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Notch1 and 2 cooperate in limb ectoderm to receive an early Jagged2 signal regulating interdigital apoptosis

Yonghua Pan [a], Zhenyi Liu [a], Jie Shen [b], Raphael Kopan [a,*]

[a] *Department of Molecular Biology and Pharmacology, and Division of Dermatology, Department of Medicine, Washington University School of Medicine, Box 8103, 660 S. Euclid Avenue, St. Louis, MO 63110, USA*

[b] *Center for Neurologic Diseases, Brigham and Women's Hospital, Program in Neuroscience, Harvard Medical School, Boston, MA 02115, USA*

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Abstract

Spontaneous and engineered mutations in the Notch ligand *Jagged2* produced the *Syndactylism* phenotype [Jiang, R.L., Lan, Y., Chapman, H.D., Shawber, C., Norton, C.R., Serreze, D.V., Weinmaster, G., Gridley, T., 1998. Defects in limb, craniofacial, and thymic Development in *Jagged2* mutant mice. Genes Dev. 12, 1046–1057; Sidow, A., Bulotsky, M.S., Kerrebrock, A.W., Bronson, R.T., Daly, M.J., Reeve, M.P., Hawkins, T.L., Birren, B.W., Jaenisch, R., Lander, E.S., 1997. Serrate2 is disrupted in the mouse limb-development mutant syndactylism. Nature 389, 722–725]. Given that additional ligands may be expressed in the developing limb bud, it was possible that loss of *Jagged2* disabled only part of Notch function in the limb. In addition, it is not clear from the expression pattern of *Jagged2* in the apical ectodermal ridge (AER) whether the ectodermal or mesenchymal compartment of the limb bud receives the Jagged2 signal. To elucidate the requirement for the Notch pathway in limb development, we have analyzed single and compound Notch receptor mutants as well as γ-secretase-deficient limbs. Floxed alleles were removed either from the developing limb bud ectoderm (using *Msx2-Cre*) or from the mesenchyme (using *Prx1-Cre*). Our results confirm that *Jagged2* loss describes the contribution of the entire Notch pathway to the mouse limb development and revealed that both Notch1 and 2 are required in the ectoderm to receive the Jagged2 signal. Interestingly, our allelic series allowed us to determine that Notch receives this signal at an early stage in the developmental process and that memory of this event is retained by the mesenchyme, where Notch signaling appears to be dispensable. Thus, Notch signaling plays a non-autonomous role in digit septation.

Keywords: Notch; Limb bud; AER; *Msx2-Cre*; Syndactyly

Introduction

Vertebrate limbs form at stereotypic positions along the embryonic flank under the influence of signals defining the primary body axes. Limb development is initiated by reciprocal inductions between the lateral plate mesoderm and the overlying ectoderm, which leads to the formation of the limb bud. Signals produced from three essential organizers interplay with each other and regulate the outgrowth and patterning of the developing limb buds along proximo-distal (P–D), dorso-ventral (D–V) and anterior–posterior (A–P) axes. The P–D axis is dependent on the signals emanating from the apical ectoderm ridge (AER), a thickened ectoderm structure along the D–V boundary of the developing limb bud (Niswander, 2002). The AER signals include several members of the FGF (Fgf4, 8, 9 and 17) and the BMP (BMP2, 4 and 7) families. Of these, two FGF proteins (Fgf4 and 8) have been shown to be absolutely essential for normal limb development (Barrow et al., 2003; Moon and Capecchi, 2000; Sun et al., 2000, 2002).

Notch signaling, a conserved and essential pathway employed repeatedly by multiple developmental and cellular processes, has also been implicated in regulating vertebrate limb development. Expression studies have revealed that multiple genes in the Notch pathway are expressed in the developing vertebrate limb buds. *Notch1* and the ligand *Jagged2* are co-expressed in the AER of the chick, mouse and rat; *Notch1*, *Notch2* and *Jagged1* have also been detected in the limb mesenchyme (Myat et al., 1996; Rodriguezesteban et al., 1997; Shawber et al., 1996; Vargesson et al., 1998), as

* Corresponding author.
E-mail address: kopan@molecool.wustl.edu (R. Kopan).

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was a Notch target, *c-hairy1* (Vasiliauskas et al., 2003). Misexpression studies with *c-hairy1* and *Delta1* in chick result in abnormal skeletal limb development (Crowe et al., 1999; Vasiliauskas et al., 2003). Misexpression of Radical Fringe, a glycosyltransferase that modifies Notch to enhance activation by Delta and reduce activation by Jagged (Lei et al., 2003), can induce ectopic AER formation in chick (Laufer et al., 1997; Rodriguezesteban et al., 1997). This latter result suggested that perhaps Notch signaling established the placement of AER by participating in formation of the D/V boundary, in a manner analogous to the role *Fringe* plays in the fly wing (Irvine and Vogt, 1997). However, mice lacking both *Radical Fringe* and *Lunatic Fringe* have normal limbs (Zhang et al., 2002).

The strongest evidence supporting a direct functional role for Notch signaling in mouse limb development stems from the analysis of spontaneous and engineered *Jagged2* mutants. Mice homozygous for a hypomorphic *Jagged2* allele ($Jag2^{sm}$) or lacking *Jagged2* entirely ($Jag2^{\Delta DSL}$) suffer from fusion of the three middle digits (syndactyly: Jiang et al., 1998; Sidow et al., 1997). In $Jag2^{\Delta DSLL}$, AER hypertrophy was observed, concomitant with increased *FGF8* expression and prolonged *BMP4* expression. It was proposed that deregulated *FGF8* expression ultimately led to decreased expression of mesenchymal *BMP2* and *7* which in turn led to reduced interdigital apoptosis (Jiang et al., 1998; Sidow et al., 1997). Which receptor(s) mediated Jagged2 signals and in which germ layer these receptors acted remained to be determined. In addition, the mild defect illustrated by *Jagged2* mutants could be due to the functional redundancy with other mouse *Notch* ligands, however, mice lacking the mesenchymal ligand *Jagged1* die in utero by E11.5, precluding determination of Jagged1 function in limb mesenchyme (Xue et al., 1999).

To fully characterize the function of Notch signaling during limb development, we conducted a systematic loss of function analysis of limbs from individual and compound mutants for several *Notch* receptors or double *presenilin* mutants described earlier (Pan et al., 2004). The removal of both *presenilin* alleles either from the ectoderm or mesenchyme of the developing limb buds ensures loss of the entire canonical Notch pathway in the targeted tissue. Our results indicate that Notch signaling acts in the ectoderm and that it plays no additional roles in limb development other than those revealed by the *Jagged2* mutants. Interestingly, both gain and loss of function studies suggest that the function of Notch signaling in the regulation of digit septation may be restricted to a specific and early period during the development of the mouse limb.

