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Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse

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SUMMARY

Numerous studies, often performed on avian embryos, have implicated retinoic acid (RA) in the control of limb bud growth and patterning. Here we have investigated whether the lack of endogenous RA synthesis affects limb morphogenesis in mutant mouse embryos deficient for the retinaldehyde dehydrogenase 2 (Raldh2/Aldh1a2). These mutants, which have no detectable embryonic RA except in the developing retina, die at E9.5-E10 without any evidence of limb bud formation, but maternal RA supplementation through oral gavage from E7.5 can extend their survival. Such survivors exhibit highly reduced forelimb rudiments, but apparently normal hindlimbs. By providing RA within maternal food, we found both a stage- and dose-dependency for rescue of forelimb growth and patterning. Following RA supplementation from E7.5 to 8.5, mutant forelimbs are markedly hypoplastic and lack anteroposterior (AP) patterning, with a single medial cartilage and 1-2 digit rudiments. RA provided until E9.5 significantly rescues forelimb growth, but cannot restore normal AP patterning. Increasing the RA dose rescues the hypodactyly, but leads to lack of asymmetry of the digit pattern, with abnormally long first digit or symmetrical polydactyly. Mutant forelimb buds are characterized by lack of expression or abnormal distal distribution of *Sonic hedgehog* (*Shh*) transcripts, sometimes with highest expression anteriorly. Downregulation or ectopic anterior expression of *Fgf4* is also seen. As a result, genes such as *Bmp2* or *Hoxd* genes are expressed symmetrically along the AP axis of the forelimb buds, and/or later, of the autopod. We suggest that RA signaling cooperates with a posteriorly restricted factor such as dHand, to generate a functional zone of polarizing activity (ZPA).

Key words: Aldh1a2, *dHand*, *Hox* genes, Limb, Mouse development, Raldh2, Retinaldehyde dehydrogenase, Retinoids, Sonic hedgehog

INTRODUCTION

Vertebrate limb growth and patterning is controlled by two integrated epithelial-mesenchymal signaling centers (for review, see Johnson and Tabin, 1997). One such region is the zone of polarizing activity (ZPA), the mesoderm at the posterior limb margin, whose removal severely truncates limb outgrowth and whose anterior ectopic grafting generates anteroposterior (AP) pattern duplications. The secreted molecule sonic hedgehog (Shh) both colocalizes and is sufficient to substitute for ZPA function (Riddle et al., 1993). The second region essential for limb proximodistal (PD) outgrowth is a specialized epithelial structure, the apical ectodermal ridge (AER), which maintains the underlying mesenchmal cells in an active proliferative state. If excised, the AER function can be reconstituted by exogenously supplying various members of the fibroblast growth factor (FGF) family (Niswander et al., 1993). Molecular signaling between the ZPA and the AER are integrated to form a mutual feedback loop allowing for coordinate growth and patterning of the limb (Niswander et al., 1994; Laufer et al., 1994).

Retinoic acid (RA), the active derivative of vitamin A (retinol), is believed to be required for limb development. RA-impregnated beads placed under the anterior limb margin induce digit duplications similar to ZPA grafts (Tickle et al., 1982). Furthermore, local administration of synthetic molecules acting as RA receptor (RAR) antagonists, or inhibitors of RA synthesis, severely inhibit limb outgrowth in chick (Helms et al., 1996; Stratford et al., 1996). Nutritional vitamin A deficiency (VAD) also has marked effects on limb growth and patterning in quail embryos (Stratford et al., 1999). Evaluating the role of retinoids in mammalian limb development is complicated by the fact that severe VAD results in female infertility. Whereas classical studies of VAD rodents did not report limb defects (e.g. Wilson et al., 1953), a recent study in which severely deficient rat embryos were obtained following RA supplementation during early gestation reported relatively mild limb hypoplasia (Power et al., 1999). In the mouse, due to partial functional redundancy, limb defects only occur if two of the RARs are disrupted in combination. RARα/RARγ mutants have skeletal limb defects with no major reductions in limb size (Lohnes et al., 1994), whereas

RARβ/RARγ mutants show impaired interdigital cell death (Dupé et al., 1999). Finally, administration of teratological doses of RA to pregnant dams during embryonic limb bud differentiation generates skeletal defects (Kwasigroch and Kochhar, 1980), whereas early administration prior to gastrulation can induce the development of supernumerary hindlimbs (Rutledge et al., 1994; Niederreither et al., 1996). Thus, while the overall importance of RA to mammalian limb outgrowth is still unclear, one common outcome of both avian and rodent studies is that RA deficiency prevents the proper establishment of the *Fgf4-Shh* AER-ZPA signaling loop (Power et al., 1999; Stratford et al., 1999).

We have used *Raldh2* (*Aldh1a2* – Mouse Genome Informatics) knockout mouse mutants to investigate the contribution of RA synthesized by the embryo from maternal retinol to limb morphogenesis. The *Raldh2* gene codes for a retinaldehyde dehydrogenase that catalyzes the second oxidative step in the biosynthesis of RA from retinol (Zhao et al., 1996). Raldh2 is responsible for most of the RA-synthesizing activity during early mouse embryogenesis (E7.5-9.5), as seen from the failure of *Raldh2*$^{-/-}$ embryos to activate RA-responsive transgenes, except in the developing retina (Niederreither et al., 1999). These mutant embryos, which die at E9.5-10.5 from severe cardiac defects, exhibit axial truncation due to impaired somite growth, as well as hindbrain defects (Niederreither et al., 1999; Niederreither et al., 2000; Niederreither et al., 2001). Furthermore, they display no sign of limb bud outgrowth; however, no conclusion concerning the importance of local RA synthesis in this process can be drawn from this observation, as development of these embryos may be arrested shortly before or at the onset of limb induction. Using several modes of RA supplementation, we now demonstrate a critical role of endogenous RA synthesis in forelimb morphogenesis, and describe the morphological and molecular consequences of this endogenous RA deficiency.

MATERIALS AND METHODS

The generation of *Raldh2* null mutant mice has been described (Niederreither et al., 1999). Two procedures were used for maternal RA supplementation. All-*trans*-RA (Sigma) was either suspended in ethanol, diluted in sunflower oil (0.5 mg/ml) and administered orally (2.5 μg/g body weight) to pregnant females twice a day from E6.5 to E10.5 dpc, or RA in suspension was directly mixed into powdered food (0. 1 mg/g food at E7.5 and 0.25 mg/g food at later stages). The RA-containing food mixture (protected from light by aluminium foil) was left in the cage for the mice to feed ad libitum and renewed each day. Scanning electron microscopy and cartilaginous skeletal stainings were performed as described by Niederreither et al. (Niederreither et al., 1999) and Jegalian and De Robertis (Jegalian and De Robertis, 1992), respectively. To analyze the patterns of RA response, *Raldh2*$^{+/-}$ mutants were crossed with the *RARE-hsp68-lacZ* reporter transgenic line (Rossant et al., 1991), which harbors a tetrameric repeat of the *RARβ2* RARE linked to the hsp68 minimal promoter and is widely used as a RA-reporter transgene. Whole-mount in situ hybridizations were performed as described previously (Décimo et al., 1995), using template plasmids cloned in our Institute or kindly provided by Drs D. Duboule (University of Geneva: *Hoxd* genes), C. C. Hui (Toronto, Canada: *Gli3*), G. Martin (UCSF: *Fgf4*, *Fgf8*), A. McMahon (Harvard University: *Shh*, *Wnt7a*, *En1*), E. Olson (University Texas, Dallas: *dHand/Hand2* - Mouse Genome Informatics), M. Petkovich (Queen's University, Kingston Canada: *Cyp26B1*) and R. Zeller (Utrecht University: *Gremlin/cktsf1b1* – Mouse Genome Informatics).

