Development 126, 4817-4826 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV2443

Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of *sonic hedgehog* activity

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Accepted 14 August; published on WWW 6 October 1999

SUMMARY

Sonic hedgehog (Shh) is expressed in the posterior vertebrate limb bud mesenchyme and directs anteroposterior patterning and growth during limb development. Here we report an analysis of the pectoral fin phenotype of zebrafish *sonic you* mutants, which disrupt the *shh* gene. We show that Shh is required for the establishment of some aspects of anteroposterior polarity, while other aspects of anteroposterior polarity are established independently of Shh, and only later come to depend on Shh for their maintenance. We also demonstrate that Shh is required for the activation of posterior HoxD genes by retinoic acid. Finally, we show that Shh is required for normal development of the apical ectodermal fold, for growth of the fin bud, and for formation of the fin endoskeleton.

Key words: Sonic hedgehog, Limb, Fin, ZPA, Hox, Colinearity, Cyclopamine, Retinoic acid, Zebrafish, *Danio rerio*

INTRODUCTION

Pattern formation and growth of vertebrate limbs is controlled by three signaling centers in the developing limb bud (for recent reviews see: Duboule, 1994; Cohn and Tickle, 1996; Johnson and Tabin, 1997; Martin, 1998). One of these is the zone of polarizing activity (ZPA), a small group of cells in the posterior mesenchyme. If placed in an ectopic anterior location, the ZPA induces mirror-image duplications of limb anteroposterior (A/P) pattern (Saunders and Gasseling, 1968).

Sonic hedgehog (Shh) belongs to a family of secreted signaling proteins that control many aspects of vertebrate development (reviewed in Hammerschmidt et al., 1997). Shh has been shown to mediate the effect of the ZPA in the chick (Riddle et al., 1993; Lopez-Martinez et al., 1995) and in the mouse (Chang et al., 1994). *shh* expression colocalizes with the ZPA, and grafting of *shh*-expressing cells or Shh protein-loaded beads to the anterior limb bud results in mirror-image symmetrical duplications identical to those elicited by ZPA grafts. Like the ZPA, ectopic anterior Shh has long-range and concentration-dependent effects on limb development (Yang et al., 1997; reviewed in Johnson and Tabin, 1997). Among the genes that can be induced by anterior Shh are the 5′ members of the HoxD cluster, which are normally expressed in posterior nested domains. The induction of *shh* in the ZPA requires retinoic acid signaling (reviewed in Johnson and Tabin, 1997).

The apical ectodermal ridge (AER) is another major signaling center of the limb bud which runs along its distal margin and is the site of expression of several FGF genes (reviewed in Martin, 1998). The AER is required for proximodistal (P/D) outgrowth of the limb, and can be functionally replaced by FGF-soaked beads, suggesting that FGF-signaling mediates AER function (Niswander et al., 1993; Fallon et al., 1994). Factors from the AER and ZPA form a mutual feedback loop that allows growth and patterning in the different axes to be coordinated (Laufer et al., 1994; Niswander et al., 1994). Thus *fgf-4*, which is expressed in the posterior AER, can be induced in the anterior AER by ectopic Shh, suggesting that Shh polarizes the AER. Conversely, removal of the AER leads to loss of *shh* expression.

The zebrafish paired fins and tetrapod limbs have evolved from a common ancestral appendage, and the morphology of zebrafish fin buds is similar to that of tetrapod limb buds (Sordino et al., 1995; Grandel and Schulte-Merker, 1998). Also, the expression patterns of zebrafish genes in the fin buds, such as *shh* (Krauss et al., 1993) and members of the HoxD complex (Sordino et al., 1995), are similar to the expression of their orthologues in tetrapod limb buds.

The zebrafish *sonic-you* (*syu*) mutant disrupts the *shh* gene and belongs to a group of mutants (the *you*-type mutants) with similar phenotypes, such as the reduction of the horizontal myoseptum. They may therefore act in a common signaling pathway (van Eeden et al., 1996a; Schauerte et al., 1998).

Given the importance of Shh in the control of limb

development, it is essential to determine the consequence of removing *shh* activity from the limb bud. Here we analyze the pectoral fin phenotype of zebrafish *syu* mutants. We show that, as predicted, several posteriorly expressed genes fail to be activated in the absence of Shh. Other posteriorly expressed genes, however, are activated in the absence of Shh, suggesting that part of the A/P polarity of the pectoral fin bud is established independently of Shh. Subsequent maintenance of A/P polarity depends on Shh. We also show that Shh is required for the normal development of the apical ectodermal fold, a structure homologous to the tetrapod AER, as well as for outgrowth of the fin bud and for formation of the fin endoskeleton.

MATERIALS AND METHODS

Fish stocks

Fish maintenance, crossing and staging was performed as in Grandel and Schulte-Merker (1998). The *syu* null allele, *syu*^{t4}, was used for all experiments except for the Alcian blue stain in Fig. 9B, in which the hypomorphic mutant *syu*^{tq252} was used. Both alleles are described in Schauerte et al. (1998).

Whole-mount in situ hybridization

In situ hybridization was done as in Kishimoto et al. (1997). cDNAs used to generate antisense probes: *ptc1* (Lewis et al., 1999), *hoxd-10*, *hoxd-11*, *hoxd-12*, *hoxd-13*, *hoxa-9*, *hoxa-10*, *hoxa-11*, *hoxa-13* (Sordino et al., 1995; van der Hoeven et al., 1996), *msx-c* (Akimenko et al., 1995), *hoxc-6* (Prince et al., 1998), *shh* (Krauss et al., 1993), *dlx-2* (Akimenko et al., 1994), *bmp-2* (Kishimoto et al., 1997), *fgf-8* (Reifers et al., 1998) and *en-1* (Ekker et al., 1992).

Retinoic acid and cyclopamine treatment

All-*trans* retinoic acid treatment was performed as in Akimenko and Ekker (1995), except that embryos were exposed to 10^{-6} M retinoic acid for 1 hour at 50% epiboly.

Embryos were exposed to 200 μM cyclopamine (purified from *Veratrum californicum* by W. G.) starting at 50% epiboly and maintained in the dark until fixation.

Histological preparations and skeletal stainings

Histology and Alcian blue stains were performed as in Grandel and Schulte-Merker (1998).

BrdU labelling

BrdU labelling was performed with a Boehringer kit (catalogue no. 1758756) according to manufacturer's instructions. Embryos were injected into the yolk with 10 mM BrdU and fixed 1 to 2 hours later.

RESULTS

Expression of A/P markers in *syu* mutant fin buds

The zebrafish *sonic-you* (*syu*) mutation results in several developmental defects, including the reduction of pectoral fins (van Eeden et al., 1996b), and has been shown to be a mutation in the *shh* gene (Schauerte et al., 1998). In order to better define the role of Shh in A/P patterning of the pectoral fin, we examined the expression of several A/P markers in *syu* mutant pectoral fin buds.