Results

Msx2-Cre mediated disruption of Notch1 in the limb ectoderm produced mild syndactyly in both fore- and hindlimbs with low penetrance

The overlapping expression patterns of *Notch1* and *Jagged2* genes in AER and the syndactyly phenotypes exhibited by the $Jag2^{\Delta DSL}$ and $Jag2^{sm}$ mutants suggest that Notch1 may mediate the function of Jagged2 in mouse limb development (Jiang et al., 1998; Myat et al., 1996; Rodriguezesteban et al., 1997; Shawber et al., 1996; Sidow et al., 1997). To investigate this possibility, we needed to circumvent the early embryonic lethality of the *Notch1* (*N1*) nulls (Conlon et al., 1995; Swiatek et al., 1994). We therefore used the conditional *N1* allele (Pan et al., 2004) and the well-characterized *Msx2-Cre* transgenic line, in which Cre recombinase is expressed in AER and the limb ectoderm (Barrow et al., 2003; Sun et al., 2000, 2002) and the skin (Pan et al., 2004). Mice homozygous for the floxed *N1* allele and the *Msx2-Cre* transgene ($N1^{flox/flox}$; *Msx2-Cre* or *N1*CKO) had obvious skin and hair phenotypes which has been reported elsewhere (Pan et al., 2004). In addition, the *N1*CKO mice exhibited syndactyly of both fore- and hindlimbs that affected digits 2, 3 and 4 with variable penetrance. Of 65 pairs of hindlimbs, 6 (9.2%) were completely wild type, and 55 (84.6%) displayed a weak phenotype ranging from barely detectable soft tissue webbing (Fig. 1D1, arrow) to partial fusions of three middle digits on each foot (Figs. 1D2–D4). Only 4 pairs of hindlimbs (6.2%) displayed a severe phenotype, completely affecting all three middle digits (Fig. 1D5). These limbs were only superficially similar to the *Jagged2* mutants since skeletal preparation did not reveal any osseous abnormalities (Fig. 1E). Interestingly, the forelimbs had much less severe phenotypes than the hindlimbs: 22/35 (64%) of the mutants have completely normal forelimbs; the rest either had very slight webbing (not shown) or had partial fusion on one side of the forelimbs (Fig. 1C, arrow). The milder syndactyly of *N1* mutants compared to that of *Jagged2* mutants suggested that Notch1 might not be the only receptor that mediates *Jagged2* signaling in AER.

Loss of both Notch1 and 2 recapitulates the limb phenotypes of Jagged2 mutants

Since syndactyly in *Jagged2* mutant mice is often accompanied by primary chondrogenic and secondary osseous fusions (Jiang et al., 1998; Sidow et al., 1997), we wondered whether the existence of other Notch paralogs could compensate for the loss of *Notch1*. Removal of *Notch2* (in $N2^{flox/flox}$; *Msx2-Cre* mice), *Notch3* or *Notch4* alone produced no phenotype (our own observation and Krebs et al., 2000, 2003). However, the Notch1 and Notch2 double conditional KO ($N1^{flox/flox}$; $N2^{flox/flox}$; *Msx2-Cre* or *N1N2*CKO; Pan et al., 2004) produced a more severe limb phenotype than *N1*CKO mice. Gross morphological examination of *N1N2*CKO mutants revealed uniform syndactylism phenotype with complete fusions of the three middle digits on each hindlimb in all individuals ($n = 16$). Despite the complete bilateral penetrance, the phenotype had variable severity. While in some hindlimbs three middle digits were still distinguishable by their distal, individually distinct nails (Fig. 2B′), limbs with complete fusion of two adjacent digits appeared to have 2 fused nails (Fig. 2C′, arrow). The most severe fusions appeared to lack one digit as only two nails were detected (Figs. 2D′ and E′, arrow). Interestingly, although still much milder than the hindlimb phenotypes, forelimb syndactyly was frequently observed in *N1N2*CKO (Figs. 2B–E): only 1 of 16 mutants examined had completely wild type forelimbs, the rest displayed various

