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dackel acts in the ectoderm of the zebrafish pectoral fin bud to maintain AER signaling

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SUMMARY

Classical embryological studies have implied the existence of an apical ectodermal maintenance factor (AEMF) that sustains signaling from the apical ectodermal ridge (AER) during vertebrate limb development. Recent evidence suggests that AEMF activity is composed of different signals involving both a sonic hedgehog (Shh) signal and a fibroblast growth factor 10 (Fgf10) signal from the mesenchyme. In this study we show that the product of the *dackel* (*dak*) gene is one of the components that acts in the epidermis of the zebrafish pectoral fin bud to maintain signaling from the apical fold, which is homologous to the AER of tetrapods. *dak* acts synergistically with Shh to induce *fgf4* and *fgf8* expression but independently of Shh in promoting apical fold morphogenesis. The failure of *dak* mutant fin buds to progress from the initial fin induction phase to the autonomous outgrowth phase causes loss of both AER and Shh activity, and subsequently results in a proximodistal truncation of the fin, similar to the result obtained by ridge ablation experiments in the chicken. Further analysis of the *dak* mutant phenotype indicates that the activity of the transcription factor engrailed 1 (En1) in the ventral non-ridge ectoderm also depends on a maintenance signal probably provided by the ridge. This result uncovers a new interaction between the AER and the dorsoventral organizer in the zebrafish pectoral fin bud.

Key words: Pectoral fin, Limb, Dorsoventral axis, Zebrafish, *dackel*

INTRODUCTION

Tetrapod limb development relies on three signaling centers within the limb bud. They are established as a consequence of limb induction and confer developmental autonomy to the bud once it has been induced (reviewed in Duboule, 1994; Cohn and Tickle, 1996; Johnson and Tabin, 1997; Martin, 1998). At the boundary between dorsally and ventrally specified tissues, the apical ectodermal ridge (AER) is induced. It is the source of several fibroblast growth factors (Fgfs) and promotes proliferation in the underlying mesenchyme that constitutes the bud (reviewed in Martin, 1998). The AER becomes structurally distinct as the thickened ectodermal margin of the limb bud. Ablation of the AER in the chick causes an arrest in limb-bud outgrowth and results in a limb that is truncated along the proximodistal (P/D) axis (Saunders, 1948). In concert with mesenchymal factors from the lateral plate, the AER induces transcription of *shh* in the posterior limb-bud mesenchyme (Niswander et al., 1994; Laufer et al., 1994; Ros et al., 1996; Grieshammer et al., 1996). Shh mediates the functions of the zone of polarizing activity (ZPA; Riddle et al., 1993), a second signaling center of the bud (Saunders and Gasseling, 1968). It also maintains, polarizes and activates the expression of genes, among others, from the *hoxa* and *hoxd* clusters, and thus stabilizes and propagates anteroposterior (A/P) polarity in the bud (Riddle et al., 1993; Laufer et al., 1994; Neumann et al., 1999). In addition, Shh signals to the ectoderm to maintain Fgf-signaling from the AER (Martin, 1998; Zúñiga et al., 1999). In the zebrafish, Shh is necessary to activate *fgf8* expression in the apical ectoderm (Neumann et al., 1999). A positive feed back loop between Shh and Fgfs coordinates growth and patterning along the A/P and P/D axes and is, at least in part, responsible for the developmental autonomy of the bud (Niswander et al., 1994; Laufer et al., 1994). Experimental or genetic ablation of either one of the two signaling centers causes the other one to stop signaling (Vogel and Tickle, 1993; Niswander et al., 1994; Chiang et al., 1996; Neumann et al., 1999). Classical experiments have suggested that a mesenchymal apical ectodermal maintenance factor (AEMF) acts to maintain signaling from the AER during limb development (Zwilling, 1961). Recent results indicate that AEMF activity consists of at least two independent signals. One is Shh, which maintains *fgf* expression in the AER (Zúñiga et al., 1999), the other is Fgf10, which is expressed in the distal limb bud mesenchyme, and activates and maintains *fgf8* expression in the AER (Ohuchi et al., 1997).

The third signaling center resides in the non-ridge ectoderm, which imposes its dorsoventral (D/V) polarity onto the

underlying mesenchyme (Poutou, 1977; Parr and McMahon, 1995). Wnt7a secreted from the dorsal ectoderm instructs the underlying mesenchymal cells to differentiate dorsal structures (Parr and McMahon, 1995), whereas En1 in the ventral ectoderm inhibits *wnt7a* expression and promotes development of ventral structures by default (Loomis et al., 1996).

The zebrafish (*Danio rerio*) pectoral fin bud is homologous to the tetrapod forelimb bud (Grandel and Schulte-Merker, 1998). In 26 h (hours postfertilization) embryos, a small pectoral fin bud has formed. The epidermis covering this bud thickens and forms the apical fold by 36 h in a position that is equivalent to the one taken by the tetrapod AER. After 48 h, the apical fold expands and is invaded by mesenchyme, thereby giving rise to the fin fold that will develop into the visible fin of the adult fish. Tetrapods do not develop any structure homologous to the fin fold. The mesenchyme of the bud gives rise to two endoskeletal elements, a proximal shoulder girdle and a distal endoskeletal disc from which the fin fold extends distally. Thus, zebrafish fin buds become structurally distinct from tetrapod limb buds only after 48 h of development when fin fold formation occurs.

During early pectoral fin development, the expression patterns of the orthologs of some of the genes described above show striking similarities to the tetrapod condition. *shh* (Krauss et al, 1993), *fgf8* (Fürthauer et al., 1997; Reifers et al, 1998) and *en1* (Ekker et al., 1992) are each active in the expected tissues of the bud, and thus probably exert an equivalent function as in the chicken or mouse. *fgf8*, however, which is the earliest AER-marker in tetrapods, becomes detectable only in the apical epidermis long after the fin bud has been established in zebrafish (Reifers et al., 1998). The analysis of the zebrafish *syu* mutant (Neumann et al., 1999), in which the zebrafish *shh* gene is mutated (Schauerte et al., 1998), has already shown important homologies to the tetrapod condition. As in the tetrapod limb bud, Syu mediates most aspects of A/P polarity and also is required for progressive development in the apical epidermis. As in the tetrapod limb bud, members of the *hoxa* and *hoxd* clusters are active in zebrafish fin buds (Sordino et al., 1995), and have been shown to be regulated by Syu signaling (Neumann et al., 1999).

Ten other mutants with a specific phenotypic alteration of pectoral fin development have been described (van Eeden et al., 1996). In this collection, the *dackel* (*dak*) mutant is unique for a specific proximodistal truncation of the fin and an early downregulation of *syu* expression (van Eeden et al., 1996).

In the present study, we investigate the fin phenotype of the *dak* mutant by combining mutant analysis and experimental embryology. We show that *dak* acts in the epidermis to initiate apical fold formation, to induce expression of *fgf4* and *fgf8*, and to maintain expression of *en1* in the ventral non-ridge ectoderm.

MATERIALS AND METHODS

Fish stocks and maintenance

Fish maintenance was as described in Grandel and Schulte-Merker (1998). The *dak* alleles *to273b* and *tw25e*, the hypomorphic *syu* allele *tq252*, and the *syu* null allele *t4* were used.

