are formed. Similarly, in the mouse embryo the forelimbs develop adjacent to somites 8-12, and limited maternal RA treatment that includes the development of the first 4-8 somites in *Aldh1a2*-mutant mice rescues initiation of the forelimb field (Mic et al., 2004). Considering the variation inherent to rescue studies in the mouse in general, and that most RA may not be cleared immediately, limb field induction in mice can be viewed as taking place concurrently with the emergence of flanking somites. This observation holds particularly true for the chick embryo, where forelimb induction has occurred by the 20-25 somite stage, and the wing bud develops adjacent to somites 15-20 (reviewed by Martin, 1998). We therefore propose a general mechanism by which somite formation and forelimb field induction are coupled through a window of RA responsiveness.

In zebrafish, the pelvic fins develop more than 2 weeks after completion of somitogenesis, in a position ventrolateral to the ninth and tenth myotome (Grandel and Schulte-Merker, 1998). If RA is equally required to induce pelvic fin development, pelvic fin induction may either occur shortly after the formation of the corresponding somites, or may be concomitant with the formation of the mesenchymal layer present in the prospective pelvic area that is present during the third week postfertilization (Grandel and Schulte-Merker, 1998). In the latter case, a heterochronic shift in signaling from RA alone or in conjunction with another signal may have enabled the delay in pelvic fin development in zebrafish. Alternatively, RA-mediated induction may not be in place for the hindlimbs. Appropriate RA rescue and inhibition assays will have to be performed to test the extent of conservation in the molecular mechanisms governing fore- and hindlimb induction.

## RA is a permissive factor for fin development

Unlocalized exogenous RA rescues *tbx5*-expressing fin buds in *nls* or RA-depleted embryos to an extent indistinguishable from wild type (Fig. 1). RA thus cannot be an instructive signal, but has to act as a permissive factor whose ectopic presence does not interfere with fin formation.

As RA is sufficient to rescue fin induction in the absence of somitic mesoderm, other somite-derived signals are not required to allow fin field induction. This finding appears to be in disagreement with a previously proposed role of *wnt2b*, which acts genetically upstream of *tbx5*. *wnt2b* is expressed in the ventral part of somites flanking the pectoral fin bud at 22 hpf (Ng et al., 2002), several hours after fin induction. Morpholino-induced inactivation of *wnt2b* leads to a failure of expression of *tbx5*, as well as of *fgf10* and *fgf24*, in the fin bud mesenchyme, which can be rescued by injection of *tbx5* mRNA (Ng et al., 2002; Fischer et al., 2003). Our findings therefore imply that *wnt2b* may be expressed outside the somites during pectoral fin field induction, in LPM or IM. Unfortunately, *wnt2b* is only weakly expressed, hampering detection of expression outside the somites or during the early segmentation period (not shown).

## RA acts over a short range during pectoral fin induction

The somitic mesoderm and LPM are in close proximity during pectoral fin field induction, separated by the IM. Diffusion of RA from forming somites must therefore travel through IM or the ectoderm to the LPM. The occurrence of one transplant with considerable contribution of wild-type cells to the dorsal myotome that fails to rescue fin induction suggests that RA may diffuse over a short distance only (Fig. 8). In agreement with the temporal