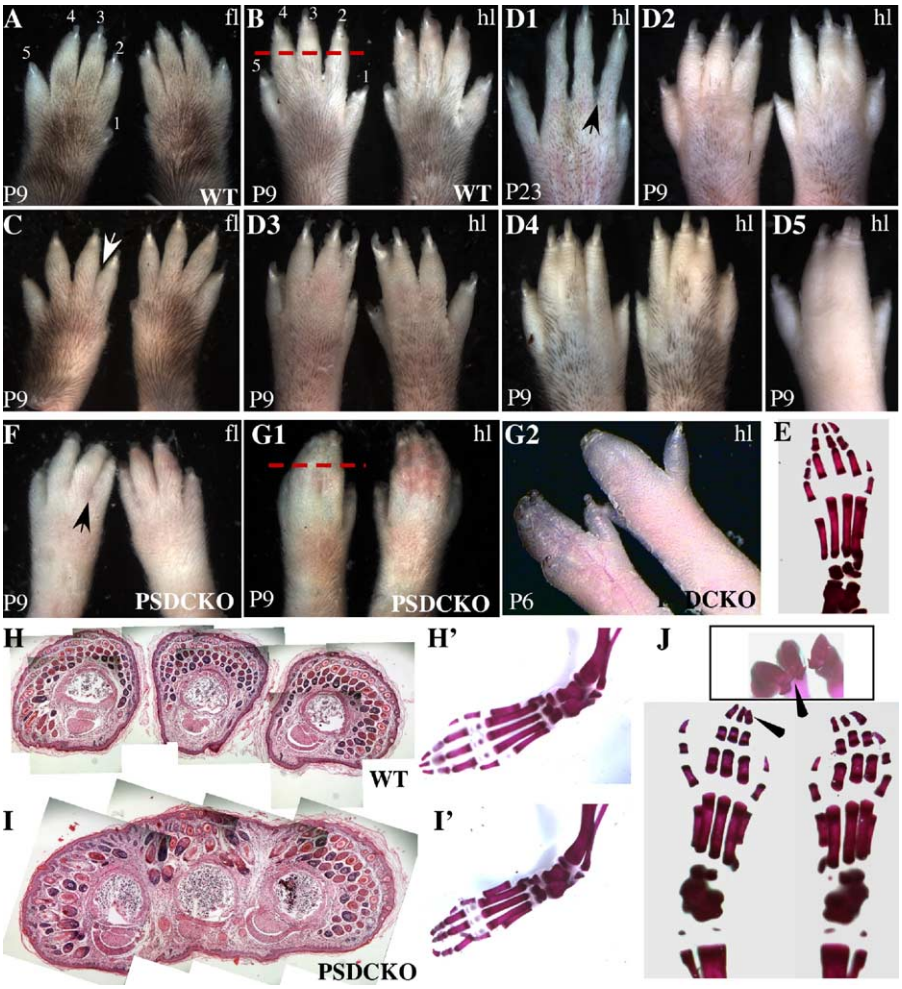

Fig. 1. *N1*CKO and *PS*DCKO limbs display syndactyly with different penetrance. (A–D) Gross limb morphology of wild type (A,B) and representative *N1*CKO mutants (C–D). fl: forelimb; hl: hindlimb. (G) Alizarin Red staining of skeletal preparation of the limb shown in panel D5, stained with Alizarin Red. (E–F) Gross limb morphology of representative *PS*DCKO mutants. (H, I) H&E staining of cross sections from hindlimbs from wild type (B) and a completely fused *PS*DCKO limb (G1), respectively. Dashed lines in panels B, G1 indicate the section plane. Panels H′, I′, J are Alizarin Red staining of skeletal preparations from the limbs shown in panels H, I, G2, respectively.

degrees of fusions ranging from barely detectable webbing to partial fusion (Figs. 2B–C). Occasionally, forelimbs displayed complete fusions of all middle digits (Fig. 2D, arrow). Importantly, the severity of the hindlimb syndactyly correlated with the extent of forelimb digit fusions within the same animal (compare Figs. 2D–E to B–C). Given the mixed genetic background of these animals, this variability could reflect the presence of genetic modifiers affecting Notch in both fore- and hindlimbs. Alternatively, it reflects variation in the timing of *Msx2-Cre* expression (see below).

To define the temporal window of Notch loss in the *N1N2*CKO animals, we analyzed the accumulation of NICD in the limb at E11.5 using antibodies to the epitope generated by γ-secretase after cleavage of N1, VLLS (Schroeter et al., 1998). In wild type animals, NICD is detected in non-AER ectodermal cells, in the AER and in a fraction of mesenchymal cells (Fig. 3A). However, in Msx2-Cre expressing animals, NICD was no longer detected in the AER in both the fore- and the hindlimb after E11.5 (Figs. 3C, E). While this examination indicates complete loss of N1 protein in the AER by this age, it cannot determine when N2 protein was lost (but see below).

Examination of the limb skeletal preparations of the *N1N2*CKO mutants revealed that, while none of the forelimbs exhibited skeletal defects (Fig. 2D, arrow and not shown), frequent (8 out of 12) osseous fusions of digital phalanges were observed in the hindlimbs. These involved either fusion of adjacent p3 (the distal phalanx, marked in Fig. 2F; Figs. 2G–H, arrowhead), or p2 (the middle phalanx, marked in Fig. 2F; Fig. 2K, right dashed circle) or p1 (the proximal phalanx, marked in Fig. 2F; Figs. 2I–K, left dashed circle). When two adjacent p1 were fused, only one p2 and one p3 existed so that the two digits became one (Figs. 2I–K, left hindlimb). Thus, the hindlimb phenotypes of *N1N2*CKO mice closely resembled those of *Jagged2* mutants: the three middle digits were tightly fused by soft tissues and often involved osseous fusions of the distal phalanges. We therefore concluded that the *N1N2*CKO mutants phenocopy the *Jagged2* mutants, indicating that both Notch 1 and 2 participate in receiving the Jagged2 signal during limb development. One phenotypic

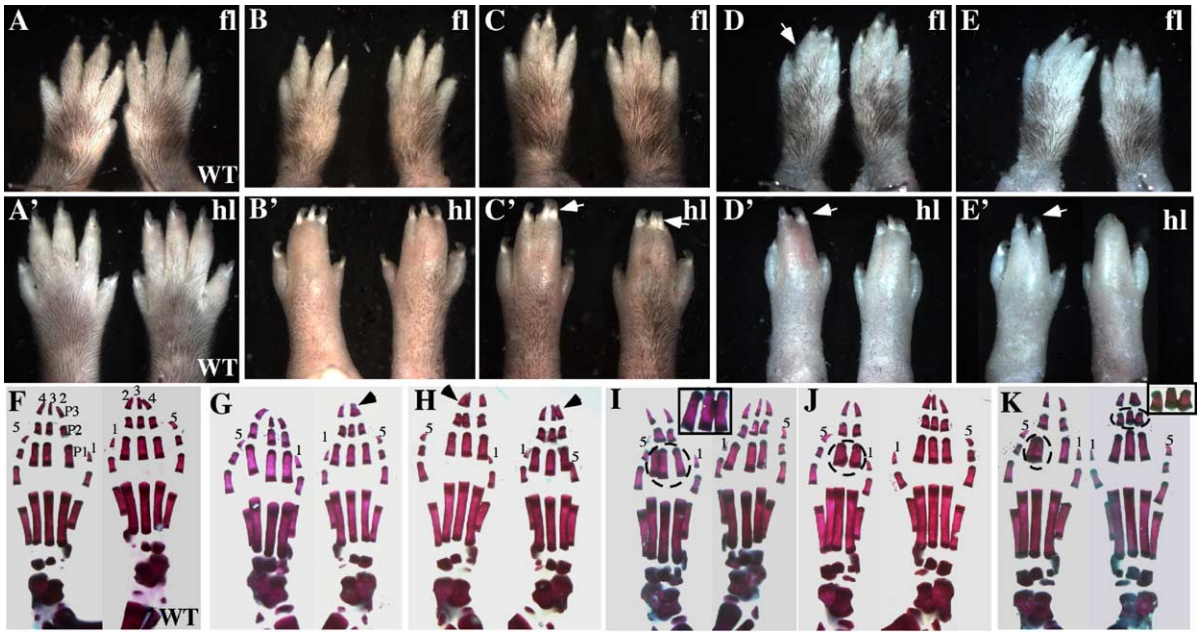

Fig. 2. *N1N2*CKO recapitulate the limb syndactyly of *Jagged2* mutants. (A–E) Gross limb morphology of P9 wild type and *N1N2*CKO mutants. Forelimbs (fl) are presented in panels A–E, and their corresponding hindlimbs (hl) are presented in panels A′–E′. (F–K) Alizarin Red staining of hindlimb skeletal preparations of P9 wild type (F) and representative *N1N2*CKO mutant limbs of variable severity (G–K).

detail (split terminal phalanges) seen in some of the $Jag2^{\Delta DSL}$ hindlimbs (Jiang et al., 1998) was not detected in N1N2CKO mice, perhaps due to a frequency lower than 1/16 in our genetic background.

