

## A role for *hairy1* in regulating chick limb bud growth

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### Abstract

Limb growth in higher vertebrate embryos is initially due to the outgrowth of limb buds and later continues as a result of elongation of the skeletal elements. The distal limb mesenchyme is crucial for limb bud outgrowth. Members of the Hairy/Enhancer of Split family of DNA binding transcriptional repressors can be effectors of Notch signaling and often act to maintain cell populations in an undifferentiated, proliferating state, properties predicted for the distal limb mesenchyme. We find that a member of this family, *c-hairy1*, is expressed in this region and that two alternatively spliced isoforms, *c-hairy1A* and *c-hairy1B*, of this gene are produced, predicting proteins that differ in their basic, DNA binding, domains. Viral misexpression of *c-hairy1A* causes a reduction in size of the limb and shortened skeletal elements, without affecting the chondrocyte differentiation program. Misexpression of *c-hairy1B* leads to a significantly lesser shortening of the bones, implying functional differences between the two isoforms. We conclude that *c-hairy1* regulates the size of the limb, suggesting a role for Notch signaling in the distal mesenchyme.

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### Introduction

Limb growth in higher vertebrates starts by outgrowth of the limb buds, and later continues as a result of elongation of the skeletal elements during chondrogenesis and subsequent ossification. The “progress zone model” (Summerbell et al., 1973) proposes that a population of naïve mesenchymal cells at the distal margin of the early limb bud (the progress zone) is maintained in an undifferentiated, proliferating state by signals from the overlying apical ectodermal ridge (AER). Proliferation of progress zone cells leads to the displacement of some cells away from the influence of the AER, causing them to start to differentiate and to acquire proximodistal identity. According to this model, proximodistal identity is specified by the length of time that a particular cell has spent under the influence of the AER: the

longer the exposure, the more distal will be its identity. An alternative model (Dudley et al., 2002; Stark and Searls, 1973; Sun et al., 2002) proposes that cell populations giving rise to different proximodistal structures of the limb are specified within distal mesenchyme early in limb bud development. These cell populations do not expand uniformly, but in sequence, such that the cells of proximal structures start to proliferate first and cells of the distal structures last. Both models imply the existence of a special zone of undifferentiated cells in the distal limb mesenchyme (which we will call the “distal zone”).

Several components of the Notch signaling pathway are expressed during limb bud development from the earliest stages, including *Serrate 1* (*Jagged1*) in the distal mesenchyme (Lindsell et al., 1996; Myat et al., 1996; Shawber et al., 1996), *Serrate 2* (*Jagged2*) in the AER (Laufer et al., 1997; Myat et al., 1996; Shawber et al., 1996), and *Notch1* in the anterior two-thirds of the limb mesenchyme and in the AER (Myat et al., 1996; Vargesson, 1998; Vargesson et al., 1998). *Radical fringe*, a glycosyltransferase that modulates interactions between Notch and its ligands (Bruckner et al., 2000; Fleming et al., 1997; Hicks et al., 2000; Moloney et al., 2000; Panin et al., 1997), is expressed at the boundary between dorsal and ventral ectoderm and acts to position the

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AER (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Mutations in mouse *Serrate* (*Jagged2*) (Jiang et al., 1998; Sidow et al., 1997) result in an abnormal AER leading to syndactyly. These observations provide strong evidence that Notch signaling is involved in development of the AER. Later in development, it has been implicated in the regulation of cartilage differentiation (Crowe et al., 1999). However, the functional significance of the expression of members of this pathway in the distal mesenchyme is not well understood.

*c-hairy1* is a member of the Hairy/Enhancer of split (H/E(spl)) family of bHLH transcriptional repressors. Vertebrate H/E(spl) family genes are implicated in a number of developmental processes, such as neurogenesis (Kageyama et al., 1997), immune system development (Staal et al., 2001), and segmentation of the mesoderm (Pourquié, 2001). A significant body of evidence has accumulated suggesting that the mouse homolog of *c-hairy1*, *Hes1*, is upregulated by and mediates Notch signaling (Jarriault et al., 1995; Ohtsuka et al., 1999; Takebayashi et al., 1994). Notch is a transmembrane protein involved in the general maintenance of precursor cell populations and the generation of cell type diversity in different systems (reviewed in Artavanis-Tsakonas et al., 1999; Lewis, 1998; Mumm and Kopan, 2000).

