Published in final edited form as:
Dev Dyn. 2010 February ; 239(2): 665. doi:10.1002/dvdy.22188.

Retinoic Acid Controls Expression of Tissue Remodeling Genes *Hmgn1* and *Fgf18* at the Digit-Interdigit Junction

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Abstract

Previous studies on retinoic acid receptor (RAR) mutants suggested that retinoic acid (RA) is required for loss of interdigital mesenchyme during digit formation. Here, we report that the RA-generating enzyme retinaldehyde dehydrogenase-2 (*Raldh2*) is expressed in the interdigital mesenchyme whereas *Cyp26b1*, controlling RA degradation, is expressed in digits, limiting autopodal RA action to the interdigital zones. E13.5 *Raldh2*$^{-/-}$ mouse embryos lose expression of the *RARE-lacZ* RA-reporter transgene and matrix metalloproteinase-11 (*Mmp11*) throughout the interdigital mesenchyme, while expression of *RARb*, *Fgf18*, and high mobility group N1 (*Hmgn1*) is lost at the digit-interdigit junction. *Raldh2*$^{-/-}$ autopods exhibit reduced interdigital apoptosis associated with loss of *Bmp7* expression, but *Bmp2*, *Bmp4*, *Msx2*, and *Fgf8* were unaffected. Although interdigital expression of *Hmgn1* was greatly down-regulated in *Raldh2*$^{-/-}$ autopods, complementary expression of *Sox9* in digit cartilage was unaffected. Regulation of *Hmgn1* and *Fgf18* at the digit-interdigit junction suggests RA controls tissue remodeling as well as apoptosis.

Keywords

Retinoic acid signaling; interdigital; *Raldh2*; *Hmgn1*; *Fgf18*; *Bmp7*; *Mmp11*; *Sox9*; *RARb*; *Cyp26b1*

INTRODUCTION

Retinoic acid (RA) plays a role in cell-cell signaling during organogenesis by functioning as a ligand for nuclear RA receptors (RAR) that control transcription of key genes (Duester, 2008). Recent studies indicate that RA is essential for induction of forelimb buds, but that RA is unnecessary for both hindlimb induction and establishment of anteroposterior or proximodistal patterning of limb buds (Zhao et al., 2009). However, at later stages of limb development when the autopod begins to form digits, a role for RA signaling in interdigital morphogenesis has been proposed based on studies of mouse *RARb/RARg* double null embryos that exhibit interdigital webbing at E15.5 that would normally have been lost by this stage (Ghyselinck et al., 1997). Further studies on autopods of *RARb/RARg* double mutants revealed a loss of apoptosis in the interdigital necrotic zone as well as down-regulation of tissue transglutaminase (*Tgm2*; tTG), a cross-linking enzyme implicated in apoptosis, and down-regulation of matrix metalloproteinase-11 (*Mmp11*; stromelysin-3) (Dupé et al., 1999). These findings suggest that RA is required for cell death in the interdigital mesenchyme to prevent interdigital webbing.

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Digit formation in mouse forelimbs and hindlimbs is also associated with complementary expression of two high mobility group (HMG) domain transcription factors encoded by *Sox9*, expressed in digit cartilage, and *Hmgn1*, expressed at the digit-interdigit boundary between E12.5-E14.5 (Furusawa et al., 2006). Analysis of null mutants in mice has demonstrated that *Sox9* is essential for cartilage formation (Bi et al., 1999). Analysis of *Hmgn1*$^{-/-}$ limb bud micromass cultures has shown that HMGN1 inhibits *Sox9* expression and retards differentiation, thus suggesting a role for HMGN1 in controlling digit formation (Furusawa et al., 2006). An additional gene of note, *Fgf18*, is expressed in the perichondrium (outer surface of the developing bone) and interdigital mesenchyme where it is proposed to function as a regulator of chondrogenesis and osteogenesis (Liu et al., 2002). Thus, *Hmgn1* and *Fgf18* play roles in tissue remodeling at the digit-interdigit junction during digit formation.