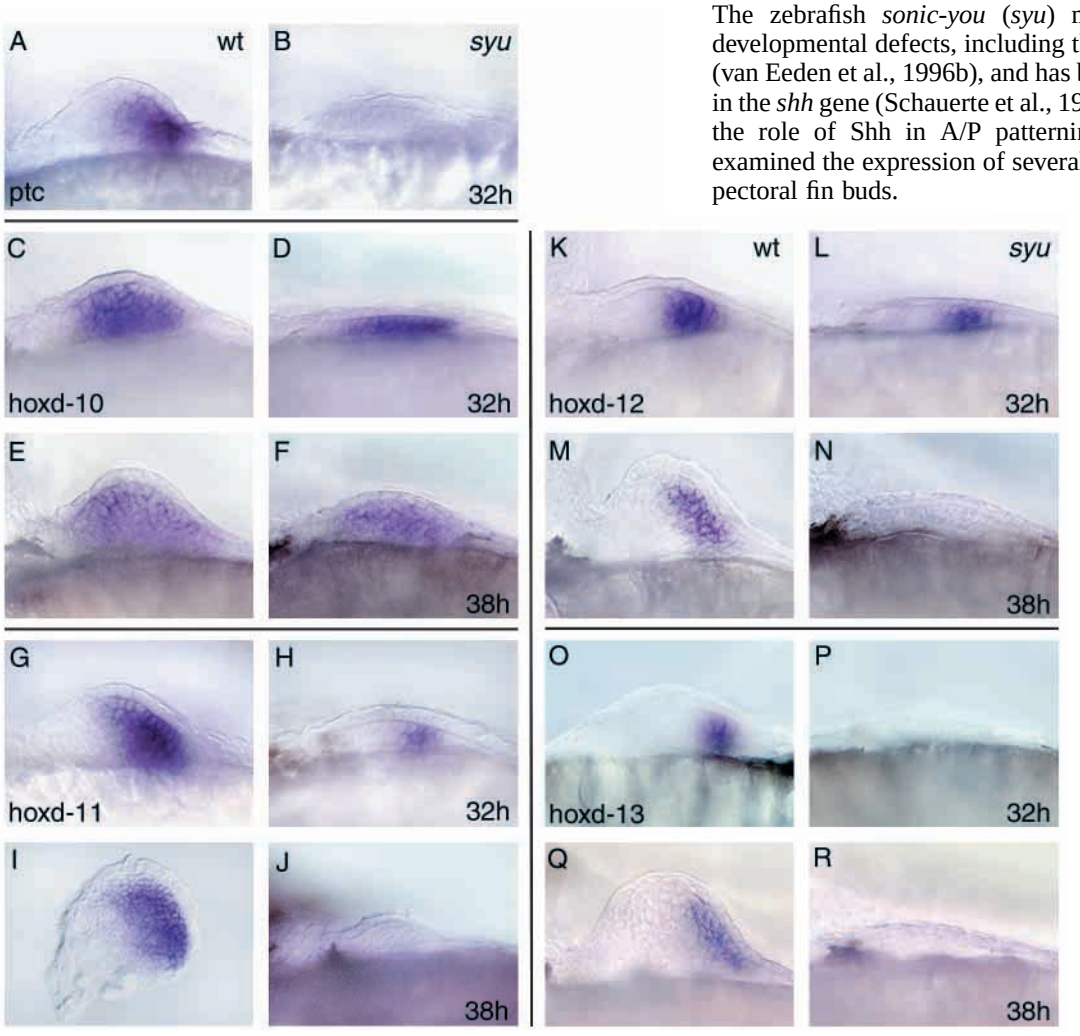


Fig. 1. Expression of *ptc1* and *hoxd* genes in wild-type and *syu* pectoral fin buds. Whole-mount in situ hypbridizations. Anterior is to the the left and distal is to the top. (A-D,G,H,K,L,O,P) 32 hour pectoral fin buds. (E,F,I,J,M,N,Q,R) 38 hour pectoral fin buds. (A,C,E,G,I,K,M,O,Q) Wild-type fin buds; (B,D,F,H,J,L,N,P,R) *syu* fin buds. (A,B) *ptc1* RNA; (C-F) *hoxd-10* RNA; (G-J) *hoxd-11* RNA; (K-N) *hoxd-12* RNA; (O-R) *hoxd-13* RNA. At 32 hours, *ptc1* and *hoxd-13* are not expressed in *syu* buds, while *hoxd-10* appears normal and *hoxd-11* and *hoxd-12* show a partial reduction. At 38 hours only expression of *hoxd-10* remains in *syu* mutant buds.

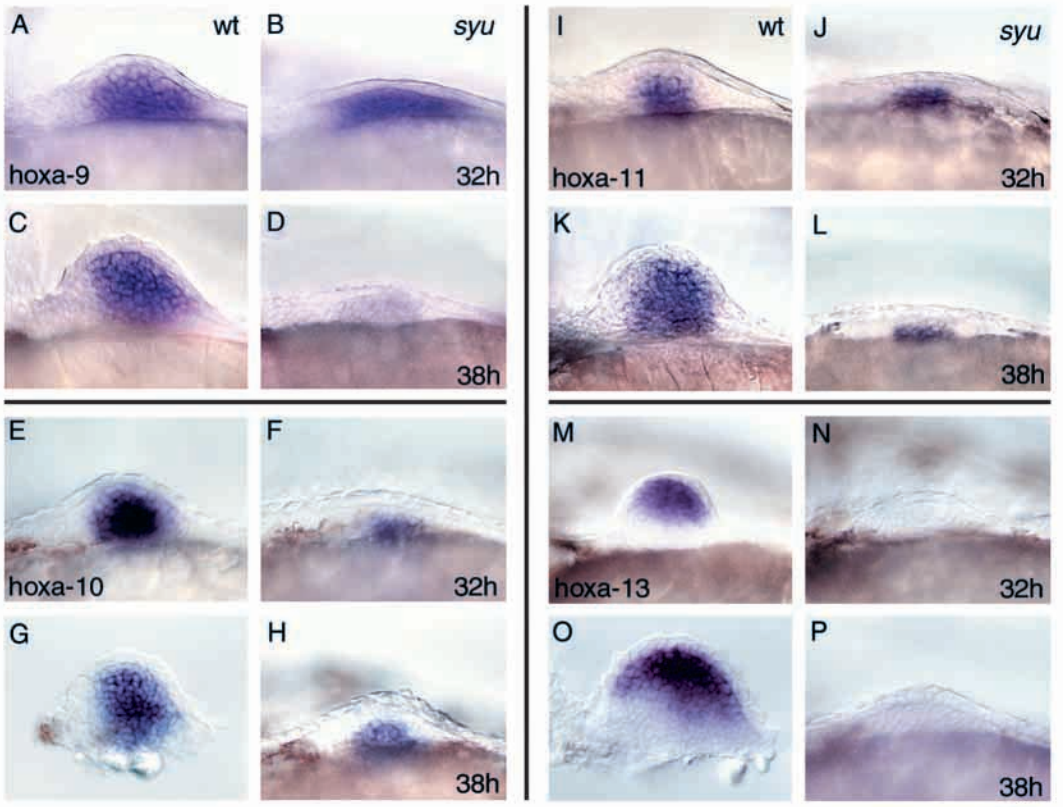
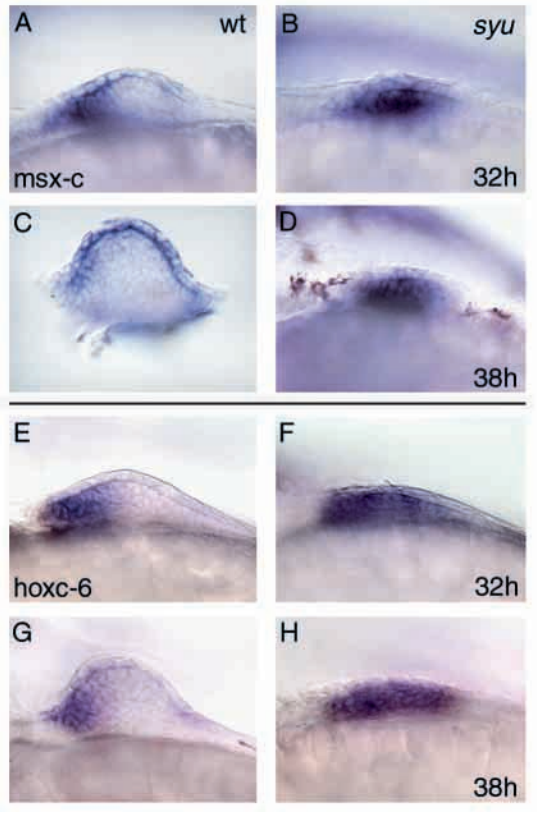