Here, we address the role of *c-hairy1* in limb development. First, we describe the expression of *c-hairy1* during limb development, and report that it is expressed in the distal mesenchyme from a very early stage. Ectopic expression of *c-hairy1* causes shortening of the limb, and we suggest an involvement of Notch signaling in the distal mesenchyme, controlling the rate at which cells leave this mesenchyme to begin their differentiation. In addition, we have uncovered the existence of two splice variants of *c-hairy1*, with distinct activities.

## Materials and methods

### Egg incubation

Fertile hens' eggs (White Leghorn; SPAFAS, CT) were incubated at 38°C in a humidified environment to give embryos at stages 16–39 (Hamburger and Hamilton, 1951).

### In situ hybridization

Whole-mount in situ hybridization using DIG-labeled RNA probes was performed as described previously (Stern, 1998; Streit et al., 1997). A range of concentrations of proteinase K (10–30 µg/ml) (Sigma) was used for optimal results. For all probes, hybridization and posthybridization washes were done at 68–70°C.

For section in situ hybridization, wings were dissected in PBS and fixed flattened (between the lid and inverted bottom of a 35-mm plastic petri dish) in 4% paraformaldehyde in PBS at 4°C overnight. After fixation, they were embed-

ded in paraffin before cutting 12-µm sections. Dewaxed and rehydrated sections were treated with 20 µg/ml Proteinase K for 8 min at 37°C, postfixed in 4% paraformaldehyde at 4°C, and acetylated for 10 min in freshly prepared 0.1 M triethanolamine, pH 8.0, 0.25% acetic anhydride solution. Prehybridization and hybridization were done in 50% formamide, 5× SSC, pH 7.0, 5× Denhardt's Solution, 250 µg/ml yeast total RNA, 500 µg/ml salmon sperm DNA at 65°C. Posthybridization washes were done in 0.2× SSC at 65°C. Signal was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and the NBT/BCIP reaction.

Chick probes used were: *c-hairy1*, 0.6 kb (Palmeirim et al., 1997; kind gift of Dr. Olivier Pourquié); *Indian hedgehog*, 0.56 kb (Vortkamp et al., 1996; kind gift of Dr. Andrea Vortkamp); *Collagen II*, 0.65 kb (Nah et al., 1988; kind gift of Dr. Andrea Vortkamp); *Collagen X*, 2.2 kb (Linsenmayer et al., 1991; kind gift of Dr. Andrea Vortkamp).

### Cloning and RT-PCR

RNA was isolated by using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's specifications. Reverse transcription was performed by using M-MuLV reverse transcriptase and primed with poly(dT).

To clone the full-length open reading frame of *c-hairy1*, primers (5'-ACGTCTCCCATGCCCGCCGACACGGGC-3' and 5'-AAACTGCAGTCACTACCAGGGGCGCCAGAC-3') were designed to include the most 5' and most 3' sequences of the *c-hairy1* ORF as well as convenient restriction sites. PCR amplification was performed with Platinum Pfx DNA Polymerase (Gibco BRL). The inserts were sequenced to confirm the identity of the products.

Based on the *c-hairy1* (Palmeirim et al., 1997), *Cynops pyrrhogaster HES1* (Shimizu-Nishikawa et al., 1999) and zebrafish *her9* (Leve et al., 2001) cDNA sequences, PCR primer pairs were designed that would span the codons encoding the second and third amino acids of the basic domain (the presumptive exon1–exon2 junction point) of each gene (for *c-hairy1*: 5'-CACCGGCCAGCGCCAGT-CACACG-3' and 5'-CGCGTCCAGGATGAGCATCT-TCAG-3'; for *cpHES1*: 7'-ACAGTCTCGCCCATG-GCAGGAG-3' and 5'-GGCCAGGCTCTCGTTGATGC-3'; for *her9*: 5'-AGCCGATAATATGGAGAAGCAGAC-3' and 5'-TTTTTAAGAGCATCAAGAAT). PCR was performed by using genomic DNA of the appropriate species (*Cp* DNA a kind gift of Dr. Kazuhito Takeshima) as a template and Taq Polymerase (Promega). The inserts were sequenced to ascertain that the desired intron had been cloned.

