# Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation

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## SUMMARY

Endochondral bone development begins with the formation of a cartilage template. Chondrocytes within this template undergo a progressive program of maturation from proliferative to prehypertrophic chondrocytes to hypertrophic chondrocytes. The progression of cells through these steps of differentiation must be carefully controlled to ensure coordinated growth. Because the Delta/Notch signaling system is known to regulate cell fate choices, we sought to determine if these molecules might be involved in the progressive cell fate decisions that chondocytes undergo. Here we demonstrate in the chick that Delta/Notch signaling negatively regulates progression from the prehypertrophic to hypertrophic state of differentiation. *Delta-1* is expressed specifically in the hypertrophic chondrocytes while *Notch-2* is expressed in chondrocytes at all stages. Misexpression of *Delta-1* using a replication-competent retrovirus blocks chondrocyte maturation. Prehypertrophic cells form normally but do not undergo differentiation to hypertrophic cells, resulting in shortened skeletal elements that lack ossification. We conclude that Delta-1 acts during chondrogenesis to inhibit the transition from prehypertrophic chondrocytes to hypertrophic chondrocytes, thus defining a novel mechanism for the regulation of the chondrocyte maturation program. In addition, these results reveal a new role for Delta/Notch signaling in regulating the progression to a terminally differentiated state.

Key words: Cartilage differentiation, Chondrogenesis, Delta, Notch, Chick

## INTRODUCTION

The Delta/Notch and Serrate/Notch ligand/receptor pairs are highly conserved signaling systems involved in many embryonic cell fate decisions (reviewed in Artavanis-Tsakonas et al., 1995; Robey et al., 1996; Kimble and Simpson, 1997). The transmembrane ligands, Delta (Dl) and Serrate (Ser), activate their receptor, Notch (N), on neighboring cells. Dl or Ser activation of N results in signaling that can be either inductive or inhibitory (reviewed in Kimble and Simpson, 1997). In either circumstance, Dl and N regulate the differentiation state of individual cells. Our most detailed understanding of Dl/N- and Ser/N-mediated signaling comes from genetic and molecular analyses in systems in which both inductive and inhibitory signaling have been characterized. Examples of inductive signals include specification of the R7 photoreceptor in the *Drosophila* eye, and blastomere specification in *C. elegans* (reviewed in Greenwald and Rubin, 1992; Kimble and Simpson, 1997). In these same organisms, examples of lateral inhibitory signals include *Drosophila* sensory organ precursor development and the *C. elegans* anchor cell/ventral uterine cell fate decision (reviewed in Greenwald and Rubin, 1992; Kimble and Simpson, 1997; Simpson, 1997). In vertebrates, multiple *N, Dl* and *Ser* homologs have been isolated. Mutagenesis and

misexpression of N and its ligands in vertebrates have revealed numerous roles for this signaling pathway during all stages of development. In these studies, Notch has been implicated in neurogenesis, myogenesis, somitogenesis, hematopoiesis, tooth development, craniofacial development and skin appendage development (references within Robey, 1997; Henrique et al., 1997; Kusumi et al., 1998; Mitsiadis et al., 1995, 1997; Jiang et al., 1998; Chen et al., 1997; Crowe et al., 1998; Crowe and Niswander, 1998; Viallet et al., 1998). Gene expression patterns suggest that N, Ser, and Dl may play multiple roles during limb development (Hayashi et al., 1996; Myat et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Vargesson et al., 1998). In the case of *Ser-2 (Jagged-2),* the recent positional cloning of this gene in the mouse limb mutant *syndactylism*, as well as analysis of the *Ser-2* knockout phenotype, both strongly point to a specific role for this gene in limb development (Sidow et al., 1997; Jiang et al., 1998). However, gene targeting studies of Dl-1 and N-1 have not uncovered a role for these proteins in the limb due to early embryonic lethality (Swiatek et al., 1994; Conlon et al., 1995; de Angelis et al., 1997). To directly address the question of whether Notch and Delta play a role in limb development, we have used gain-of-function experiments. In this study, we have misexpressed *Dl-1* in the chick embryonic limb bud and have defined a new role for

Delta in regulating chondrocyte differentiation during endochondral bone formation.

Endochondral bone formation begins with the appearance of a mesenchymal condensation (reviewed in Erlebacher et al., 1995). This condensation expresses *Bone morphogenetic protein receptor IB* (*BmpRIB*) and, as it begins to differentiate, *Type II Collagen* (*Col-II*) (Zou et al., 1997; Hyun-Duck et al., 1988). Around each cartilage condensation, a thin layer of spindle-shaped cells forms and differentiates into the perichondrium. The perichondrium inhibits chondrocyte proliferation and maturation, thereby helping to control the growth and differentiation of the forming cartilage element (Vortkamp et al., 1996; Long and Lisenmayer, 1998). Genes expressed in the chick perichondrium include *Bone morphogenetic Protein 7* (*Bmp7*) and *Parathyroid hormone/parathyroid hormone-related peptide receptor* (*PTH/PTHrP receptor*) (Vortkamp et al., 1996; Macias et al., 1997; Zou et al., 1997). As the cartilage element grows, different zones can be distinguished that demarcate the progressive differentiation of the chondrocytes (see Fig. 7 for zones). Cells at the ends of the elements are immature and undergo rapid proliferation. Adjacent to the proliferation zone are the larger and more sparsely distributed prehypertrophic chondrocytes. In the chick, cells of the prehypertrophic zone express *Indian Hedgehog* (*Ihh*), *PTH/PTHrP receptor* and *Bone Morphogenetic Protein Receptor IA* (*BMPRIA*) (Vortkamp et al., 1996; Zou et al., 1997). In the center of the cartilage element are the terminally differentiated, enlarged hypertrophic cells. These cells express a unique form of collagen, *Type X Collagen* (*Col-X*) (Linsenmayer et al., 1991). The hypertrophic cells eventually undergo programmed cell death and are replaced by osteoblasts, which mature into osteocytes and secrete bone matrix, forming the mature skeleton.

Recently, the modulation of chondrocyte growth and maturation has been attributed to the signaling molecules mentioned above. Misexpression of *Ihh* or constitutively active *BMPRIA,* genes normally expressed in the prehypertrophic chondrocytes, leads to a delay in maturation of the proliferating chondrocytes to the prehypertrophic state (Vortkamp et al., 1996; Zou et al., 1997). Misexpression of *Parathyroid hormone-related peptide* (*PTHrP*) or constitutively active *PTH/PTHrP receptor,* normally expressed in the perichondrium at the cartilage ends (periarticular perichondrium) and the prehypertrophic cells respectively, leads to a similar chondrocyte delay (Lee et al., 1995; Weir et al., 1996; Schipani et al., 1997). Conversely, mice mutant for PTHrP or PTH/PTHrP receptor show the opposite phenotype in that chondrocyte differentiation and ossification are accelerated (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996). These initial studies prompted a model in which IHH produced in the prehypertrophic chondrocytes activates its receptor Patched (PTC), which is expressed in the surrounding perichondrium (Vortkamp et al., 1996). IHH and PTC then act through BMPs in the perichondrium, which in turn activate one of their receptors, BMPRIA, in the prehypertrophic chondrocytes and the periarticular region (Zou et al., 1997). IHH and BMP signaling ultimately results in periarticular PTHrP expression and activation of the PTH/PTHrP receptor (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997). This feedback mechanism serves to negatively regulate the number of cells committing to a prehypertrophic fate.

In this study, we show that *Dl-1* and *N-2* are expressed in chondrocytes during their maturation, and that *Dl-1* is specifically expressed in hypertrophic chondrocytes. Misexpression of *Dl-1* using a replication-competent retrovirus results in a block in chondrocyte maturation from the prehypertrophic to hypertrophic state and leads to a shortening of the cartilage elements. Chondrocytes of *Dl-1*-infected limbs express normal prehypertrophic markers, but do not differentiate further and do not express the hypertrophic marker *Col-X*. This developmental block leads to a lack of ossification of the skeletal elements. In addition, the perichondrium of *Dl-1*-misexpressing limbs is morphologically aberrant and expresses perichondrial markers at decreased levels. These studies define a new step in the regulation of chondrocyte maturation in progression from the prehypertrophic to hypertrophic state of differentiation.