The phenotypes of γ-secretase-deficient limbs are less severe than that of N1N2CKO limbs

We anticipated that, when all the canonical Notch signaling was removed in the mice lacking both presenilins and are thus γ-secretase-deficient (Mizutani et al., 2001; Saxena et al., 2001), the phenotype would be identical to or more severe than that seen with *N1CKO* and *N1N2*CKO. Indeed, $PS1^{flox/flox}$; $PS2^{-/-}$; *Msx2-Cre* or *PS*DCKO (Saura et al., 2004; Pan et al., 2004) mice exhibited much higher frequency of complete fusions (23 out of 43 pairs of hindlimbs; 53.5%) than *N1*CKO mutants (compare Fig. 1, G1–G2 to D5 for hindlimb, Fig. 1, F to C for forelimb). To our surprise, however, the phenotype of fused *PS*DCKO limbs was not as severe as that of *N1N2*CKO limbs (Figs. 2D′–E′, left hindlimb). Skeletal preparations identified only 2 of 23 fused hindlimbs with slight osseous fusions at adjacent p3 (Fig. 1J, arrowhead); the rest only contained soft tissue fusions (Figs. 1I, I′). Functional γ-secretase was no longer detected in the AER by E11.5 in *PS*DCKO mutants as indicated by lack of V1744 staining (Fig. 3B). In these mice, both N1 and N2 activities are lost simultaneously when functional γ-secretase is removed from the AER; the milder phenotypes observed in *PS*DCKO limbs may be due to the relatively long half life of *PS1* mRNA and protein, leading to more persistent enzymatic γ-secretase activity after Msx2-Cre-mediated conditional inactivation of *presenilin* during a critical period before E11.5 (Wines-Samuelson et al., 2005 and Discussion). Indirectly, this observation also confirms that deletion of *N2* must have been complete prior to E11.5 in *N1N2*CKO mutants to account for the more severe phenotype. The limb phenotypes of loss of function mutants created by *Msx2-Cre* are summarized in Table 1.

Prx1-Cre-mediated loss of Notch signaling from the limb mesenchyme did not produce limb defects associated with fusion but uncovered a role for presenilin in distal phalanx formation

The fact that loss of Notch signaling in the ectodermal-derived AER only caused minor late onset defects in the autopod (digit formation) raised the possibility that the main function of this signaling pathway is in the developing mesenchyme. Both the mesenchymal expression patterns of Notch pathway components (*Notch1* and *2*, *Jagged1*, *c-hairy1* and *Cux1*) and the results from overexpression studies in chick supported this possibility (Myat et al., 1996; Shawber et al., 1996; Tavares et al., 2000; Vasiliauskas and Stern, 2001). Staining with anti-Val1744 antibody did not detect Notch activation in the cells immediately adjacent to the ectoderm, a result inconsistent with a model in which Jagged2 in the AER signals to the mesenchyme. However, sporadic NICD accumulation was detected in limb mesenchyme (Fig. 3, arrows point to the mesenchymal NICD).

To address the role of the Notch signaling in limb mesenchyme (LM), we removed Notch signaling from the developing limb bud mesenchyme with *Prx1-Cre* transgenic line (Logan et al., 2002; insets in Figs. 4B′, D′). Some uncertainty exists in the literature regarding the spatial and temporal pattern of conditional gene inactivation mediated by this Cre line; however, removal of floxed alleles with Prx1-Cre