Retinaldehyde dehydrogenase-2, encoded by *Raldh2* (*Aldh1a2*), oxidizes the vitamin A metabolite retinaldehyde to RA, and thus controls much of the endogenous synthesis of RA during vertebrate organogenesis (Duester, 2008). Loss of RA signaling in *Raldh2*$^{-/-}$ mouse embryos leads to defects in body axis extension and heart development that result in major developmental abnormalities beyond E8.5 with no sign of limb development (Niederreither et al., 1999; Mic et al., 2002). Limited maternal dietary RA supplementation of *Raldh2*$^{-/-}$ embryos rescues early lethality (Niederreither et al., 2001), with forelimbs consistently exhibiting retarded growth compared to hindlimbs which appear normal at E10.5 (Zhao et al., 2009). These differences between forelimb and hindlimb development in rescued *Raldh2*$^{-/-}$ embryos demonstrate that RA action in the body axis from E7.5-E8.5 is required for induction of forelimbs (most likely through RA repression of axial *Fgf8* expression), but that RA is not required for hindlimb induction at E9.5, nor for patterning of either forelimbs or hindlimbs (Zhao et al., 2009). As *Raldh2* expression later initiates in the interdigital mesenchyme at E12.5 (Niederreither et al., 1997; Kuss et al., 2009), examination of hindlimbs in *Raldh2*$^{-/-}$ rescued mutants at later stages establishes a genetic loss-of-function model for examining a later role of RA in the interdigital zones.

Here, we use rescued *Raldh2*$^{-/-}$ embryos to examine the late effects of RA signaling in the E13.5 mutant hindlimb autopod, which appears relatively normal from an external view, but which completely lacks RA synthesis. Our findings demonstrate that RA generated by *Raldh2* is required for interdigital apoptosis. Our studies also show that RA induces expression of *Hmgn1* and *Fgf18* specifically at the digit-interdigit junction, thus placing RA upstream of genes that define the digit periphery during interdigital tissue remodeling.

RESULTS

Raldh2 Null Model for Studying Interdigital RA Signaling

Expression of *Raldh2* in mouse hindlimb autopods was examined by whole-mount in situ hybridization at E12.5-E13.5. At E12.5, *Raldh2* mRNA was detected throughout the interdigital zone, whereas at E13.5 *Raldh2* mRNA was detected mostly at the digit-interdigit junction (Fig. 1A-B). These findings suggest that in addition to playing a role in the induction of apoptosis throughout the interdigital mesenchyme as previously suggested (Dupé et al., 1999), RA signaling may play another role specifically at the digit-interdigit junction.

To examine the function of RA during interdigital development, we examined rescued *Raldh2*$^{-/-}$ embryos by performing maternal dietary RA supplementation from E6.75-E9.25 followed by return to a normal diet from E9.25 onwards. At E12.5-E13.5, rescued *Raldh2*$^{-/-}$ embryos consistently exhibited a stunted forelimb and a relatively normal-sized hindlimb (Fig. 1C-D). The *RARE-lacZ* RA-reporter transgene present in these embryos demonstrated that rescued mutants lack expression of *RARE-lacZ* in the interdigital zones of both forelimbs and hindlimbs (Fig. 1C-D). As mutant forelimbs always exhibited growth retardation, all further

studies were performed on hindlimbs from wild-type and rescued *Raldh2*$^{-/-}$ embryos, with the latter referred to simply as *Raldh2*$^{-/-}$ embryos.

Effect of *Raldh2* Loss-of-Function on Interdigital RA Signaling, *Mmp11* Expression, and Apoptosis

At E13.5 we observed that hindlimb autopodal *RARE-lacZ* expression is normally very high in the interdigital zones, but this expression was completely absent in hindlimbs from *Raldh2*$^{-/-}$ embryos (Fig. 2A-B). We also examined expression of *Cyp26b1* encoding a P450 enzyme that catalyzes RA degradation in several regions of the embryo including limb buds (MacLean et al., 2001). *Cyp26b1* mRNA was localized in developing digit cartilage but not interdigital mesenchyme in wild-type E13.5 hindlimbs, and this pattern was still observed in *Raldh2*$^{-/-}$ embryos although expression was somewhat reduced (Fig. 2C-D). These findings suggest that RA synthesis controlled by *Raldh2* in the interdigital mesenchyme coupled with RA degradation controlled by *Cyp26b1* in the digits, results in the confinement of autopodal RA signaling to the interdigital zones.

As previous studies on *RARb/RARg* double mutants had demonstrated that loss of these two RA receptors results in loss of interdigital expression of *Mmp11*, we examined this gene in autopods lacking RA synthesis. In *Raldh2*$^{-/-}$ hindlimbs at E13.5, we observed loss of interdigital *Mmp11* expression (Fig. 2E-F). Thus, our findings are consistent with the *RARb/RARg* loss-of-function model, providing evidence that *Mmp11* is an important target gene for RA action in the interdigital zone.