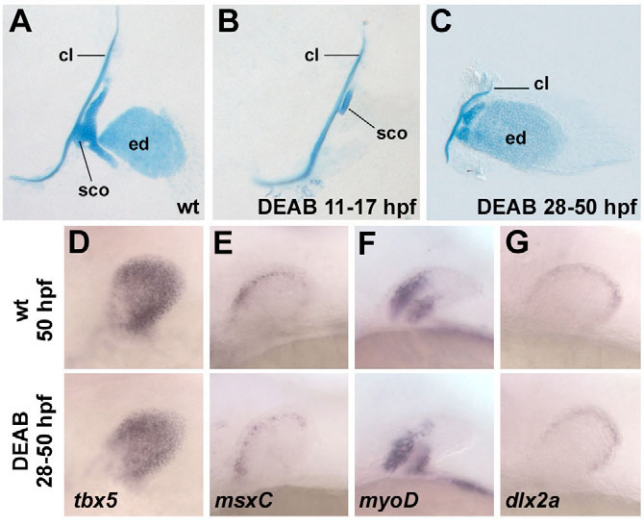


**Fig. 7. RA requirement in pectoral fins after 28 hpf.** (**A-C**) Alcian Blue stains of larval pectoral fin and girdle at 6 (A,B) and 4,5 dpf (C). Treatment with 10 μM DEAB from 10-17 hpf inhibits formation of the scapulocoracoid (sco) (B), and from 28-50 hpf affects differentiation in the proximal skeletal elements of the pectoral girdle (C). (**D-G**) In situ hybridizations of pectoral fins at 50 hpf; differentiation of the fin mesenchyme (D,E), appendicular muscles (F) and apical fold (G) appear unchanged upon treatment with 10 μM DEAB. cl, cleithrum; ed, endoskeletal disc.

requirement of RA, *aldh1a2*-expressing cells posterior to the eighth somite either emerge too late or are located too far from the fin field for induction. Alternatively, the LPM may have lost the competence to respond to RA once the embryo has passed the eight-somite stage.

In the absence of somites, the molecular targets of RA are likely to be found in the LPM. Of the possible target genes that RA may act on, *tbx5* is the earliest marker expressed in cells of the LPM that contribute to the fin bud. Expression of *tbx5* starts at the seven-somite stage, in two paraxial domains that flank the anterior head and trunk of the embryo (Tamura et al., 1999; Begemann and Ingham, 2000; Ruvinsky et al., 2000). The temporal requirement for RA signaling coincides with the onset of *tbx5* expression in the LPM, therefore RA may directly induce *tbx5* expression in the presumptive fin field. However, we have been unable to identify sequences of putative binding sites for RA receptors (RA-responsive elements) in the putative promoter region of human *Tbx5* (the genomic sequence of the zebrafish *tbx5* region is currently incomplete) that could mediate direct regulation of *tbx5*. Agarwal et al. (Agarwal et al., 2003) have proposed that in the mouse an axial signal may exist that serves to support *Tbx5* expression, or that might confer competence to the LPM to induce forelimb bud outgrowth by *Tbx5*. Our experiments in zebrafish suggest that RA is synthesized at the right time and place to qualify as such a signal.

## Limb initiation through axial signals

Axial signals that regulate limb development are likely to be derived from paraxial or intermediate mesoderm (IM). The finding that *tbx5* expression, and thus the presumptive fin field, is absent in *ntl/spt* embryos, identifies somitic *aldh1a2* expression, rather than expression in the IM, as the earliest genetically confirmed source of an axial signal that triggers fin field initiation.

A role of IM in limb induction is still debated. Experiments using foil barriers and ablation of IM tissue in the chick embryo have suggested that the IM provides important signals for limb

development (Stephens and McNulty, 1981; Strecker and Stephens, 1983; Geduspan and Solursh, 1992). However, this view has been challenged by the observation that wing buds form in experiments blocking chick mesonephric differentiation, and more specifically IM-expressed FGF8 is dispensable for mouse limb induction (Fernandez-Teran et al., 1997; Boulet et al., 2004); finally, the loss of IM cells does not affect normal development of limbs in *Pax2*$^{-/-}$/*Pax8*$^{-/-}$ mouse embryos (Bouchard et al., 2002). Although we did not observe transplanted wild-type cells in the IM of *nls* mutants, the rescue of fin development by somites alone suggests that IM-derived RA signaling is not required for pectoral fin initiation in wild type.

## Continuous RA signaling prior to fin outgrowth is required for AP-pre-patterning and apical fold formation

Taking advantage of the relative ease with which RA signaling can be manipulated using exogenous agents in the zebrafish, we have uncovered that following RA-mediated induction of the pectoral fin field at 12-13 hpf, RA remains to be required continuously throughout the pre-budding stage to establish posterior cell fates in the fin field. Importantly, this RA dependency occurs several hours prior to the development of a visible mesenchymal condensation and of apical fold formation. This is exemplified by the absence of *shh* expression in fin buds devoid of RA prior to 22 hpf (Fig. 6M-P). As *aldh1a2* is not expressed within the fin field until a visible bud emerges at 26-28 hpf, the strong expression of *aldh1a2* in adjacent somites is most likely to provide RA to the developing pectoral fins.