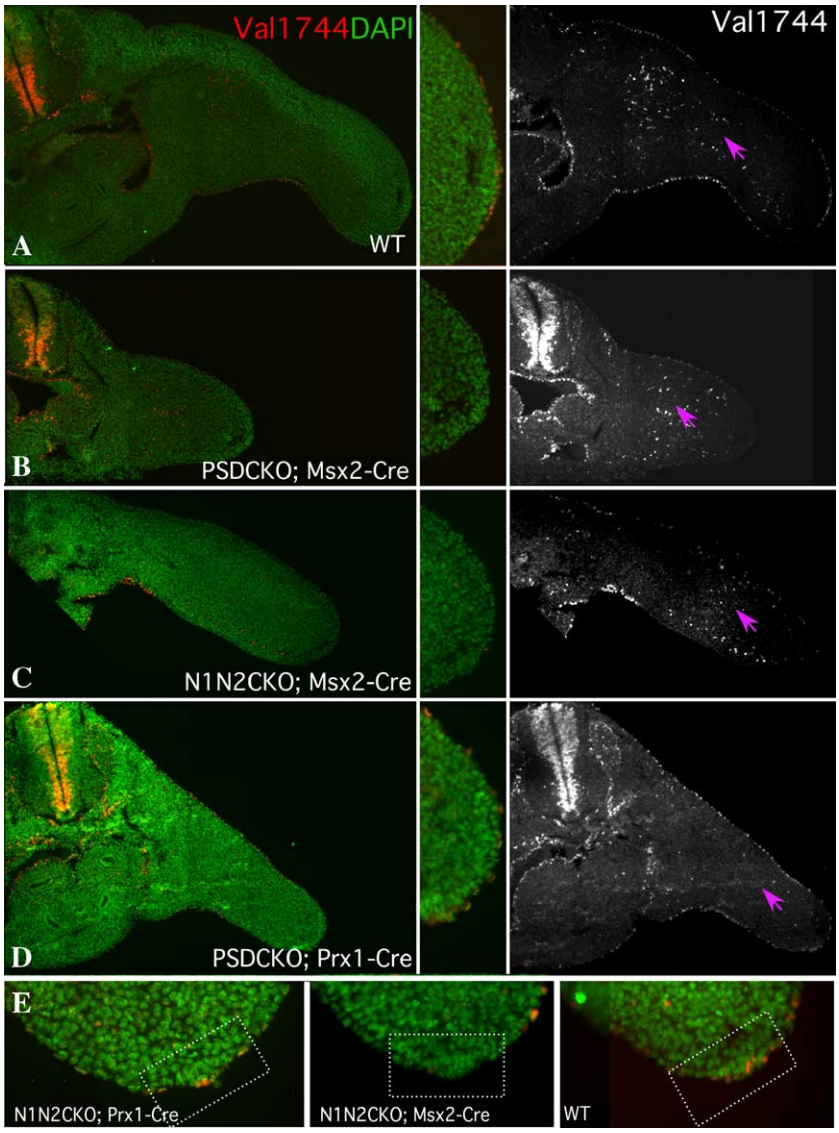

Fig. 3. Cre lines delete Notch in the AER and the mesenchyme by E11.5. (A–D) Evaluation of Cre-mediated deletion in the hindlimb using Val1744 antibody. In these panels, merged images with Val1744 staining and DAPI-counterstained nuclei are on the left, close-up views of the AER are in the middle, and the Val1744 channels are on the right. (E) Distribution of NICD in the forelimb. Ectodermal Val1744 is abundant in WT and Prx1-Cre mutant limbs but missing in AER of the Msx2-Cre mutant limbs (compare Figs. 3A and D with B and C, and also the three panels in E). In panel E, note that, in the Msx2-Cre forelimb bud, complete absence of NICD is seen in the ventral (left) and AER ectoderm, but only partial loss of NICD is observed in dorsal ectoderm (right). As control for antibody staining, the ventral neural tube, where strong Notch activation is seen, is included. The purple arrows indicate sporadic V1744 staining in mesenchyme. The dashed line outlines the perimeter of the limb.

can result in penetrant limb phenotypes (see Discussion). Since we cannot account for the timing of N2 loss, we examined *PS1*$^{flox/flox}$*; PS2*$^{-/-}$*; Prx1-Cre* or LM-*PS*DCKO limbs for loss of γ-secretase. We observed a reduction of mesenchymal NICD accumulation in the LM-*PS*DCKO hindlimb by E11.5 (Fig. 3D, forelimb not shown), suggesting that Prx1-Cre is active in most cells by this time. No obvious phenotypes was observed in external appearance, skeletal preparation (Figs. 4B–B′′ and D–D′′) or function of either fore- or hindlimbs in neonatal or adult single and compound *Notch; Prx1-Cre* mutants animals. Collectively, these results suggested that the major function of Notch1 and Notch2 in skeletal patterning is in the ectoderm prior to E11.5.

Table 1
Limb phenotypes in loss of function Notch mutants

Genotype	Mutant phenotype		
	Complete	Partial	WT
*N1*CKO (65)	4 (6.2%)	55 (84.6%)	6 (9.2%)
*PS*DCKO (43)	23 (53.5%)	18 (41.9%)	2 (4.6%)
*N1N2*CKO (16)	16 (100%)	0 (0%)	0 (0%)
*N1*CKO-fl (35)	0 (0%)	13 (36%)	22 (64%)
*N1N2*CKO-fl (16)	0 (0%)	15 (93.7%)	1 (6.3%)

The limbs of LM-*PS*DCKO mice lacked digit fusion. Skeletal patterning seems to be perfectly normal in LM-*PS*DCKO forelimbs (Fig. 5B′), however, we noticed several abnormalities associated with loss of *presenilin* from limb mesenchyme that were never seen in *N1N2*CKO or in *Jagged2*

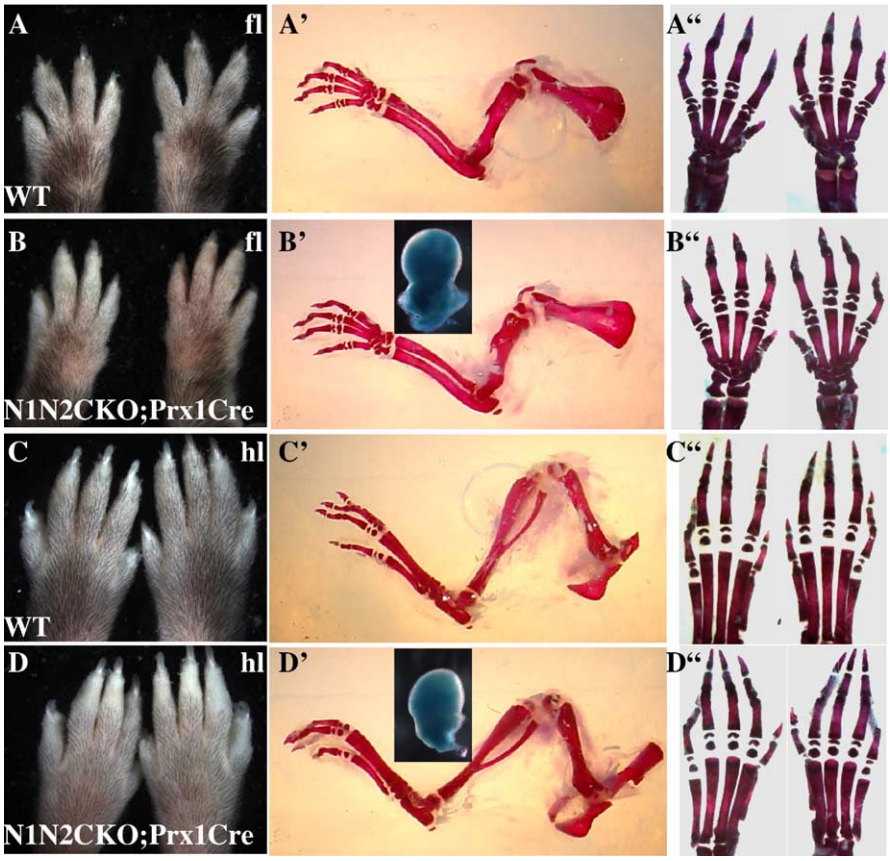

Fig. 4. *Prx1-Cre*-mediated removal of *Notch1* and *2* from limb mesenchyme has no impact on limb development. (A–D) Gross limb morphology. (A′–D′) Alizarin Red staining of the limbs shown in panels A–D. (A″–D″). Close-up of the autopod from panels A′–D′: note that the limbs of N1N2 mutants are indistinguishable from the wild type control. Inset in panels B′ and D′: X-gal staining of limb buds from the fore- (B′) and hindlimb (D′) of E11.5 embryo (genotype: *Prx1-Cre/+; Rosa26R/+*).

mice. Forelimbs digits were clinched (Fig. 5B, arrowheads). In the hindlimb, however, some digits appeared to be truncated (Figs. 5D1–D3, limbs numbered in red); skeletal preparations revealed that the distal phalanx was missing from these digits (p3; Figs. 5D1′–D3′). One animal (out of 7) showed a severe morphological defect (red arrow in 5D3), with almost all the phalanges missing and a deformed metatarsal skeleton (Fig. 5D3′, dashed circle). These phenotypes were not observed in *N1N2*CKO mice and may reflect γ-secretase-independent activity of presenilin which we recently described in the somite (Huppert et al., 2005), another endochondral bone-producing structure. This activity seems to influence the ability of cadherins, PDGF receptors and perhaps other receptors to productively activate Erk and AKT (Baki et al., 2004; Kang et al., 2005; Kim et al., 2005). The possibility that *presenilin* deficiency in mesenchyme affects endochondral skeletal differentiation in a Notch-independent manner is under investigation and will not be further explored in this study.