Fig. 2. Expression of *hoxa* genes in wild-type and *syu* pectoral fin buds. Whole-mount in situ hybridizations. Anterior is to the left and distal is to the top. (A,B,E,F,I,J,M,N) 32 hour pectoral fin buds; (C,D,G,H,K,L,O,P) 38 hour pectoral fin buds. (A,C,E,G,I,K,M,O) Wild-type fin buds; (B,D,F,H,J,L,N,P) *syu* fin buds. (A-D) *hoxa-9* RNA; (E-H) *hoxa-10* RNA; (I-L) *hoxa-11* RNA; (M-P) *hoxa-13* RNA. At 32 hours, *hoxa-13* is not expressed in *syu* mutant buds, while *hoxa-9* and *hoxa-11* are expressed normally and *hoxa-10* shows a partial reduction. At 38 hours, only a strong proximal central expression of *hoxa-10* and *hoxa-11* and a weak expression of *hoxa-9* is detectable in *syu* mutant buds.

As in other vertebrates, two zebrafish *patched* (*ptc*) genes are expressed in the posterior fin mesenchyme, and in *syu* mutant fin buds expression of both *ptc* genes is lost (Lewis et al., 1999; Fig. 1A,B). Since *ptc1* and *ptc2* expression is a general response to all known hedgehog family members (Lewis et al., 1999), this suggests a complete absence of hedgehog signaling in *syu* mutant fin buds (see also below).



The 5′ members of the zebrafish HoxD complex are expressed in the pectoral fin bud in a colinear pattern (Sordino et al., 1995; Fig. 1). *hoxd-10* is expressed throughout the bud mesenchyme and is activated normally in *syu* mutant fin buds (Fig. 1D). *hoxd-11* and *hoxd-12* are activated slightly later than in wild types, but are clearly expressed in posterior domains in *syu* fin buds at 32 hours of development (Fig. 1H,L and data not shown). *hoxd-13*, the most 5′ member, is not expressed in *syu* fin buds at any stage (Fig. 1P,R and data not shown). At 38 hours of development, expression of *hoxd-10* is still present in *syu* mutant buds, while expression of *hoxd-11* and *hoxd-12* is no longer detectable (Fig. 1F,J,N).

The 5′ members of the HoxA complex are also expressed in the zebrafish pectoral fin bud (Sordino et al., 1995; Fig. 2). The most 3′ member of this group, *hoxa-9*, is expressed throughout the bud mesenchyme at 32 hours of development, and is expressed normally in *syu* fin buds at this stage (Fig. 2A,B). The expression domain of *hoxa-10* shows a posterior bias in wild-type and *syu* mutant pectoral fin buds at 32 hours but, while *hoxa-10* is expressed in both proximal and distal

Fig. 3. Expression of *msx-c* and *hoxc-6* in wild-type and *syu* pectoral fin buds. Whole-mount in situ hybridizations. Anterior is to the left and distal to the top. (A,B,E,F) 32 hour pectoral fin buds; (C,D,G,H) 38 hour pectoral fin buds. (A,C,E,G) Wild-type fin buds; (B,D,F,H) *syu* fin buds. (A-D) *msx-c* RNA; (E-H) *hoxc-6* RNA. *msx-c* and *hoxc-6*, which are normally restricted to the anterior mesenchyme, are expressed throughout the mesenchyme of *syu* mutant fin buds.

mesenchymal cells in wild-type buds, it is restricted to proximal cells in *syu* mutants (Fig. 2E,F). *hoxa-11* is expressed in a proximal central domain both in wild-type and *syu* fin buds at 32 hours (Fig. 2I,J). *hoxa-13* is expressed in a posterior distal domain in wild-type pectoral fin buds at 32 and 38 hours, and fails to be expressed in *syu* mutant buds at any stage (Fig. 2M-P and data not shown).

At 38 hours of development, *hoxa-9* expression is excluded from the most anterior region of wild-type pectoral fin buds, and is strongly reduced in *syu* mutant buds, being expressed weakly throughout the mesenchyme (Fig. 2C,D). In wild-type buds at 38 hours, *hoxa-10* is excluded from a slightly larger anterior domain than *hoxa-9* (Fig. 2G). In *syu* mutant buds, *hoxa-10* is expressed in a proximal central region that lacks a posterior bias (Fig. 2H). *hoxa-11* is expressed similarly to *hoxa-10* in 38 hour wild-type pectoral fin buds, and is also reduced to a proximal central domain in *syu* mutant fin buds (Fig. 2K,L).

The *bmp-2* gene is expressed in the apical epidermis and the posterior mesenchyme of the zebrafish pectoral fin bud at 32 hours of development (Fig. 6K). Posterior mesenchymal expression of *bmp-2* is present in *syu* fin buds at 32 hours (Fig. 6L) but, while *bmp-2* is still expressed weakly in posterior mesenchymal cells in wild-type buds at 38 hours, this expression is lost in *syu* mutant buds at the same stage (Fig. 6M,N).

The zebrafish *msx-c* gene is expressed in the anterior mesenchyme of 32 hour wild-type pectoral fins (Akimenko et al., 1995; Fig. 3A). In *syu* mutant buds, *msx-c* is expanded throughout the mesenchyme, although expression appears to be slightly weaker at the posterior margin (Fig. 3B). At 38 hours, *msx-c* is restricted even further anteriorly in wild-type buds, but is still expressed throughout the mesenchyme of *syu* fin buds (Fig. 3C,D). Zebrafish *hoxc-6* shows a similar restriction to the anterior pectoral fin bud mesenchyme as *msx-c* (Molven et al., 1990; Fig. 3E,G), and is also expanded throughout the mesenchyme in *syu* mutant fin buds at 32 and 38 hours of development (Fig. 3F,H).

Taken together, these data show that *shh* activity is required for the posterior activation of *ptc*, *hoxd-13* and *hoxa-13*, and for the posterior repression of *msx-c* and *hoxc-6* in the pectoral fin bud. The maintenance of posterior aspects of expression of *hoxa-9* and *hoxa-11* also depends on *shh* activity. In contrast, the posterior activation of *hoxd-11*, *hoxd-12*, *hoxa-10* and *bmp-2* does not depend on *shh* activity, although it is slightly delayed and weaker than in wild-type fin buds. After the Shh-independent activation of *hoxd-11*, *hoxd-12*, *hoxa-10* and *bmp-2*, the maintenance of these genes becomes dependent on Shh.

Evidence that Shh is the only hedgehog acting in the early fin bud

Two other hedgehog family members, *echidna hedgehog* (*ehh*) and *tiggy winkle hedgehog* (*twhh*) have been characterized in the zebrafish, and it has been proposed that they might partially compensate for Shh in *syu* mutants (Schauerte et al., 1998). To address this possibility, we assayed *ehh* and *twhh* expression in early fin buds. Neither gene is expressed detectably in pectoral fin buds at 32 hours (Fig. 4E,F).

We further addressed this issue by treating embryos with cyclopamine, which has been shown to inhibit hedgehog signal transduction (Incardona et al., 1998). Cyclopamine-treated embryos develop many phenotypes similar to *syu* mutants, such as loss of the horizontal myoseptum, but also show cyclopia (data not shown). While *syu* null mutants show a partial reduction of *ptc* expression at the midline (Lewis et al., 1999; Fig. 4B), cyclopamine-treated embryos show no detectable *ptc* expression (Fig. 4C). Since *ptc* induction is a general response to all hedgehogs (Lewis et al., 1999), this indicates that cyclopamine inhibits signaling by multiple hedgehog family members and supports the proposal that signaling by other hedgehogs in the zebrafish, such as *ehh* and *twhh*, contributes to midline pattering in *syu* embryos.