As our rescued *Raldh2*$^{-/-}$ mutant embryos do not survive beyond E13.5, we were unable to determine if older embryos retain interdigital webbing as shown for E15.5 *RARb/RARg* double mutants (Ghyselinck et al., 1997). The *RARb/RARg* phenotype is also associated with a large reduction in interdigital apoptosis at E13.5 (Dupé et al., 1999). TUNEL assays of wild-type and *Raldh2*$^{-/-}$ hindlimbs at E13.5 show that a loss of RA also results in a large reduction in interdigital apoptosis (Fig. 2G-H).

Loss of Interdigital RA Synthesis Results in Loss of *Hmgn1* Expression but *Sox9* Expression is Unchanged

Our observation that *Raldh2* expression progressively becomes limited to the digit-interdigit junction by E13.5 led us to examine expression of genes known to play a role in digit formation. As previously reported, *Hmgn1* and *Sox9* exhibit complementary expression in the autopod, with *Hmgn1* limited to the digit-interdigit boundary and *Sox9* found in the digits (Furusawa et al., 2006). Here, we demonstrate that the E13.5 hindlimb autopod normally exhibits very high expression of *Hmgn1* limited to the digit-interdigit junction (not throughout the interdigital mesenchyme), but that *Raldh2*$^{-/-}$ hindlimbs exhibit greatly reduced *Hmgn1* mRNA in this domain (Fig. 3A-B). In contrast, *Sox9* mRNA normally expressed at high levels in the digits is unchanged in the *Raldh2*$^{-/-}$ hindlimbs (Fig. 3C-D). Alcian blue staining of cartilage also shows that E13.5 wild-type and *Raldh2*$^{-/-}$ hindlimbs have comparable digit cartilage formation (Fig. 3E-F). Together, these findings suggest that RA acts at the digit-interdigit junction to maintain a high level of *Hmgn1* expression, but loss of *Hmgn1* in *Raldh2* mutants does not appear to allow *Sox9* expression to expand into the interdigital zone.

Effect of RA Signaling on Interdigital Expression of *RARb* and *Fgf18*

In order to extend our gene expression analyses at the digit-interdigit junction we examined expression of two additional genes previously shown to be expressed in the interdigital zone, i.e. *RARb* (Mendelsohn et al., 1991) and *Fgf18* (Liu et al., 2002). Previous studies have shown that *RARb* is RA inducible and contains an RA response element in its promoter (Hoffmann et al., 1990), and *Fgf18* expression in the caudal body axis is lost in *Raldh2*$^{-/-}$ embryos (Vermot

et al., 2005; Zhao and Duester, 2009). Here, we found that *RARb* mRNA localized primarily to the digit-interdigit junction (rather than throughout the interdigital zone) in E13.5 hindlimbs, and this domain was lost in *Raldh2*$^{-/-}$ embryos (Fig. 4A-B). Concurrently, we found that *Fgf18* mRNA was expressed predominately in the developing joints and at lower levels in the interdigital zone in E13.5 hindlimbs; although interdigital expression was low it is interesting to notice that such expression is localized primarily to the digit-interdigit junction rather than being spread evenly across the interdigital zone (Fig. 4C). In E13.5 *Raldh2*$^{-/-}$ hindlimbs, the digit-interdigit expression domain of *Fgf18* mRNA was lost, whereas expression in joints was unchanged (Fig. 4D). These observations provide evidence that a major function of interdigital RA signaling is regulation of FGF signaling at the digit-interdigit junction during tissue remodeling.

Bmp Expression in *Raldh2* Mutant Hindlimbs

Bone morphogenetic protein (BMP) signals are thought to control interdigital cell death perhaps by regulating events in the apical ectodermal ridge such as FGF signaling or *Msx2* expression (Pajni-Underwood et al., 2007; Maatouk et al., 2009). *Msx2*, expressed in the apical ectodermal ridge and interdigital mesenchyme, has been found to be required for regression of interdigital mesenchyme (Marazzi et al., 1997; Satokata et al., 2000). Here we found that expression of *Fgf8* and *Msx2* in autopods of E13.5 *Raldh2*$^{-/-}$ hindlimbs is not significantly altered (Fig. 4E-H). Thus, loss of RA signaling may not control interdigital apoptosis through effects on the apical ectodermal ridge.