Skeletal abnormalities resulting from overactivation of Notch signaling in the limb ectoderm are also more severe in the hindlimb than in the forelimb

Since loss of Notch signaling in the limb AER and ectoderm resulted in the mesenchymal phenotypes described here, the impact on the mesenchyme must reflect an indirect effect. If so, ectopic activation of Notch signaling exclusively in the ectoderm should also lead to disruption of limb mesenchyme. To examine this possibility, we utilized a transgenic line in which the *NICD* (constitutively activated Notch1 intracellular domain) transgene is expressed under the control of the relatively strong (but mosaic) chicken β-actin promoter and a floxed "stop cassette" (*Msx2-Cre; CALSL-NICD*; Yang et al., 2004). Msx2-Cre-mediated NICD expression produced dramatic effect on the limb skeletal development, but, again, as seen with the loss of function analysis, the penetrance and severity of these defects varied between fore- and hindlimbs: more severe phenotypes were observed in hindlimbs (Fig. 6A, compare enlarged lower boxes to the upper ones on the right side). Some *Msx2-Cre; CALSL-NICD* mice had extensive hindlimb truncations including complete loss of zeugopods and autopods (Fig. 6B, arrowhead); others only had partial zeugopods with tibia only (Fig. 6C, arrow) and an autopod missing a few digits and/or phalanges (Fig. 6C). Some mice had normal zeugopod but no digit 5 (autopod defect, Fig. 6D). Interestingly, the most severe forelimb defects only involved soft tissue syndactyly (not shown) and occasional osseous fusions of phalanges (Fig. 6B, circle). When we examined another transgenic line that expresses NICD within the weaker, but

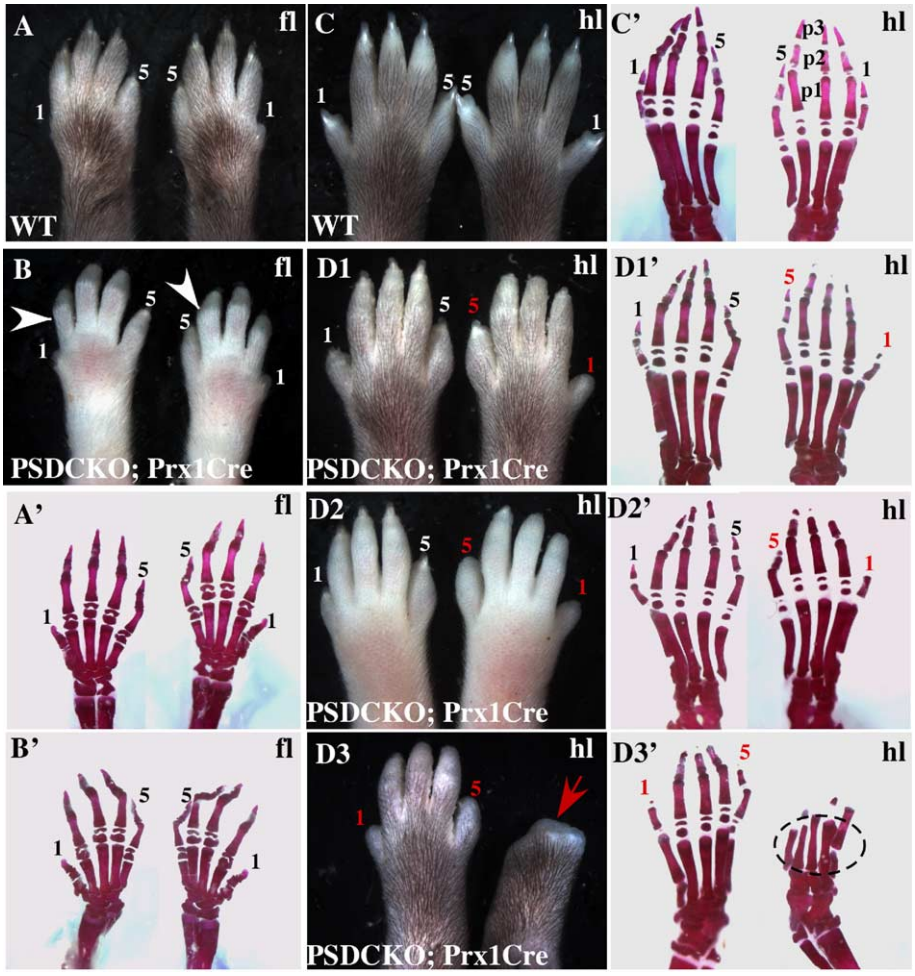

Fig. 5. *Prx1-Cre*-mediated loss of presenilin from limb mesenchyme is associated with abnormal phalanx development. (A–D) Gross fore- (fl) and hindlimb (hl) morphology of limbs from wild type (A, C) and PSDCKO (B, D1–D3) mice. (A′–D′) Corresponding skeletal preparations stained with Alizarin Red from the limb shown in panels A–D. Red numbers indicate phalanx that are reduced or missing.

uniformly expressed, ROSA locus (Murtaugh et al., 2003), milder disruption of limb development was observed relative to the CALSL-NICD and again more severe in hind- vs. forelimbs (not shown).

FGF8 is upregulated in AER upon loss of Notch signaling from limb ectoderm

In the *Jagged2* mutants, *FGF8* expression was upregulated in AER while *BMP2, 7* expressions were downregulated in the interdigital mesenchyme. If *Notch1* and *Notch2* mediate *Jagged2* signaling, we would expect similar impact on *FGF8* upon loss of Notch receptors. Indeed, *FGF8* expression was upregulated in the AER of *N1N2*CKO hindlimbs (Fig. 7D, arrowhead), while the forelimbs of the same animals did not display a noticeable change in *FGF8* level (Figs. 7A, B). *PS*DCKO and *N1*CKO limb buds, which display less severe phenotypes, had a lesser disruption in *FGF8* expression (not shown). Thus, perturbations in *FGF8* expression correspond to the severity of the phenotypes displayed by individual mutants.