If the Shh-independent activation of posterior *hox* genes in the fin bud were due to the activity of another hedgehog, one would expect to lose this expression following cyclopamine treatment. While fin buds of cyclopamine-treated embryos fail to express *ptc* (Fig. 4H), they show clear posterior activation of *hoxd-11* and *hoxd-12* (Fig. 4I and data not shown), and thus phenocopy *syu* mutant buds. Taken together, these data suggest that Shh is the only hedgehog acting in the early fin bud.

Interestingly, *shh* expression is initially normal in fin buds of cyclopamine-treated embryos, but becomes weaker at 38 hours and is lost by 48 hours (Fig. 4G,J-L), indicating that Shh activity is required for maintenance of *shh* expression. This is likely due to the effect of Shh on the apical fold (see below).

Shh is required for ectopic activation of posterior genes by retinoic acid

Application of retinoic acid-soaked beads to the anterior chick limb bud leads to activation of *shh* and 5′ genes of the HoxD complex, and to the duplication of posterior structures (reviewed in Johnson and Tabin, 1997).

We found that treatment of zebrafish embryos with retinoic acid during epiboly leads to the formation of pectoral fin buds that are elongated along their A/P axis and shifted anteriorly from their normal position flanking somites 2 and 3 to a position next to the abnormal head structures of these embryos (Fig. 5).

To further characterize the role of Shh in A/P patterning of the fin bud, we compared the effect of retinoic acid on wild-type and *syu* mutant buds. Fin buds of wild-type embryos treated with retinoic acid express *shh*, *hoxd-11* and *hoxd-12* throughout the whole mesenchyme at 32 hours (Fig. 5B,E and data not shown), suggesting that they have a uniform posterior character. This effect is distinct from that observed by Akimenko and Ekker (1995), in which retinoic acid treatment between 24 and 28 hours of development leads to expression of *shh* at the anterior margin of the pectoral fin bud by 48 to 56 hours.

Pectoral fin buds of *syu* mutant embryos treated with retinoic acid are also elongated along their A/P axis, shifted to the anterior, and express *hoxd-10* at 32 hours, like their wild-type counterparts (Fig. 5C,D). However, buds of *syu* embryos treated with retinoic acid fail to show an anterior expansion of *hoxd-11* and *hoxd-12*, which are instead expressed in a few cells at the posterior margin of the fin bud (Fig. 5F and data not shown).

These data indicate that *shh* activity is required for the anterior activation of *hoxd-11* and *hoxd-12* by retinoic acid and that retinoic acid is not sufficient to expand the *shh*-independent expression of *hoxd-11* and *hoxd-12*.

Apical ectodermal fold development in *syu* mutant pectoral fin buds

As in tetrapods, zebrafish fin buds are first covered by an unstructured epidermis (Fig. 7A). At 36 hours, the epidermis forms a specialized structure along the apical margin of the bud, the apical fold (Fig. 7C), which is homologous to the tetrapod AER. In order to characterize the effect of *syu* on the apical fold, we analyzed the expression of several markers expressed in the apical fold.

Zebrafish *dlx-2* is expressed in the apical epidermis (Akimenko et al., 1994), and is expressed normally in *syu* mutant fin buds at 32 hours, but expression becomes weaker and restricted to fewer cells than in wild-type embryos as development proceeds (Fig. 6A-F). By 48 hours, about 50% of *syu* embryos fail to show *dlx-2* staining and, at 56 hours, no *dlx-2* expression can be detected in *syu* mutant pectoral fin buds (data not shown). Zebrafish *bmp-2* is also activated normally in the apical epidermis of *syu* mutant fin buds, but expression subsequently becomes weaker (Fig. 6K-P). Both *dlx-2* and *bmp-2* show elevated expression in the posterior apical epidermis of wild-type fin buds at 38 hours (Fig. 6C,M), but are expressed in a non-polar manner along the A/P axis of the apical epidermis in *syu* buds (Fig. 6D,N).

Zebrafish *fgf-8* is activated relatively late in the apical epidermis, at 36 hours (Reifers et al., 1998; Fig. 6G), coincident with apical fold formation. In *syu* mutant fin buds, *fgf-8* expression fails to be activated (Fig. 6H,J; data not shown).

The establishment of the AER has been shown to occur at the interface between ventral cells that express *engrailed-1* (*en-1*) and dorsal cells that do not (reviewed in Zeller and Duboule, 1997). In *syu* mutant pectoral fin buds, the expression of *en-1* appears normal both at 32 and 48 hours (Fig. 6Q-T).

shh expression depends on FGF signaling from the AER in the chick, as it is lost upon AER removal and can be rescued by FGF application (Laufer et al., 1994; Niswander et al., 1994). *shh* expression can thus be taken as a marker for ridge activity. In cyclopamine-treated wild-type embryos, *shh* is activated normally and is strongly expressed at 32 hours (Fig. 4G). At 38 hours, expression has become weak and, at 48 hours, it is undetectable (Fig. 4J-L). This coincides with the reduction of apical fold markers in *syu* mutants and further suggests that apical fold activity is reduced at 38 hours and lost by 48 hours in the absence of Shh activity.

To further analyze the role of Shh in apical epidermal fold development, we examined histological sections of *syu* mutant pectoral fin buds. At 38 hours, an apical fold has formed in wild-type pectoral fin buds, but is not present in *syu* mutant fin buds (Fig. 7C,D). At 48 hours, 9 out of 9 *syu* buds showed an apical fold while, at 60 hours, this number was 2 out of 8 (data not shown). Since some *syu* buds still have an apical fold at 72 hours (Fig. 7F) this suggests that the apical fold, if it is formed, is variably lost in *syu* mutants. Starting at 48 hours, the apical fold is invaded by mesenchyme in wild-type fin buds, accompanied by an expansion of the fold, which leads to the formation of the fin fold (Grandel and Schulte-Merker, 1998). Fin fold formation does not occur in *syu* mutant pectoral fin buds (Fig. 7E,F).

Taken together, these data suggest that initial specification of the apical epidermis, reflected by expression of *en-1*, *dlx-2* and *bmp-2* at early stages, occurs normally in the absence of Shh. Subsequent developmental progression of the apical epidermis is impaired in the absence of Shh, as the activation of later markers does not occur, and as the expression of early markers becomes weaker after 32 hours. Consistent with these observations, *shh* expression, which depends on FGFs from the apical epidermis, is normal at early stages but is subsequently lost in cyclopamine-treated wild types. Also, apical fold formation is delayed and is not followed by fin fold formation in *syu* mutants.

Absence of larval pectoral fin structures and reduced proliferation in *syu* mutants

The zebrafish pectoral fin bud gives rise to a larval fin with a cartilaginous fin endoskeleton (Sordino et a., 1995; Grandel and Schulte-Merker, 1998). In *syu* null mutant larvae, none of the endoskeletal elements of the pectoral fin are formed (Fig. 9C). Consistent with this observation, no signs of cartilage condensation can be detected in *syu* null mutant fin buds at earlier stages (Fig. 7E,F). The most proximal part of the fin endoskeleton, the scapulocoracoid, contacts a bone derived from the body wall, the cleithrum (Sordino et al., 1995; Grandel and Schulte-Merker, 1998; Fig. 9A). In *syu* null mutants, a cleithrum is present, although it is small and mishapen (Fig. 9C). In a *syu* hypomorphic allele, syu^{tq252}, the elements of the pectoral fin endoskeleton are formed, but are smaller than in wild-type larvae (Fig. 9B).