## MATERIALS AND METHODS

### RNA probes

Antisense RNA in situ probes were prepared as described: *Dl-1, RCAS* (Crowe et al., 1998), *BmpRIA* (Zou et al., 1997), *N-1, Ser-1* and *Ser-2* (Myat et al., 1996), *N-2* (Crowe et al., 1998), *Ihh* and *PTH/PTHrP receptor* (Vortkamp et al., 1996), *Col II* (Hyun-Duck et al., 1988), *Col X* (Ninomiya et al., 1986), *Bmp7* (Francis-West et al., 1995), *Wnt-5a* (Dealy et al., 1993), *Evx-1* (Burrill et al., 1997), *Msx-1* and *Msx-2* (Robert et al., 1991), *Hox d11* and *Hox d13* (Izpisua-Belmonte et al., 1991), and *PTHrP* (Schermer et al., 1991). A *Gdf-5* probe was kindly provided prior to publication by F. Luyten. Digoxigenin and $^{33}$P labeling were done according to the manufacturer's directions.

### In situ hybridization

White Leghorn chicken eggs were obtained from SPAFAS (Norwich, CT) and incubated at 39°C for noted lengths of time. Embryos were fixed and processed for either whole-mount in situ hybridization (Henrique et al., 1995) or for paraffin sectioning and RNA section in situ hybridization (digoxigenin-label, Neubuser et al., 1995; radioactive label, Manova et al., 1990). Modifications to the whole-mount procedure were as in Crowe et al. (1998). Modifications to the radioactive protocol: use of [$^{33}$P]UTP-labeled probes and 50% formamide in the hybridization mix.

### Viral infection

RCASBP(A)/c-*Delta-1* was kindly provided by D. Henrique (1997). Transfection and growth of RCAS viruses were performed as described by Morgan and Fekete (1996). Concentrated virus with a titer of $3.7\text{-}5 \times 10^7$ pfu/ml was injected into the presumptive forelimb or hindlimb region of Hamburger and Hamilton (1951) (HH) stage 13-16 embryos. At stage/day 30/7 or older, the injected limb was shorter >90% of the time in >500 injections. Injections that encompassed the entire limb region resulted in extensive viral infection to all cartilage elements and soft tissue, as assayed by expression of viral or exogenous *Dl-1* transcripts. Injection into either the anterior or posterior part of the presumptive limb region resulted in localization of viral transcripts to the anterior or posterior cartilage elements, whereas viral transcripts were more widespread within the soft tissues. Infected cartilage elements in both cases showed phenotypes whereas uninfected elements served as an internal control and were similar to the elements of uninjected contralateral limbs.

### Skeletal staining

Alcian green staining of cartilage was done as previously described in Niswander et al. (1993). Alcian blue/Alizarin red staining was modified from Otto et al. (1997). Alcian blue concentration was

increased to 0.75 mg/ml and all staining and wash steps were shortened. Safranin O staining of sections was as described in Prophet et al. (1994).

### Bromodeoxyuridine (BrdU) labeling

Proliferating cells were detected using the Amersham BrdU labeling kit. Day 7 embryos, virally infected at day 2, were injected in the amnion near the heart with 250 μl of BrdU. They were reincubated for another 2.5 hours, killed, fixed in carnoy fixative and embedded in paraffin. BrdU-labeled cells were detected as per manufacturer's instructions.

### TUNEL staining

Apoptotic cells were labeled by the TUNEL method. The protocol was modified from Shen et al. (1997). Briefly, paraffin sections were rehydrated to PBS, fixed in 4% PFA and then rinsed in PBS. Slides were then incubated for 15 minutes in 20 μg/ml proteinase K, rinsed in PBS and refixed in 4% PFA. After incubating 5 minutes in TdT buffer, slides were placed at 37°C for 1.5 hours in TdT buffer plus DIG-UTP and TdT enzyme. Slides were then rinsed in PBS and blocked in 1% BBR (Boehringer-Mannheim) in MAB before incubation overnight at 4°C in 1:5000 anti-DIG/AP in 1% BBR. The next day, slides were washed several times in TBS before overnight incubation with the BM purple substrate (Boehringer-Mannheim).

## RESULTS

### *Delta-1* misexpression causes skeletal abnormalities

Replication-competent retrovirus-mediated misexpression of *Dl-1* in the presumptive limb region of HH stage 13-16 chick results in a severe shortening of the limb skeletal elements (Fig. 1A,C). Although individual embryos were affected to differing extents (illustrated by error bars in Fig. 2), shortening of the limb cartilage elements was observed in >90% of the cases. The difference in length of the cartilage elements was first detectable at day 6-7 of incubation (HH stage 29-31). This difference in size became more apparent on subsequent days

such that by embryonic day 8 (E8) the infected elements averaged 65% the length of the contralateral control elements (Fig. 2). Lethality of *Dl-1*-infected embryos beginning at E5 made the collection of highly infected older embryos (E9 and older) difficult. The reason for this lethality is unknown. Despite this complication, a few live embryos obtained at E9-10 showed extreme shortening, with some elements as small as 40% the length of the contralateral control (Figs 1A,B, 5A). Although the size of the skeletal elements was dramatically affected, skeletal patterning appeared relatively normal and all elements were present within the limb (Fig. 1).

In addition to the shortening of the limb skeleton, other cartilage phenotypes were observed. Frequently, individual cartilage elements were bent and no longer remained within one plane (arrows in Fig. 1B,D; 68%; $n=19$). In extreme cases, cartilage elements were severely twisted. Also observed in *Dl-1*-infected limbs were gaps within the cartilage elements where it appeared to have split (Fig. 7A; see below; 24%; $n=33$). *Dl-1*-infected limbs also displayed aberrant digit **2** formation such that the element consisted of one unjointed condensation, unattached at the wrist (arrowhead in Fig. 1D and 7Bc; see below; 45%; $n=29$). In addition, changes in matrix production were occasionally observed, as reflected in a reduction in Alcian blue staining and *Col-II* expression levels in some elements (radius in Fig. 5A and H compared to D).

### *Delta-1* misexpression does not interfere with early limb growth or patterning

The progress zone is a region of undifferentiated and highly proliferative mesenchyme cells at the distal tip of the growing limb bud, beneath the apical ectodermal ridge (AER). Cells within the progress zone gain progressive positional information as the limb bud grows. Cells that leave the progress zone early contribute to proximal structures while those that remain longer form more distal structures, thus resulting in proper proximodistal patterning of the limb. Summerbell et al.
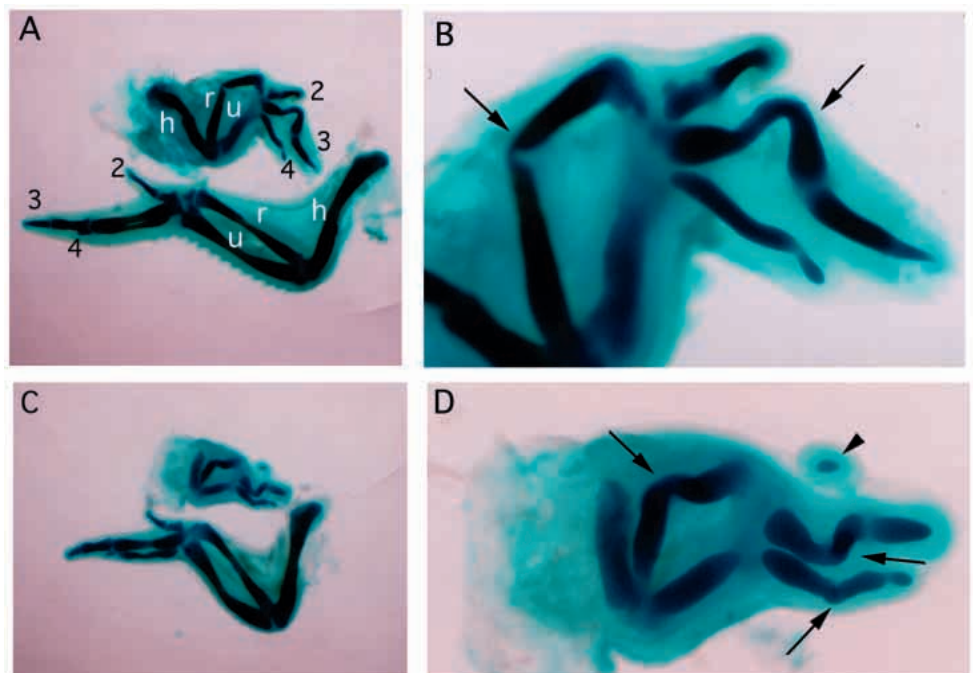