We also examined expression of several BMPs in the autopod at E13.5. We found that *Bmp2*, expressed primarily in the interdigital region, was unaffected by loss of RA in the *Raldh2*$^{-/-}$ mutant (Fig. 5A-B). We also found that *Bmp4* expression in the apical ectodermal ridge and digit mesenchyme was unaffected by loss of RA in the *Raldh2*$^{-/-}$ mutant, and expression in the interdigital region was maintained at the same level in both wild-type and mutant (Fig. 5C-D). However, *Bmp7* expression in the interdigital region was completely lost in the *Raldh2*$^{-/-}$ mutant (Fig. 5E-F). Similar results for *Bmp2/4/7* expression were obtained for *RARb/RARg* mutants (Dupé et al., 1999). Together, these findings suggest that RA signaling results in the induction of interdigital *Bmp7* expression which may be important for inducing interdigital cell death. Although our results suggest that RA does not regulate *Bmp2* and *Bmp4*, these genes have also been shown to be important for interdigital cell death (Maatouk et al., 2009) via their receptor Bmpr1a expressed in the apical ectodermal ridge (Pajni-Underwood et al., 2007).

DISCUSSION

Following limb induction and patterning, RA synthesis initiates in the autopod by E12.5 and RA signaling becomes confined to the interdigital zones by a combination of interdigital *Raldh2* expression and *Cyp26b1* expression in the developing digits. Analysis of *Raldh2*$^{-/-}$ autopods has identified several genes whose interdigital expression is either completely lost (*Mmp11*, *RARb*, *Fgf18*, *Bmp7*) or greatly down-regulated (*Hmgn1*). In contrast, expression of *Sox9* and *Bmp4* in digit cartilage is unaffected by a loss of RA. Similarly, while *Fgf18* expression is lost in the interdigital zone of *Raldh2*$^{-/-}$ autopods, we find that it is maintained in digits (particularly joints) consistent with RA signaling not occurring in digit cartilage due to *Cyp26b1* expression. A precedent exists for RA regulation of certain *Fgf18* expression domains as it has been shown that *Fgf18* expression at the somite/presomitic mesoderm border requires RA synthesis controlled by *Raldh2* (Vermot et al., 2005; Zhao and Duester, 2009). Our observation that RA signaling is normally absent in developing digit cartilage and apparently unnecessary for skeletogenesis is consistent with previous observations that addition of RA or loss of *Cyp26b1* inhibits cartilage formation or ossification (Biddulph et al.,

1988; Laue et al., 2008; Kuss et al., 2009; Maclean et al., 2009). In addition, recent studies have demonstrated that although RARg expression in the bone growth plate is required for bone growth, endogenous RA is undetectable in the growth plate, and further functional experiments demonstrated that RARg functions as a ligand-less repressor during bone growth (Williams et al., 2009).

RA synthesis controlled by *Raldh2* expression initially occurs throughout the interdigital zone by E12.5. At E13.5 we find that *RARE-lacZ* (an RA-reporter transgene) and *Mmp11* (an endogenous RA target gene) are expressed throughout the interdigital zone and both require RA synthesis for expression. From previous studies on *RARb/RARg* mutant mice it appears that RA must act throughout the interdigital zone to induce *Mmp11* and to initiate apoptosis that will eventually eliminate this tissue by E15.5 (Dupé et al., 1999). We found that *Raldh2*$^{-/-}$ autopods also have a large reduction in interdigital apoptosis, thus providing evidence that both ligands and receptors for RA signaling are crucial for interdigital apoptosis. *Mmp11* presumably functions in tissue remodeling during the apoptotic process through degradation of extracellular matrix as do other members of the matrix metalloproteinase family (Bai et al., 2005). We also found that *Raldh2*$^{-/-}$ autopods have a large reduction in interdigital *Bmp7* expression which may be crucial for BMP-mediated induction of apoptosis. However, we found that endogenous RA does not regulate expression of *Bmp2* or *Bmp4* in the apical ectodermal ridge which has also been associated with interdigital apoptosis (Pajni-Underwood et al., 2007; Maatouk et al., 2009), and we observed no requirement for endogenous RA to regulate *Msx2* or *Fgf8* expression in the apical ectodermal ridge.

The findings presented here suggest that in addition to induction of interdigital apoptosis, RA also functions to define the digit-interdigit junction where tissue remodeling occurs. By E13.5 we observed that expression of *Raldh2* is limited to the digit-interdigit junction rather than being expressed throughout the interdigital zone. In contrast to *Mmp11*, interdigital expression of *RARb, Hmgn1*, and *Fgf18* in E13.5 autopods occurs specifically at the digit-interdigit junction rather than throughout the interdigital zone, thus correlating with *Raldh2* expression in this domain. *Hmgn1* and *Fgf18* have previously been shown to be expressed in the interdigital zone and both are implicated in regulation of digit formation (Liu et al., 2002; Furusawa et al., 2006). We propose that RA synthesis becomes more predominately localized at the digit-interdigit junction as autopod development proceeds, leading to *RARb* upregulation which intensifies RA signaling at this junction, resulting in up-regulation of *Hmgn1* and *Fgf18*. Thus, RA-induced FGF signaling specifically at the digit-interdigit interface may be necessary for normal tissue remodeling during digit formation. In summary, our new model for interdigital RA action suggests that RA signaling acts upstream of *Hmgn1* and *Fgf18* to initiate the tissue remodeling process that will lead to formation of the digit-interdigit junction, ultimately defining the edge of the domain that is slated for elimination during interdigital apoptosis.