The outgrowth of *syu* mutant fin buds is strongly reduced, as there are fewer cells in *syu* buds than in wild-type buds at all stages from 28 hours on. Consistent with this observation, *syu* fin buds show reduced BrdU labelling at all stages examined between 28 hours and 41 hours (Fig. 8 and data not shown). The reduction in BrdU labelling appears most severe after 38 hours (Fig. 8B,D).

These results indicate that *shh* activity is required for cell proliferation in the pectoral fin bud at all stages and already well before the observed reduction of apical fold activity. Shh is also required for the formation of the fin endoskeleton.

DISCUSSION

Shh-dependent and Shh-independent aspects of A/P polarity in the early zebrafish pectoral fin bud

We have shown that the posterior activation of *ptc*, *hoxd-13* and *hoxa-13* and the posterior repression of *msx-c* and *hoxc-6* in the zebrafish pectoral fin bud is absolutely dependent on Shh, as it does not occur in *syu* null mutant embryos (Figs 1, 2, 3). This suggests that Shh directs the expression of these genes in the A/P axis of the limb, consistent with the observation that ectopic Shh can activate posteriorly expressed genes.

In contrast, *hoxd-11*, *hoxd-12*, *hoxa-10* and *bmp-2* are initially activated in posterior domains in *syu* null mutant fin buds (Figs 1, 2, 6). Since ectopically expressed Shh is able to activate *hoxd-11* and *bmp-2* in the anterior chick limb bud (Riddle et al., 1993; Laufer et al., 1994), these data suggest that Shh is sufficient, but not absolutely necessary for their posterior activation. Our data imply that there must be an additional factor that activates the posterior expression of these genes, and thereby generates a Shh-independent A/P polarity of the fin bud at this stage.

What might be the molecular nature of the factor(s) controlling early A/P polarity in the pectoral fin bud

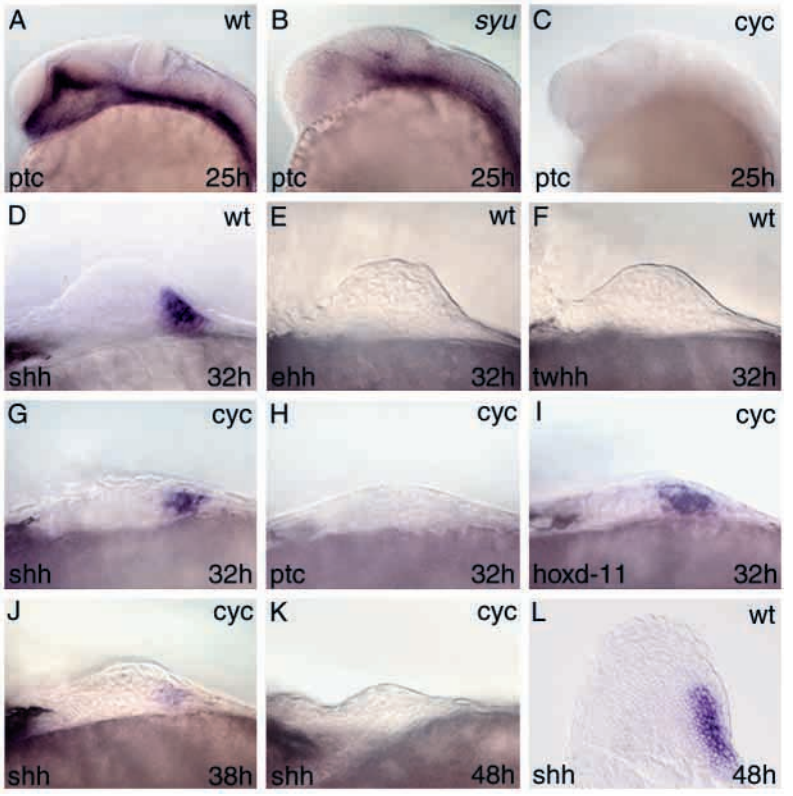


Fig. 4. Effect of cyclopamine on midline *ptc1* expression and on fin development. Whole-mount in situ hybridizations. Anterior to the left. (A-C) Dorsal to the top, 25 hour embryos; (D-L) distal to the top: 32 hour pectoral fin buds (D-J); 48 hour pectoral fin buds (K,L). (A,D-F,L) Wild type; (B) *syu*; (C,G-K) wild type treated with cyclopamine. (A-C,H) *ptc1* RNA; (D,G,J-L) *shh* RNA; (E) *ehh* RNA; (F) *twhh* RNA; (I) *hoxd-11* RNA. Note the partial reduction of midline *ptc1* expression in *syu* mutants and the complete absence in cyclopamine-treated embryos (A-C). *ehh* and *twhh* are not expressed in 32 hour pectoral fin buds (E,F). Cyclopamine-treated fin buds fail to express *ptc1* (H), but activate *shh* and *hoxd-11* (G,I). *shh* expression subsequently becomes weaker (J) and is lost (K). cyc, cyclopamine.

independently of Shh? At present, there is no answer to this question, although it is clear that there is already some A/P polarity present in the pectoral fin-forming region even before the first signs of fin development. For example, the anterior expression boundaries of several genes of the *hoxb* and *hoxc* clusters are found in the lateral plate mesoderm of this region (Prince et al., 1998).

Our results argue against the possibility that the Shh-independent A/P polarity might be due to another hedgehog. Firstly, Shh is the only characterized zebrafish hedgehog expressed in the early fin bud (Fig. 4D-F). Secondly, *ptc* expression is induced by all zebrafish hedgehog genes, indicating that it is a general response to hedgehog signaling (Lewis et al., 1999). The *syu* null mutant shows a complete absence of *ptc* expression in the pectoral fin buds (Fig. 1B; Lewis et al., 1999), suggesting that there is no hedgehog signaling in *syu* buds. Thirdly, cyclopamine generates a stronger midline phenotype than the *syu* null mutant (Fig. 4A-C and data not shown), suggesting that it inhibits signaling by multipe hedgehogs expressed in the midline that compensate partially for the loss of Shh. In the fin bud, however, cyclopamine generates a phenocopy of the *syu* null mutant (Fig. 4G-I,K and data not shown). Taken together, these results suggest that, in the zebrafish, there is partial redundancy between *shh* and other hedgehogs (such as *ehh* and *twhh*) at the midline, but that *shh* is the only hedgehog acting in the early fin bud. This could expain why the mouse *shh* null mutant has a more severe midline phenotype than the zebrafish *syu* null mutant (Chiang et al., 1996; Schauerte et al., 1998), while the P/D truncation of pectoral fins in zebrafish *syu* mutants appears to be at least as severe as the limb truncation in the mouse *shh* mutant. However, in contrast to the mouse *shh* mutant, cyclopamine-treated zebrafish embryos express medial floorplate markers (data not shown), consistent with the proposal that hh signaling does not induce medial floorplate in the zebrafish (J. Odenthal and C. N. V., unpublished data).