Discussion

Removal of Notch receptors or γ-secretase from the limb AER/ectoderm phenocopies the *Jagged2* mutant phenotypes (Jiang et al., 1998; Sidow et al., 1997), arguing that the latter only activates ectodermal Notch signaling. In the absence of Notch receptors, even the most extreme malformations are still restricted to a few phalanges and interdigital soft tissue in the autopod. Therefore, the role of Notch signaling in the limb bud is restricted to the autopod where it contributes to the regulation of mesenchymal apoptosis during digit formation. In this respect, the vertebrate limb differs from the insect wing, where Notch provides critical organizer activity. Interestingly, while *Msx2-Cre*-mediated removal of Notch signaling occurs at an early patterning stage (E9.5–10.5: Sun et al., 2000, 2002) and is completed well before visible formation of the autopod in either fore- or hindlimbs, the defects in digit septation process (E12.5–E13.5) are consistently more severe in the hindlimb. This would be consistent with the notion that Notch signaling acts indirectly and during a precise temporal window to impact this process.

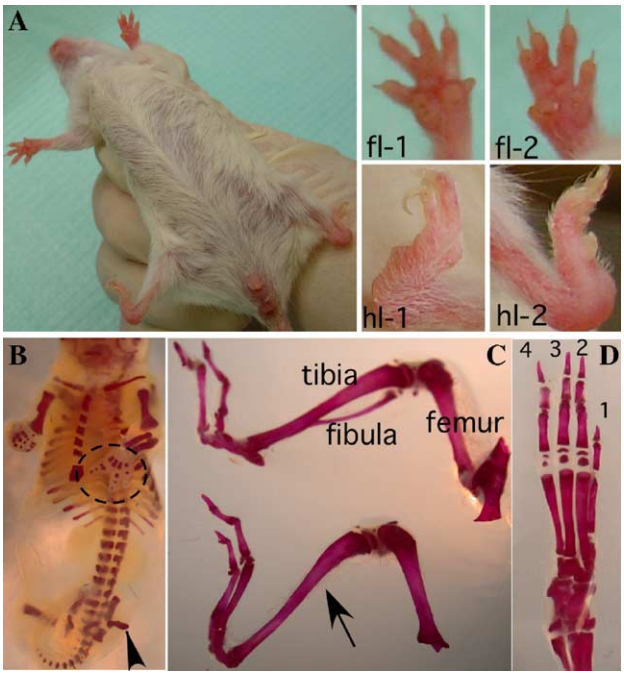

Fig. 6. *Msx2-Cre*-mediated overactivation of Notch signaling in limb ectoderm results in severe hindlimb malformations. (A) Adult transgene *Msx2-Cre: CALSL-NICD* mouse containing a floxed, truncated Notch1 (NICD) under the control of chicken β-actin promoter. The hindlimbs are more severely affected than the forelimbs from the same animal despite the continuous presence of activated Notch1. (B–C) Representative Alizarin-stained skeletal preparations of limbs from *Msx2-Cre: CALSL-NICD* mutant mice harvested at different ages.

Notch signaling acts in the ectoderm to affect limb mesenchymal development

Jagged2 is expressed in the AER of developing limb bud (Jiang et al., 1998), which could signal to either ectodermal or adjacent mesenchymal cells. We could not detect mesenchymal NICD accumulation immediately adjacent to the ectoderm (where *Jagged2* is expressed). Since Notch is activated by short-range signals, this could suggest that the mesenchymal Notch signals are induced by a mesenchymal ligand (such as *Jaggged1*). Analysis of *Prx1-Cre; Sox9^{flox}/Sox9^{flox}* line (Akiyama et al., 2002) demonstrated near complete deletion of this floxed/floxed allele. We report here that LM-*PS*DCKO limbs have reduced mesenchymal Val1744 staining at E11.5 compared with both wild type and *PS*DCKO limbs (Fig. 3). Given the sporadic patterns of Notch1 activation in the mesenchyme and the complicated interpretation of gene deletion due to the invasion of Pax3-positive, Prx1-Cre negative myoblasts into the limb (Bober et al., 1994; Martin and Olson, 2000), we cannot unequivocally demonstrate a complete mesenchymal deletion of our targeted alleles by E11.5. Nonetheless, significant reduction in γ-secretase activity in the hindlimb did not result in any *Jagged2*-like phenotypic consequences. Thus, despite the expression patterns of some Notch pathway components in the limb mesenchyme and the prominent patterning defects observed in overexpression studies conducted here and in the chick, loss of function analysis clearly demonstrated that the *Notch1* and *Notch2* are to a large extent dispensable in the mesenchyme. Therefore, it is the ectodermal activity of *Notch1* and *2* that impacts mesenchyme development. The subtle skeletal defects seen in the mice with mesenchymal loss of *presenilin* could be due to either the impact on other substrates of γ-secretase (Kopan and Ilagan, 2004) or to their functions regulating protein trafficking (Wrigley et al., 2004), PI3K/AKT/GSK3β activity (Baki et al., 2004; Kang et al., 2005) and Erk activation (Kim et al., 2005), any of which could contribute to the formation of distal phalanx. Collectively, these results highlight the inherent problem with gain of function studies.

Notch signaling is required in a narrow developmental window

Previous investigators established that the initiation of Msx2-Cre activity in the hindlimb occurs shortly *before* AER formation in the hindlimb but shortly *after* AER formation in the forelimb (Barrow et al., 2003; Sun et al., 2002). All authors report that this Cre line is active throughout the AER and the ventral ectoderm of both fore- and hindlimbs from E11 onwards. We confirmed that to be the case in our study as well (Fig. 3). Thus, one likely explanation for the difference we observe between fore- and hindlimbs of Msx2-Cre mutants could be the timing of Cre activation. Genes whose function is required before or during AER formation could still be transiently functional in the forelimb; the same gene would be completely inactivated in the hindlimb. Apoptosis begins in the autopod at E12.5, at a stage when the AER and the ventral ectoderm are affected to an equal degree in all limbs. Therefore, the different phenotypes caused by loss of Notch signaling in fore- vs. hindlimb in our study must reflect a requirement for Notch signaling in the AER or the ectoderm prior to E11.5.