RESULTS

Limb growth and patterning in RA-rescued *Raldh2*$^{-/-}$ embryos

Initial experiments aimed at rescuing *Raldh2*$^{-/-}$ mutant embryos involved subteratogenic administration of RA by oral gavage at 12 hour intervals from E6. 75 to E10.5 (see Niederreither et al., 1999). Under these conditions, only a fraction of the mutant embryos (~25%) survived until E12.5-13.5. Scanning electron micrographs showed a striking reduction in the outgrowth of the forelimbs, which could be reduced to small rod-like structures (Fig. 1A-C). Left forelimbs were consistently more affected than the right ones (Fig. 1B; see below). In contrast, the mutant hindlimbs had a normal appearance (data not shown). Thus, all surviving mutant embryos had forelimb defects, yet these were frequently accompanied by a range of malformations including exencephaly and craniofacial defects (Fig. 1B, and data not shown).

To investigate whether a more steady RA supplementation may improve mutant forelimb development, a food administration protocol was developed. This mode of administration has been shown to result in a better rescue of cardiac development, thus improving survival of the *Raldh2*$^{-/-}$ mutants at fetal stages (Niederreither et al., 2001). Preliminary trials were carried out to determine the maximal dose of RA which could be administered without teratogenic effects in wild-type and heterozygote littermates, along with the minimal RA treatment period allowing *Raldh2*$^{-/-}$ fetus survival.

When RA was provided ad libitum at a concentration of 100 μg/g maternal food from E7.5 to E8.5, about 5% of the *Raldh2*$^{-/-}$ mutants survived until E18.5. These mutants exhibited alterations in craniofacial morphology, lack of cephalic flexure and cardiovascular defects (Fig. 1D, and data not shown). Fetal forelimb outgrowth was strikingly reduced, whereas the hindlimbs appeared normal (Fig. 1D). Closer examination of left mutant 'minilimbs' revealed what appeared to be a single digit with a nail-like structure (Fig. 1D, inset). Two digits could be formed on the right side under these conditions (data not shown).

We next determined the extent to which we could rescue mutant forelimb development under maximal subteratogenic RA supplementation. RA was administered at 100 μg/g food from E7.5 to E8.5, and then at 250 μg/g food from E8.5 to E14.5. A marked increase in mutant forelimb size was observed, and in some cases the right forelimb appeared close to its wild-type size (Fig. 1E and data not shown). However, the number and pattern of digits formed was variable. In some cases 4 or 5 digits were seen. Some mutants exhibited mirror-image digit duplications. Fig. 1E shows an example of complete digit duplication on the left side, whereas the right side limb has a 'lobster claw' defect (arrowhead). Other mutants exhibited less severe alterations, such as polydactyly with 6 symmetrical digits (Fig. 1F), or five digits with an abnormally long first digit (Fig. 1G, arrowhead). No abnormalities of the forelimbs were observed in wild-type or heterozygote littermates. Increasing RA concentration to 400

Table 1. Skeletal patterns of the *Raldh2*$^{-/-}$ forelimbs (E14.5) according to the RA-rescue protocol

	RA (stage/dose*)											
Experimental group:	E7.5-8.5/100 (*n*=3)† 1		E7.5-9.5/100 (*n*=5)† 2		E7.5-9.5/250 (*n*=12) 3		E7.5-10.5/100 (*n*=6)† 4		E7.5-10.5/250 (*n*=15) 5		E7.5-10.5/250+ gavage E8‡ (*n*=12) 6	
	L	R	L	R	L	R	L	R	L	R	L	R
Scapula												
Normal§	0	0	1	0	**6**	**8**	**4**	**6**	**15**	**15**	**12**	**12**
Hypoplastic	**3**	**3**	**4**	**5**	**6**	4	2	0	0	0	0	0
Humerus												
Normal§	0	1	1	1	**9**	**12**	1	2	**10**	**14**	**8**	**12**
Hypoplastic/absent	**3**	**2**	**4**	**4**	3	0	**5**	**4**	5	1	4	0
Radius/ulna												
Normal§	0	0	1	1	2	**8**	1	1	**8**	**10**	5	**7**
Single ulna	0	1	1	**2**	**8**	4	2	2	4	5	**7**	5
Indistinct cartilage	**3**	**2**	**3**	**2**	2	0	**3**	**3**	3	0	0	0
Digits												
6	0	0	0	0	0	1	0	1	3	2	**4****	2
5 (abnormal)¶	0	0	0	0	4	**5**	1	0	**4**	4	2	1
5	0	0	1	0	0	3	0	0	0	3	2	**5**
4	0	0	1	1	**6**	3	1	1	**4**	**6**	3	4
3	0	0	0	**2**	1	0	0	**2**	**4**	0	1	0
2	0	1	**2**	**2**	1	0	**3**	1	0	0	0	0
1	**3**	**2**	1	0	0	0	1	1	0	0	0	0

The most frequently observed patterns are indicated in bold.
*μg RA per gram of dry food. The RA dose was kept at 100 μg/g from E7.5-8.5 to avoid teratogenic effects, and eventually raised to 250 μg/g from E8.5.
†Many *Raldh2*$^{-/-}$ mutants died before E14.5 under these conditions.
‡See text for a full description of this experiment.
§Not taking into account an overall size reduction, or slight alterations in shape.
¶With abnormally long first digit, in contrast to the normally shorter wild-type thumb.
**Including one case with 7 digits.

μg/g food from E8.5-E14.5 did not improve forelimb growth or patterning, but did result in teratogenic effects in both wild-type and mutant embryos, including exencephaly and lumbosacral truncations, and embryonic lethality in some mothers (data not shown). No further experiment was therefore conducted at concentrations higher than 250 μg/g. RA supplementation was also started at E6.5 (100 μg/g) to test whether abnormal forelimb development in mutants could be due to lack of RA prior to E7.5. These early treatments did not improve the mutant forelimb phenotype (data not shown).

In order to determine the critical period for RA-dependent forelimb outgrowth, a range of RA treatment levels and times where tested (Table 1). In all cases RA was administered from E7.5 to E8.5 at 100 μg/g food, as these levels are necessary to rescue heart development in the mutants (lower levels at this stage severely reduced mutant survival, whereas higher levels resulted in teratogenic effects). Embryos were collected at E14.5 and skeletal patterns were analyzed after whole-mount Alcian Blue cartilage staining. The RA rescue of mutant forelimb development appeared to be both stage- and dose-dependent (see Table 1 and Fig. 2A for examples of forelimb patterns obtained under various treatment conditions). Short-term treatments (E7.5-E8.5: experimental group 1, Table 1) resulted in highly truncated mutant forelimbs. These contained a minute scapular blastema, no recognizable humerus, a single, indistinct stylopodal/zeugopodal element, and 1 or 2 rudimentary digits (Fig. 2A). Extending the duration of RA treatment to E9.5 (100 μg/g food: group 2, Table 1) had little effect on overall forelimb growth, while development along the AP axis was improved, as seen by the occasional presence of a humeral and/or of two separate radial and ulnar cartilages (Table 1). Most mutants, though, exhibited no (or a highly hypoplastic) humerus and a single ‘radial/ulnar’ zeugopodal cartilage (Fig. 2A,C,D), and their dactyly was slightly improved (2-3 digits; Table 1, Fig. 2A,D). Interestingly, increasing RA to the maximal non-teratogenic tolerated dose (250 μg/g food from E8.5 to E9.5: group 3, Table 1) provided a better rescue of skeletal patterning along both the PD and AP axes. Most forelimbs had well formed scapula and humerus; however, the resulting limbs remained truncated and many cases of single, ulna-like zeugopodal cartilages were seen, whereas dactyly was usually rescued to 4-6 digits (Table 1, Fig. 2A,D).

As a general trend, the growth and skeletal defects were often more severe in left-side than in right-side forelimbs. For example, several mutants from group 3 had a single ulnar cartilage on the left side and two zeugopodal cartilages on the right side, and were hypodactylous only on the left side (see Table 1 for the left-right distribution of the abnormalities).