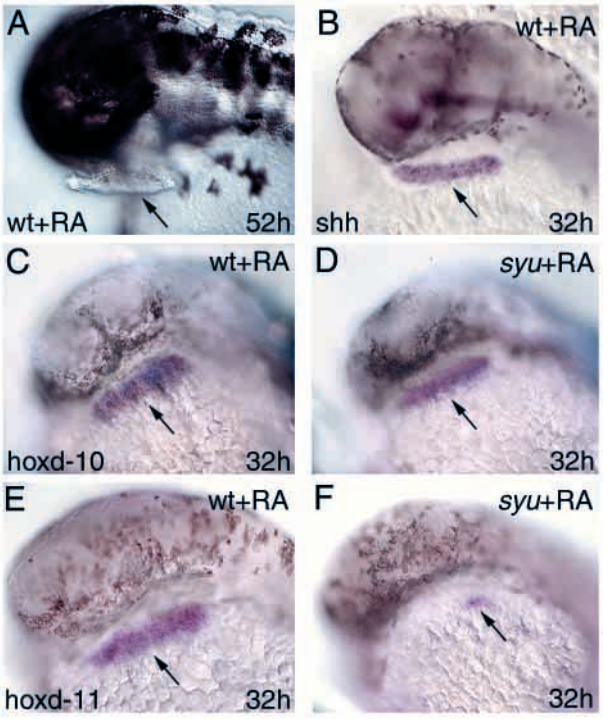


Fig. 5. Effect of retinoic acid treatment on wild-type and *syu* pectoral fin buds. (A-F) retinoic acid-treated embryos. (A) Embryo at 52 hours; (B-F) whole-mount in situs of embryos at 32 hours of development. (A,B,C,E) Wild-type embryos treated with retinoic acid; (D,F) *syu* embryos treated with retinoic acid. (B) *shh* RNA; (C,D) *hoxd-10* RNA; (E,F) *hoxd-11* RNA. The pectoral fin buds are elongated and located next to the heavily pigmented anterior end of both wild-type and *syu* embryos treated with retinoic acid (arrows in A to E). Note also that the posterior markers *shh* and *hoxd-11* are expressed throughout the fin bud mesenchyme in wild-type embryos (arrows in B and E), but not in *syu* embryos treated with retinoic acid, which instead show a patch of *hoxd-11* expression at the posterior margin of the fin bud (arrow in F).

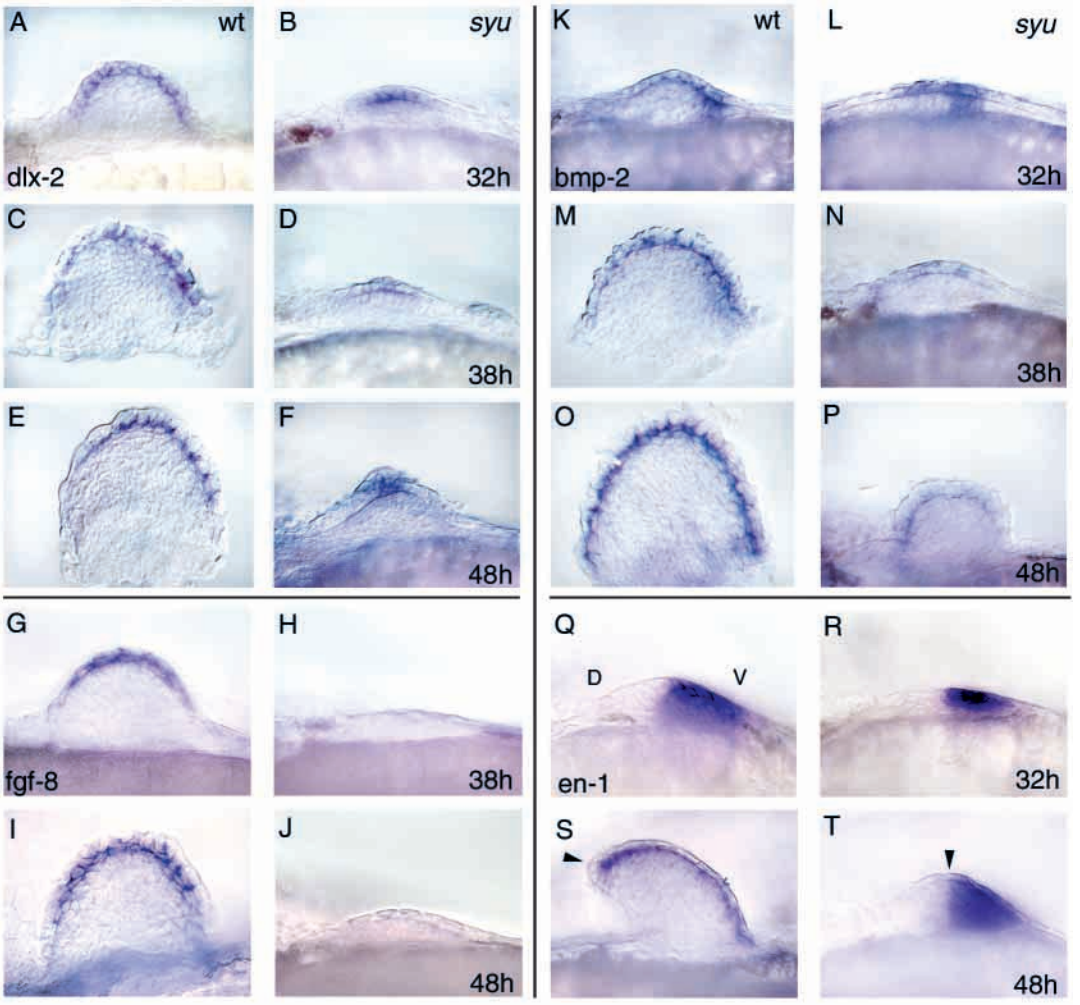


Fig. 6. Expression of apical epidermal fold markers and *en-1* in wild-type and *syu* mutant pectoral fin buds. Whole-mount in situ hybridizations. Anterior is to the left and distal to the top, except in (Q-T), where dorsal is to the left and distal the top. (A,B,K,L,Q,R) 32 hour pectoral fin buds; (C,D,G,H,M,N) 38 hour pectoral fin buds; (E,F,I,J,O,P,S,T) 48 hour pectoral fin buds. (A,C,E,G,I,K,M,O,Q,S) Wild-type fin buds; (B,D,F,H,J,L,N,P,R,T) *syu* fin buds. (A-F) *dlx-2* RNA; (G-J) *fgf-8* RNA; (K-P) *bmp-2* RNA; (Q-T) *en-1* RNA. *dlx-2* and *bmp-2* expression appears normal in the apical epidermis of *syu* buds at 32 hours, but is reduced at 38 hours and 48 hours. Note that *dlx-2* and *bmp-2* expression is stronger in the posterior apical epidermis of wild-type, but not *syu* buds at 38 hours. *bmp-2* is expressed in posterior mesenchyme at 32 hours in *syu* buds, but this expression is lost by 38 hours. *fgf-8* expression is not detectable in *syu* fin buds. *en-1* is expressed normally in *syu* fin buds at 32 and 48 hours. Note the apical thickening in the wild-type bud (arrowhead in S), which is not visible in the *syu* bud (arrowhead in T).

The gene affected in the chick *limbless* mutant has been shown to be required for the formation of the AER (Carrington and Fallon, 1988). Due to the dependence of *shh* expression on the AER (Laufer et al., 1994; Niswander et al., 1994), *limbless* mutant buds have no detecable *shh* expression, but nevertheless show weak posterior activation of *hoxd-11* and *hoxd-12* in the wing bud and *hoxd-13* in the leg bud (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). While one cannot rule out the possibility that *limbless* mutant buds express *shh* weakly or transiently, these data suggest that the posterior activation of several *hoxd* genes occurs independently of Shh both in the zebrafish fin bud and the chick limb bud.

Shh and retinoic acid in the fin bud

It has been proposed that retinoic acid acts upstream of *shh* activation in the ZPA (reviewed in Johnson and Tabin, 1997). Our data confirm and extend this proposal: *shh* is ectopically expressed in fin buds of retinoic-acid-treated embryos, and the ectopic activation of *hoxd-11* and *hoxd-12* by retinoic acid requires *shh* activity (Fig. 5B,E,F and data not shown), demonstrating that Shh mediates the activation of these posterior genes by retinoic acid. Our data also indicate that retinoic acid is not sufficient to cause the anterior expansion of the Shh-independent expression of *hoxd-11* and *hoxd-12* (Fig. 5F and data not shown). Ogura et al. (1996) have proposed that retinoic acid and Shh cooperate in generating ZPA activity, suggesting that the role of retinoic acid may not only be to activate *shh*.