In situ hybridization

Whole-mount in situ hybridization was carried out and the same antisense probes were used as described in Neumann et al. (1999), with the exception of the *fgf8* probe, which was from Fürthauer (1997). P. Sordino (1995) provided *hoxd* and *hoxab* probes. The zebrafish *fgf4* gene was cloned by B. W. D. (Draper et al., 1999). A detailed characterization will be reported elsewhere. The amino acid sequence has been submitted to GenBank (accession number AF283555).

Fgf4 bead implantation

Fgf4 beads were prepared according to the method of Reifers et al. (2000). Zebrafish embryos were manually dechorionated and placed into a drop of Low Melting Point Agarose 1.5% (Gibco BRL), which was allowed to dry and subsequently fenestrated to manipulate the fin bud. The epidermis was digested away with a drop of Light White Mineral Oil (Sigma) at the ventral edge of the fifth somite. A bead was inserted into the lateral plate mesenchyme and pushed anteriorly below the epidermis into the fin bud mesenchyme with electrophoretically sharpened tungsten needles. Manipulation and further incubation was carried out in Ringer's plus 20 μg/ml Gentamycin (Gibco BRL), which was replaced by E3 after 12 h.

Transplantation

Donor embryos were injected with 0.625% rhodamin-dextran and 0.625% biotin-dextran (Molecular Probes, Eugene, OR) at the one-cell stage. Transplantations were performed at late blastula stages between sphere stage to 30% epiboly, with stage matched donors and hosts – the manipulation is particularly easy at this stage and we were unable to perform transplantations when fin buds had formed. The choice of site for transplantation was guided by the fate map of Kimmel et al. (1990), which shows that ectodermal cell fates are located between 0% to 80% latitude at the 50% epiboly stage on the ventral side of the embryo. Because the dorsal and ventral sides cannot be distinguished during blastula stages, we implanted donor cells into two opposing areas roughly 30% latitude away from the animal pole to ensure that the implanted cells from one of the two locations would give rise to epidermis. In accordance with the map, we observed mainly epidermal and neuroectodermal clones. The frequency with which the transplanted cells populated the fin bud ectoderm in greater numbers was 6.5%. The manipulations were carried out in Ringer's plus gentamycin. Embryos were allowed to develop until 60 h, fixed and prepared for staining. Biotin-labeled donor cells were detected with a rabbit anti-biotin primary antibody (kindly provided by Heinz Schwarz, MPI für Entwicklungsbiologie, Tübingen) and fluorescently labeled with a secondary goat anti-rabbit-Cy3 coupled antibody (Jackson).

RESULTS

Phenotypic description of *dak* mutants

Among the 11 genes that have been identified by mutant phenotype to be involved in zebrafish pectoral fin development, recessive alleles of *dackel* (*dak*) lead to the loss of functional pectoral fins in homozygous mutant larvae (van Eeden et al., 1996; Fig. 1A,B). Four morphologically indistinguishable alleles of *dak* have been found (van Eeden et al., 1996). The two alleles chosen in the present study also behave alike with regard to molecular markers. Despite the dramatic loss of the larval fin, pectoral fin buds form normally in *dak* mutants. During the first 10 hours of fin development (26-36 h), it is not possible to identify living *dak* embryos by phenotype. Only after formation of the apical fold in sibling embryos, can *dak* mutants be identified by the lack of this structure (Fig. 1C,D) as well as by the smaller size of the fin buds, which have stopped growing after 32 h. The vast majority of *dak* mutant

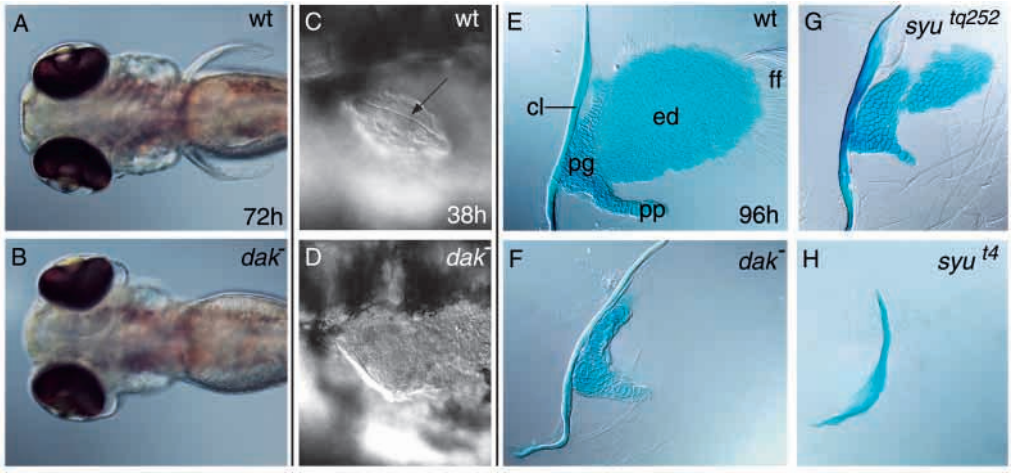

Fig. 1. Phenotype of *dak* and *syu* mutants at 72 h (A,B), 38 h (C,D) and 96 h (E-H): (A,C,E) Wild-type (wt), (B,D,F) *dak*$^{-}$, (G) *syu*^{tq252}, (H) *syu*^{t4}. (A,B) anterior is towards the left, (C,D) anterior is towards the left, view from distal. Note that the wt apical fold in C produces a 'line' (arrow) where dorsal and ventral epidermis become juxtaposed. This 'line' is absent in the *dak*$^{-}$ bud in D. (E-H) distal is towards the right. Living *dak*$^{-}$ larvae lack functional pectoral fins at 72 h and fail to develop an apical fold at 38 h. At 96 h *dak*$^{-}$ larvae have developed a pectoral girdle that distinguishes them from *syu* hypomorphic and null mutants. cl, cleithrum; ed, endoskeletal disc; ff, fin fold; pg, shoulder girdle; pp, postcoracoid process.