RA treatments were also extended to E10.5 at low (100 μg/g food) and high (250 μg/g food) doses (groups 4 and 5, Table 1). The forelimb phenotypes of mutants treated at the lower dose did not markedly differ from those of group 2, which received the same dosage until E9.5 (see Table 1, and data not shown). Further improvement of the overall forelimb growth was obtained under high RA concentration (Fig. 2A, group 5). Most mutant limbs in this group had a well formed scapula and humerus, as well as distinct radius and ulna cartilages (Table

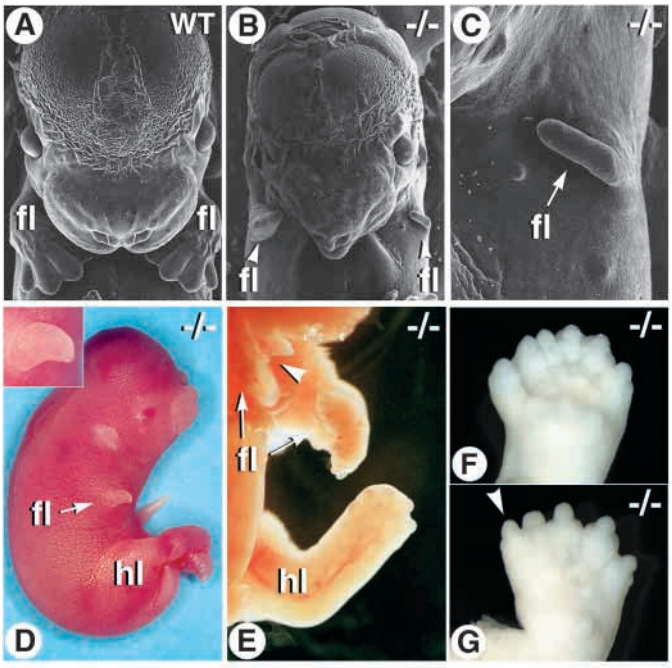

Fig. 1. Morphology of the *Raldh2*$^{-/-}$ forelimbs. (A-C) Scanning electron micrographs of wild-type (A) and *Raldh2*$^{-/-}$ (B,C) embryos collected at E13.5 after RA supplementation by oral gavage from E6.75 to E9.25. (C) A close-up of the left mutant forelimb. (D) *Raldh2*$^{-/-}$ fetus collected at E18.5 after RA supplementation via the maternal food supply (100 μg/g food) from E7.5 to 8.5. An enlargement of the forelimb rudiment is shown (inset). (E) *Raldh2*$^{-/-}$ fetus collected at E18.5 after RA supplementation via maternal food (100 μg/g food from E7.5 to 8.5 and 250 μg/g food from E8.5 to E14.5). The left forelimb shows mirror-image digit duplications and the right forelimb has an abnormal digital cleft (arrowhead). (F,G) Abnormal digit patterning in two *Raldh2*$^{-/-}$ fetuses collected at E18.5 after the same conditions of supplementation. The arrowhead in G points to the enlarged first digit. hl, hindlimb; fl, forelimb.

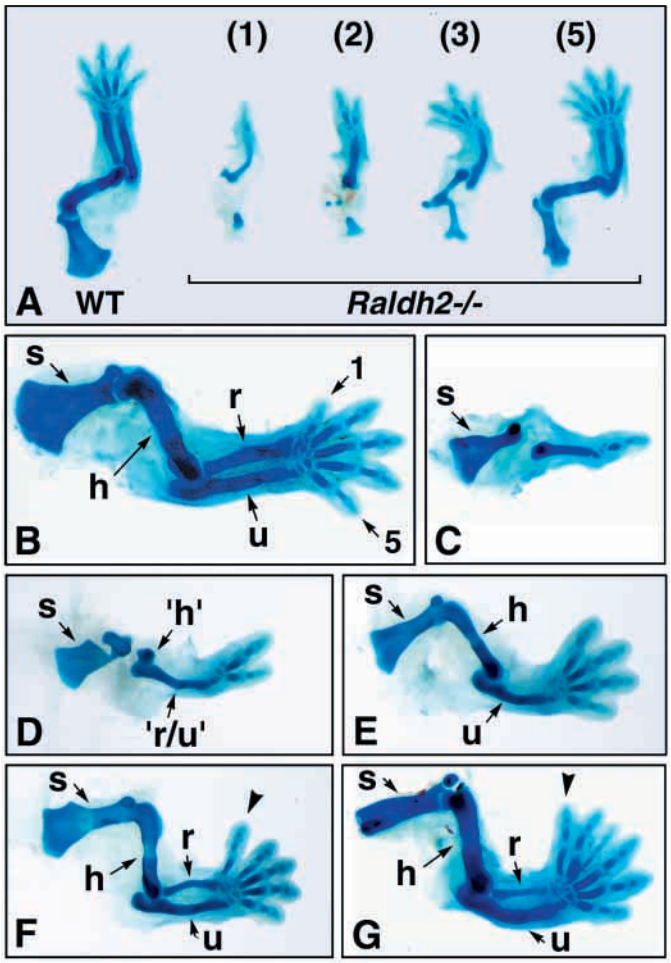

Fig. 2. Skeletal defects of the *Raldh2*$^{-/-}$ forelimbs. (A) comparative view of the right forelimbs of control (left) and *Raldh2*$^{-/-}$ embryos after four different conditions of RA supplementation (experimental group numbers as in Table 1). Note the progressive improvement of limb size and skeletal patterning according to the experimental group. (B) Skeletal pattern of a wild-type forelimb. (C-G) Examples of the *Raldh2*$^{-/-}$ abnormal forelimb patterns. The embryos shown were from experimental groups 1 (C), 2 (D), 4 (E) and 5 (F,G). See Text for a full description of the skeletal patterns. In F and G, an arrowhead points the abnormally long and triphalangeal first digit (compare with B). h, humerus; r, radius; s, scapula; u, ulna; 1 and 5: digit number.

1, Fig. 2A,F,G). The digit number ranged from 3 (on the left side) to 6 digits (Table 1). Mutant forelimbs with 5 digits showed a characteristic pattern alteration: the first digit was as long as the other digits (Fig. 2F), while it is normally shorter (two phalanges) in wild type (Fig. 2B). Such a symmetrical arrangement of all digits was also seen in polydactylous mutant limbs (Fig. 2G).

In both chick and mouse embryos, *Raldh2* expression is strongly induced in both lateral plate and somitic trunk mesoderm shortly before limb buds are induced (Niederreither et al., 1997; Swindell et al., 1999). This may possibly generate a surge of RA critical for forelimb bud outgrowth, consistent with HPLC data indicating elevated RA levels in the chick wing bud (Thaller and Eichele, 1987). To reproduce such a RA surge, an additional series of experiments was performed (group 6, Table 1) in which a RA dose was administered by maternal oral gavage (30 μg/g body weight at E8 – 8:00 am) in addition to food administration under the same conditions as group 5. RA administered by maternal gavage has been shown to act in the embryo within 2-4 hours (e.g. Mendelsohn et al., 1994). Thus, the RA surge is expected to occur at ~E8.5 (noon), when embryos are at the 5-8 somite stage. Although this condition did not fully restore normal humerus and radius/ulna patterning, it resulted in a lower incidence of hypodactylous mutant forelimbs (Table 1). Again, some of the mutants were polydactylous or had abnormal first digits.

Molecular alterations in the RA-rescued *Raldh2*$^{-/-}$ forelimbs

Numerous molecular signals act in coordination to control limb outgrowth and patterning (for a review, see Johnson and Tabin, 1997). Molecular defects underlying altered *Raldh2*$^{-/-}$ forelimb development were investigated both under severe growth deficiency conditions (Table 1, groups 1,2) or under a condition (Table 1, group 3) that better rescues forelimb growth, but results in patterning defects. In all cases, RA administration was stopped at least 24 hours before molecular analysis (e.g. at E9.5 for an E10.5 analysis).