**Fig. 1.** *Delta-1* misexpression results in shortened and bent limb skeletal elements. (A,C) *Dl-1*-injected limb elements (top) are reduced in length as compared to the contralateral uninfected control (bottom) but all elements are present. (B,D) Higher magnification of *Dl-1*-infected limbs shown in A, C to highlight bent cartilage elements (arrows) and aberrant changes in digit 2 (arrowhead). Embryos in A, B fixed at E9 and C, D fixed at E8; h, humerus; r, radius; u, ulna; 2,3,4, digits **2**, **3**, **4**.
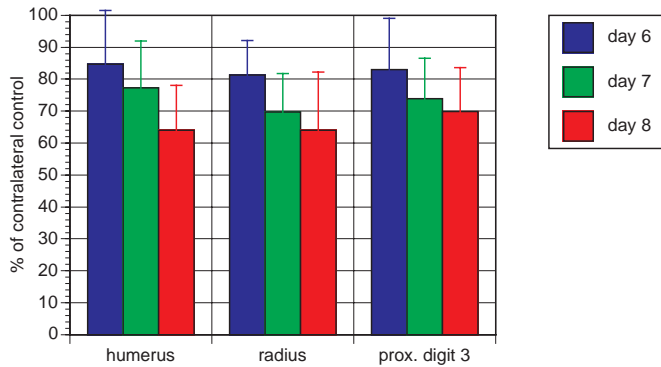
**Fig. 2.** The length of *Dl-1*-infected limb elements is progressively reduced. Reduction in the size of *Dl-1*-infected skeletal elements is first observed at E6 and becomes more apparent with increasing age. Bars represent the mean length of the *Dl-1*-infected elements as a percentage of the contralateral control element. The measurements are derived from 6-8 embryos examined on each day. The error bars indicate standard deviation. Large deviations in the data set result from the variable level of infection of each embryo, which leads to variable degrees of phenotype. prox. digit 3, the proximal most phalange of digit **3**.

(1973) proposed a model in which a reduction in size of the progress zone would result in a properly patterned limb skeleton in which each element is reduced in size. Expression patterns of *Notch* and *Ser* are consistent with a potential role for these molecules in the progress zone. *Ser-1* transcripts are found in the distal and posterior mesenchyme of the growing limb bud from at least stage 21 to 26 (Myat et al., 1996; and data not shown). Expression of *Ser-2* and *N-1* is observed in the AER during HH stages 20-26 (Myat et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997 and data not shown). At these stages, *N-2* is ubiquitously expressed at low levels throughout the limb (data not shown). *Dl-1* expression is not detected during early limb bud stages (HH 19-25; data not shown). Despite this, it is possible that *Dl-1* misexpression may interfere with or mimic a role normally performed by Serrate in regulating limb outgrowth.

To address whether misexpression of *Dl-1* may be affecting the size of the progress zone either directly or indirectly, we examined the expression patterns of *Ser* and *N*, and of proposed progress zone markers, in *Dl-1*-infected limbs. *N-1* transcripts were upregulated rapidly upon *Dl-1* misexpression (within 48 hours (E4)), 2 days prior to observable size differences between contralateral and injected limbs (Fig. 3A,B; 80% of embryos, *n*=20). This indicates that ectopic Dl-1 is actively signaling prior to the appearance of a visible phenotype. Other genes in the Notch pathway (*N-2*, *n*=12; *Ser-1*, *n*=24; *Ser-2*, *n*=5) showed no detectable change in RNA expression (data not shown). A number of genes are expressed in the distal limb mesenchyme including the progress zone: *Wnt-5a, Evx-1, Msx-1, Msx-2, Hox d11* and *Hox d13* (Dealy et al., 1993; Niswander and Martin, 1993; Robert et al., 1991; Izpisua-Belmonte et al., 1991). The expression of these markers were examined in *Dl-1*-infected limbs (*n*= at least 14 for each gene; a range

of stages were tested) but only the late interdigital expression pattern of *Msx-1* and *Msx-2* showed a detectable change (Fig. 3C,D). At E7 (HH stages 30-32), *Msx* expression remained in the distal mesenchyme and did not progress to the interdigital regions (*Msx-1*, 67%, *n*=9; *Msx-2*, 78%, *n*=9). As this change was subtle and occurred late, and as expression of other markers was not altered, we concluded that Dl-1 most likely did not affect the size of the progress zone. This idea was also supported by the relatively late effect on limb size. Prior to E6, we did not detect a difference between the overall size of the *Dl-1*-infected and the contralateral control limbs. However, by E6, the *Dl-1* limbs started to be smaller, a difference that became more pronounced as development proceeded. For a reduced progress zone to play a role in the Dl-1 limb reduction, a difference in size of the limb should be observed during the stages that the elements are specified within the distal tip mesenchyme. At E6, all of the limb cartilage elements have condensed and Summerbell et al. (1973) showed that the limb has already attained all of the positional information necessary for full proximodistal outgrowth. For this reason, we sought to determine if Dl-1 was acting during chondrogenesis to alter skeletal length.

### *Dl-1* and *N-2* are expressed during chondrocyte differentiation

To determine if Dl-1 plays a role in chondrogenesis, we examined the expression patterns of *Dl-1, Ser-1* and *Ser-2*, and *N-1* and *Ser-2* in the cartilage elements of wild-type embryos. Chondrocytes in each forming cartilage element follow a progressive differentiation program resulting in three zones of cells: proliferating, prehypertrophic and hypertrophic. To determine in which zones these genes may be expressed, we compared the expression patterns of the Notch family of receptors and ligands in differentiating chondrocytes, with the known expression patterns of *Ihh*, a prehypertrophic
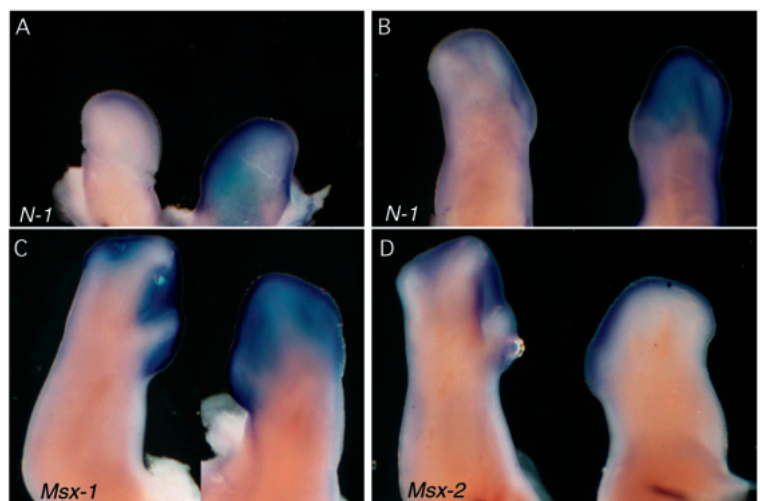


**Fig. 3.** Misexpression of *N-1, Msx-1* and *Msx-2* in *Dl-1*-infected limbs. In all panels, contralateral limbs are on the left and injected limbs on the right. (A,B) *Notch-1* expression is upregulated upon *Dl-1* misexpression prior to the time of an observable phenotype. *N-1* induction 48 hours (A) and 96 hours (B) after *Dl-1* injection. (C,D) *Msx-1* and *Msx-2* expression remained in the distal tip mesenchyme and did not progress into the interdigital tissue (fixed at E 7).

chondrocyte marker and *Col-X*, a hypertrophic marker (Linsenmayer et al., 1991; Vortkamp et al., 1996). While *Ser-1, Ser-2* and *N-1* transcripts were not detected in the chondrocytes or perichondrium, (data not shown, examined at E8 and E10, times at which all developmental stages of maturation are represented), both *Dl-1* and *N-2* are expressed. At E6, the radius and ulna of the forelimb have differentiated into all three zones. At this stage, the *Ihh* and *Col-X* expression domains overlap in the center of the element (Fig. 4C,D). Analysis of alternate sections shows *Dl-1* transcripts colocalize with *Col-X* in the center region (Fig. 4B). This is in contrast to *N-2*, which is ubiquitously expressed in all chondrocytes (Fig. 4A). *Dl-1* expression remains colocalized with *Col-X* in the hypertrophic chondrocytes at all stages examined (E6-10; Fig. 4G and data not shown). At E8, ossification of the radius and ulna is observed at the center of each element. At this stage, *Col-X* expression decreases in the ossifying regions but persists in regions flanked by the *Ihh* expression domains (Fig. 4F,H). In contrast, *Dl-1* transcripts at this stage are detected in the center of the element, with expression most strong in the hypertrophic *Col-X* domain (Fig. 4G). *N-2* expression at this stage remains ubiquitous (Fig. 4E). Thus, *N-2* is expressed in chondrocytes at all stages of maturation, whereas *Dl-1* expression specifically marks the hypertrophic chondrocytes.