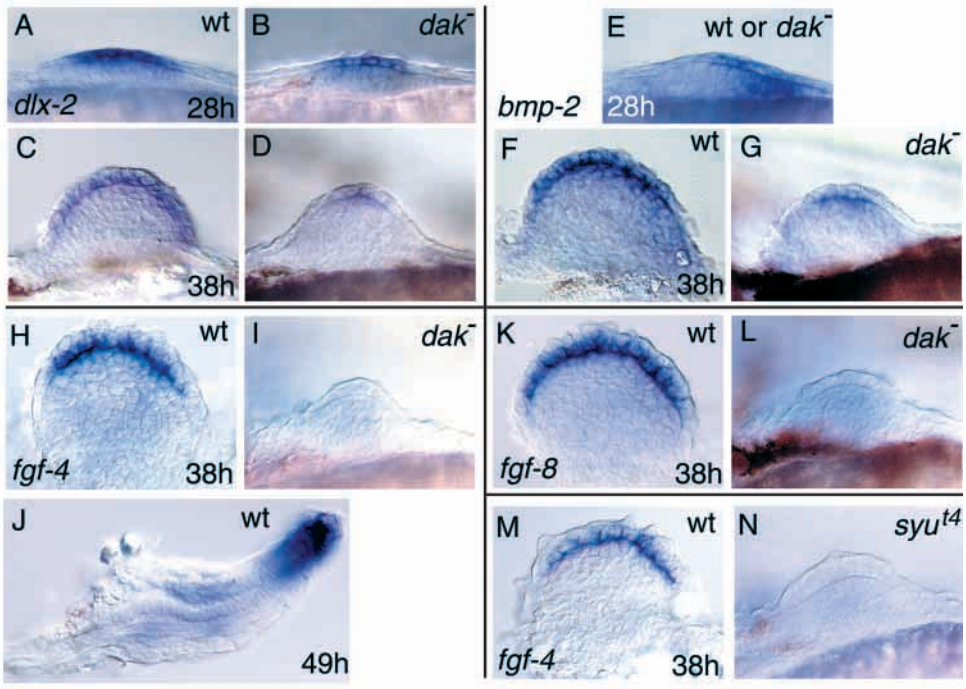

Fig. 2. Expression of apical ectodermal markers at 28 h (A,B,E) and 38 h (C,D,F,G,H,I,K-N). Anterior is towards the left, distal is towards the top. (J) shows the expression at 49 h: distal is towards the right, dorsal is towards the top. (A,C,F,H,J,K,M) wt, (B,D,G,I,L) *dak*$^{-}$, (E) wt and *dak*$^{-}$ are indistinguishable, (N) *syu*^{t4}. (A-D) expression of *dlx2*. (E-G) expression of *bmp2*. (H-J,M,N) expression of *fgf4*. (K,L) expression of *fgf8*. *dlx2* and *bmp2* are activated at 28 h but their expression is strongly reduced at 38 h in *dak*$^{-}$. Both *fgf*s are not properly activated in *dak*$^{-}$. *fgf4* is not expressed in *syu*^{t4}.

embryos (94%; 185 of 196 buds from 11 independent egglays) fail to form an apical fold, even at later stages. *dak* homozygotes also do not form a fin fold. In those cases where a small apical fold can be observed at 60 h (6%, 11 of 196), the underlying mesenchyme has not invaded the fold. Skeletal preparations of hatched *dak* larvae show that not all pectoral fin structures are lost in the mutant (Fig. 1F). *dak* mutants retain the pectoral girdle, the proximalmost pattern element of the fin. The girdle itself appears mildly affected as well, as the postcoracoid process is shorter than in siblings and is sometimes absent. All *dak* alleles are larval lethals (van Eeden et al., 1996).

dak mutants impede progression of apical epidermal development

As the *dak* mutant fin phenotype is suggestive of a defect caused in apical ridge signaling, we tested the expression of four apical ectodermal markers. At 28 h, *dlx2* and *bmp2b* were both detected in *dak* mutants (Fig. 2A,B,E). But while *bmp2b* was expressed at wild-type levels, the expression of *dlx2* was already reduced. At 38 h, after the apical fold has formed in sibling embryos, the activity of both markers was downregulated severely in *dak*$^{-}$ fin buds, becoming undetectable at even later stages in the case of *dlx2* (Fig. 2C,D,F,G and data not shown)

As the apical fold forms, expression of *fgf8* starts in the apical ectoderm at 36 h (Reifers et al., 1998). This contrasts with tetrapods where *fgf8* is the earliest AER marker. We report expression of *fgf4* in the apical fold at the same time (Fig. 2H). The onset of *fgf4* expression correlates well with the situation in tetrapods, where *fgf4* marks the apical ridge at later developmental stages (Niswander and Martin, 1992; Laufer et al., 1994). However, we could not detect a posterior bias of the

fgf4 expression domain typical of tetrapods. Another difference concerns its later expression in the myogenic mesenchyme of the fin at 48 h (Fig. 2J), which has not been reported in tetrapods. (A manuscript on *fgf4* function and expression is in preparation by B. W. D.).

At 38 h, *fgf8* was not detectable in most *dak*⁻ embryos (42 of 47), or was present at very weak levels only (5 of 47; Fig. 2K,L). At the same stage, *fgf4* remained inactivated in a similar manner (41 of 45 showed no detectable expression, Fig. 2H,I). At 48 h and at 60 h, *fgf4* could not be detected at all in 16 and 62 cases, respectively, and *fgf8* was seen in minor amounts in only 8 of 42 and 2 of 18 cases, respectively (data not shown).

The lack of an apical fold in *dak* mutant fin buds is a structural difference to *syu* null mutant fin buds, which are able to form an apical fold with some delay. However, *syu* null mutant buds, like *dak*⁻, do not express either *fgf* (Neumann et al., 1999; Fig. 2M,N).

The analysis of epidermal gene expression in *dak*⁻ fin buds clearly shows a requirement for a functional *dak* gene product already at the earliest developmental stage tested. This contrasts with the wild-type appearance of living *dak*⁻ fin buds at the same stage. Clearly, at later stages *dak* is indispensable for normal growth, epidermal morphogenesis and for initiation of *fgf4* and *fgf8* expression, two genes proposed to play key roles in mediating AER function in tetrapods.

dak does not act downstream of *syu* in the pectoral fin bud

Transcription of the *shh* gene in the posterior mesenchyme requires a functional AER signal in the tetrapod limb bud (Niswander et al., 1994; Laufer et al., 1994; Ros et al., 1996; Grieshammer et al., 1996). We thus probed *dak* mutants for the presence of *syu* transcript in the fin buds in order to test for the presence of ridge activity. van Eeden et al. (1996) described the early reduction and later loss of *syu* transcript in the pectoral fin buds of *dak* mutants. We have confirmed these results (Fig. 3A-F). At 28 h, only 2 hours after the first appearance of the bud, *dak* mutant buds showed reduced levels of *syu* expression. The reduction was mild at first, but enhanced at later stages, with *syu* mRNA levels finally falling below the level of detection. This behavior points to the early presence of apical ridge activity in *dak*⁻ fin buds as the induction of *syu* expression does occur.

As Syu positively feeds back on its own expression (Neumann et al., 1999), we assayed the activity of the pathway downstream of *syu* in *dak* mutants, and analyzed the expression of *patched1* (*ptc1*), a target gene of Syu signaling (Lewis et al., 1999; Fig. 3G-L). While *ptc1* expression was not detected in *syu* null mutants, its expression was detected at 28 h and 38 h in *dak* mutants, although at reduced levels. At 48 h, *ptc1* expression had been lost along with *syu* transcript. *hoxa13* and *hoxd13* are likewise targets of Syu signaling in the fin bud, as they are not activated in *syu* null mutant buds (Neumann et al., 1999). As in the case of *ptc1*, their expression was initiated in *dak* mutant buds but lost at 38 h (see Figs 5R-U, 6R-U). These results suggest that the pathway downstream of *syu* is functional and that *dak* directly or indirectly maintains *syu* expression. There are three additional observations indicating that downregulation of the Syu signaling pathway in the fin bud is insufficient to cause the *dak* mutant phenotype.

When we tested *dak* mutants for *en1* expression (Fig. 4A-C), we found wild-type levels of expression at 28 h, but a reduction of the expression level and the size of the expression domain at 38 h and later. As *en1* expression was not reduced in *syu* null mutant fin buds at 38 h and 48 h (Neumann et al., 1999; Fig. 4D,E), this aspect of the phenotype cannot be explained by reduction of *syu* signaling.