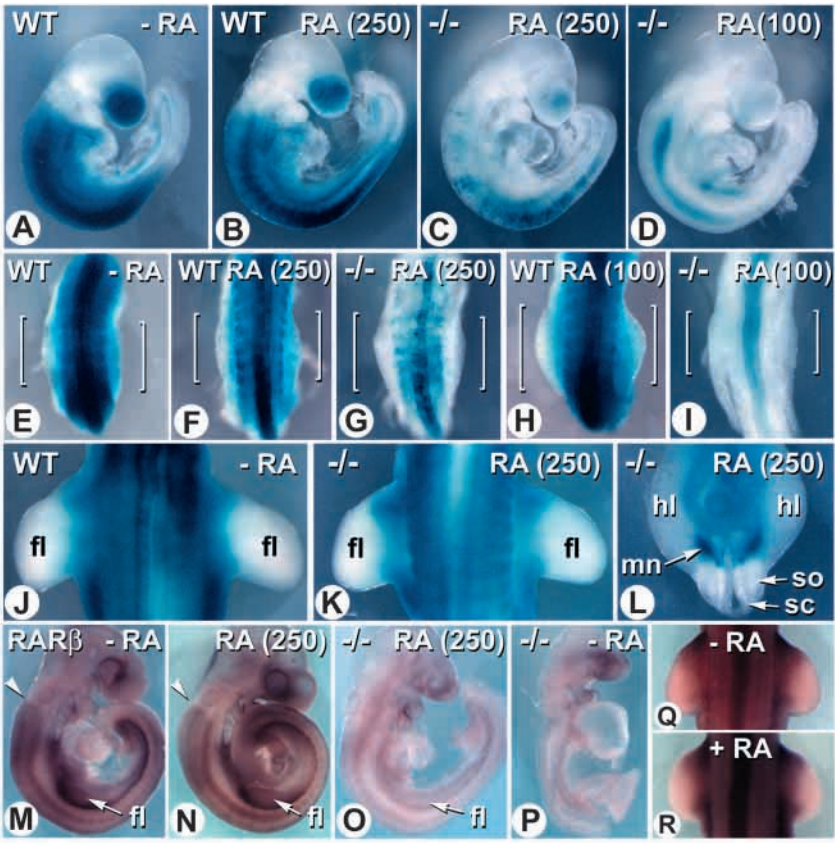

Fig. 3. (A-L) Analysis of the patterns of embryonic RA response during maternal RA supplementation. Embryos carrying the *RARE-hsp68-lacZ* RA reporter transgene, collected from RA-supplemented or untreated mothers, were concomitantly X-gal stained. (A-D) Profile views of E9.5 embryos. (E-I) Dorsal views of the forelimb bud region of E9.5 embryos. Brackets indicate the outgrowing forelimb buds. (J,K) Dorsal views of the forelimbs of E10.5 embryos. (L) Transverse section of an E10.5 embryo at the level of the hindlimb buds. WT, wild-type embryos; –/–, *Raldh2*$^{-/-}$ embryos; –RA, untreated embryos. The embryos in B-D and F-I were RA-treated from E7.5 to E8.5 with 100 μg/g food and from E8.5-E9.5 with 100 μg/g food or 250 μg/g food, as indicated in the panels (100 and 250, respectively). The embryo in K was RA-treated until E10.5, and the one in L until E9.5 (250 μg/g food). (M-P) Detection of *RARβ* transcripts in E9.5 wild-type (M,N) and *Raldh2*$^{-/-}$ (O,P) embryos that were untreated (M,P) or RA-treated from E7.5 to E9.5 (250 μg/g food) (N,O). Arrowheads point to the similar rostral expression boundaries in the hindbrain of untreated and RA-treated wild-type embryos. Profile views. (Q,R) *RARβ* transcript distribution in the forelimbs of E10.5 wild-type (Q) and *Raldh2*$^{-/-}$ (R) embryos that received RA from E7.5 to E10.5 (250 μg/g food). Dorsal views. fl, forelimb buds; hl, hindlimb buds; mn, mesonephros; sc, spinal cord; so, somite.

We first analyzed whether the administered RA might interfere with the expression pattern of an RA-responsive (*RARE-hsp68-lacZ*) (Rossant et al., 1991) reporter transgene (Fig. 3A-L) or with endogenous expression of an RA-responsive gene (*RARβ*; Fig. 3M-R). The expression pattern of the *RARE-hsp68-lacZ* was not detectably altered in wild-type embryos analyzed at E9.5 after dietary RA supplementation from E7.5 to E9.5 (250 μg/g food; Fig. 3B; 100 μg/g food: data not shown), when compared to untreated embryos (Fig. 3A). During initiation of limb budding, the reporter transgene was expressed at lower levels in lateral plate mesoderm than in somitic mesoderm or neural tube, in both untreated (Fig. 3E) and RA-treated (Fig. 3F) wild-type embryos. Transgene activity became restricted to the proximal limb mesenchyme as soon as outgrowth started (Fig. 3H). At E10.5, proximally restricted transgene expression was similarly seen in untreated (Fig. 3J) and RA-treated (E7.5 to E10.5; Fig. 3K) wild-type embryos. However, the RA-responsive transgene was clearly downregulated in the RA-rescued *Raldh2*$^{-/-}$ embryos. E9.5 mutants treated with 250 μg/g food exhibited weak and/or patchy expression in both the spinal cord and trunk mesoderm (Fig. 3C,G), whereas mutants treated at a lower dose (100 μg/g food) showed almost complete downregulation of the transgene (Fig. 3D,I); note the persistent transgene activity in the ventral region of the spinal cord and hindbrain neuroepithelium, which may indicate the presence of another RA-generating activity (K. N., J. V., P. C. and P. D., unpublished data). *Raldh2*$^{-/-}$ embryos treated with RA until E10.5 (250 μg/g) exhibited weak transgene activity in the trunk and proximal forelimb mesoderm (data not shown). However, the reporter transgene was selectively activated in the mesonephric area, near the base of the hindlimb buds (Fig. 3L), suggesting the presence of another local RA-producing activity (see Discussion).

Consistent with the RA-reporter transgene data, no detectable ectopic expression of the endogenous RARβ gene was found in RA-supplemented wild-type embryos (Fig. 3N). No displacement of the rostral expression boundary was seen, for instance, in the postotic hindbrain, and the expression level was higher along the proximal forelimb bud and flank mesoderm of both untreated and RA-treated E9.5 embryos (Fig. 3M and N, respectively). In contrast, endogenous RARβ expression was markedly downregulated throughout the trunk region of the RA-rescued *Raldh2*$^{-/-}$ embryos (Fig. 3O). Expression levels were thus comparable to those seen in non-rescued E9.5 *Raldh2*$^{-/-}$ embryos (Fig. 3P). RARβ transcript distribution was also analyzed in the forelimb buds of untreated (Fig. 3Q) and RA-treated (Fig. 3R) E10.5 wild-type embryos. Similar proximally-restricted expression was observed in both cases.

Fibroblast growth factors (FGFs) are key effectors of limb growth and AER function. *Fgf8* is specifically expressed in the surface ectoderm, and subsequently in the AER of the developing limb buds, and its conditional mutation in mouse severely impairs limb development (Lewandowski et al., 2000; Moon and Capecchi, 2000). *Raldh2* mutant embryos under short-term (E7.5-E8.5) RA supplementation showed a reduced forelimb domain of *Fgf8* expression, which did not define a proper AER domain as in control littermates (Fig. 4A-C). Upon longer (E7.5-E9.5) RA rescue, *Raldh2*$^{-/-}$ embryos exhibited a range of AER alterations: FGF8 was often expressed at higher levels in the anterior portion of the mutant AER (whereas its