To detect expression of the two isoforms of *c-hairy1*, the same primers used to clone the *c-hairy1* intron were used in RT-PCRs (see above).

GenBank Accession Nos. are as follows: *c-hairy1A* ORF (AY225439), *c-hairy1B* ORF (AY225440), *c-hairy1* presumptive first intron (AY225441), *CpHES1* presumptive

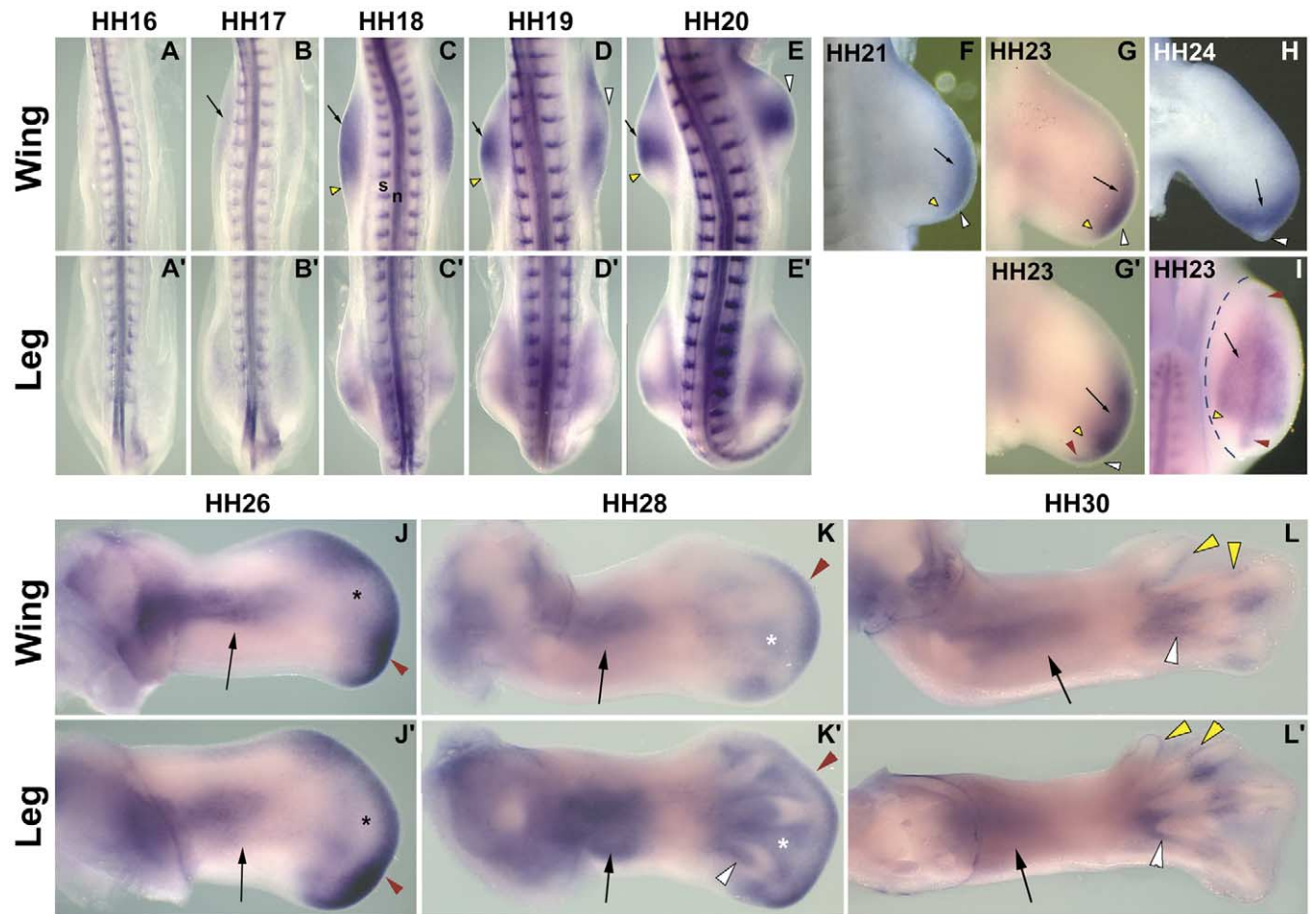


Fig. 1. Expression of *c-hairy1* in developing chick limbs. (A–E, A'–E') *c-hairy1* begins to be expressed (arrows) in limb buds at stage 17 (B, B'). Subsequently, the expression domain is restricted to the anterior two-thirds of the limb bud (anterior to yellow arrowhead, C–E, C'–E') and is strongest in the middle third. No expression is detected in the AER (white arrowheads). *c-hairy1* is also expressed in the neural tube (n in C) and in posterior somites (s in C). (F–I) After stage 21 expression is restricted to the distal limb buds, in a thin stripe immediately underneath the AER (burgundy arrowheads in G' and I) and a broader domain (arrows, F–I) in the anterior distal mesenchyme (yellow arrowheads mark the posterior boundary of this domain), but not in the AER (white arrowheads). The longitudinal section (H) shows expression in distal mesenchyme (arrow) and absence of expression in the AER (white arrowhead). (J–L, J'–L') Expression in the distal mesenchyme immediately beneath the AER (burgundy arrowheads) is maintained until stage 28 (J, J', K, K'). Further proximally, in the autopod, *c-hairy1* is downregulated from the condensing digits (black asterisks) from stage 26 (J, J') and is restricted to interdigital regions (white asterisks) by stage 28 (K, K'). Expression is also apparent dorsally and ventrally in the digits, associated with developing tendons (white arrowheads, K' L, L') and