Structural comparisons were made of the endoskeletal elements of the fins in *dak* and *syu* mutants. In larvae carrying the *syu* null allele, no endoskeleton formed at all, while in carriers of the hypomorphic *syu* allele, both endoskeletal elements, girdle and disc were formed but were variably reduced (Neumann et al., 1999; Fig. 1G,H). As *dak* mutants do not phenocopy either *syu* mutant, the phenotypic difference indicates the involvement of the two genes in different processes. Another observation to indicate that *dak* does not act solely via the *syu* pathway comes from the analysis of *hox* expression patterns.

At 28 h, wild-type levels of expression of the 3′ located member of the *hoxd* cluster, *hoxd10*, could be detected in *dak* mutants (Fig. 5A). At the same time, reduced expression of *hoxd11* and *hoxd12* was seen in *dak* mutants (Fig. 5F,G,L,M). *hoxd13* was not stably expressed at this stage in wild-type fin buds, but a strong reduction of its expression was seen at 32 h in *dak*⁻ fin buds (Fig. 5R,S). At later stages, the expression of all four *hoxd* genes was further downregulated, having dropped below detection level by 38 h (*hoxd13*; Fig. 5T,U) and 48 h (*hoxd10*, *hoxd11* and *hoxd12*; Fig. 5D,E,J,K,P,Q). Though the

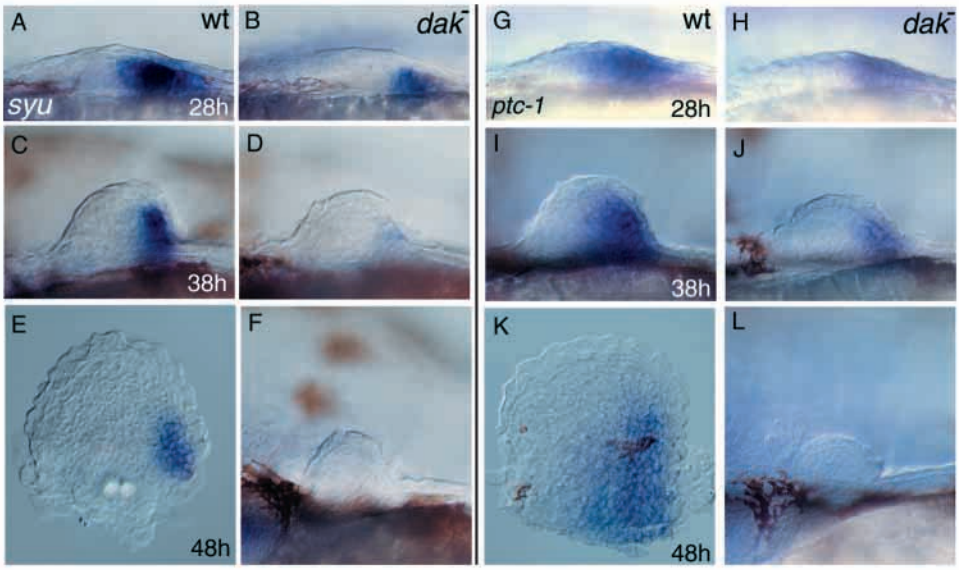

Fig. 3. Expression of *syu* and *ptc1* at 28 h (A,B,G,H), 38 h (C,D,I,J) and 48 h (E,F,K,L). (A,C,E,G,I,K) wt and (B,D,F,H,J,L) *dak*⁻ fin buds. Anterior is towards the left, distal is towards the top. The expression of both genes is reduced at 28 h and 38 h. No expression is detected at 48 h.

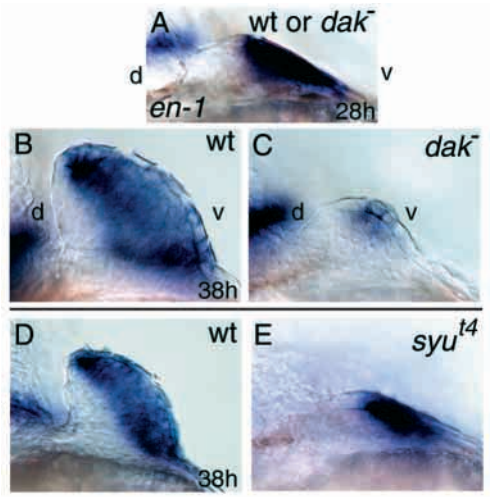

Fig. 4. *en1* expression in *dak*⁻ and *syu*^{t4} at 28 h (A) and 38 h (B-E). (B,D) wt, (C) *dak*⁻ and (E) *syu*^{t4}. (A) wt and *dak*⁻ are indistinguishable. Dorsal is to the left and ventral is to the right. At 28 h, *dak*⁻ shows wt levels of *en1*. At 38 h, expression of *en1* is downregulated in *dak*⁻ but not in *syu*^{t4}.

late loss of the three more 5′ located *hoxd* genes can be explained by the loss of *syu* signaling in *dak* mutant fin buds, downregulation of *hoxd10* at 38 h is not seen in *syu* null mutants at the same stage (Neumann et al., 1999).

Reduced levels of expression of *hoxa9*, *hoxa10* and *hoxa13* were detected at 28 h in *dak*⁻ embryos, while transcript levels of *hoxa11* were not affected at this time (Fig. 6A,B,G,H,M,R,S). During further development, mRNA of *hoxa9* and *hoxa10* dropped below the detection level, while *hoxa11* and *hoxa13* could show very low levels of expression in some *dak* mutant buds at 48 h (Fig. 6E,F,K,L,P,Q,V,W). We further noticed the loss of *hoxa10* expression in the posterior and distal mesenchyme at 38 h, which leaves the bud with an apolar patch of *hoxa10* at that time, reminiscent of the situation found in the *syu* null mutant fin buds (Fig. 6I,J). But again, the early reduction of the 3′ member in the cluster, *hoxa9*, distinguishes *dak* mutant fin buds from those of the *syu* null mutant.

We have presented several differences in the behavior of *dak* and *syu* mutants that all suggest that *dak* is involved in other processes besides maintaining *syu* signaling. In view of the fact that the apical epidermis is more strongly affected in *dak* mutants than in *syu* mutants, our results suggest that the primary defect is a loss of apical ridge function in *dak* mutants.

Application of Fgf4-soaked beads to *dak* mutant fin buds rescues mesenchymal and early epidermal gene expression

Beads soaked in either Fgf4 or Fgf2 are equally potent in replacing an ablated AER in the chick embryo when stapled onto or transplanted into the ridge denuded bud (Niswander et al., 1993; Fallon et al., 1994). We have implanted Fgf4-soaked