### *Dl-1* misexpression blocks chondrocyte maturation from the prehypertrophic to hypertrophic state

Since *Dl-1* and *N-2* are expressed in the maturing chondrocytes, we hypothesized that the cartilage phenotype associated with *Dl-1* misexpression might stem from an effect on chondrocyte proliferation, apoptosis or differentiation. To test for an effect on proliferation or cell death, we examined embryos at E7 (HH stage 31), a stage at which the cartilage shortening phenotype is readily observed. BrdU labeling showed that *Dl-1*-infected cartilage elements contain two zones of proliferating cells similar to those of controls. The overall size of the proliferation zones and number of BrdU incorporating cells were also similar between control and *Dl-1*-infected limbs (data not shown; $n=10$). Using the TUNEL assay to detect apoptotic cells, we noted that, overall, *Dl-1*-infected limbs showed more cell death than contralateral control limbs. However, this cell death was distributed randomly throughout the limbs and did not correspond specifically to the chondrocytes or the perichondrium (data not shown; $n=4$). We conclude from these results that any possible reduction in cell proliferation or increase in cell death cannot fully account for the observed dramatic size difference between the injected and contralateral control limb cartilage elements.

Next we examined chondrocyte differentiation in the *Dl-1*-injected limbs.

The zones of chondrocyte differentiation are distinguishable both morphologically and molecularly. In an uninfected limb, the small and closely packed proliferating cells are found at the ends of the cartilage elements. Just adjacent, the prehypertrophic chondrocytes are larger and spaced further apart. The prehypertrophic cells specifically express markers such as *Ihh* and *PTH/PTHrP receptor* (Vortkamp et al., 1996). Both proliferating and prehypertrophic cells are marked by expression of *Col-II* (Hyun-Duck et al., 1988). The mature, hypertrophic cells, at the center of the element, are flanked by the two zones of prehypertrophic cells. The hypertrophic cells do not express *Col-II*, but do express *Col-X* (Linsenmayer et al., 1991). The hypertrophic chondrocytes eventually undergo programmed cell death, and are replaced by osteoblast cells that secrete bone matrix. Regions of the differentiating cartilage element that have been replaced by bone are stained by Alizarin red, whereas the chondrogenic regions stain with Alcian blue, Alcian green and Safranin O.

Alizarin red staining of *Dl-1*-infected limbs revealed greatly reduced levels of ossification. Proximal elements (i. e. the humerus and femur) stain very little with Alizarin red as compared to the control, while more distal elements are often completely devoid of bone (Fig. 5A; $n=8$). Safranin O staining of the cartilage matrix shows that the cells of *Dl-1*-infected cartilage elements are small and closely packed, suggesting a lack of hypertrophic cells (Fig. 5F,G versus B,C). Together, the histological analyses show that chondrocyte differentiation and
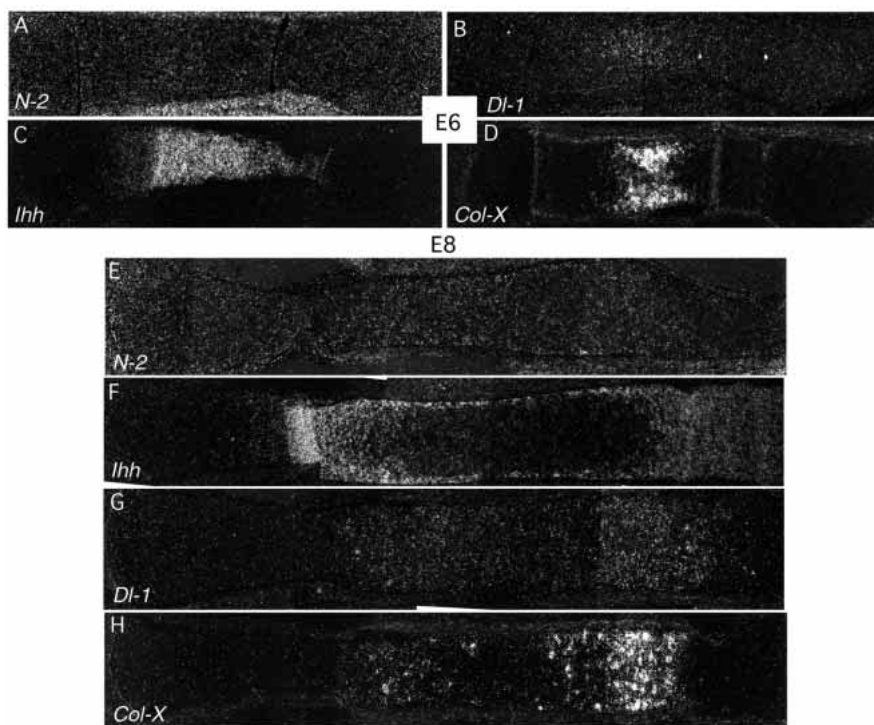