EXPERIMENTAL PROCEDURES

Generation of Rescued *Raldh2*$^{-/-}$ Embryos

Generation of heterozygous *Raldh2*$^{+/-}$ adult mice were previously described (Mic et al., 2002). Rescue of *Raldh2*$^{-/-}$ early lethality (which occurs at E9.5) was performed by supplementing the maternal diet with RA as follows. From E6.75-E9.25 pregnant females were provided food with 0.1 mg all-*trans*-retinoic acid (catalog #R2625, Sigma Chemical Co., St. Louis, MO, USA) per g standard mouse chow. RA-supplemented food was prepared by mixing 50 μl of a 50 mg/ml suspension stock solution of RA in 100% ethanol with 450 μl corn oil, then evenly distributing 200 μl RA/ethanol/oil suspension into 10 g mouse chow (ground with mortar and pestle) to achieve a final RA concentration is 0.1 mg/g mouse chow. The food was put in a deep glass Petri dish and changed twice a day (once in the morning and once in the evening) in a cage where all other food has been removed. RA was provided at E6.75 (evening

of day 6), E7.25 (morning of day 7), E7.75, E8.25, and E8.75. At E9.25, mice were put back on normal food until embryos were collected at E12.5-E13.5. *Raldh2*$^{-/-}$ embryos were identified by the stunted forelimb (hindlimb normal) or by genotyping using PCR analysis of yolk sac DNA. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

Whole-mount in situ Hybridization, *lacZ* Detection, Cartilage Staining, and TUNEL Assay

Whole-mount in situ hybridization (ISH) was used to detect *Raldh2* mRNA and other mRNAs as previously described (Mic et al., 2002). In order to assess *RARE-lacZ* expression, β-galactosidase activity was detected by performing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining for 6-24 h as previously described (Rossant et al., 1991). Alcian blue staining of cartilage was performed as described (Hogan et al., 1994). Apoptosis was detected using the DeadEnd Colorimetric TUNEL System (Promega Corp.) following the manufacturer's protocol. Wild-type and *Raldh2*$^{-/-}$ embryos were treated under identical hybridization or staining conditions and stained for the same length of time. For each analysis (ISH, β-galactosidase stain, Alcian blue stain, or TUNEL), at least three *Raldh2*$^{-/-}$ embryos and three wild-type embryos at a similar stage were examined, all with comparable results.

Acknowledgments

We thank the following individuals for mouse cDNAs used to prepare *in situ* hybridization probes: M. Bustin (*Hmgn1* and *Sox9*), V. Giguère (*RARb*), B. Harfe (*Msx2*), B. Hogan (*Bmp4*, *Bmp7*), J. C. Izpisua-Belmonte (*Bmp2*), N. Itoh (*Fgf18*), G. Martin (*Fgf8*), M. Petkovich (*Cyp26b1*), M.-C. Rio (*Mmp11*). We also thank J. Rossant for providing *RARE-lacZ* reporter mice. G.D. was funded by a National Institutes of Health grant.

Grant sponsor: The National Institutes of Health; Grant number: GM062848

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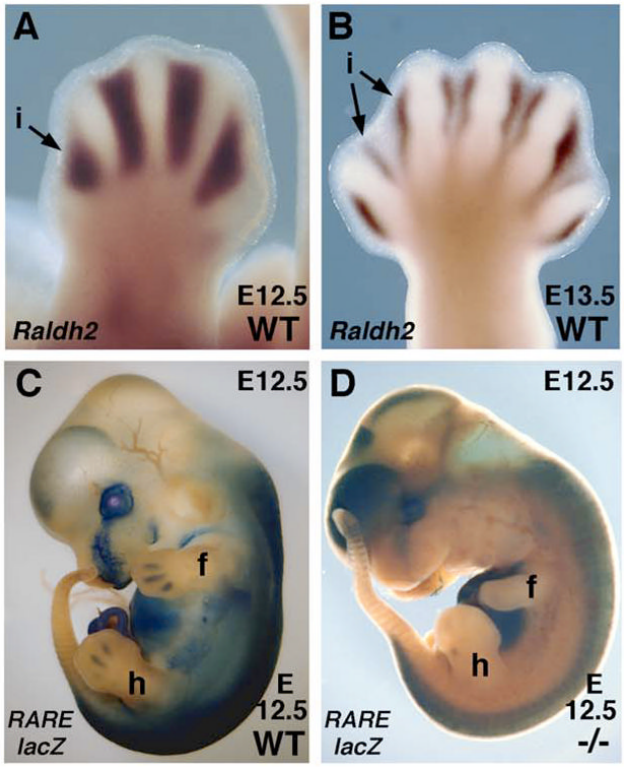

Fig. 1.