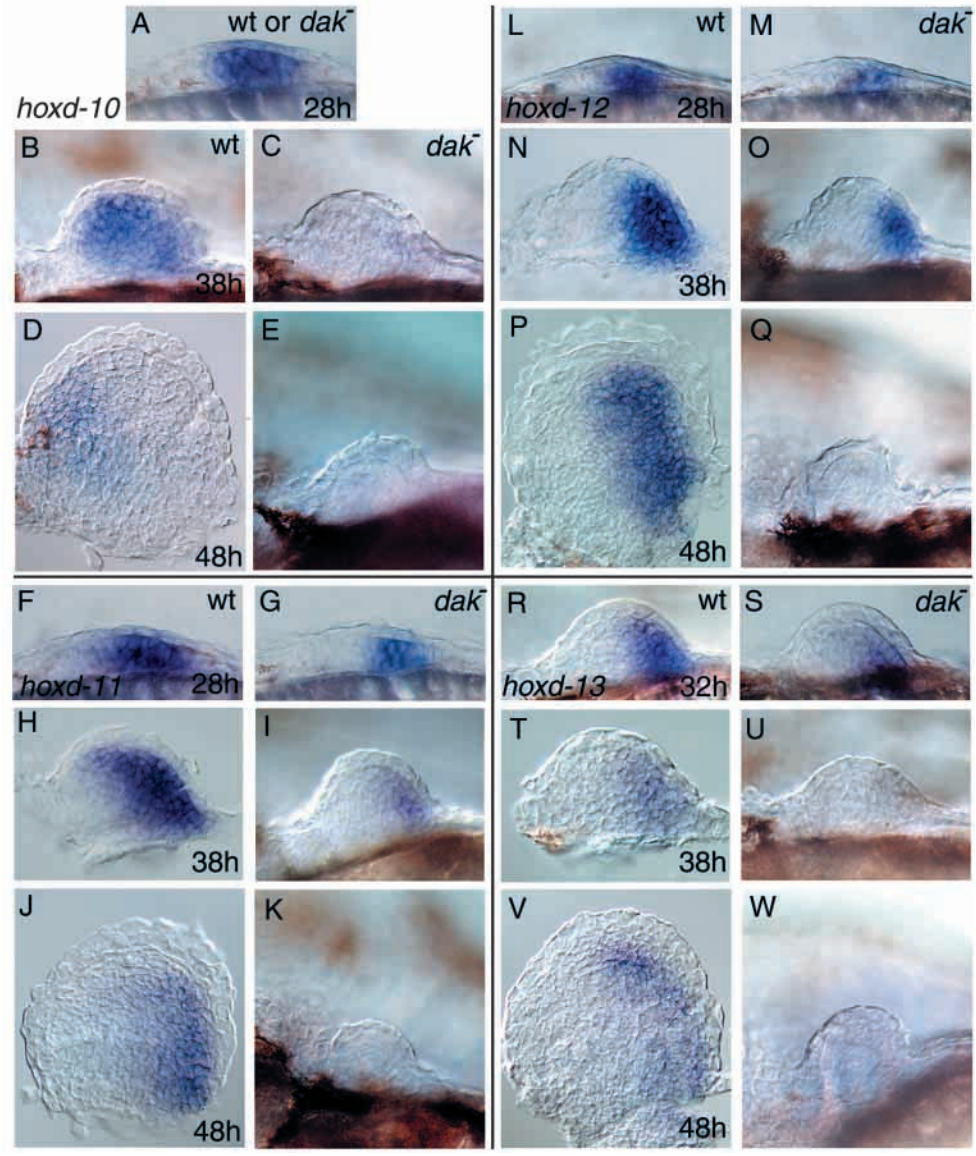

Fig. 5. Expression of *hoxd* genes at 28 h (A,F,G,L,M), 32 h (R,S), 38 h (B,C,H,I,N,O,T,U) and 48 h (D,E,J,K,P,Q,V,W). Anterior is towards the left, distal is towards the top. (B,D,F,H,J,L,N,P,R,T,V) wt and (C,E,G,I,K,M,O,Q,S,U,W) *dak*⁻. (A) *dak*⁻ and wt are indistinguishable. (A-E) expression of *hoxd10*. (F-K) expression of *hoxd11*. (L-Q) expression of *hoxd12*. (R-W) expression of *hoxd13*. *Hoxd* gene expression is lost at 48 h in all cases. Note the downregulation of *hoxd10* at 38 h which is not seen in *syu*^{t4}.

beads into *dak* mutant buds to investigate whether *dak* interferes with the bud's reception of the Fgf signal from the apical fold.

The implantation was carried out within a time window between 35 h and 37 h, which surrounds the timepoint of endogenous *fgf4* and *fgf8* activation in wild-type fin buds. Of the 62 mutant fin buds that received an Fgf4 bead, all showed further growth than usually seen. 24 hours after the operation the treated buds were about two to three times the size of the untreated buds on the contralateral side (Fig. 7). We found expression of *syu*, *hoxd11* and *hoxa13* to be reactivated in the operated buds, while no expression or only slight expression was seen on the contralateral side (Fig. 7A-F). As indicated in Table 1, detection of slight expression in the control side buds of some of the marker genes tested was expected from in situ analyses of untreated *dak* mutants at the same stage. All three mesenchymal markers showed the posterior bias characteristic of *syu*, *hoxd11* and of the early phase of expression of *hoxa-13*, irrespective of the position of the bead. In a total of 42 cases, PBS-soaked beads were implanted into *dak* mutant fin buds as controls. PBS-beads had no effect on bud size nor did they lead to activation of marker gene expression above the usual level at 60 h (Fig. 7L,M).

We also examined expression of ectodermal markers. The early apical ectodermal markers *bmp2b* and *dlx2* were activated. They showed a posterior bias in 6 of 7 cases and in 7 of 9 cases, respectively (Fig. 7G,H; Table 1, and data not shown). The late apical ectodermal markers *fgf4* and *fgf8*, however, could not be detected in Fgf4-treated fin buds (Fig. 7I,J; Table 1, and data not shown). Apical fold formation was not readily detected in vivo. We therefore sectioned six of the bead-treated fin buds and found two individuals with a small apical fold, while the other four did not show any sign of apical fold formation. Somewhat surprisingly, the expression of *en1* was upregulated again in its normal domain upon the Fgf signal (Fig. 7K).

As the fin bud mesenchyme is able to respond to the Fgf4 signal by activating *syu*, *hoxd11* and *hoxa13*, we conclude that *dak* does not act downstream of the Fgf signal. The *dak* ectoderm, however, is only partially able to respond to Fgf4 as it activates only early epidermal markers but neither *fgf* expression nor significant apical fold formation are observed. *dak* thus behaves as if it were required to promote progression of apical ectodermal development.

Transplantation of wild-type cells into the epidermis of *dak*⁻ fin buds rescues *fgf8* expression and apical folding and leads to activation of *syu*

Recombination of ectoderm and mesenchyme of mutant and wild-type donor and host limb buds has been carried out successfully in the chick limb bud in order to better define the capacities of each tissue in mutants (Ros et al., 1996). We have carried out a similar experiment by injecting lineage-labeled wild-type cells into *dak* mutant embryos during early epiboly stages and screened for their presence in the fin buds at 60 h. 46 *dak* mutant embryos survived the procedure, which allowed for inspection of 92 fin buds. 16 buds showed wild-type cells in the fin bud ectoderm. Among these, 10 buds had received only one or a few wild-type cells and were phenotypically *dak*⁻. Six buds showed larger clusters of transplanted cells in