In addition, *hoxc6a*, a marker of the anterior pectoral mesenchyme, is expanded posteriorly in RA-depleted fins. We interpret this as a consequence of loss of *shh*, which is required to repress the posterior expression of *hoxc6a* (Neumann et al., 1999). By contrast, posterior expression of *hoxd11a* and *hoxd12a* is independent of *shh* (Neumann et al., 1999), yet both genes are never expressed in fin buds lacking RA. Similarly, *hand2* is not expressed in the absence of RA, and forelimbs in both zebrafish and mice that are mutant for *hand2*/*Hand2* resemble limbs that develop without RA, in that they never express *hoxd11a* and *hoxd12a* in the fin mesenchyme (Yelon et al., 2000) or *Shh* in the limb bud (Charité et al., 2000).

*hand2* expression in the LPM has been implicated in regulating AP prepatterning prior to the formation of the ZPA and is required for early expansion of the presumptive fin field (Yelon et al., 2000). Similarly, in *Aldh1a2*$^{-/-}$ mutant mice, *Hand2* is not expressed in forelimbs, but expression can be rescued by RA (Niederreither et al., 2002; Mic et al., 2004). Our results thus suggest that continued RA signaling from somites acts via *hand2* expression to establish a prepattern in the fin field mesenchyme. This process occurs during early somitogenesis and advocates a requirement of somite-derived RA for the maintenance of *hand2* expression in the LPM (Fig. 8).

It has been suggested that *Aldh1a2* expression in proximal limb mesoderm of the mouse establishes a proximodistal gradient needed for AER formation in the forelimb buds (Mic et al., 2004). We find that zebrafish embryos blocked immediately after the initiation stage (starting at 14 hpf) infrequently formed incomplete apical folds, while fin buds that developed during a RA block starting at 16 hpf did not exhibit apical fold defects (not shown and Fig. 6K,L). At this early stage, somite-derived RA-signaling is unlikely to form a gradient in the fin mesenchyme, and has been detected throughout the pre-budding mouse forelimb (Mic et al., 2004). This suggests that RA signaling is required between 14-16 hpf, when *dlx2a* is first expressed in the fin field (Akimenko et al., 1994), for full AP extension of *dlx2a* expression in the mature apical fold. Likewise, AER-formation in mammalian forelimbs is more likely to be a consequence of early deficits in full RA signaling, rather than the failure to establish a gradient of RA activity over the outgrowing limb bud. This notion is further supported by the expression of zebrafish *aldh1a2* throughout the entire fin bud mesenchyme during the outgrowth phase.

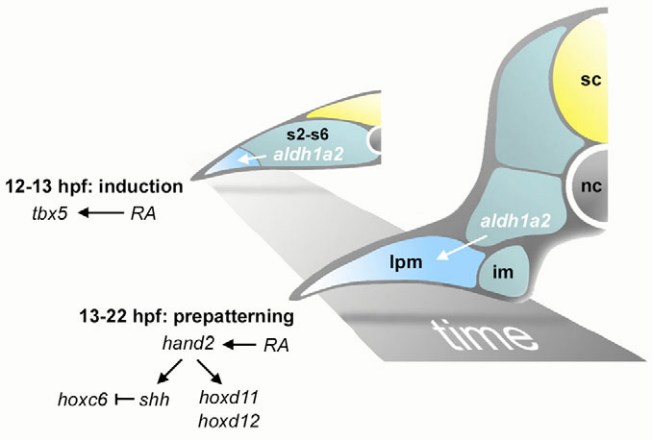


**Fig. 8. Model of RA signaling during pectoral fin development.** Expression of *aldh1a2* during the formation of somites 2-7 is required to induce *tbx5* in the lateral plate mesoderm (lpm) between 12-13 hpf, after which RA signaling is maintained from ventral somites flanking the pectoral fin field to establish a prepattern of posterior fates in the developing fin buds. Arrows indicate genetic interactions between somite-derived RA and fin mesenchyme, and are based on this and previous studies (Neumann et al., 1999; Yelon et al., 2000).