peridigitally (yellow arrowheads, L, L'). *c-hairy1* is also observed in a proximal domain (arrows) by stage 26. This expression domain is only present ventrally and is maintained at least until stage 30 (J–L). Hamburger and Hamilton (HH) developmental stages are indicated. Images in (A–G) are dorsal, (I) is apical, (J–I) are ventral; (except in H, I) anterior, top; distal, right. In (I) anterior, top; dorsal, right; distal points out of the plane of page; dashed line marks the ventral base of the limb; T, tail. (H) A thick longitudinal section. Distal, bottom-right; dorsal, top-right.

first intron (AY225442), and *ZfHer9* presumptive first intron (AY225443).

#### Retroviral constructs and infection

The *c-hairy1A* and *c-hairy1B* open reading frames were inserted into RCAS-BP(A) and/or RCAS-BP(B) vectors with an intermediate cloning step in the pSlax13 shuttle vector (Morgan and Fekete, 1996). Replication-competent RCAS-BP(A) and/or RCAS-BP(B) retroviruses encoding *c-hairy1A*, *c-hairy1B*, or myristylated enhanced GFP (*MKGFP*-virus) (Pagan-Westphal and Tabin, 1998) were

grown in primary chick embryo fibroblasts or a DF1 chick fibroblast cell line and concentrated as described by Morgan et al. (1992) and Fekete and Cepko (1993). As additional controls, we used several other retroviruses that were in current use in the laboratory and were known to produce no limb phenotype (“neutral virus”). Infection of chick limb buds was performed in ovo. Viruses were injected at stage 17–19 into entire wing and leg buds. Embryos were incubated for up to 8.5 days, then fixed and processed for skeletal staining or sectioning. Viral infectivity was assessed 48 h after the infection by whole-mount in situ hybridization with a probe against viral transcript or by