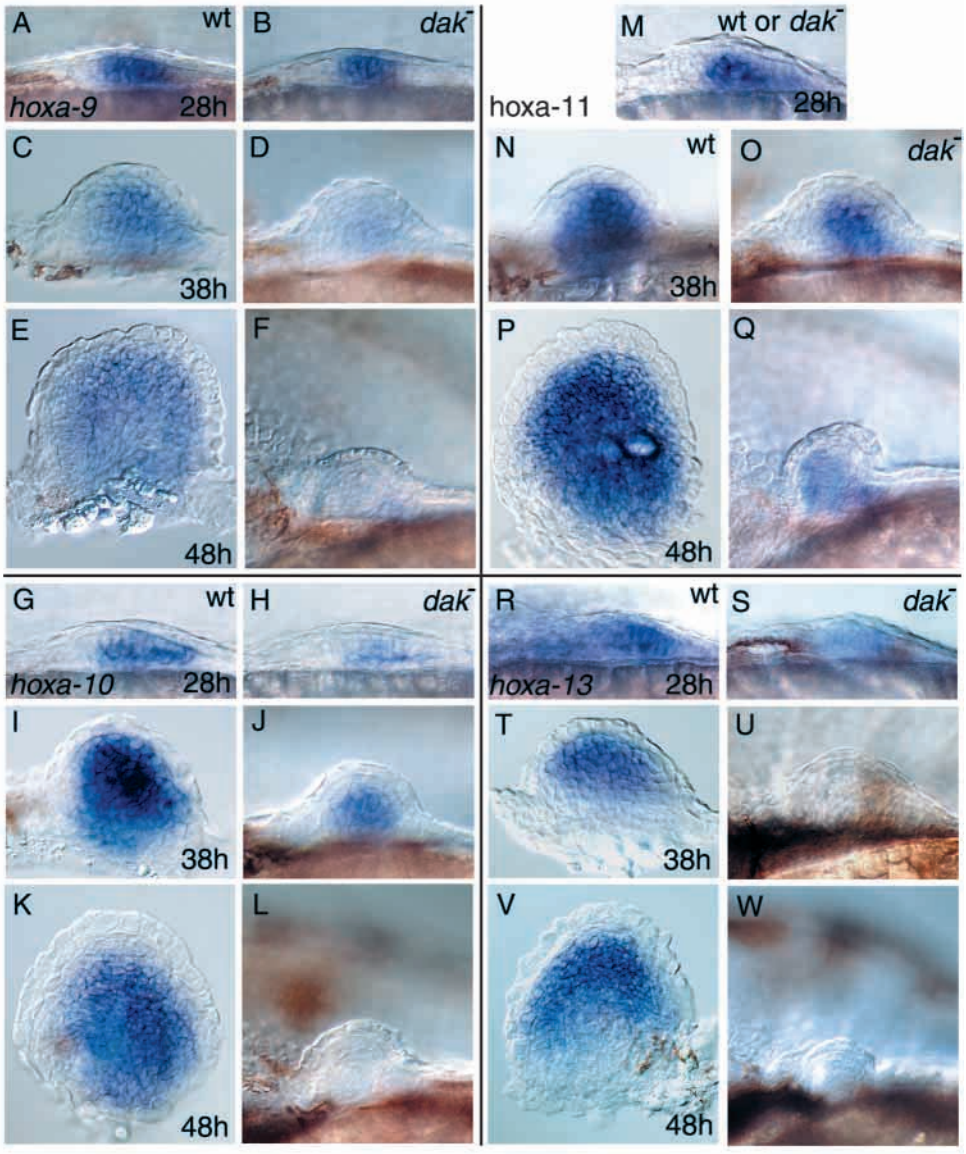

Fig. 6. Expression of *hoxa* genes at 28 h (A,B,G,H,M,R,S), 38 h (C,D,I,J,N,O,T,U) and 48 h (E,F,K,L,P,Q,V,W). Anterior is towards the left, distal is towards the top. (A,C,E,G,I,K,N,P,R,T,V) wt and (B,D,F,H,J,L,O,Q,S,U,W) *dak*⁻. (M) *dak*⁻ and wt are indistinguishable. (A-F) expression of *hoxa9*. (G-L) expression of *hoxa-10*. (M-Q) expression of *hoxa-11*. (R-W) expression of *hoxa13*. *hoxa9* and *hoxa10* are lost at 48 h, while slight *hoxa11* and *hoxa13* expression may still be detectable.

the epidermis. These buds were larger than unmanipulated *dak*$^{-}$ buds and all had developed an apical fold distally.

We examined the distribution of wild-type cells in the six fin buds, which had received larger contributions of wild-type cells (Table 2). In all cases, wild-type cells could be observed in the apical fold (Fig. 8). All buds had also received different amounts of cells in the ventral epidermis (Fig. 8). In all but one case, wild-type cells were located in the dorsal epidermis as well.

We also have carried out in situ hybridizations in the experimental fin buds and found, among the six larger buds, *fgf8* expression in the apical fold in three cases (Fig. 8) and *syu* expression in the other three cases (Table 2; data not shown). Thus, all mutants that had received wild-type cells in the epidermis and were phenotypically scored to show a limited 'rescue' of the *dak* mutant phenotype showed activation of the marker gene tested. All other *dak*$^{-}$ embryos had neither activated *fgf8* nor *syu*.

We conclude from these observations that a functional *dak* gene product is required in the epidermis of the fin bud for induction of *fgf8* and for apical fold morphogenesis. The nonautonomous activation of *syu* in the posterior mesenchyme provides evidence for the presence of ectodermal signaling to the mesenchyme during fin development.

Table 1. Fgf4 bead implantation into *dak*$^{-}$ fin buds: marker gene expression

Gene	Strength of expression	Fgf4 bead implanted into the left fin bud		PBS beads implanted into the left fin bud		Untreated dak^{-}
		Left bud	Right bud	Left bud	Right bud	
syu	Strong	8	0	0	0	0
	Weak	1	1	0	1	0
	Undetectable	0	7	6	5	48
hoxd11	Strong	8	0	0	0	0
	Weak	0	4	4	3	4
	Undetectable	0	4	2	3	10
hoxa13	Strong	6	0	0	0	0
	Weak	0	6	4	3	10
	Undetectable	0	0	1	2	6
dlx2	Strong	8	0	0	0	0
	Weak	1	5	3	1	4
	Undetectable	0	4	2	4	12
bmp2	Strong	7	0	0	0	0
	Weak	0	3	3	1	8
	Undetectable	0	4	1	3	24
fgf4	Strong	0	0	0	0	0
	Weak	0	0	0	0	0
	Undetectable	5	5	5	5	36
fgf8	Strong	0	0	0	0	0
	Weak	1	1	0	0	2
	Undetectable	9	9	5	5	16
en1	Strong	8	0	0	0	0
	Weak	0	8	5	5	15
	Undetectable	0	0	1	1	3

DISCUSSION

dak mutants lose the apical ridge signal during development

The formation of a boundary between dorsally and ventrally specified territories is a prerequisite for AER induction in the limb bud (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). The early wild-type expression of *en1* in the ventral ectoderm of *dak* mutant buds indicates that the initial specification of cell fates along the D/V axis occurs normally in the mutant. The presence of *bmp2b* and *dlx2* in the apical epidermis shows that ridge induction has occurred in *dak* mutant fin buds at 28 h, though ridge activity is reduced already at this early stage. This is indicated by reduced levels of *dlx2* expression and by lower amounts of *syu* transcript. Despite the reduced signal from the apical epidermis, living *dak* mutants cannot be distinguished from wild-type siblings by their phenotype prior to 36 h – up to 10 hours after bud formation. This indicates that during the first 10 hours of fin development, the fin bud behaves normally with respect to growth and shape. Likewise, the expression of mesenchymally expressed genes can be detected in their wild-type domains at 28 h, though many of them show lower