Raldh2 and interdigital RA signaling. (A-B) Detection of *Raldh2* mRNA in the mouse hindlimb autopod at E12.5 and E13.5; by E13.5 *Raldh2* expression is more concentrated at the digit-interdigit junction. (C-D) *RARE-lacZ* RA-reporter expression at stage E12.5 for wild-type (WT) or a rescued *Raldh2*$^{-/-}$ mutant (−/−); note retarded forelimb growth in the mutant compared to the hindlimb which is relatively normal in size, and loss of interdigital *RARE-lacZ* expression. i, interdigital region.

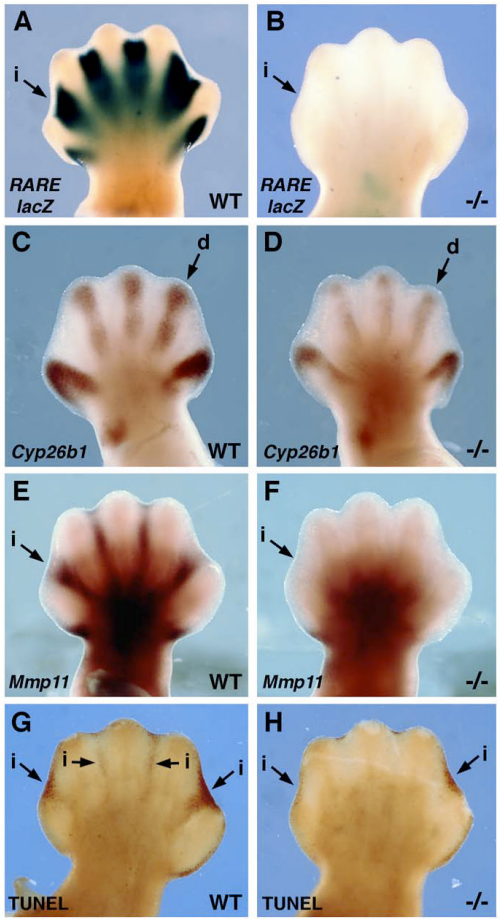

Fig. 2.

Loss of interdigital RA synthesis results in loss of *Mmp11* expression and reduced apoptosis. (A-B) *RARE-lacZ* expression in a wild-type autopod is limited to the interdigital region, and all RA activity is lost in an autopod from an *Raldh2*$^{-/-}$ mutant. (C-D). *Cyp26b1* mRNA is limited to autopod cartilage including digits, and expression is somewhat reduced in the *Raldh2*$^{-/-}$ mutant. (E-F) *Mmp11* mRNA in the interdigital region is completely lost in the *Raldh2*$^{-/-}$ mutant. (G-H) TUNEL assay demonstrating less interdigital apoptosis in the *Raldh2*$^{-/-}$ mutant. All autopods shown are E13.5 hindlimbs. d, digit; i, interdigital region.