Shh maintains A/P polarity and developmental progression in the pectoral fin bud

Following the Shh-independent activation of *hoxd-11, hoxd-12* and *bmp-2* at 32 hours of development, no expression of these genes can be detected in *syu* mutant pectoral fin buds at 38 hours, and the posterior bias of *hoxa-10* expression is also lost (Figs 1, 2, 6), suggesting that Shh is required for the posterior maintenance of these genes. The only sign of A/P polarity left in the absence of Shh signaling at 38 hours is the weak expression of *shh* in posterior cells in cyclopamine-treated embryos (Fig. 4J), suggesting that Shh controls most of the A/P polarity of the pectoral fin bud at this stage.

The expression domains of *hoxa-9* and *hoxa-11* develop a posterior bias by 38 hours in wild-type pectoral fin buds, but this does not occur in *syu* mutant fin buds (Fig. 2C,D,K,L).

This suggests that Shh not only maintains A/P polarity, but is also required for the temporal progression of patterning in the pectoral fin bud. This proposal is further supported by the observation that the activation of *hoxd-11* and *hoxd-12* is slightly delayed and that the most 5′ members of the *hoxa* and *hoxd* clusters, *hoxa-13* and *hoxd-13*, which are the latest to be expressed, are not activated at all in *syu* mutant pectoral fin buds (Figs 1, 2). Finally, the observation that early apical fold markers, such as *dlx-2*, but not late apical fold markers, such as *fgf-8*, are expressed in *syu* mutant buds, also supports the proposal that Shh is required for developmental progression (Fig. 6).

Four zebrafish genes (the *you*-type mutants) have been placed in a group with *syu* because they share several phenotypes, such as reduction of the horizontal myoseptum, and have been proposed to function in a common signaling pathway (van Eeden et al., 1996a). It is therefore interesting to compare the pectoral fin phenotypes of the *you*-type genes with that of *syu*. Apart from *syu*, only *chameleon* shows a reduction of pectoral fins (van Eeden et al., 1996a). The other *you*-type genes, including *you-too*, which codes for the Shh target *gli2* (Karlstrom et al., 1999), do not affect the pectoral fins. This suggests that specific responses to Shh in different contexts may be mediated by distinct genes. A detailed knowledge of the *syu* pectoral fin phenotype will be helpful in analyzing other *you*-type genes in this context, and in analyzing other fin mutants.

Colinearity and the dependence of *hox* gene expression on Shh

At 32 hours of development, the most 3′ members of the *hoxa* and *hoxd* complexes, *hoxa-9* and *hoxd-10*, are expressed normally in *syu* mutant pectoral fin buds (Figs 1, 2). The adjacent 5′ genes, *hoxa-10*, *hoxd-11* and *hoxd-12*, are expressed at reduced levels and expression is slightly delayed in *syu* mutant fin buds, and the most 5′ members, *hoxa-13* and *hoxd-13*, fail to be expressed at all in *syu* mutant pectoral fin buds (Figs 1, 2). There is thus a clear correlation between gene position in the *hox* clusters and the degree of dependence on Shh, with more 5′ genes showing a greater dependence on Shh for activation in the pectoral fin bud than 3′ genes. It should be noted, however, that the more 5′ genes are activated later, and so their failure to be expressed in *syu* fin buds could be due to the reduced developmental progression of these buds.

Shh is required for apical fold development

The expression of early apical fold markers, such as *dlx-2* and *bmp-2*, appears normal in *syu* mutant pectoral fin buds at 32 hours (Fig. 6A,B,K,L), suggesting that Shh is not required for the specification of the apical fold. At 38 hours, however, apical fold expression of *dlx-2* and *bmp-2* starts to become weaker in *syu* mutant pectoral fin buds (Fig. 6C,D,M,N). In addition, *fgf-8* expression, which is activated in the apical fold at 36 hours, is not detectable in *syu* pectoral fin buds (Fig. 6G-J), suggesting that the development of the AER comes to depend on Shh at this stage. Consistent with this proposal, an apical fold can be detected in wild-type fin buds at 38 hours, but not in *syu* buds (Fig. 7C,D).

Anterior expression of Shh induces ectopic *fgf-4* in the chick AER, suggesting that Shh polarizes the AER (Laufer et al., 1994; Niswander et al., 1994). Consistent with these data, *dlx-2* and *bmp-2* expression, which is normally stronger in the posterior apical fold of the zebrafish pectoral fin at 38 hours, is uniform throughout the apical epidermis in *syu* mutants (Fig. 6C,D,M,N).

At 48 hours, expression of marker genes in the apical fold of *syu* mutant pectoral fin buds is strongly reduced compared to wild-type buds (Fig. 6), and apical fold formation is delayed in *syu* mutants, and fails to be followed by fin fold formation (Fig. 7E,F). These results suggest that in addition to polarizing the apical fold, Shh is required for development of the apical fold along its entire A/P axis.

shh expression is dependent on the activity of the AER as it is lost upon ridge removal and can be rescued by FGF-application (Laufer et al., 1994; Niswander et al., 1994). In cyclopamine-treated embryos, *shh* is activated normally in the fin buds, but becomes weaker at 38 hours and is lost by 48 hours (Fig. 4G,J-L). This reduction coincides with the reduction of apical fold markers in *syu*, and thus further supports the proposal that apical fold activity is initially present, but subsequently lost in the absence of Shh activity.

Shh is required for growth of the pectoral fin bud and for the formation of the fin endoskeleton

There is a clear reduction in cell proliferation in *syu* pectoral fin buds already as early as 28-30 hours (Fig. 8A,B). Since the observed reduction of apical fold markers and of the apical-fold-dependent expression of *shh* is first observed at 38 hours, this suggests that the effect of Shh on fin bud proliferation is at least partially independent of the apical fold. However, the more severe reduction in proliferation at 39-41 hours (Fig. 8C,D), which coincides with the reduction of apical fold markers in *syu*, suggests that apical fold activity contributes to reduced growth in *syu* buds. The fin endoskeleton formed by a *syu* hypomorph has a reduction of cell number in both the A/P and P/D axes (Fig. 9B), suggesting that Shh is required for growth in both axes.

The phenotype of a mouse *shh* mutant has been reported by Chiang et al. (1996), and although a detailed analysis of the limb phenotype of this mutant is not yet available, it is interesting to compare the mouse *shh* limb phenotype to the zebrafish *shh* fin phenotype. The limb skeleton of mice lacking Shh consists of a proximal bone, the humerus in the forelimb and the femur in the hindlimb. This suggests that only distal structures of the mouse limb require Shh, consistent with the observation that an ectopic ZPA or Shh-expressing cells only duplicate the autopod and part of the zygopod in the chick (Saunders and Gasseling, 1968; Riddle et al., 1993). In contrast, the pectoral fin buds of zebrafish lacking Shh fail to develop endoskeletal structures along the whole P/D axis, including the girdle (Fig. 9C). This difference could be due to the fact that tetrapods have a well-developed somatopleure, thus allowing proximal parts of the limb to be formed by the body wall, while zebrafish only have a very thin layer of somatopleure (Grandel and Schulte-Merker, 1998), so that all pectoral fin structures are derived from the fin bud itself. Consistent with this proposal, fate-mapping studies of the mouse hindlimb have shown that only structures distal to the femur are derived from the limb bud proper (Muneoka et al., 1989).

Although there thus appear to be some aspects of the *shh* fin/limb phenotype that are different between the zebrafish and the mouse, our data support the proposal that Shh is the major

determinant of A/P polarity in the late fin bud, as little or no A/P polarity is detectable in *syu* mutant fin buds at 38 hours. It will be interesting to determine whether the dependence of the apical fold on Shh has been conserved during evolution.