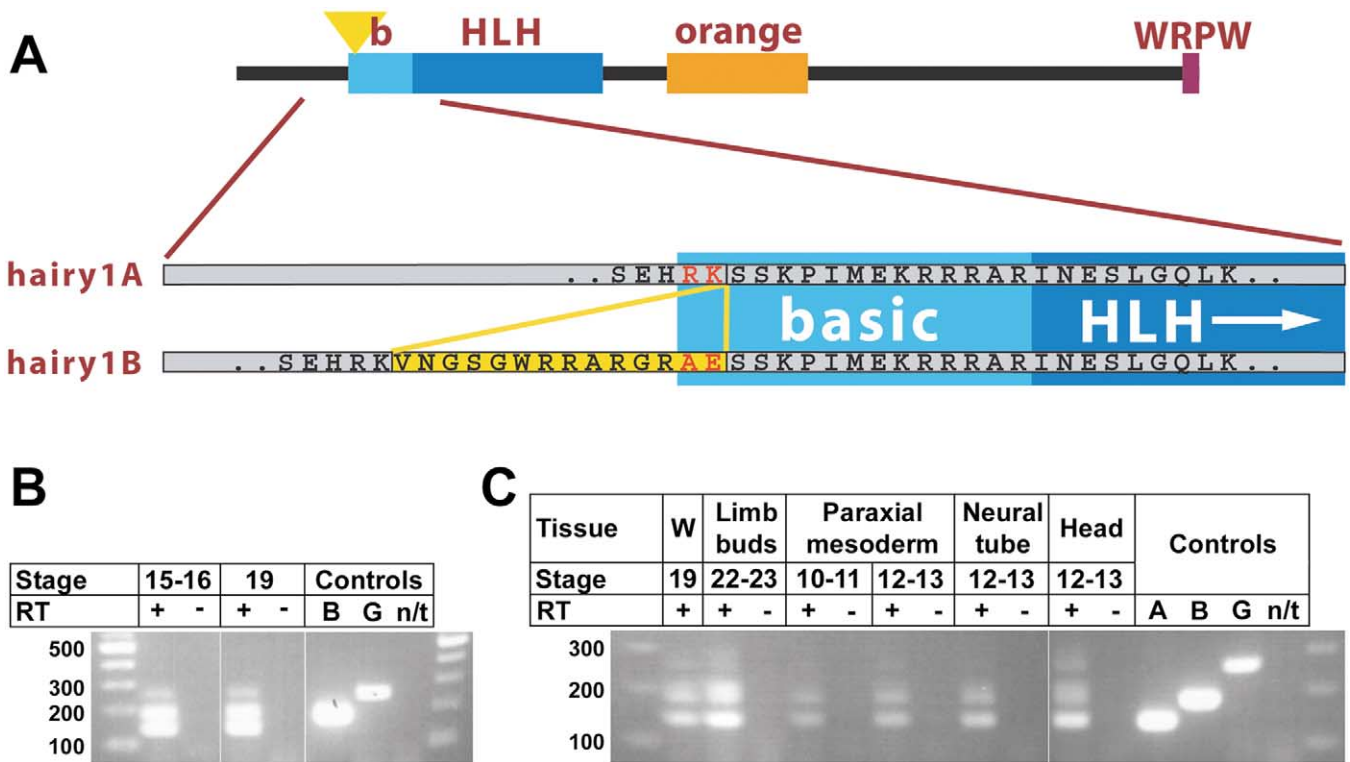


Fig. 2. Alternative splicing of *c-hairy1*. (A) (Top) Schematic representation of *c-hairy1* protein with colored blocks indicating domains conserved among H/E(spl) family members: basic (b), helix–loop–helix (HLH), orange, and WRPW. Yellow triangle indicates the position of the additional 14 amino acids introduced by the alternative splicing. (Bottom) Sequence differences between *c-hairy1A* and *c-hairy1B* proteins. The additional 14 amino acids of *c-hairy1B* are highlighted in yellow. The pair of amino acids that differ in the basic regions of the two proteins is shown in red. (B, C) Expression of two alternatively spliced isoforms of *c-hairy1*. RT-PCR was performed with primers spanning the putative exon1–exon2 junction. Total RNA was isolated from whole embryos (B) and from various embryonic tissues (C) as indicated above the lanes (W, whole embryo). As a positive control, plasmids with cloned *c-hairy1A* (lane A) and *c-hairy1B* (lane B), and chick genomic DNA (lane G), were used as templates to generate RNA. In all stages and tissues tested, bands representing the A and B isoforms of *c-hairy1* were detected (same sizes as in lanes A and B, respectively). In some cases, an unspliced *c-hairy1* mRNA band of the same size as the genomic band (lane G) was also detected. Presence or absence of reverse transcriptase in the first-strand synthesis reaction is indicated with + and –, respectively. lane n/t, “no template” control.

staining sectioned tissue with antibodies raised against viral epitopes.