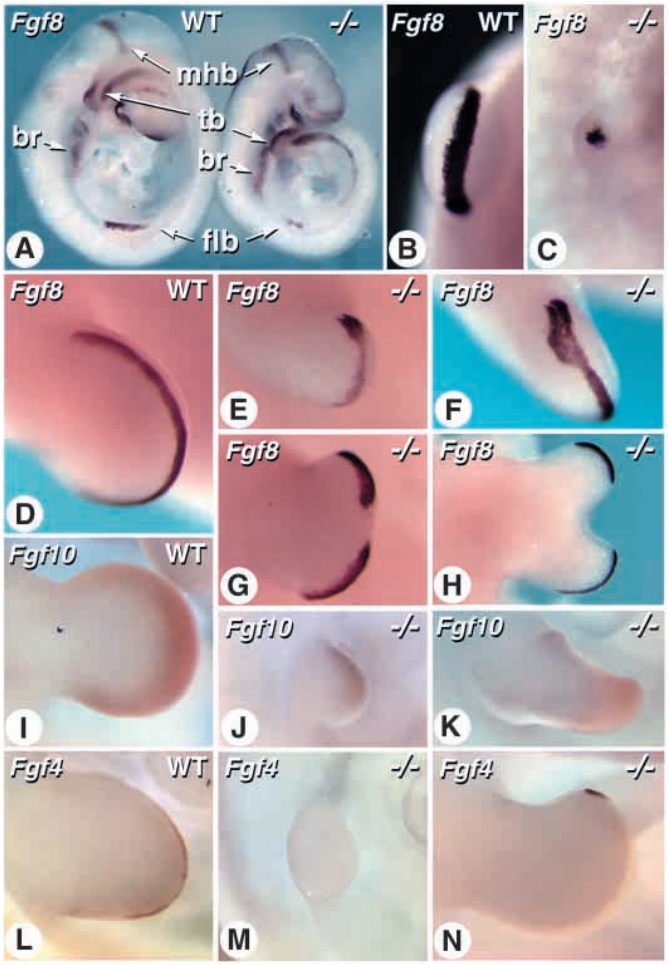

Fig. 4. Abnormal patterns of *Fgf* expression in the *Raldh2*$^{-/-}$ forelimbs. (A) Comparative view of E9.5 wild-type (left) and *Raldh2*$^{-/-}$ embryos treated with RA from E7.5 to E8.5. Note comparable expression levels in various expression domains, except in forelimb ectoderm. br, branchial arches; flb, forelimb buds; mhb, mid-hindbrain region; tb, tail bud. (B,C) Details of *Fgf8* expression in the forelimb ectoderm of E9.5 wild-type (B) and *Raldh2*$^{-/-}$ (C) embryos after RA supplementation from E7.5-8.5. (D) *Fgf8* expression pattern in an E10.5 wild-type forelimb AER. (E-H) Altered patterns of *Fgf8* expression and AER morphology in E10.5 *Raldh2*$^{-/-}$ forelimbs, after RA treatment from E7.5 to E9.5 (250 μg/g). (I-K) *Fgf10* expression in the distal mesoderm of E10.5 wild-type (I) and *Raldh2*$^{-/-}$ (J,K) forelimbs. Whereas *Fgf10* is expressed throughout the distal mesoderm in (I), a preferential anterior distribution is seen in (J) and an abnormal anterior tissue outgrowth with specific *Fgf10* expression is found in (K). (L-N) *Fgf4* expression in wild-type (L) and *Raldh2*$^{-/-}$ (M,N) E10.5 forelimb buds. *Fgf4* is expressed in the posterior two-thirds of the AER in wild type. No expression is detectable in the bud in M, whereas the one in N shows an ectopic patch of expression in the anterior part of the AER.

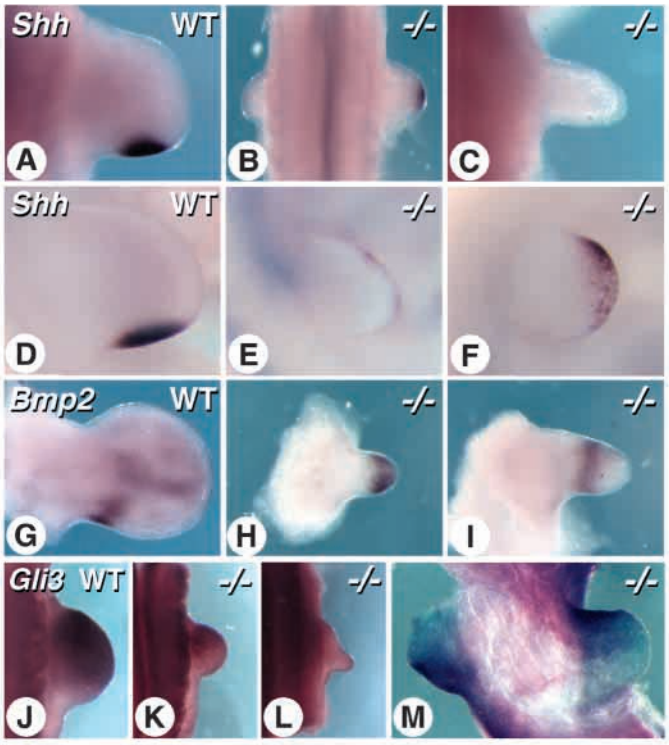

Fig. 5. Altered expression patterns of *Shh*, *Bmp2* and *Gli3* in *Raldh2*$^{-/-}$ forelimbs. (A-C) *Shh* transcripts in E9.5 wild-type (A) and *Raldh2*$^{-/-}$ (B,C) forelimb buds after short-term (E7.5 to E8.5) RA supplementation. (D-F) *Shh* transcripts in E10 wild-type (D) and *Raldh2*$^{-/-}$ (E,F) forelimb buds after long-term (E7.5 to E9.5) RA supplementation. (G-I) *Bmp2* transcripts in E10.5 wild-type (G) and *Raldh2*$^{-/-}$ (H,I) forelimb buds after short-term (H) and long-term (I) RA supplementation, respectively. (J-M) *Gli3* transcripts in E9.5 wild-type (J) and *Raldh2*$^{-/-}$ (K-M) forelimb buds analyzed at E9.5 after short-term (J-L) and long-term (M) RA supplementation, respectively.

expression is more prominent posteriorly in the E10.5 wild-type AER; Fig. 4D), which sometimes appeared wider or abnormally bifurcated (Fig. 4E,F). In some mutants, the AER was disrupted centrally (Fig. 4G), leading in extreme cases to the development of two separate distal outgrowths (Fig. 4H).

Mesodermal expression of *Fgf10* (Fig. 4I), which is also crucial for limb outgrowth (Sekine et al., 1999), was present in the distal mesoderm of the RA-supplemented *Raldh2*$^{-/-}$ forelimbs even under severe deficiency conditions. However, its expression was biased anteriorly in some mutants (Fig. 4J) or was restricted to the mesenchyme underlying the two abnormal distal outgrowths (Fig. 4K). *Fgf4*, which is normally expressed in the posterior two thirds of the AER (Fig. 4L), is thought to control limb growth through a positive signaling feedback loop with Sonic hedgehog (Niswander et al., 1994; Laufer et al., 1994). Conditional mutagenesis of *Fgf4* in the AER, though, does not alter limb development (Moon et al., 2000; Sun et al., 2000). *Fgf4* expression was downregulated or absent in severely deficient *Raldh2*$^{-/-}$ forelimb buds (Fig. 4M). Interestingly, ectopic expression towards the anterior margin was observed in some of the less severely affected mutants (Fig. 4N). These ectopic patches of *Fgf4* expression, which correlated with regions of high *Fgf8* expression (compare Fig. 4E and N), were associated with abnormal outgrowth of the anterior portion of the footplate (Fig. 4N).

Sonic hedgehog (Shh) is a key molecular determinant of AP limb patterning and its expression has been shown to be retinoid-dependent in other VAD systems (Stratford et al., 1996; Stratford et al., 1999; Power et al., 1999). Wild-type *Shh*

expression is specific to the posterior mesenchyme of the developing limbs, i.e. the ZPA region (Fig. 5A). *Shh* expression was either undetectable, or markedly reduced, in *Raldh2*$^{-/-}$ forelimb buds expected to develop as minilimbs (Fig. 5B,C). When present, its expression was seen along the distal margin instead of being posteriorly restricted (Fig. 5B). Even under better rescue conditions, *Shh* was expressed in distal mesenchyme at lower levels than in wild type (Fig. 5D-F), and reversal of its transcript distribution, with highest expression towards the anterior limb margin, was observed in some mutants (Fig. 5E,F). We also analyzed *Bmp2* expression, whose posterior expression in wild-type limbs (Fig. 5G) is dependent on Shh (Drossopoulou et al., 2000; Chiang et al., 2001). *Bmp2* was expressed at appropriate levels in *Raldh2*$^{-/-}$ minilimbs, but showed no posterior asymmetry (Fig. 5H). Mutant limbs that achieved better outgrowth showed a ring-like *Bmp2* transcript pattern along the AP axis (Fig. 5I).