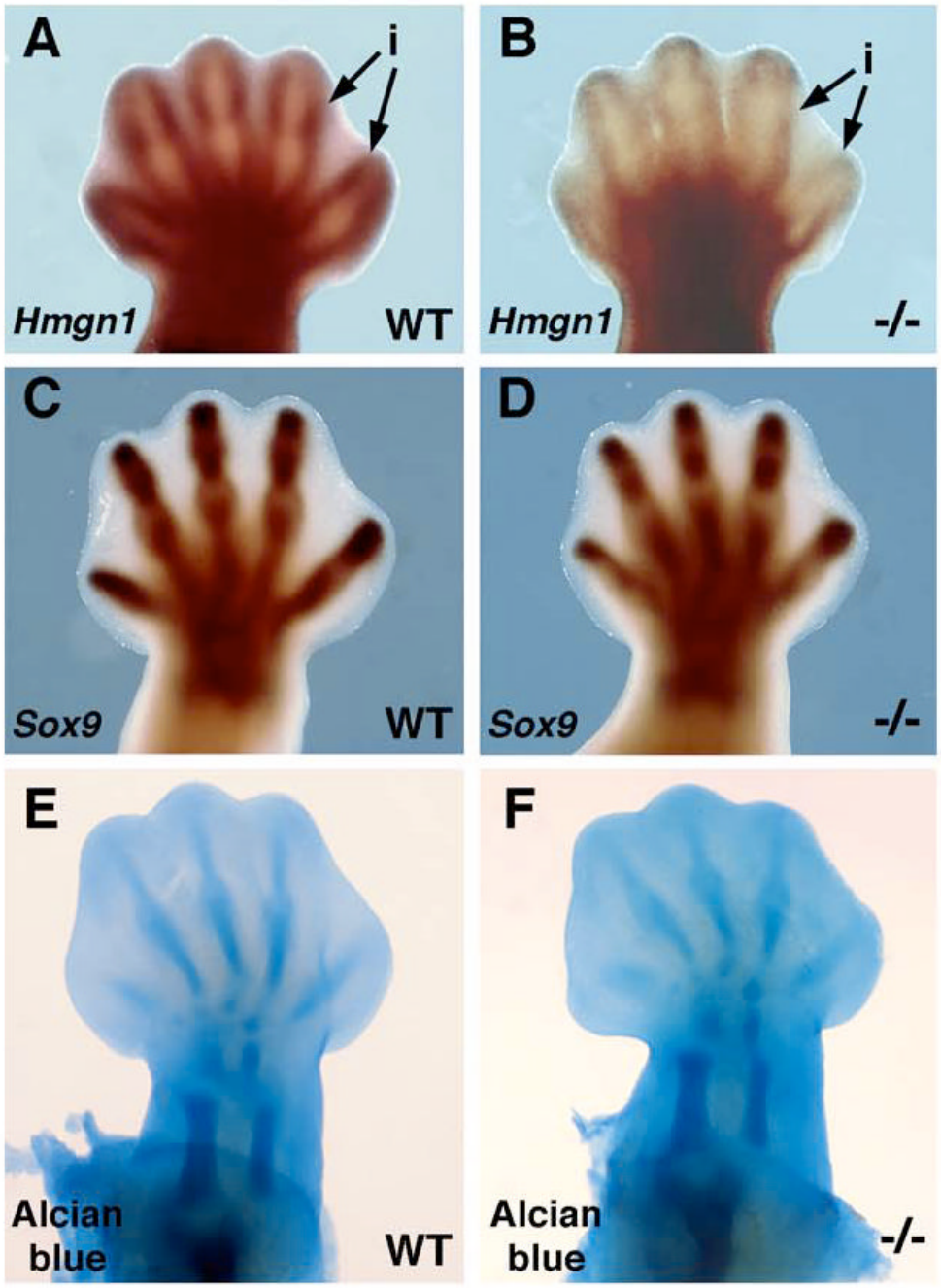

Fig. 3.

Loss of interdigital RA signaling results in loss of *Hmgn1* expression but not *Sox9*. (A-B) Interdigital expression of *Hmgn1* mRNA is highest at the digit-interdigit junction and is greatly down-regulated in the *Raldh2*$^{-/-}$ mutant. (C-D) *Sox9* mRNA is limited to autopod cartilage including digits and is unaffected by loss of autopodal RA signaling. (E-F) Alcian blue staining of cartilage demonstrates that a loss of interdigital RA signaling does not affect digit cartilage formation. All autopods shown are E13.5 hindlimbs. i, interdigital junction.