**Fig. 4.** Endogenous expression of *Notch-2* and *Delta-1* during cartilage maturation. (A-D) Alternate sections of an E6 forearm skeletal element. (E-H) Alternate sections of an E8 forearm element. (A,E) *N-2* is ubiquitously expressed in the cartilage at all stages of differentiation. (B-D) At E6, *Dl-1* transcripts overlap those of *Ihh* and *Col-X*. (F,G) At E8, *Ihh* (F) is expressed in the two zones of prehypertrophic chondrocytes and slightly overlaps the domain of *Col-X* (H) expression in hypertrophic chondrocytes. *Dl-1* (G) is expressed in the *Col-X* domain but also remains on in hypertrophic cells that have downregulated *Col-X*.
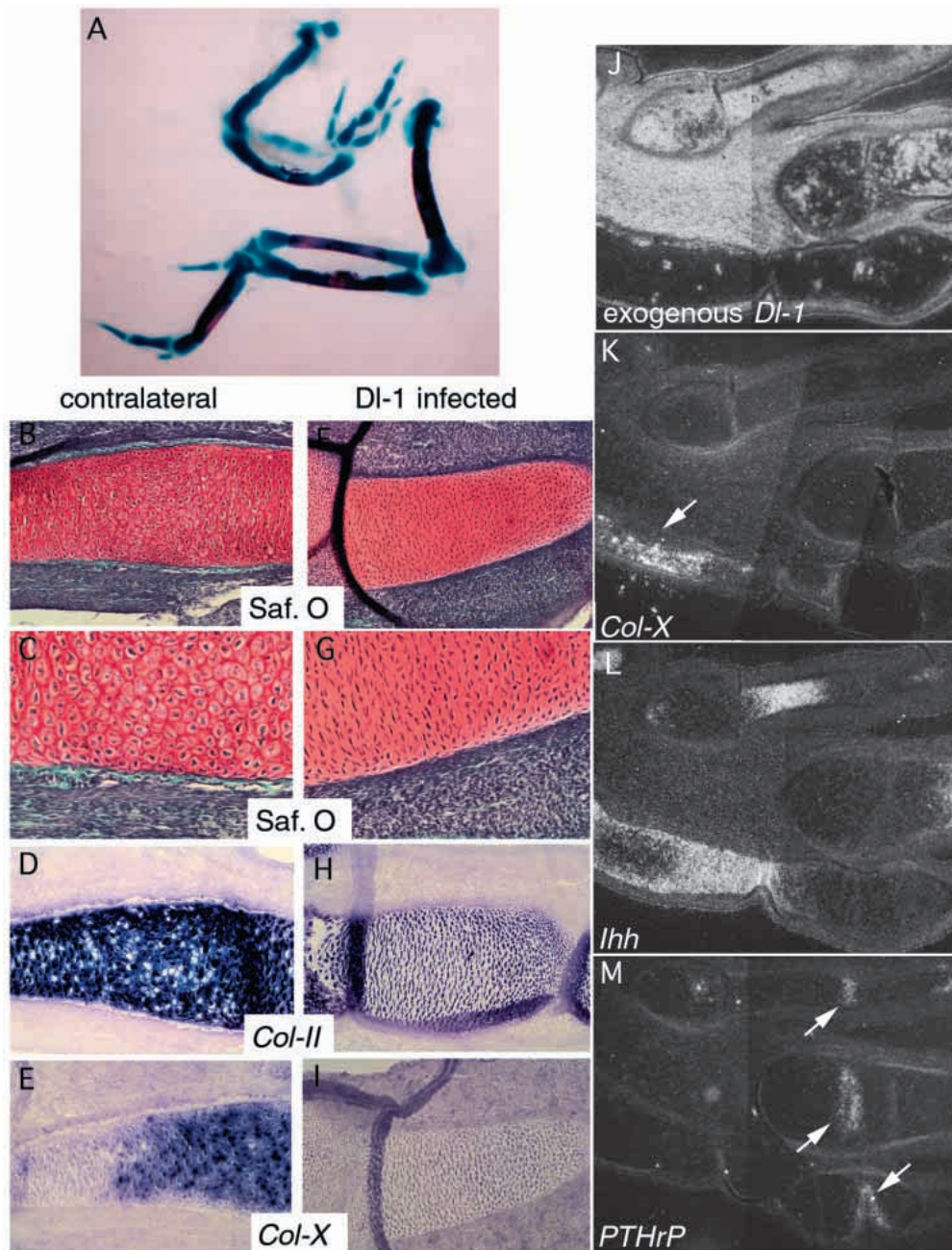
**Fig. 5.** Hypertrophic differentiation and ossification are blocked following *Dl-1* misexpression but prehypertrophic differentiation occurs normally. (A) Ossification is greatly reduced or absent in E10 *Dl-1*-infected limbs (top limb) as determined by lack of Alizarin red staining in the radius and digits and reduced staining in the humerus and ulna. Note the reduced Alcian blue staining of the radius. (B-I) Sections through digit region of contralateral control (B-E) and *Dl-1*-infected (F-I) wings fixed at E8. (B,C,F,G) Safranin O staining shows absence of hypertrophic cells in the infected limb. (C,G) Higher magnification of center of element. (D,E,H,I) RNA probes for proliferating/prehypertrophic (*Col-II*) and hypertrophic (*Col-X*) chondrocytes confirms the absence of cells undergoing hypertrophic differentiation (compare H to D and I to E respectively). Note that *Col-II* expression appears reduced in the *Dl-1*-infected element (H versus D). (J-M) Radioactive in situ hybridization of serial sections through the digit region of a *Dl-1*-infected leg fixed at E8 demonstrates that prehypertrophic differentiation has occurred normally. (J) Hybridization with a *Dl-1* probe to detect exogenous transcripts. The upper two digits and surrounding soft-tissue are highly infected whereas the lower digit is relatively uninfected. (K,L) Hypertrophic differentiation has proceeded normally in the uninfected digit as shown by *Col-X* expression (not shown) and downregulation of *Ihh* in the center of the element but has not occurred in the infected digit. (L) The prehypertrophic chondrocyte-specific markers *Ihh* and *PTHrP receptor* (not shown) are expressed in both the infected and uninfected digits. (M) *PTHrP* transcripts are detected in the periarticular and forming joint regions of both infected and uninfected elements.

ossification are affected in *Dl-1*-infected limbs. To pinpoint the step at which cartilage differentiation was affected, we

analyzed expression of markers of the prehypertrophic (*Col-II*) and hypertrophic (*Col-X*) chondrocytes. All *Dl-1-*

misexpressing cartilage elements (alternate sections hybridized with viral probe) are *Col-II* positive throughout and do not express *Col-X* (Fig. 5H,I and data not shown; observed in all cases; *n*>8 limbs). In contrast, in the contralateral control, *Col-II* is downregulated in the hypertrophic center of the element where *Col-X* is expressed (Fig. 5D,E). To determine if the block to hypertrophic differentiation is a direct result of *Dl-1* expression in the chondrocytes and not an overall delay in limb development, we examined limbs in which infection spread throughout the limb soft tissue, but was limited to only some of the cartilage elements (e.g. anterior elements in Fig. 5J). In this scenario, the uninfected elements serve as an internal control. When alternate sections were probed for *Col-X* and exogenous *Dl-1*, it became obvious that, while infected elements lacked *Col-X* expression, uninfected elements showed normal accumulation of *Col-X*-positive hypertrophic chondrocytes (Fig. 5J,K; in all cases, *n*=6 limbs). This correlation of the virus with the phenotype rules out the possibility that the infected limb was delayed overall in development. Our results demonstrate that hypertrophic cells marked by *Col-X* are absent in *Dl-1*-infected elements at least 3 days after these cells form in control elements. However, the lethality imposed by *Dl-1* misexpression made it impossible to determine whether cartilage differentiation was blocked or just delayed. In sum, the histological and molecular results demonstrate a lack of hypertrophic chondrocytes in *Dl-1*-infected limbs and an absence of ossification.

Having shown that *Dl-1* misexpression inhibits hypertrophic differentiation, we next sought to determine whether this block occurs before or after the differentiation of the prehypertrophic chondrocytes. To do this, we analyzed expression of prehypertrophic markers. *Ihh* and *PTH/PTHrP receptor* are both expressed in *Dl-1*-infected chondrocytes (Fig. 5J,L and data not shown; 100%; *Ihh n*=8; *PTH/PTHrP receptor n*=4), suggesting that prehypertrophic chondrocytes form normally. However, there was only a single region of prehypertrophic cells, instead of the two normally separated by hypertrophic cells (compare top infected digit to lower uninfected digit in Fig. 5J,L). In addition, expression of *PTHrP* in the periarticular (joint) region appears normal (Fig. 5M; 100%; *n*=3). Therefore, *Dl-1* misexpression does not appear to interfere with the IHH/PTHrP regulatory loop, thought to control the progression from the proliferating to prehypertrophic fate. Instead, our results indicate that Dl-1 signaling negatively regulates progression from prehypertrophic to hypertrophic differentiation.

## Delta-1 misexpression disrupts the perichondrium and results in cartilage abnormalities

The perichondrium is important in regulating chondrocyte growth and differentiation (Long and Lisenmayer, 1998). During normal cartilage maturation, *PTH/PTHrP receptor* and *Bmp7* are both expressed in the perichondrium, a distinct band of cells around the cartilage elements (Vortkamp et al., 1996; Macias et al., 1997) (Fig. 6F,H). In approximately 40% of *Dl-1*-infected elements, we noted aberrant perichondrial formation. Unlike normal perichondrial cells, which are spindle shaped and form a sheath around the cartilage (Fig. 6C versus B), the aberrant cells are rounded and form a broad, disorganized ring around the cartilage elements (Fig. 6A). In addition, *PTH/PTHrP receptor* and *Bmp7* expression are