In summary, our data show that some A/P polarity is established independently of Shh in the fin bud. As development proceeds, control of A/P polarity shifts to Shh, and thus becomes autonomous to the bud. Shh is also required

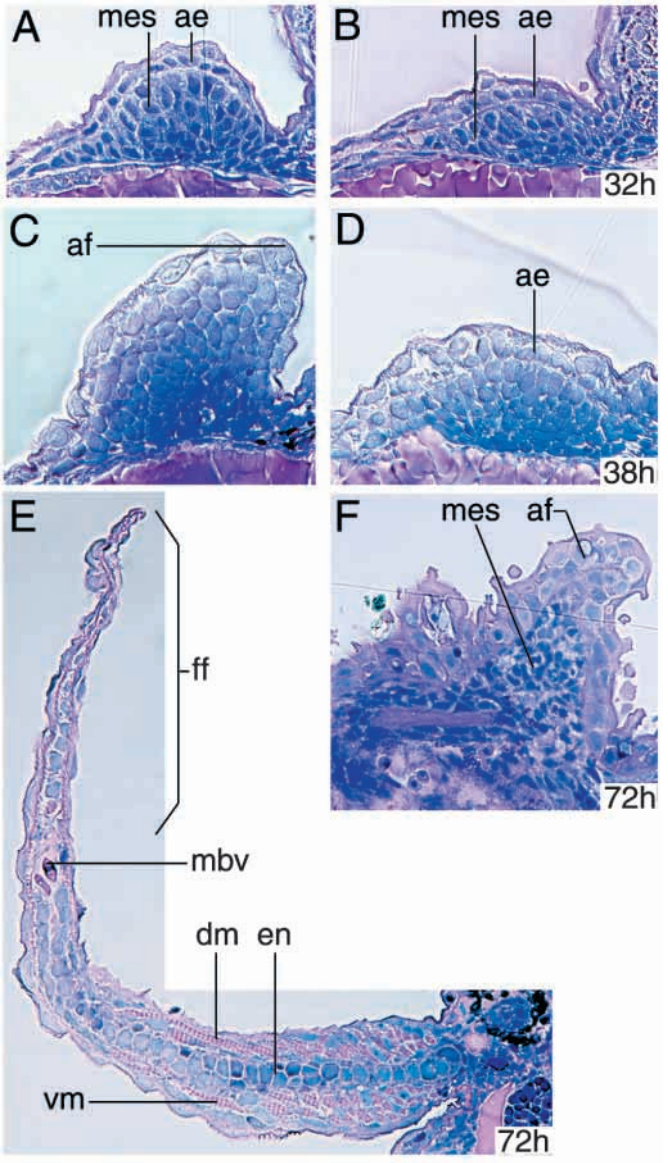


Fig. 7. Morphology of wild-type and *syu* pectoral fin buds. Cross sections through fin buds performed perpendicular to the P/D axis of the fin bud. (A,B) Pectoral fin buds at 32 hours; (C,D) pectoral fin buds at 38 hours; (E,F) pectoral fin buds at 72 hours. (A,C,E) Wild-type fin buds; (B,D,F) *syu* fin buds. An apical fold (af) is not present in *syu* buds at 38 hours (D), but is present in some *syu* buds at 72 hours (F). Note the reduced size of *syu* buds compared to wild-type buds. The *syu* apical fold does not develop into a fin fold, which is well developed in wild-type fins at 72 hours (E,F). Note the absence of differentiated tissues in *syu* buds at 72 hours. ae, apical epicermis; af, apical fold; dm, dorsal musculature; en, endoskeletal disc; ff, fin fold; mbv, marginal blood vessel; mes, mesenchyme; vm, ventral musculature.

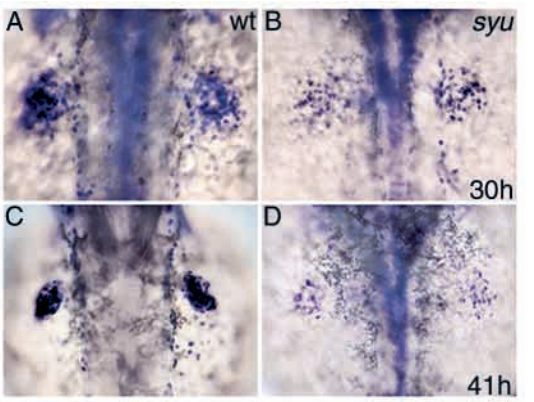


Fig. 8. Reduced proliferation in *syu* pectoral fin buds. Dorsal view of embryos at the level of the pectoral fin buds. Anterior is to the top. (A,C) Wild type; (B,D) *syu*. (A,B) Injected with BrdU at 28 hours and stained at 30 hours; (C,D) injected with BrdU at 39 hours and stained at 41 hours. Note the reduced BrdU incorporation in *syu* fin buds.

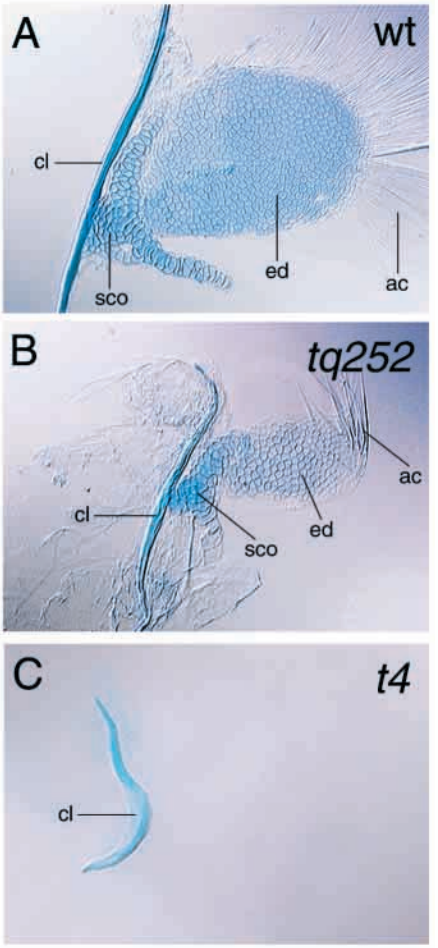


Fig. 9. Larval pectoral fin endoskeleton formed in wild-type and *syu* mutants. Alcian blue stains of the pectoral girdle and fin of larvae on day 6. (A) Wild-type pectoral fin endoskeleton. (B) *syu* hypomorphic allele *tq252*. (C) *syu* null allele *t4*. *syu* null mutants do not form any elements of the fin endoskeleton and show a reduced cleithrum (cl). *syu*^{tq252} hypomorhic mutants variably show reductions of the fin endoskeleton and are able to form all elements including the scapulocoracoid (sco), endoskeletal disc (ed) and the actinotrichs (ac), but show a reduction in cell number, which is most apparent in the endoskeletal disc. ac, actinotrichs; cl, cleithrum; ed, endoskeletal disc; sco, scapulocoracoid.

for development and activity of the apical fold, and for formation of the fin endoskeleton. Finally, Shh is required for proliferation in the fin bud at all stages, and thus already before the reduction of the apical fold, suggesting that Shh-dependent proliferation is at least partially independent of the apical fold.

We would like to thank Marie-Andrée Akimenko, Mark Ekker, Yasuyuki Kishimoto, Stefan Krauss, Kate E. Lewis, Victoria Prince, Frank Reifers and Paolo Sordino for cDNAs. C. J. N. was supported by a DFG fellowship (NE 701/1-1) and an EMBO fellowship (ALTF 458-1997). Further support came from the DFG to S. S. M. (Schwerpunktprogramm: Evolution entwicklungsbiologischer Prozesse, SCHU 1228).

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