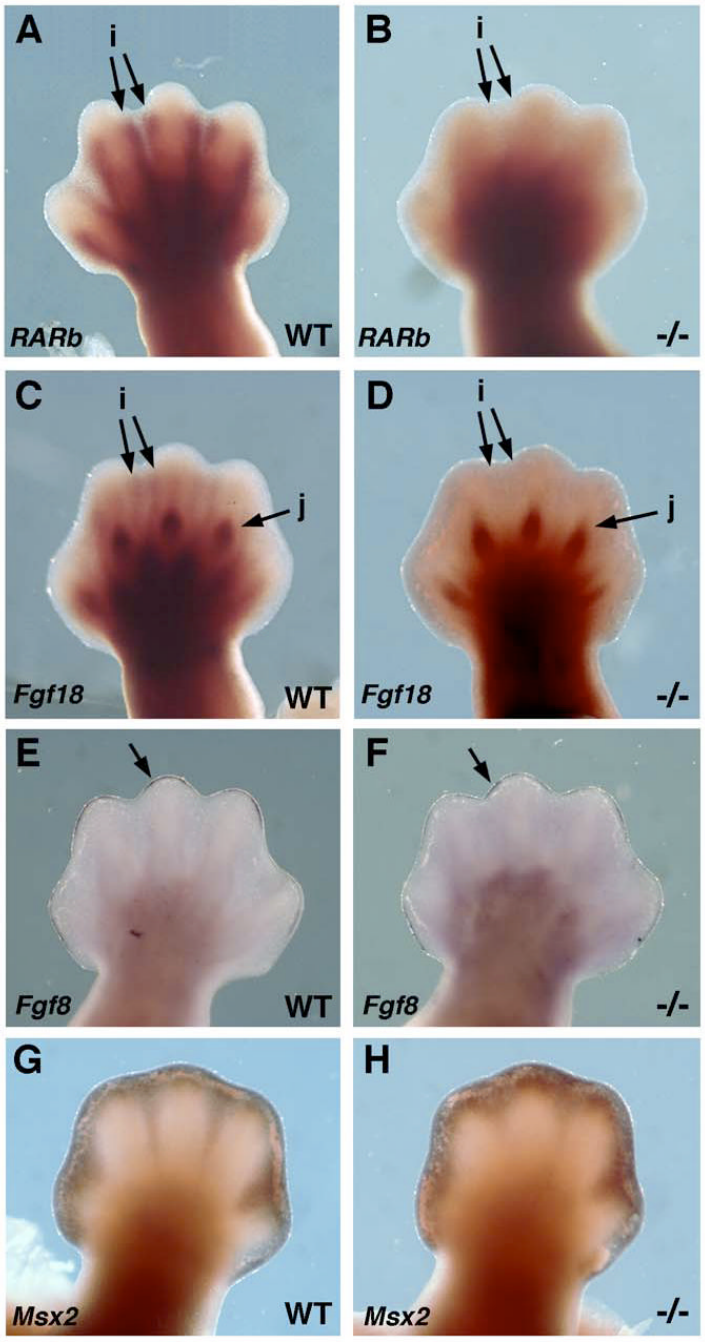

Fig. 4.

Loss of RA eliminates interdigital expression of *RARb* and *Fgf18*. (A-B) Interdigital expression of *RARb* mRNA is highest at the digit-interdigit junction and this expression domain is completely lost in the *Raldh2*$^{-/-}$ mutant. (C-D) *Fgf18* mRNA localized at the digit-interdigit junction is lost in the mutant, but expression in the phalange joints is unaffected by loss of autopodal RA activity. (E-F) *Fgf8* expression in the apical ectodermal ridge is unaffected by loss of RA. (G-H) *Msx2* expression in the apical ectodermal ridge and interdigital mesenchyme is unaffected by loss of RA. All autopods shown are E13.5 hindlimbs. i, interdigital junction; j, joint region.

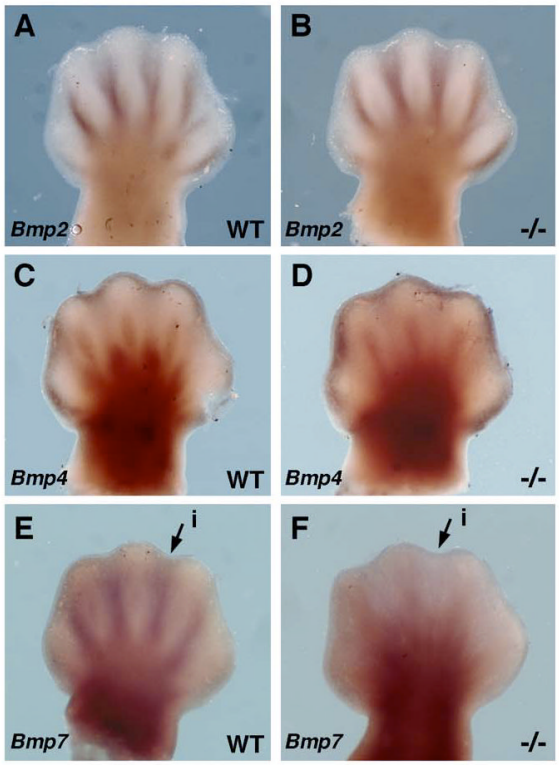

Fig. 5.

Effect of loss of RA on Bmp expression in autopods. (A-B) *Bmp2* expression in the interdigital region is unaffected by loss of RA in the *Raldh2*$^{-/-}$ mutant. (C-D) *Bmp4* expression in the autopod is unaffected by loss of RA in the *Raldh2*$^{-/-}$ mutant. (E-F) *Bmp7* expression in the interdigital region is completely lost in the *Raldh2*$^{-/-}$ mutant. All autopods shown are E13.5 hindlimbs. i, interdigital region.