detected at lower levels and in an indistinct and patchy manner around the cartilage element (Fig. 6E,G,I). However, these striking perichondrial abnormalities are not observed around all *Dl-1*-infected cartilage elements. Some infected elements, which lack *Col-X* expression, have an apparently normal perichondrium, based on histology and gene expression (Fig. 5F-I; data not shown). Thus, we conclude that the observed perichondrium disruption is a secondary effect of *Dl-1*
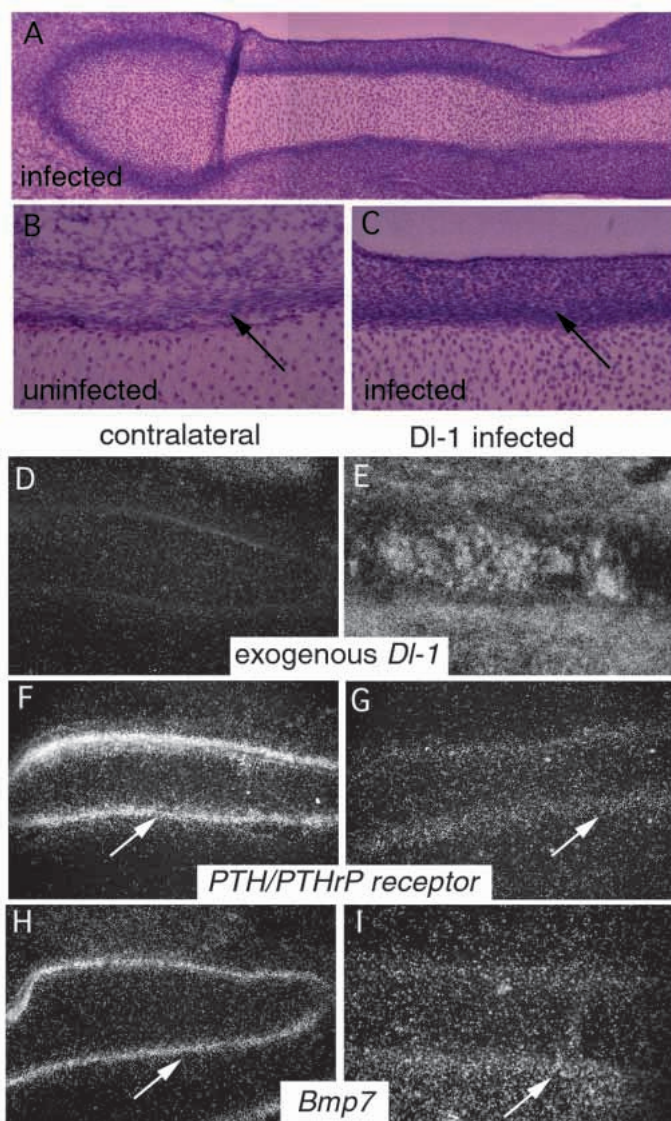


**Fig. 6.** Aberrant perichondrial morphology and gene expression in *Dl-1*-infected limbs. (A-C) Hematoxylin- and eosin-stained sections through the digit region of *Dl-1*-infected (A,C) and uninfected (B) legs. In some infected limbs, the perichondrial cells lose their characteristic spindle-shaped morphology and they appear disorganized relative to control perichondrium (compare arrows in B and C). (D-I) Radioactive in situ hybridization of serial sections through forearm region of contralateral control (D,F,H) and *Dl-1*-infected (E,G,I) wings fixed at E7. (D,E) Misexpression of *Dl-1* is shown with a *Dl-1* probe. Perichondrial markers including *PTH/PTHrP* receptor (F,G) and *Bmp7* (H,I) are expressed diffusely and at lower levels in *Dl-1*-infected limbs relative to contralateral controls (arrows point to perichondrium).

misexpression and is most likely not the cause for the block in chondrocyte maturation.

Another phenotype associated with *Dl-1* infection is apparent 'breaks' in the cartilage elements (observed by Alcian blue staining in E7 and older embryos). At the region of the breaks, the two halves taper off and separate, appearing as two elements rather than one (arrowheads in Fig. 7Aa). The perichondrium associated with such split elements seems to grow around each half to encircle it (Fig. 7Ab,c). Histological sections through these regions demonstrate that chondrocytes at the level of the break still express *Col-II*, but are no longer surrounded by cartilage matrix, judged by lack of Safranin O staining (arrow Fig. 7Ad,e). Chondrocytes at the breakpoint become closely packed and seem to represent a final bridge before separation of the two halves (Fig. 7Ad,e). One explanation is that the cartilage breaks may represent regions of ectopic joint formation. Therefore, we used whole-mount and section in situ hybridization to examine the expression of *Gdf-5*, which normally marks and is involved in forming the joint regions (Storm and Kingsley, 1996; F. Luyten and P. Francis-West, personal communication), prior to and during the time when complete breaks can be observed (E6-E9). In *Dl-1*-injected limbs, *Gdf-5* expression in the joints demonstrates the shortened length of the elements (arrows mark ulna region in Fig. 7Ba – infected limb is on the right). Nevertheless, we did not detect ectopic *Gdf-5* expression in the regions where the breaks occurred (Fig. 7Ba,b and data not shown; *n*=13). However, because the 'broken' phenotype occurs infrequently (24%; *n*=33), and may occur over a short window of time that does not correspond with the timing of normal joint formation, we cannot definitively rule out the possibility of ectopic *Gdf-5* expression.

In addition to the forming joints, *Gdf-5* is normally expressed in the interdigital tissue (Storm and Kingsley, 1996; F. Luyten and P. Francis-West, personal communication). Strikingly, we did observe a change in *Gdf-5* expression in the
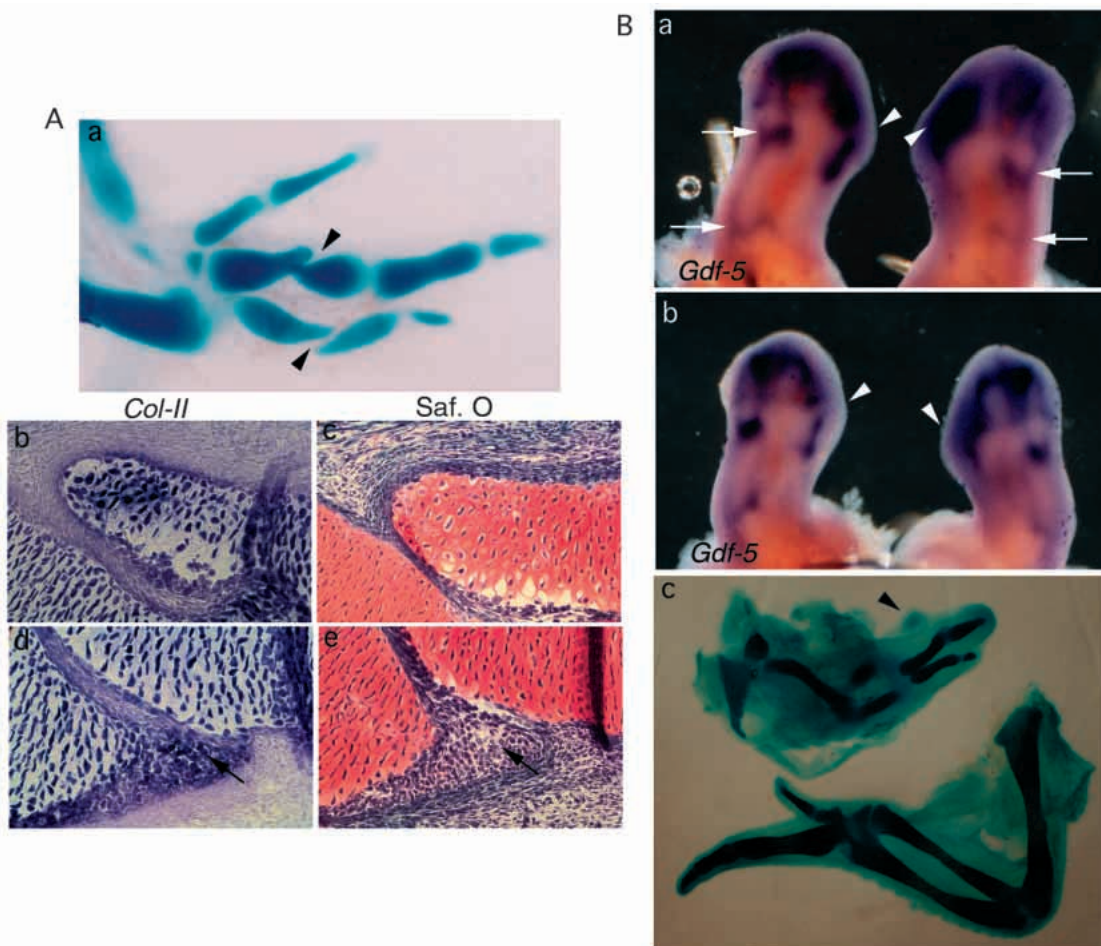