Several lines of evidence indicate that *Shh* expression is prevented in the anterior limb mesenchyme by repressing activity of the zinc finger factor Gli3 (Büscher et al., 1997; Masuya et al., 1997). *Gli3* was expressed at abnormally low levels in the *Raldh2*$^{-/-}$ minilimbs (Fig. 5K,L). Although its anterior distribution was usually maintained (Fig. 5K), an example of reversed AP *Gli3* pattern was observed in one of the *Raldh2*$^{-/-}$ forelimbs (Fig. 5M). Thus, the abnormal anterior expansion of *Shh* may correlate with downregulation (or reversal) of the *Gli3* expression pattern.

The basic helix-loop-helix factor dHand has been implicated in the establishment of the limb ZPA and the induction of posterior *Shh* expression (Fernandez-Teran et al., 2000; Charité et al., 2000). *dHand* is first expressed along the whole lateral plate mesoderm and then, during limb outgrowth (E9.5), is selectively downregulated in the anterior region of the buds (see Fig. 6D). In wild-type E10.5 forelimb buds, *dHand* is expressed along the posterior mesoderm in a domain that outflanks that of *Shh* (Fig. 6A). Instead of being posteriorly restricted, we found that *dHand* was expressed along the whole distal margin of the severely deficient *Raldh2*$^{-/-}$ forelimbs (Fig. 6B). In mutant limbs that underwent better growth, *dHand* was expressed throughout the mesoderm (Fig. 6C). To see whether these abnormal patterns result from misregulation during early limb budding, mutant embryos were analyzed at E9-E9.5. Interestingly, *dHand* downregulation occurred normally during the initial phase of limb budding in mutants (Fig. 6E, bracket). Thus, a graded posterior to anterior expression pattern could be established in the *Raldh2*$^{-/-}$ buds (Fig. 6F). However, owing to their size deficiency, mutant limb buds expressed *dHand* in a domain encompassing a larger portion of the buds, compared to wild type (compare Fig. 6D and F). Thus, in most severely deficient buds, only the proximal, anterior margin was devoid of *dHand* transcripts (Fig. 6G, arrow), which accounts for *dHand* expression throughout the distal mesoderm during further outgrowth (Fig. 6C).

The *Meis2* homeobox gene (*Mrg1* – Mouse Genome Informatics) is a determinant of proximal limb structures (Capdevila et al., 1999; Mercader et al., 2000). Experiments performed on chick embryos have strongly suggested that RA produced by Raldh2 is responsible for the induction of *Meis2* expression in the proximal limb mesoderm (see Fig. 7A), while distal inhibition of RA synthesis by the FGF pathway prevents its distal expression (Mercader et al., 2000). Although *Meis2*

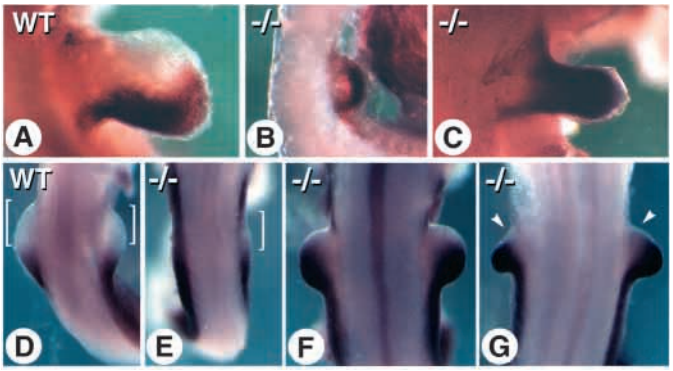

Fig. 6. Expression of *dHand* in *Raldh2*$^{-/-}$ forelimbs. (A-C) Wild-type (A) and *Raldh2*$^{-/-}$ (B,C) forelimb buds analyzed at E10.5 after short-term and long-term RA supplementation, respectively. Note the lack of posterior asymmetry of *dHand* expression in mutant forelimbs. (D-G) Early expression pattern of *dHand* in wild-type (D) and *Raldh2*$^{-/-}$ (E-G) forelimb buds analyzed at E9-9.5 after short-term (D,E,G) and long-term (F) RA supplementation. Although *dHand* transcripts are posteriorly restricted during early forelimb outgrowth in mutants (brackets), only the anteroproximal forelimb margin remains free of *dHand* transcripts during further limb growth (arrowheads).

may be expressed at lower levels in the trunk mesoderm of the *Raldh2*$^{-/-}$ embryos that were rescued at a low RA concentration (100 μg/g food; Table 1, group 2; compare Fig. 7A and B), its transcripts clearly extended into the proximal mesoderm of the growth-deficient forelimb buds (Fig. 7B). Under higher RA rescue conditions (250 μg/g food; Table 1, group 3), the distal boundary of *Meis2*-expressing cells appeared similar in wild-type and mutant forelimbs (as far as their different morphology allows such a comparison): in both wild-type and mutants, *Meis2* transcripts extended to the groove that separates the prospective stylopod and zeugopod regions of the developing forelimbs (Fig. 7C,D, arrowheads). Thus, exogenously administered RA did not result in a distal deregulation of *Meis2* expression, suggesting that FGF signaling is efficient in *Raldh2*$^{-/-}$ limbs (see Fig. 4). To demonstrate that FGF signaling could operate in mutant limbs, we analyzed *Sprouty4* gene expression. *Sprouty* genes encode membrane-bound antagonists of FGF signaling, which are themselves induced by FGFs in several regions of the embryo, including the distal limb mesoderm (Minowada et al., 2000) (see Fig. 7E). No *Sprouty4* induction was seen in highly truncated mutant forelimb buds (Fig. 7F). However, *Sprouty4* could clearly be induced in the distal mesoderm of mutant limbs under better rescue conditions (Fig. 7G), indicating that FGF signaling is effective in these limbs.

At least two types of proteins could intervene in the tissue distribution of RA: the cellular retinoic acid binding proteins (CRABPs) and the RA-metabolizing enzymes Cyp26A1 and Cyp26B1 (Donovan et al., 1995; MacLean et al., 2001). Owing to its graded expression pattern, CRABP I has been suggested to mediate RA gradients within the limb buds (Maden et al., 1989) (see Fig. 7H). However, genetic data indicate that its absence (as well as that of CRABP II) results in essentially normal limbs (Gorry et al., 1994; Lampron et al., 1995). *CRABP I* transcripts were expressed according to a distal to

proximal (rather than anterior to posterior) gradient in the *Raldh2*$^{-/-}$ minilimbs (Fig. 7I). This may reflect the growth retardation and/or developmental delay of these limbs (*CRABP I* is expressed distally at earlier stages in wild-type limb buds) (Dollé et al., 1989) rather than AP pattern alterations. Indeed, *CRABP I* was expressed almost normally (i.e. maximally in the anterior mesoderm) in less truncated mutant forelimbs (Fig. 7J). Likewise, the RA-metabolizing enzyme *Cyp26B1* was expressed at comparable levels in the distal region of the *Raldh2*$^{-/-}$ and control forelimb buds (data not shown).

Although the forelimbs of the RA-rescued *Raldh2*$^{-/-}$ mice do not show obvious dorsoventral (DV) patterning defects (e.g. Fig. 1E-G), we analyzed the expression of *Wnt7a* and *Engrailed-1* (*En1*), two determinants of limb DV polarity that are specifically expressed in the dorsal and ventral limb bud ectoderm, respectively (Kimmel et al., 2001), and whose expression is misregulated in the wing buds of VAD quail embryos (Stratford et al., 1999). *Wnt7a* was properly expressed along the dorsal ectoderm of the *Raldh2*$^{-/-}$ forelimb buds; however, the presence of cells ectopically expressing *Wnt7a* was detected in the anteriormost ventral ectoderm (Fig. 7K), especially in the most severely growth-deficient limbs (Fig. 7L). In contrast, *En1* expression was not detectable in the prospective ventral forelimb ectoderm of E9.5 *Raldh2*$^{-/-}$ mutants (compare Fig. 7M and N; note also the diminished expression in dermomyotome). At E10.5, *En1* was expressed throughout the ventral ectoderm of the *Raldh2*$^{-/-}$ forelimb buds (Fig. 7N, inset) while, at this stage, its expression became restricted to the AER in wild-type littermate embryos (Fig. 7M, inset). En1 acts both as a repressor of *Wnt7a* ventral expression and as a determinant of proper AER formation (Kimmel et al., 2000). Its delayed timing of activation may contribute to the AER defects seen in *Raldh2*$^{-/-}$ forelimb buds (Fig. 4), and to the abnormal anterior expression of *Wnt7a*.