The ability to manipulate endogenous RA levels in zebrafish embryos at any stage of development has made it possible to reveal a link between somite-maturation and pectoral fin field induction, and has shown that the molecular mechanisms leading to apical fold formation and patterning of the posterior limb originate from RA signaling at the earliest stages of fin field formation. In the future, it should be possible to apply this approach successfully to a variety of RA-dependent developmental processes.

We thank I. May and R. Hellmann for fish care; L. Bernard, N. Dascaleanu, M. Jahnke, E. Spies and K. Webb for technical assistance; and C. Neumann for comments on the manuscript. We are grateful to Bristol Myers Sqibb for the kind gift of BMS493. This work was funded by grants from Konstanz University to A.M., a Landesgraduiertenstipendium to Y.G. and a grant from the Deutsche Forschungsgemeinschaft to G.B. (BE 1902 / 3-1).

### References

**Agarwal, P., Wylie, J. N., Galceran, J., Arkhitko, O., Li, C., Deng, C., Grosschedl, R. and Bruneau, B. G.** (2003). Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. *Development* **130**, 623-633.

**Ahn, D. G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K.** (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. *Nature* **417**, 754-758.

**Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M.** (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-3486.

**Amacher, S. L., Draper, B. W., Summers, B. R. and Kimmel, C. B.** (2002). The zebrafish T-box genes no tail and spadetail are required for development of trunk and tail mesoderm and medial floor plate. *Development* **129**, 3311-3323.

**Begemann, G. and Ingham, P. W.** (2000). Developmental regulation of Tbx5 in zebrafish embryogenesis. *Mech. Dev.* **90**, 299-304.

**Begemann, G., Schilling, T. F., Rauch, G. J., Geisler, R. and Ingham, P. W.** (2001). The zebrafish *neckless* mutation reveals a requirement for *raldh2* in mesodermal signals that pattern the hindbrain. *Development* **128**, 3081-3094.