*Alcian Blue and Alizarin Red staining of cartilage elements*

For Alcian Blue staining, embryos were fixed in 5% trichloroacetic acid (w/v in water) overnight, rinsed in acid alcohol (1 volume glacial acetic acid, 4 volumes 95% ethanol), and stained in 150 µg/ml Alcian Blue (Sigma) in acid alcohol for at least 24 h at room temperature. After washing and dehydrating, embryos were cleared overnight in benzyl alcohol:benzyl benzoate (1:2).

For Alcian Blue/Alizarin Red staining, embryos were fixed in 95% ethanol, stained in 150 µg/ml Alcian Blue (Sigma) in acid alcohol for at least 24 h at room temperature, washed in 95% ethanol for 1 h, and precleared with 1% KOH for 12 h before staining in 75 µg/ml Alizarin Red S (Sigma) in 1% KOH for 24 h. Embryos were then cleared in 20% glycerol, 1% KOH, which was replaced daily until clearing was complete.

**Results**

*Expression of c-hairy1 in the developing limb buds*

We examined *c-hairy1* expression in developing chick limb buds between stages 16 and 30 by whole-mount nonradioactive in situ hybridization using a previously described *c-hairy1* clone (Palmeirim et al., 1997). *c-hairy1* is first expressed in the developing wing and leg buds at stage 17 (Fig. 1A–E, A'–E'). By stage 18, the signal is strong throughout the limb mesenchyme, except posteriorly. During stages 19 and 20, strong *c-hairy1* expression becomes concentrated to the central third (along the AP axis) of the mesenchyme. As the limb bud elongates (Fig. 1F–I), expression of *c-hairy1* is maintained distally, directly abutting the AER. From stage 22, this distal expression consists of two domains: a thin stripe beneath the AER extending throughout the distal margin (which is particularly clear in the leg buds; Fig. 1G' and I) and a wider region of expression extending proximally. When viewed from a distal aspect, the latter

domain is triangular with an anterior apex and is excluded from the posterior mesenchyme (Fig. 1I).

Later in development, expression of *c-hairy1* is found in two domains (Fig. 1J–L, J'–L'). The distal domain is restricted to the autopod portion of the limb bud and later evolves into an elaborate pattern that is associated with digit formation. Expression is restricted to the interdigital regions surrounding the developing condensations. From stage 28, *c-hairy1* is also expressed in regions associated with tendon formation and in restricted regions along the digits. Until stage 29, *c-hairy1* is also expressed in a narrow zone abutting the AER. A second, proximal, domain is apparent by stage 26 (Fig. 1J–L, J'–L'). Expression is observed only ventrally in a central (along the AP axis) region of the limb and extends distally in a thin stripe.

#### *c-hairy1* RNA is alternatively spliced

RT-PCR amplification of the entire *c-hairy1* open reading frame from chick stage 19 RNA produced two products. One represents the published sequence (Palmeirim et al., 1997) and the other contains an extra 42 bases within the *c-hairy1* ORF. The conceptual translation of the new cDNA sequence predicted a 14-amino-acid insertion within the basic (DNA binding) domain of *c-hairy1* (Fig. 2A); this causes the amino acids in the first two positions of the basic domain to change from RK to AE, possibly affecting the DNA binding properties of the protein.

The intron–exon structure of mouse *H/E(spl)* genes as well as *Drosophila hairy* is largely conserved (Sakagami et al., 1994). Specifically, the first intron always falls between the codons encoding the second and the third amino acids of the basic region, the location of the insertion within *c-hairy1B*. To investigate the possibility that the second isoform of *c-hairy1* results from an alternative splicing event, we used PCR primers spanning the putative first intron of *c-hairy1* to amplify the corresponding genomic fragment. The sequence of the amplified fragment confirmed that *c-hairy1* has an intron at the same location as a first intron of a number of other *H/E(spl)* family members. We identified two potential splice-donor sites separated by 42 bases identical in sequence to the insertion within *c-hairy1B* (not shown). Therefore, it is likely that *c-hairy1* RNA is alternatively spliced.