The *AbdominalB*-related Hoxd genes (*Hoxd9-d13*) are sequentially activated along both the PD and AP axes of the limb buds and, thus, control the morphogenesis of defined skeletal elements (Zakany and Duboule, 1999). Before limb budding, only *Hoxd9* is expressed along the flank mesoderm up to the prospective forelimb level. *Hoxd9* expression was detected in the flank of E9.5 *Raldh2*$^{-/-}$ embryos even in the absence of any RA supplementation. However, these embryos showed no increase of *Hoxd9* expression in the putative forelimb territory, as normally seen in wild-type embryos (data not shown). *Hoxd9* upregulation took place in the forelimb buds of the RA-supplemented mutant embryos, even if highly hypoplastic (data not shown). Likewise, *Hoxd11*, *d12* and *d13* were expressed at normal levels in mutant forelimb buds (Fig. 8, and data not shown). However, their spatial transcript distributions were abnormal. In E9.5 mutant buds, lack of posterior restriction was observed for both *Hoxd11* (compare Fig. 8A and B) and *Hoxd12* (Fig. 8F and G). Over the next day, expression of these genes is specifically upregulated in the posterodistal region of the autopod in wild type (Sordino et al., 1995), leading to an apparent 'double expression domain' for *Hoxd11* (Fig. 8C) and 2 regions with different signal intensities for *Hoxd12* (Fig. 8H). This autopodal upregulation was clearly present in mutant embryos (Fig. 8D,E,I,J). It was seen, however, along the whole margin of the autopod (see the strong *Hoxd12* expression at the level of the abnormal anterior outgrowth: Fig. 8J, arrowhead) or was even reversed in polarity (see *Hoxd11* strong expression along the anterior margin, and weak expression along the posterior margin, of the autopod in Fig. 8D and E).

DISCUSSION

Embryonic RA synthesis is necessary for proper forelimb growth and anteroposterior patterning

We have used *Raldh2*-null mutants to analyze the RA-dependency of early limb growth and patterning. The critical stages and molecular events that depend on Raldh2-mediated RA synthesis were investigated under different conditions of RA supplementation. We found that forelimb development critically requires endogenous RA synthesis: the mutant forelimb buds develop as highly truncated rudiments (along both their PD and AP axes) following RA rescue of the embryos by oral gavage. Moreover, forelimb growth was improved by providing a more steady RA supply to the mutant embryos *via* maternal food. Thus, RA was found to be critically required between E8.5 and E9.5: increasing the RA dose at these stages rescues mutant forelimb development more efficiently than extending the RA supplementation until E10.5. These data implicate RA, which is normally synthesized by Raldh2 along the lateral plate mesoderm and later in the proximal limb bud mesoderm (Niederreither et al., 1997; Swindell et al., 1999), as a crucial signal for sustained forelimb growth. Within a given mutant embryo, the left-side forelimb often appeared to be less efficiently rescued than the right-side one. There is no obvious explanation for this observation, as we found no evidence of abnormal left-right axis specification in rescued or unrescued *Raldh2*$^{-/-}$ mutants (see Niederreither et al., 2001). In this respect, we note that certain *Hox* gene knockouts also result in phenotypes (e.g. asymmetric vertebral transformations) that are more prevalent on the left side of the embryo (e.g. Fromental-Ramain et al., 1996).

While exogenously supplied RA could ensure forelimb growth in the rescued mutants (most likely because FGF signaling is operational; see below), it was unable to ensure normal AP patterning. Specific activation of *Shh* in the posterior limb bud mesoderm is an essential feature of the ZPA, the main determinant of AP limb patterning (Riddle et al., 1993). Although some of the severely affected *Raldh2*$^{-/-}$ forelimbs lacked detectable *Shh* expression, most often they showed a delocalized expression towards the distal tip of the limb rudiment and, sometimes, a reversed pattern with higher levels towards the anterior limb margin. *Fgf4*, the maintenance of which depends on Shh in a positive feedback loop (Zuniga et al., 1999; Chiang et al., 2001), was either downregulated or expressed ectopically at the anterior margin. Accordingly, the *Raldh2*$^{-/-}$ forelimbs exhibited marked AP patterning defects. In its most severe form, the *Raldh2*$^{-/-}$ phenotype consisted of a single undefined stylopodal/zeugopodal element and a digit rudiment. This phenotype is remniscent of, although not similar to, that of *Shh*$^{-/-}$ null mutants, which have a polarized stylopodal element (humerus) and a distinct, non-polarized zeugopodal element and single digit (Chiang et al., 2001; Kraus et al., 2001). We therefore propose that the *Raldh2*$^{-/-}$ forelimb abnormalities result from a lack of *Shh* asymmetrical posterior expression, rather than from a lack of *Shh* function. This interpretation is supported by the different outcomes seen

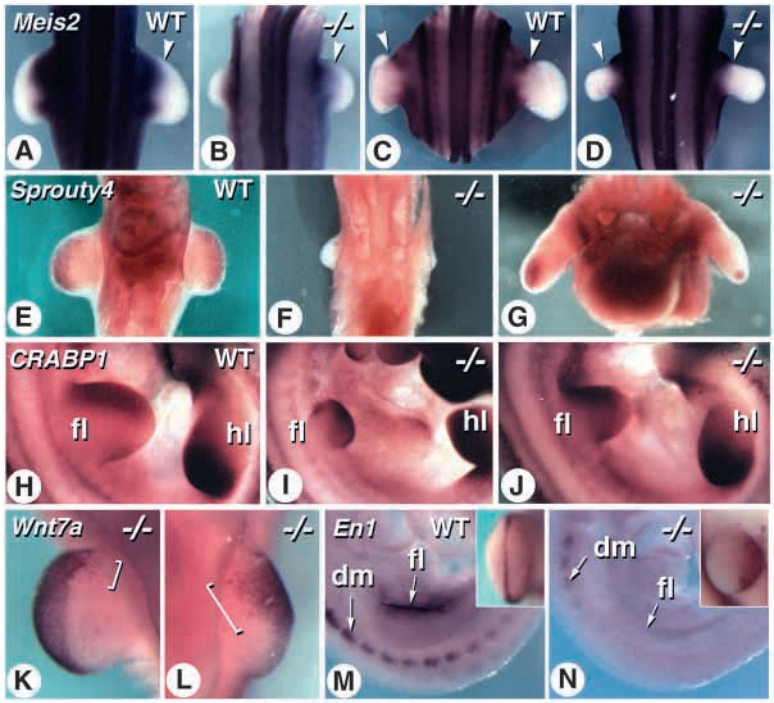

Fig. 7. *Meis2*, *Sprouty4*, *CRABP I*, *Wnt7a* and *En1* transcript distributions in wild-type (A,C,E,H,M) and *Raldh2*$^{-/-}$ (B,D,F,G,I,J,K,L,N) limb buds. All embryos are at E10-E10.5, except in M,N. (A-D) Dorsal views of the forelimb buds of *Meis2*-hybridized embryos. (E-G) Ventral views of the forelimb buds of E10.5 *Sprouty4*-hybridized embryos. (H-J) Profile views of *CRABP I*-hybridized embryos. (K,L) Ventral views of *Wnt7a*-hybridized embryos, where brackets indicate patches of ectopically expressing ventral cells. (M,N) Profile views of E9.5 *En1*-hybridized embryos, with insets showing *En1* expression in E10.5 forelimb buds. dm, dermomyotome.

in both mutants on the expression of downstream genes: *Bmp2* expression in posterior mesenchyme is substantially reduced in *Shh*$^{-/-}$ mutants (Chiang et al., 2001), whereas its expression expands symmetrically in anterior and posterior mesenchyme in *Raldh2*$^{-/-}$ mutants. Likewise, *Hoxd11* and *Hoxd12* expression is severely reduced in *Shh*$^{-/-}$ limb buds, whereas these genes are expressed along both the anterior and posterior margins – sometimes at higher levels at the anterior margin – of the *Raldh2*$^{-/-}$ forelimb autopod.