**Begemann, G., Gibert, Y., Meyer, A. and Ingham, P. W.** (2002). Cloning of

zebrafish T-box genes tbx15 and tbx18 and their expression during embryonic development. *Mech. Dev.* **114**, 137-141.
**Begemann, G., Marx, M., Mebus, K., Meyer, A. and Bastmeyer, M.** (2004). Beyond the neckless phenotype: influence of reduced retinoic acid signaling on motor neuron development in the zebrafish hindbrain. *Dev. Biol.* **271**, 119-129.
**Berggren, K., McCaffery, P., Dräger, U. and Forehand, C. J.** (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. *Dev. Biol.* **210**, 288-304.
**Bouchard, M., Souabni, A., Mandler, M., Neubuser, A. and Busslinger, M.** (2002). Nephric lineage specification by Pax2 and Pax8. *Genes Dev.* **16**, 2958-2970.
**Boulet, A. M., Moon, A. M., Arenkiel, B. R. and Capecchi, M. R.** (2004). The roles of *Fgf4* anf *Fgf8* in limb bud initiation and outgrowth. *Dev. Biol.* **273**, 361-372.
**Capdevila, J. and Izpisua Belmonte, J. C.** (2001). Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Dev. Biol.* **17**, 87-132.
**Charité, J., McFadden, D. G. and Olson, E. N.** (2000). The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. *Development* **127**, 2461-2470.
**Chen, Y., Pollet, N., Niehrs, C. and Pieler, T.** (2001). Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of Xenopus embryos. *Mech. Dev.* **101**, 91-103.
**Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C.** (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.
**Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R.** (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-136.
**Dupé, V. and Lumsden, A.** (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.
**Ekker, M., Akimenko, M. A., Bremiller, R. and Westerfield, M.** (1992). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* **9**, 27-35.
**Emoto, Y., Wada, H., Okamoto, H., Kudo, A. and Imai, Y.** (2005). Retinoic acid-metabolizing enzyme Cyp26a1 is essential for determining territories of hindbrain and spinal cord in zebrafish. *Dev. Biol.* **278**, 415-427.
**Fernandez-Teran, M., Piedra, M. E., Simandl, B. K., Fallon, J. F. and Ros, M. A.** (1997). Limb initiation and development is normal in the absence of the mesonephros. *Dev. Biol.* **189**, 246-255.
**Fischer, S., Draper, B. W. and Neumann, C. J.** (2003). The zebrafish fgf24 mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation. *Development* **130**, 3515-3524.
**Garrity, D. M., Childs, S. and Fishman, M. C.** (2002). The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129**, 4635-4645.
**Geduspan, J. S. and Solursh, M.** (1992). A growth-promoting influence from the mesonephros during limb outgrowth. *Dev. Biol.* **151**, 242-250.
**Gibson-Brown, J. J., Agulnik, S. I., Chapman, D. L., Alexiou, M., Garvey, N., Silver, L. M. and Papaioannou, V. E.** (1996). Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev.* **56**, 93-101.
**Goering, L. M., Hoshijima, K., Hug, B., Bisgrove, B., Kispert, A. and Grunwald, D. J.** (2003). An interacting network of T-box genes directs gene expression and fate in the zebrafish mesoderm. *Proc. Natl. Acad. Sci. USA* **100**, 9410-9415.
**Grandel, H. and Schulte-Merker, S.** (1998). The development of the paired fins in the zebrafish (Danio rerio). *Mech. Dev.* **79**, 99-120.
**Grandel, H., Lun, K., Rauch, G. J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Kuchler, A. M., Schulte-Merker, S., Geisler, R. et al.** (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* **129**, 2851-2865.
**Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C. and Izpisúa-Belmonte, J. C.** (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891-900.
**Keegan, B. R., Feldman, J. L., Begemann, G., Ingham, P. W. and Yelon, D.** (2005). Retinoic acid signaling restricts the cardiac progenitor pool. *Science* **307**, 247-249.
**Kimmel, C. B., Kane, D. A., Walker, C., Warga, R. M. and Rothman, M. B.** (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* **337**, 358-362.
**Kimmel, C. B., Warga, R. M. and Schilling, T. F.** (1990). Origin and organization of the zebrafish fate map. *Development* **108**, 581-594.
**Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F.** (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
**Krauss, S., Johansen, T., Korzh, V. and Fjose, A.** (1991). Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. *Development* **113**, 1193-1206.
**Krauss, S., Concordet, J. P. and Ingham, P. W.** (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.
**Kudoh, T., Wilson, S. W. and Dawid, I. B.** (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.
**Linville, A., Gumusaneli, E., Chandraratna, R. A. and Schilling, T. F.** (2004). Independent roles for retinoic acid in segmentation and neuronal differentiation in the zebrafish hindbrain. *Dev. Biol.* **270**, 186-199.
**Logan, M.** (2003). Finger or toe: the molecular basis of limb identity. *Development* **130**, 6401-6410.
**Majumdar, A., Lun, K., Brand, M. and Drummond, I. A.** (2000). Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. *Development* **127**, 2089-2098.
**Martin, G. R.** (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12**, 1571-1586.
**Melby, A. E., Warga, R. M. and Kimmel, C. B.