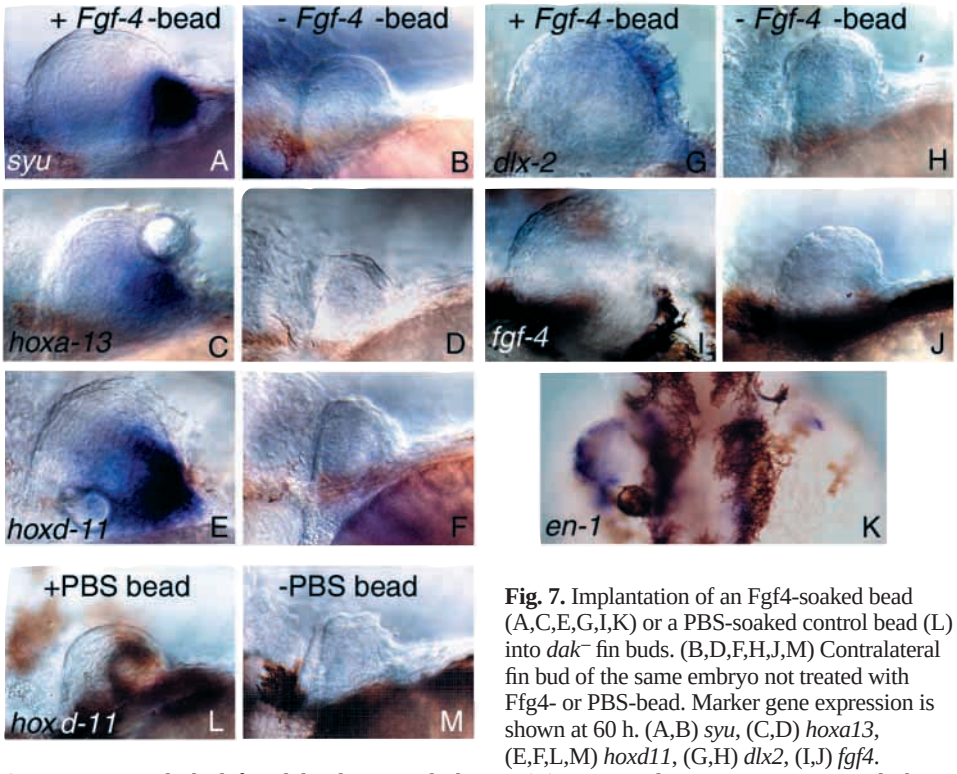

Fig. 7. Implantation of an Fgf4-soaked bead (A,C,E,G,I,K) or a PBS-soaked control bead (L) into *dak*$^{-}$ fin buds. (B,D,F,H,J,M) Contralateral fin bud of the same embryo not treated with Ffg4- or PBS-bead. Marker gene expression is shown at 60 h. (A,B) *syu*, (C,D) *hoxa13*, (E,F,L,M) *hoxd11*, (G,H) *dlx2*, (I,J) *fgf4*. Anterior is towards the left and distal is towards the top. (K) *en1*. Distal view, anterior is towards the top. All Fgf4 bead-treated buds are larger than the untreated buds on the contralateral side. All mesenchymal markers tested are activated. In the apical ectoderm only early markers are activated (*dlx2*, *bmp2*; see text) whereas late markers are not activated (*fgf4*, *fgf8*; see text). Note that *en1* is activated. PBS-bead implantation does not lead to marker gene activation of control buds.

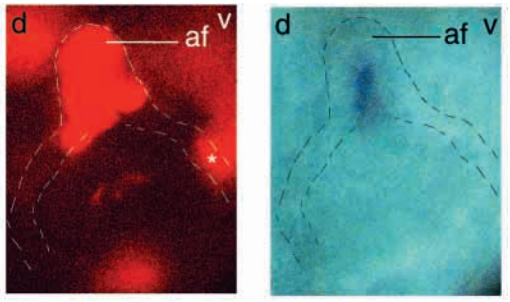

Fig. 8. Transplantation of wt cell into a *dak*⁻ fin bud. Distal is towards the top, dorsal towards the left and ventral towards the right. Fluorescent image (left) shows biotin-labeled wt cell in the apical fold (af) and in the ventral non-ridge ectoderm (*). Bright-field image (right) shows expression of *fgf8* in the apical fold. The outline of the bud and the epidermal basal lamina are indicated by the broken lines.

levels of expression. In the case of *ptc1*, *hoxa10*, *hoxa12*, *hoxa13*, *hoxd11*, *hoxd*12 and *hoxd13* the observed reduction of expression can be understood in terms of their dependence on Syu and thus as an indirect consequence of the reduced ridge signal. *hoxa9* and *hoxd10*, however, show a more direct dependence on *dak*/AER function. At 38 h, expression of *dlx2* and *bmp2b* have been strongly downregulated in *dak* mutant fin buds and induction of *fgf4* and *fgf8* expression, as well as apical fold formation, do not occur properly. The failure of progressive development in the apical epidermis and the successive loss of *syu* expression leads us to conclude that the early weak AER signal has been lost by 38 h in *dak* mutant fin buds. The early presence and later loss of the AER signal also is reflected by the later truncation of the fin along the proximodistal axis. This behavior points to a qualitative change between 28 h and 38 h in the mechanisms controlling the function of the apical epidermis and promoting its developmental progression into the apical fold phase: although the *dak* gene is necessary at both stages for normal development, it is not until later that its product becomes indispensable.

dak is not part of the Syu/Fgf feedback loop in the fin bud

As a feedback loop interconnects Fgf signaling from the AER and Shh signaling from the ZPA in the tetrapod limb bud (Niswander et al., 1994; Laufer et al., 1994), it could be possible that the cause of the observed loss of AER signal in *dak* mutant buds lies in the involvement of the *dak* gene in this feedback loop. However, our analyses of gene expression, the phenotypic comparison of *dak* mutants with the *syu* mutants and the Fgf-bead implantation experiment suggest that this is not the case.

We have shown by in situ hybridization that *syu* signal transduction is functional in *dak* mutants as the mesenchymal targets of *syu* signaling *ptc1*, *hoxa13* and *hoxd13* are induced in *dak* mutant fin buds. The phenotypic difference of the fin endoskeletons in *dak* and *syu* mutants likewise suggests the involvement of *dak* and *syu* in different genetic pathways. By implantation of Fgf4-soaked beads, we further showed that the reception of the Fgf-signal from the apical fold by the *dak*⁻ fin bud mesenchyme was not hampered as *syu*, *hoxa13*, and *hoxd11* are induced. In addition to these results that suggest

Table 2. Transplantation of wt cells into *dak*⁻ fin buds: growth, apical fold formation and expression of *fgf8* and *syu*

Pectoral fin is larger	Presence of the apical fold	Wild-type cells in apical fold	Wild-type cells in dorsal ectoderm	Wild-type cells in ventral ectoderm	Gene expressed
+	+	+	+	+	*fgf8*
+	+	+	+	+	*fgf8*
+	+	+	–	+	*fgf8*
+	+	+	+	+	*syu*
+	+	+	+	+	*syu*
+	+	+	+	+	*syu*

+, present; –, absent.