**Fig. 7.** *Dl-1* infection causes breaks in the cartilage and aberrant digit **2** development. (A) Alcian blue staining illustrates aberrant separation of the cartilage. (a) In an E10 embryo, the digit **3** and **4** metacarpals are separated into two halves, which taper off at the broken ends (arrowheads). (b-e) Alternate sections through two regions of an E8 digit exhibiting aberrant breakage. (b,c) The perichondrium surrounds the split elements. (d,e) Cells in the region of the break express *Col-II* but they are densely packed and have little cartilage matrix as determined by lack of Safranin O staining. (B) *Dl-1* misexpression affects digit **2** formation. (a,b) *Gdf-5* is misexpressed in the region of digit **2** condensation (arrowheads) in *Dl-1*-infected limbs (fixed at E6, infected limb on the right). In the contralateral limb, *Gdf-5* expression is excluded from the digit **2** condensation (left). The reduced size of the ulna is also highlighted by the shortened distance between the two regions of *Gdf-5* expression in the elbow and wrist joints (arrows in a). (c) Digit **2** in *Dl-1*-infected limbs is often small, unjointed and unattached to the wrist (arrowhead in c; infected limb is on top).

area of digit **2** condensation. In *Dl-1*-misexpressing limbs, *Gdf-5* expression was observed at E6-E7 throughout the presumptive digit **2** region (70%; *n*=11). This contrasts with its normal expression, around digit **2** in the contralateral limb (arrowheads in Fig. 7Ba,b). This ectopic *Gdf-5* expression could in some way be related to later changes that we observe in digit **2**. Digit **2** is often quite small in *Dl-1*-infected limbs, consisting of one unjointed condensation, which is not attached at the wrist (Figs 1D, 7Bc; 45%; *n*=29). The observed misexpression of *Gdf-5* may cause the digit **2** phenotype, or alternatively, a Dl-1-induced delay in digit **2** condensation may result in an expansion of *Gdf-5* expression as a secondary effect. Although we cannot be sure of the cause of the phenotype, it is interesting to note that Vargesson et al. (1998) have reported endogenous expression of *N-1* in the hand plate, most strongly in the anterior region near digit **2**. This leaves open the possibility that ectopic Dl-1 may affect the normal function of N-1 in this area.

## DISCUSSION

Recent work by a number of groups has detailed the process of chondrocyte maturation, in particular the regulation of the progression of cells from the proliferative to the prehypertrophic state. This process involves an intricate regulatory mechanism linking three different signaling pathways: IHH, BMP and PTHrP (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997). In this report, we have uncovered a novel level of chondrocyte regulation, controlling the progression from the prehypertrophic to the terminally differentiated hypertrophic state. We have demonstrated that this step in the maturation program is negatively regulated by Delta-1.

*Dl-1* and *N-2* are expressed in chondrocytes as they differentiate (Fig. 4). *N-2* is ubiquitously expressed throughout the limb, including all chondrocytes. *Dl-1,* on the contrary, is specifically expressed in the hypertrophic chondrocytes as they form and continues to be expressed in these cells even after they have downregulated *Col-X*.

To discover if the localized expression of *Dl-1* is important, *Dl-1* was misexpressed in the developing cartilage elements. The *Dl-1*-misexpressing limbs are reduced in size, but do not exhibit this phenotype until day 6-7 of development. This observation is consistent with the timing of hypertrophic cell differentiation in control limbs (Vortkamp et al., 1996; Fig. 4). Hypertrophic cells that express *Col-X* are first noted in the radius and ulna of control limbs at about E6. This is not the case in *Dl-1*-infected limbs. The hypertrophic cell marker, *Col-X* was not detected in *Dl-1*-infected cartilage elements, even at E9, and the cells did not morphologically resemble enlarged hypertrophic chondrocytes. As a result of the failure to differentiate hypertrophic chondrocytes, *Dl-1*-infected elements failed to form bone, as assessed by Alizarin red staining (Fig. 5). Although *Dl-1*-infected chondrocytes do not terminally differentiate to the hypertrophic state, earlier stages of differentiation appear to be unperturbed. Proliferation rates were similar between injected and uninjected limbs and both limbs exhibited normal expression of the prehypertrophic markers *Ihh* and *PTH/PTHrP receptor*. Similarly, the periarticular expression of *PTHrP* was unaffected, indicating that the prehypertrophic regulatory loop was not disturbed.

During normal development, differentiation of the hypertrophic chondrocytes results in a dramatic increase in long-bone size due to an increase in both the size of the cells and the amount of surrounding cartilage matrix. Hypertrophic cells increase up to ten times in cytoplasmic volume during their terminal differentiation and the rate of hypertrophic cell



**Fig. 8.** Molecular regulation of chondrocyte differentiation. Schematic representation of zones of cartilage differentiation and corresponding molecular markers. Cartilage differentiation occurs progressively as chondrocytes mature from a population of highly proliferative cells, to prehypertrophic chondrocytes, to hypertrophic chondrocytes. Previous studies indicate that molecular signals within the prehypertrophic chondrocytes (including IHH and BMPR-IA) negatively regulate the progression of proliferative chondrocytes to the prehypertrophic state of differentiation. This negative regulation is mediated through PTHrP and BMPR-IA expressed in the periarticular region. The studies described here define a new step in the progressive maturation of chondrocytes from a prehypertrophic to hypertrophic state. This is negatively regulated by Dl-1 (blue), which is coexpressed with Col-X in hypertrophic chondrocytes. Dl-1 signals to its receptor N-2 (yellow), which is expressed ubiquitously in the cartilage element. Dl-1 signaling from the hypertrophic chondrocytes serves to inhibit prehypertrophic cells from entering this final stage of differentiation.
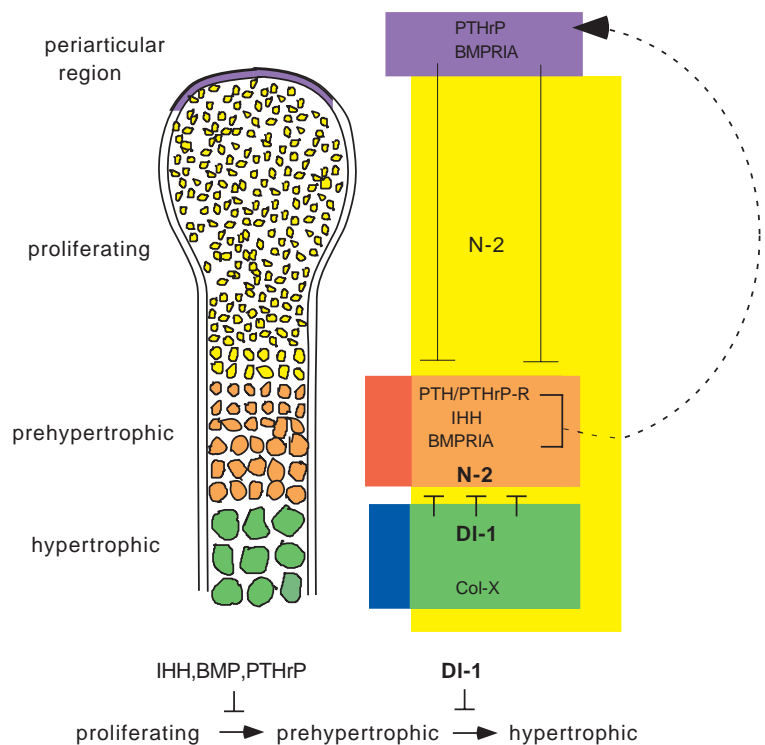
volume increase is proportional to the rate of longitudinal bone growth (Breur et al., 1991). An absence of hypertrophic cells would thus result in a dramatic shortening of the cartilage elements. This corresponds to what we observe in the *Dl-1*-misexpressing limbs. Therefore, as *Dl-1*-infected cartilage elements showed relatively normal levels of proliferation and cell death, the dramatic reduction in element size may be due, in large part, to the lack of hypertrophic differentiation.