** (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* **122**, 2225-2237.
**Mic, F. A. and Duester, G.** (2003). Patterning of forelimb bud myogenic precursor cells requires retinoic acid signaling initiated by Raldh2. *Dev. Biol.* **264**, 191-201.
**Mic, F. A., Sirbu, I. O. and Duester, G.** (2004). Retinoic acid synthesis controlled by Raldh2 is required early for limb bud initiation and then later as a proximodistal signal during apical ectodermal ridge formation. *J. Biol. Chem.* **279**, 26698-26706.
**Molotkov, A., Molotkova, N. and Duester, G.** (2005). Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. *Dev. Dyn.* **232**, 950-957.
**Molven, A., Wright, C. V., Bremiller, R., De Robertis, E. M. and Kimmel, C. B.** (1990). Expression of a homeobox gene product in normal and mutant zebrafish embryos: evolution of the tetrapod body plan. *Development* **109**, 279-288.
**Morriss-Kay, G. M. and Ward, S. J.** (1999). Retinoids and mammalian development. *Int. Rev. Cytol.* **188**, 73-131.
**Neumann, C. J., Grandel, H., Gaffield, W., Schulte-Merker, S. and Nusslein-Volhard, C.** (1999). Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of sonic hedgehog activity. *Development* **126**, 4817-4826.
**Ng, J. K., Kawakami, Y., Buscher, D., Raya, A., Itoh, T., Koth, C. M., Rodriguez Esteban, C., Rodriguez-Leon, J., Garrity, D. M., Fishman, M. C. et al.** (2002). The limb identity gene Tbx5 promotes limb initiation by interacting with Wnt2b and Fgf10. *Development* **129**, 5161-5170.
**Niederreither, K., McCaffery, P., Dräger, U. C., Chambon, P. and Dolle, P.** (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67-78.
**Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dolle, P.** (2002). Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. *Development* **129**, 3563-3574.
**Odenthal, J., Haffter, P., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J. et al.** (1996). Mutations affecting the formation of the notochord in the zebrafish, Danio rerio. *Development* **123**, 103-115.
**Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Mikawa, T., Nohno, T. and Noji, S.** (1995). An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochem. Biophys. Res. Commun.* **209**, 809-816.
**Rallis, C., Bruneau, B. G., Del Buono, J., Seidman, C. E., Seidman, J. G., Nissim, S., Tabin, C. J. and Logan, M. P.** (2003). Tbx5 is required for forelimb bud formation and continued outgrowth. *Development* **130**, 2741-2751.
**Ruvinsky, I., Oates, A. C., Silver, L. M. and Ho, R. K.** (2000). The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev. Genes Evol.* **210**, 82-91.
**Smith, D. M., Torres, R. D. and Stephens, T. D.** (1996). Mesonephros has a role in limb development and is related to thalidomide embryopathy. *Teratology* **54**, 126-134.
**Sordino, P., van der Hoeven, F. and Duboule, D.** (1995). Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature* **375**, 678-681.
**Stafford, D. and Prince, V. E.** (2002). Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. *Curr. Biol.* **12**, 1215-1220.
**Stafford, D., White, R. J., Kinkel, M. D., Linville, A., Schilling, T. F. and Prince, V. E.** (2006). Retinoids signal directly to zebrafish endoderm to specify *insulin*-expressing β-cells. *Development* **133**, 949-956.
**Stephens, T. D. and McNulty, T. R.** (1981). Evidence for a metameric pattern in the development of the chick humerus. *J. Embryol. Exp. Morphol.* **61**, 191-205.
**Strecker, T. R. and Stephens, T. D.** (1983). Peripheral nerves do not play a trophic role in limb skeletal morphogenesis. *Teratology* **27**, 159-167.
**Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G.** (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282-296.
**Tamura, K., Yonei-Tamura, S. and Belmonte, J. C.** (1999). Differential expression of Tbx4 and Tbx5 in Zebrafish fin buds. *Mech. Dev.* **87**, 181-184.

**Tanaka, M., Cohn, M. J., Ashby, P., Davey, M., Martin, P. and Tickle, C.** (2000). Distribution of polarizing activity and potential for limb formation in mouse and chick embryos and possible relationships to polydactyly. *Development* **127**, 4011-4021.
**Thaller, C. and Eichele, G.** (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625-628.
**Tickle, C.** (2003). Patterning systems–from one end of the limb to the other. *Dev. Cell* **4**, 449-458.
**Vandersea, M. W., Fleming, P., McCarthy, R. A. and Smith, D. G.** (1998). Fin duplications and deletions induced by disruption of retinoic acid signaling. *Dev. Genes Evol.* **208**, 61-68.
**Vogel, A., Rodriguez, C. and Izpisua-Belmonte, J. C.** (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737-1750.
**Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B.** (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271-280.
**Wendling, O., Dennefeld, C., Chambon, P. and Mark, M.** (2000). Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. *Development* **127**, 1553-1562.
**Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y. and Hamada, H.** (2004). Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. *Dev. Cell* **6**, 411-422.
**Yelon, D., Ticho, B., Halpern, M. E., Ruvinsky, I., Ho, R. K., Silver, L. M. and Stainier, D. Y.** (2000). The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. *Development* **127**, 2573-2582.