functional *syu* and *fgf* signal transduction cascades in *dak* mutants, we have made observations, which are not in agreement with a possible function of *dak* in the Syu/Fgf feedback loop. The expression patterns of *hoxd10* and *en1* in *dak* mutant fin buds differ from those observed in *syu* null mutant fin buds in which the Syu/Fgf feedback loop is abolished. Without Syu signal, *hoxd10* expression is equivalent to wild-type levels at all stages investigated. In *dak* mutant fin buds, however, *hoxd10* is downregulated at late stages, which again implies that *dak* and *syu* exert their effects through different pathways. A similar observation is made for *en1* expression, which is unaffected at early and late stages in *syu* null mutant embryos but is downregulated in *dak* mutant buds at 38 h and later. If *dak* were to act solely as a component of the Syu/Fgf feedback loop, downregulation of *hoxd10* and *en1* should not be observed in *dak* mutant fin buds.

bmp2b and *dlx2* become targets of Syu signaling at later stages of fin development (Neumann et al., 1999). Upon implantation of an Fgf4 bead into *dak* mutant fin buds, late expression of both markers is detected at high levels with a posterior bias. This suggests that the *dak* apical ectoderm is competent to respond to the prolonged Syu signal by inducing the early AER markers. Nevertheless, progressive ectodermal development is still hampered. The late apical fold markers, *fgf4* and *fgf8*, which are also targets of Syu signaling (Fig. 2M,N; Neumann et al., 1999) are not activated upon Fgf-4 bead implantation, despite the Syu signal in the experimental buds. Likewise, apical folding which is independent of *syu* signaling does not take place significantly in these buds.

The differences in the activation of early and late developmental events in the apical epidermis upon Fgf4 bead implantation in experimental *dak* mutant fin buds are probably due to a specific function of *dak* that is needed at later stages for the progression of development in the apical fold.

dak mutants reveal the existence of a *syu*-independent pathway for maintaining expression of 3′ located *hox* genes

hoxd10 is downregulated in *dak* mutant fin buds at 38 h and *hoxa9* is already expressed at lower levels at 28 h. In contrast, the *syu* null mutant does not influence the expression of *hoxd10* in the fin bud at 32 h and 38 h, nor of *hoxa9* at 32 h. This specific difference indicates the presence of a *dak*-dependent maintenance signal for these two 3′ located *hox* genes that is independent of Syu. In the chick limb bud, an AER signal is

needed in concert with Shh to initiate ectopic *hoxd* expression in the anterior limb bud mesenchyme, thus indicating an influence of the ridge on *hox* expression (Laufer et al., 1994). As will be discussed below, *dak* acts in the epidermis to maintain AER functions. It therefore seems likely that the downregulation of expression of both 3′ *hox* genes reflects the dependence of their expression on the AER.

Fgf4 is sufficient to reactivate *en1* expression in the *dak* fin bud ectoderm

The early wild-type expression of *en1* and the normal positioning of the early apical ectodermal markers indicate the correct initial specification of cell fates along the D/V axis in *dak* mutants. At 38 h and later, however, *en1* has disappeared from the ventral ectoderm except for a small distal patch. Additionally, the ridge markers are downregulated and *syu*, the mesenchymal ridge-target, is lost. These findings prompted the question whether the loss of *en1* reflects the cause for the loss of ridge activity or whether a ridge signal is needed for sustained *en1* expression. There is no evidence from chicken or mouse for either of the two possibilities. The rescue of *en1* expression by implantation of an Fgf4 bead shows that a Fgf signal is sufficient to maintain levels of En1 in the ventral ectoderm. *dak* thus reveals the later dependence of *en1* on a maintenance signal for ventral ectodermal identity. The fact that Fgf4 can provide the maintenance signal strongly suggests, that the ridge provides the signal to the ventral ectoderm to maintain *en1* expression. The loss of *en1* expression in *dak* mutants is thus due to the loss of ridge function.

This finding uncovers a new interaction among two of the three signaling centers in the bud, namely the dependence of the D/V organizer on the AER. The mouse *en1* mutant clearly shows that En1 is required for restricting the expression of the dorsalizing signal Wnt7a to the dorsal ectoderm (Loomis et al., 1996). We therefore conclude that in the zebrafish, the AER maintains the proper dorsoventral organization of the ectoderm and thus its proper patterning activity along the D/V axis via *dak* and *fgf*.

Our results extend other findings that indicate the signaling centers mutually interact to establish pattern coordinately along the three axes of the limb (Laufer et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). Yet we would also like to point out that the functions of the zebrafish apical epidermis are probably maintained by different genetic pathways. The apical ectoderm of *syu* null mutant fin buds retains the capacity to form an apical fold and *en1* expression is maintained in the ventral ectoderm (Neumann et al., 1999). Together with our data from the present analysis of the *dak* mutant fin bud, these findings suggest that the *syu* signal from the ZPA maintains only some aspects of AER integrity and signaling but not others. If our proposal is correct, that it is an Fgf signal from the AER that maintains *en1* expression in vivo, this might indicate that not all Fgfs are under the control of Syu in the zebrafish fin bud.

A functional copy of the *dak* gene is required in the fin bud ectoderm to promote apical epidermal development and to maintain *syu* expression in the mesenchyme

Upon transplantation of wild-type cells into the epidermis of *dak* mutant fin buds, *fgf8* expression and apical fold formation were observed. A wild-type copy of *dak* is thus required in the fin bud ectoderm to promote developmental progression of the apical epidermis. As wild-type cells were found in the apical fold, but also in the ventral non-fold ectoderm and often also in the dorsal non-fold ectoderm, we cannot unambiguously localize the site where the *dak* gene is required. Importantly, *syu* was transcribed in the mesenchyme of *dak* fin buds, which had received wild-type cells in the ectoderm. As *syu* expression is not observed in untreated *dak* fin buds at this stage, this result confers experimental proof of epidermal-mesenchymal signaling in the fin bud. The analysis of the *syu* null mutant (Neumann et al., 1999) has shown that the Syu signal from the ZPA is necessary to maintain strong expression of *dlx2* and *bmp2b*, and to induce expression of *fgf4* and *fgf8* in the apical ectoderm, thus demonstrating mesenchymal-epidermal signaling in the fin bud. It is thus likely that there exists, as in tetrapods, a positive feedback loop between epidermal signals and Syu.

Our results provide evidence that in the zebrafish fin bud, as in the tetrapod limb bud, different mechanisms regulate the early establishment of AER activity and its later maintenance during development. *dak* functions in the ectoderm to activate *fgf4* and *fgf8* synergistically with Syu. In addition it is required, independently of Syu, to induce apical fold formation, as well as to maintain *en1* expression in the ventral ectoderm. Although we cannot exclude a permissive function of the *dak* gene in these processes, we favor a hypothesis in which *dak* acts to transduce the signal of a Syu independent apical ectodermal maintenance factor. This interpretation is suggested by the fact that the AER relies on such a signal as it cannot maintain its activity autonomously (Zwilling, 1961).

We propose that *dak*$^{-}$ fin buds arrest development because the fin bud ectoderm fails to transduce an apical ectodermal maintenance signal. This signal is independent of *syu* signaling, as different aspects of the *dak* mutant phenotype are not in agreement with a function of *dak* downstream of Syu. Owing to the lack of AER maintenance, the feedback loop between the ZPA and the apical ectoderm is not productively established, which causes both organizers to lose activity over time. The nonautonomous effect of ectodermal wild-type cells to maintain *syu* expression in *dak* mutant mesenchyme is a clear indication that the AER activity has been restored in these buds. An attractive hypothesis for the function of *dak* is that it acts in the apical epidermis in a cell-autonomous manner to activate late apical ectodermal gene expression and to promote apical fold formation upon reception of an AEMF signal.

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