We propose that Delta/Notch signaling regulates a previously undescribed checkpoint in the process of chondrocyte maturation (Fig. 8). Previous studies have shown that the progression of cells from the proliferating to the prehypertrophic fate is negatively controlled through the actions of IHH, BMPs and PTHrP (schematized in Fig. 8). IHH in the prehypertrophic chondrocytes signals through PTC in the surrounding perichondrium to regulate PTHrP in the periarticular region (Lanske et al., 1996; Vortkamp et al., 1996). An intermediate step may involve BMP, expressed in the perichondrium and activation of BMPRIA in both the prehypertrophic and periarticular regions (Zou et al., 1997). PTHrP then activates its receptor, ultimately resulting in negative regulation of the progression of chondrocytes to a prehypertrophic fate (Amizuka et al., 1994; Karaplis et al., 1994; Lee et al., 1995; Lanske et al., 1996; Vortkamp et al., 1996; Weir et al., 1996; Schipani et al., 1997). *Dl-1* misexpression does not affect this pathway as *Ihh, PTHrP, PTH/PTHrP receptor* and *Bmp7* are all expressed in infected limbs (Figs 5, 6 and data not shown). Normally, cells that progress to the prehypertrophic state then differentiate into hypertrophic chondrocytes. Our results indicate that Dl-1 regulates this step. As *N-2* is expressed ubiquitously, it is possible that Dl-1 acts by signaling through N-2 to the less differentiated prehypertrophic cells, to other hypertrophic cells or to the perichondrium. Although all three of these scenarios are possible, we propose a model in which Dl-1 signals to the prehypertrophic chondrocytes to limit their entry to the hypertrophic fate (Fig. 8). We favor this model over one in which Dl-1 signals to the hypertrophic chondrocytes, as the hypertrophic chondrocytes do not form in *Dl-1*-misexpressing limbs. Our studies however do not allow us to uncover possible roles that Dl-1 may have in hypertrophic cells themselves. Also, we cannot rule out the possibility that Dl-1 may activate Notch in the perichondrium, triggering signaling through an uncharacterized secondary signal from the perichondrium back to the hypertrophic chondrocytes. We find this less likely however, as addition of PTH to perichondrium-free cultures can alone restore the normal rate of hypertrophic differentiation, which is accelerated following removal of the perichondrium (Long and Lisenmayer, 1998). Thus any role for the perichondrium in regulating this terminal step of chondrocyte differentiation may be minor. Dl-1 is a cell surface ligand and is thought to act on abutting cells. Chondrocytes are separated from each other by cartilage matrix, raising the possibility that Dl-1 may not signal N in a membrane-bound form. It has been suggested that an alternate ligand for N, Ser, can act as a short-range diffusible ligand (Couso et al., 1995), and the same may be true for vertebrate Dl-1, although this has not been examined. In the same respect, we cannot rule out the possibility that Dl-1 causes the upregulation or release of a secondary diffusible signal. Regardless of which cells are receiving the Dl-1 signal, and whether the effect of Dl-1 is direct or indirect, it is interesting to note that, during chondrogenesis, Delta/Notch signaling is regulating the progression of cells to a terminally differentiated state. Delta and Notch are important for numerous cell fate decisions during embryogenesis, but this usually involves a choice between alternative cell fates. During chondrogenesis, Delta does not control this classic type of cell fate decision, but instead regulates progression to a terminally differentiated state.

It is also interesting to note that both *Ihh* and *Dl-1,* negative regulators of successive steps in chondrocyte differentiation, are expressed specifically within the zone that they inhibit cells from entering. *Ihh* is expressed in the prehypertrophic chondrocytes and acts to prevent proliferating chondrocytes from entering this zone prematurely. Similarly, *Dl-1* is expressed in the hypertrophic cells, and this signal acts to prevent prehypertrophic cells from becoming hypertrophic. This expression pattern suggests that these signaling molecules are not responsible for differentiation of the chondrocytes within which they are expressed. In fact, they may become expressed as a consequence of normal differentiation of the chondrocytes. This mode of regulation suggests that chondrocytes eventually must overcome the negative influence imposed by these signals. To accomplish this, chondrocytes may either follow an intrinsic program of differentiation or they may progressively differentiate through the guidance of positive signals. If the chondrocyte program is intrinsic, then when negative factors are misexpressed, progression would be slowed but not halted, and chondrocytes would eventually overcome their block. In contrast, if chondrocytes are guided by positive signals, then misexpression of a negative factor would result in an imbalance of positive to negative signals and would then lead to a permanent block in differentiation. *Dl-1*-injected embryos do not survive long enough to analyze whether hypertrophic differentiation is delayed or blocked; but, studies in transgenic mice misexpressing PTHrP or its constitutive active receptor favor the first model. Both of these transgenic mouse lines show delayed progression from the proliferating to the prehypertrophic state (Weir et al., 1996; Schipani et al., 1997). However, this is eventually overcome and ossification of the elements occurs. A similar transgenic experiment could be done to test whether the same is true for the Dl-1 imposed block.

Another interesting level of regulation, revealed by both the Dl-1 experiments and the previous IHH and PTHrP results, is that there must be coordinate regulation of the size of the differentiation zones. For example, when *Dl-1* is misexpressed and a stall is imposed to the prehypertrophic chondrocyte progression to hypertrophy, an increase was not observed in the number of prehypertrophic cells. Similarly, when *Ihh, PTHrP* or constitutive active *PTH/PTHrP receptor* misexpression was performed, an increase in proliferating chondrocytes was not reported (Vortkamp et al., 1996; Weir et al., 1996; Schipani et al., 1997). It might have been expected that a stall in differentiation would result in an increase in cells in the previous, less differentiated zone. As this does not occur, it implies that there is regulation of zone size. There may be communication between zones or each zone may be regulated independently of the others. At least in Dl-1-infected elements, communication between zones was not apparent as misexpression of *Dl-1* did not affect expression of prehypertrophic regulators such as *Ihh* and *PTHrP*. However,

this type of regulation may occur at a level other than transcription and this remains to be tested. IHH may itself be the regulator of prehypertrophic zone size. If this is the case, a stall in a step downstream of this (Dl-1 control of hypertrophic differentiation) would leave the IHH control intact. As cells fail to leave the prehypertrophic zone to become hypertrophic, IHH levels within the prehypertrophic zone would not decrease. This would result in signaling through PTHrP and would prevent new cells from entering prehypertrophy. This would account for the normal prehypertrophic zone size in *Dl-1*-misexpressing limbs. It remains to be tested whether Dl-1 may be a regulator of hypertrophic zone size.

In addition to a block to chondrocyte maturation, *Dl-1* misexpression also results in disruption of the perichondrium. In some *Dl-1*-infected limbs, the cells in the perichondrium are rounded and disorganized (Fig. 6). This aberrant morphology may also be a consequence of Dl-1-induced changes in cell adhesion (see below). Perichondrial markers such as *Bmp7* and *PTH/PTHrP receptor* are expressed more diffusely and at lower levels in infected than in uninfected limbs (Fig. 6). The perichondrium is known to play an important role in regulating chondrocyte maturation, and thus it is possible that the effects of Dl-1 on chondrocytes may be mediated by changes in the perichondrium (Long and Lisenmayer, 1998). However, our data suggests that the effect of Dl-1 on the perichondrium is unrelated to the chondrocyte defect. The perichondrium disruption is not observed in all cases and elements devoid of *Col-X* expression often have a morphologically and molecularly normal perichondrium (Fig. 5F-I and data not shown). The use of a replication-competent retroviral expression system in this study makes it impossible to determine whether the perichondrium defect is due to *Dl-1* misexpression in the chondrocytes, leading to a defect in signaling to the surrounding perichondrium, or results from *Dl-1* misexpression in the perichondrium itself.

*Dl-1*-misexpressing cartilage elements are often severely bent and occasionally broken into two pieces (Fig. 7A). Cells at the site of the break express a chondrocyte marker, *Col-II*, but are no longer surrounded by matrix (Fig. 7). There are many possible explanations for the appearance of these breaks. On the one hand, the breaks may be a consequence of the severe cartilage bending. On the other hand, it is also possible that Dl-1 may directly affect matrix deposition, as *Col-II* expression and Alcian blue staining is often lower in *Dl-1*-infected limbs as compared to the contralateral control (Fig. 5H vs. D and the radius in Fig. 5A). This loss of extracellular matrix might affect the structural integrity of the cartilage element, leading to bends and breaks. In addition, since *Dl-1* expressing cells are known to be adhesive to one another (Fehon et al., 1990), misexpression of Dl-1 may cause changes in cell adhesion. Thus, it is possible that both the cartilage bending and breakage occur due to the effects of Dl-1 on both matrix production and cell adhesion.

In summary, we have defined a novel mechanism for the regulation of the chondrocyte maturation program. Our results indicate that progression of chondrocytes from the prehypertrophic to hypertrophic state is negatively regulated by Delta-1. Our study also reveals a new role for the Delta/Notch pathway, in regulation of cells to a terminally differentiated state.

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