Further support for the existence of a narrow temporal window stems from the genetic analysis of an allelic series (*N1*CKO, *PS*DCKO, *N1N2*CKO; Table 1), namely, that the

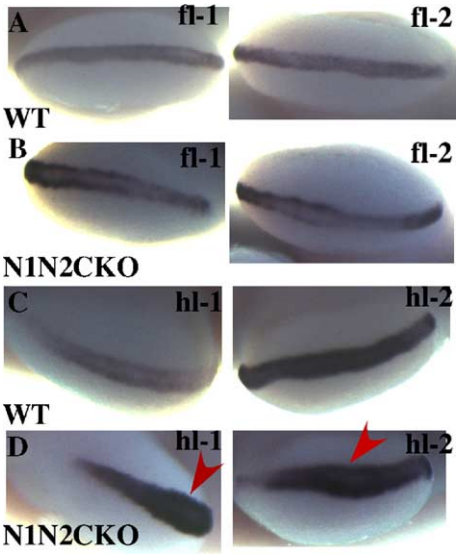

Fig. 7. *FGF8* expression in the AER is upregulated in *N1N2*CKO limbs. Limb buds were isolated from E11.5 embryos. (A–B) Limb buds from wild type embryo. (C–D) Limb buds from one representative N1N2CKO. fl: forelimb. hl: hindlimb.

phenotype of *N1N2*CKO mutant limbs is more severe than *PS*DCKO limbs. We determined that, at E11.5, *PS*DCKO limbs did not accumulate NICD and therefore lost γ-secretase activity. Analysis of hair follicle, another organ with clear P/D axis, also revealed less severe consequences to loss of γ-secretase than loss of N1 and N2 in distal portions of the hair follicle due to temporal difference in loss of activity. While *PS*DCKO mice will eventually loss all canonical Notch signaling due to loss of γ-secretase activity once presenilin proteins were degraded (Mizutani et al., 2001; Saxena et al., 2001), perdurance of presenilin (and thus γ-secretase enzymatic activity) allowed for sufficient Notch activity to persist in follicles, whereas loss of both *Notch1* and *Notch2* in the same domain abrogated all relevant Notch signals (Pan et al., 2004). It is likely that the limb P/D specification system proceeds normally in many *PS*DCKO limbs because catalytic γ-secretase functions persist long enough during a critical early stage to support proper patterning in the autopod. In contrast, *N1N2*CKO mice generated with the same Cre line display a more severe phenotype because Notch receptors act in a stoichiometric manner, and Notch signaling is sensitive to the amounts of receptor on the cell surface (Sakata et al., 2004; Shaye and Greenwald, 2002; Wilkin et al., 2004).

Alternative (or in addition to) to a role in the AER, Notch signaling may impact the autopod from the dorsal and/or ventral ectoderm. A signal emanating from the limb ectoderm during late limb bud stages could contribute to regulation of interdigital apoptosis. The differential penetrance seen between fore- and hind- and left and right limbs in the same animal could then be due to the mosaic Cre activity within the dorsal ectoderm of individual limbs (see deletion patterns in Fig. 3). Consistent with this possibility, variable yet generally larger domains of Cre expression are detected in hindlimbs (Barrow et al., 2003; Sun et al., 2002); however, in this case, *PS*DCKO limbs should resemble *N1N2*CKO limbs as they are similarly affected by the time the autopod patterns. The results from ectopic activation or loss of Notch in the AER also indicate an early window of sensitivity to Notch signaling in limb development.

Yet, another explanation is that forelimbs may have different gene products involved in autopod patterning, some of which may ameliorate the forelimb phenotype of Notch mutant. Supporting this later hypothesis is the observation that forelimbs of *Jagged2* mutant mice, which are uniformly affected throughout their developmental history, also display a milder forelimb phenotype (Jiang et al., 1998; Sidow et al., 1997). One way to distinguish these two possibilities is to remove Notch signaling by using another Cre line, *RARCre*, which is active in the forelimb ectoderm before the formation of AER but not in the hindlimb (Barrow et al., 2003; Moon and Capecchi, 2000; Moon et al., 2000). The function of Notch signaling in the forelimb AER could then be evaluated and compared to the phenotypes observed in this study.

Finally, genetic background has been implicated in modulating the severity of the *Jagged2*^{sm} phenotypes (Sidow et al., 1997). This mechanism cannot account for the penetrance seen in our mutants that are all kept on outbred background yet display a fully penetrant N1N2CKO phenotype. We therefore favor a model in which Notch activity is required during an early developmental stage that will only affect the autopod later during digit septation. A likely mechanism involves Notch promoting (or blocking) production of an ectodermal factor capable of impacting the mesenchyme. FGF8 was proposed to be such a factor (Jiang et al., 1998); this report conforms that *FGF8* perturbations correlate well to the severity of the phenotype.

Two models emerge to account for developmental patterning along the P–D axis of limb bud. The "early specification" model (Dudley et al., 2002; Sun et al., 2002) argues that early events imprint a "memory" in cells yet to form a limb bud (affecting progenitors); the "progress zone" model (Wolpert et al., 1975) posits that the duration of time mesenchymal cells exposed to the AER establishes their patterning (affecting decedents). Our observations cannot differentiate between the two models. An early period of Notch activity impacts a late process (intra-digital apoptosis), indicating that it may impact progenitors but not decedents as proposed by the "early specification" model. However, the consequence of activating Notch signaling (or losing *Notch1* and *Notch2*) in the limb in the correct temporal window could be critical to set up a process (perhaps through FGF8) that unfolds over time to impact mesenchymal cells during autopod formation.

Experimental procedure

Mice

The generation of compound mutants *PS*DCKO, *N1*CKO and *N1N2*CKO with *Msx2-Cre* and *Prx1-Cre* was done essentially as described (Pan et al., 2004).

Histology, immunohistochemical analysis, whole-mount in situ hybridization, X-gal staining and skeletal preparation

For histological analysis, hindlimbs from P9 pups were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Five-micrometer cross sections were collected for hematoxylin and eosin staining. The detection of activated Notch1 by Val1744 Ab and the dilution conditions were performed as described (Lin and Kopan, 2003; Lin et al., 2000). For whole-mount in situ hybridization, embryos were harvested at E11.5 and fixed in 4% paraformaldehyde. The in situ hybridization using digoxigenin-labeled probe was conducted as previously described (Yuan et al., 1999). For X-gal staining, *Prx1-Cre/+; Rosa26R/+* embryo was harvested at E11.5 and fixed, rinsed in PBS and incubated in X-gal solution as described (Kopan et al., 2002). Skeletal preparations of mice were carried out as described (Liu et al., 2002).

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