We suggest that the Raldh2 function in the flank and/or the proximal forelimb bud mesoderm is to synthesize RA required for efficient posterior activation of *Shh*. Interestingly, HPLC data have indicated higher RA levels in the posterior region of the chick limb bud (Thaller and Eichele, 1987) even though Raldh2 does not exhibit a preferential posterior distribution (Niederreither et al., 1997; Swindell et al., 1999). Such an uneven RA distribution could possibly be ascribed to the action of RA-metabolizing enzymes (MacLean et al., 2001). It is however likely that a posteriorly restricted factor such as dHand (Fernandez-Terran et al., 2000; Charité et al., 2000) is also required to specifically activate *Shh*. We found no misregulation of *dHand* in the flank mesoderm of E9 *Raldh2*$^{-/-}$ mutants, consistent with the idea that it acts upstream – or in parallel – with RA signaling. However, *dHand* expression encompassed a relatively larger area of the early forelimb bud mesoderm in *Raldh2*$^{-/-}$ than in wild-type embryos. This could contribute to the inability of *Raldh2*$^{-/-}$ embryos to properly restrict *Shh* expression to the posterior margin of their forelimb buds, and thus to establish a functional ZPA.

Even though the presence of a RA response element has been noted in the *Shh* promoter region (Chang et al., 1997), the effects of RA could be complex and several genes may be involved. *Hoxb8*, which is normally expressed up to the prospective posterior forelimb mesoderm, is thought to be directly controlled by RA and can be activated ectopically up to 4 hours after implantation of a RA bead in the anterior chick wing bud mesoderm (Lu et al., 1997; Stratford et al., 1997). However, we found no downregulation or misexpression of *Hoxb8* in the flank mesoderm of *Raldh2*$^{-/-}$ embryos (our unpublished data) and Stratford et al. (Stratford et al., 1999) reported that *Hoxb8* is expressed at normal levels – and is even anteriorly expanded – in the lateral mesoderm of VAD quail embryos. Interestingly, Ogura et al. (Ogura et al., 1996) have provided evidence that some of the RA effects in establishing the ZPA activity could be *Shh*-independent, as they have shown that a yet unidentified factor(s) induced by RA in P19 embryonal carcinoma cells can act in combination with Shh to generate a strong ZPA activity in a chick wing bud assay.

Exogenous RA can allow proper forelimb proximodistal patterning in *Raldh2* mutants

The homeobox gene *Meis2* has been implicated as a

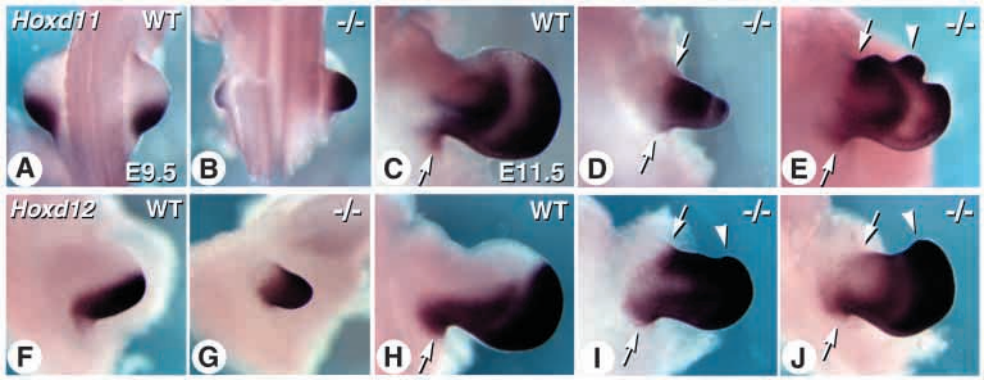

Fig. 8. Altered distributions of *Hoxd11* (A-E) and *Hoxd12* (F-J) transcripts in the *Raldh2*$^{-/-}$ forelimbs. In mutants, deregulated expression along the forelimb AP axis is seen prior to the formation of the autopod (A,B,F,G: E9.5). Later on (C-E,H-J: E11.5), both the autopodal (arrowheads) and non-autopodal (arrows) expression domains extend ectopically along the anterior margin of the mutant limbs.

determinant of proximal limb structures (Capdevila et al., 1999; Mercader et al., 2000). Mercader et al. (Mercader et al., 2000) found that addition of RA ectopically can 'reprogram' distal limb mesodermal cells to express more proximal gene combinations, including that of *Meis2*. Administration of a RAR antagonist leads to a rapid downregulation of *Meis2* expression, followed by selective lack of proximal elements. It was further postulated that proximal *Meis2* expression is antagonized – and thus prevented – in distal mesoderm through the FGF signaling pathway (Mercader et al., 2000). Although *Meis2* expression may be subtly downregulated in the trunk mesoderm of *Raldh2*$^{-/-}$ embryos that were rescued at a low RA concentration (100 μg/g food: Table 1, group 2), its transcripts were clearly detected in the proximal mesoderm of the mutant minilimbs. Under better rescue conditions, *Meis2* proximodistal expression boundary was located, as in wild-type embryos, at the level of the stylopod-zeugopod boundary. This leads us to conclude that, if RA synthesized by Raldh2 is involved in *Meis2* regulation, its lack of synthesis can be readily compensated for in mutant embryos by maternal RA supplementation. As this supplementation is unlikely to reproduce the tissue distribution of enzyme-mediated RA synthesis, it appears that the positioning of the *Meis2* expression boundary may be more critically dependent on FGF-mediated distal repression than on RA-mediated proximal activation. Several lines of evidence indicate that the FGF signaling pathway is operational in the RA-rescued *Raldh2*$^{-/-}$ forelimbs: *Fgf10* and *Fgf8* are expressed at relatively normal levels in the mesoderm and AER, respectively, of the RA-rescued limbs, and they can elicit the expression of target genes such as *Sprouty4* in distal limb mesoderm. We also note that in the mutant limbs, the gene for the BMP antagonist Gremlin is expressed distally as in wild-type embryos. Thus, in contrast to the case of the AP axis, we conclude that the control of gene expression along the limb PD axis is not critically dependent on the precise tissue distribution of Raldh2-synthesized RA, as this control can be essentially achieved in mutant embryos through exogenous RA supplementation.

Retinoic acid and hindlimb development

Hindlimb development was not detectably altered in the *Raldh2* mutants, whatever the RA rescue conditions used. This could be because (1) maternally administered RA fully rescues hindlimb development; (2) another RA-synthesizing enzyme may be critically involved in RA synthesis within the hindlimb field; (3) hindlimb development may be prominently controlled by other inducing/growth promoting factors.

We and others (Grün et al., 2000) have observed that *Raldh3* is expressed in the mesonephros of wild-type embryos, adjacent to the hindlimb buds. This expression is present in RA-rescued *Raldh2*$^{-/-}$ embryos (our unpublished data). Strong activation of the *RARE-hsp68-lacZ* reporter transgene is seen within the same region in mutant embryos (Fig. 3). Thus, Raldh3 could be responsible for the local RA synthesis required for proper hindlimb development. An alternative possibility may implicate Wnt signaling, which has recently been involved in the initiation of limb development (Kawakami et al., 2001). Whereas *Wnt2b*, the candidate for forelimb induction, is expressed at rather low levels in the forelimb field, several *Wnt* genes (including *Wnt8a/8c* or *Wnt3a*) are expressed in the region where hindlimbs are induced. Owing to a preponderant Wnt influence, hindlimb development may require lower RA levels to proceed normally, and these levels may be reached in *Raldh2* mutants by maternal supplementation. In any event, hindlimb development is unlikely to be RA independent, as both the forelimbs and hindlimbs are affected in VAD quail embryos (Stratford et al., 1999).

In conclusion, we have reported that correct patterning and outgrowth of the forelimb depends on endogenous RA production by Raldh2, which can only be partially rescued in knockout mutants by sustained RA supplementation from E7.5 to (at least) E9.5. As *Raldh2* null mutants do not survive past this stage without RA supplementation, only conditional mutation of *Raldh2* in the lateral mesoderm will indicate if forelimb outgrowth is entirely abolished, and hindlimb development is affected, in its absence.

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