To investigate whether both isoforms of *c-hairy1* are expressed at detectable levels, we performed RT-PCR on chick total embryo RNA as well as RNA obtained from several embryonic tissues (Fig. 2A and B), using an exon junction-spanning primer pair. This revealed that both isoforms of *c-hairy1* are expressed in the embryo (stage 15–16 and stage 19) and are detectable in paraxial mesoderm (stage 10–11 and stage 12–13), neural tube (stage 12–13), head (stage 12–13), and limb buds (stage 22–23). Thus, the *c-hairy1* gene does produce two splice isoforms in the chick embryo.

#### Misexpression of *c-hairy1* during limb bud development reduces limb size

The undifferentiated mesenchyme of the distal limb has been proposed to be important in limb outgrowth and proximodistal patterning (see Introduction). The distal domain of *c-hairy1* expression subjacent to the AER roughly corresponds to this distal zone. Since homologs of *c-hairy1* are involved in maintaining cells in an undifferentiated state in a number of developmental systems (Kageyama et al., 2000), we hypothesized that *c-hairy1* could be responsible for maintaining this presumptive population of distal zone cells. If so, downregulation of *c-hairy1* in regions distant from the AER could regulate this process. To investigate this, we maintained *c-hairy1* expression throughout the limb bud using a retroviral vector.

To assess whether the two splice variants of *c-hairy1* are functionally different, we constructed two vectors, encoding *c-hairy1A* and *c-hairy1B*, respectively. Embryos were separately infected with each virus in the right wing and right leg buds at stage 17–19. The resulting limbs appeared normally proportioned; however, by stage 36 (E10), the limbs infected with *c-hairy1A* virus were visibly smaller than the uninfected, contralateral controls ( $n = 20$ ), while *c-hairy1B* had a less obvious effect ( $n = 10$ ). This initial observation suggested that misexpression of *c-hairy1A* results in reduced limb size.

#### Ectopic *c-hairy1A* expression affects the length and development of skeletal elements

A reduction in overall limb size late in development could be a consequence of the shortening of the skeletal elements of the limb. To address this, *c-hairy1A* virus-infected embryos were fixed at stages 36–37 and stained with either Alcian Blue (for cartilage) alone or together with Alizarin Red (for mineralized tissue). The skeletal elements in the infected limbs are shorter than the contralateral uninfected controls (Fig. 3A). All the elements of the stylopod, zeugopod, and autopod are affected in both the wing and the leg. However, no extra or missing skeletal elements were ever ( $n = 15$ ) observed, nor did the existing skeletal elements appear to be transformed into different ones, suggesting that *c-hairy1A* misexpression does not affect limb patterning. Length measurements of the various skeletal elements (Fig. 3C) revealed that the infected ulna and radius are approximately 13% shorter than the equivalent uninfected controls, and the third metacarpal is about 20% shorter than the contralateral control metacarpal (Student's *t* test,  $P < 0.001$  in all cases).

The extent of mineralization of the skeletal elements is also severely reduced in the infected limbs (Fig. 3A and D). In infected wings, the Alizarin Red-stained regions are shortened by approximately 25% in the radius and ulna and 34% in the metacarpal in contrast to the GFP virus-infected wings or wings infected with other viruses, which present

no phenotype ( $P < 0.001$ ). We noted that the length of the mineralized region in each type of cartilage element is more severely affected than the total length of the skeletal elements ( $P < 0.005$ ; Fig. 3C and D). Qualitatively similar observations were made for other skeletal elements of the developing wings and legs.

#### *c-hairy1B* misexpression has subtle effects

Although misexpression of *c-hairy1B* in the limb buds also results in a slight shortening of the skeletal elements of the infected wing (approximately 4% in radius and ulna and 8% in the 3rd metacarpal; Fig. 3C;  $P < 0.03$  compared with GFP virus and  $P < 0.001$  compared with “neutral” virus or no virus controls), this effect is much less pronounced than that caused by *c-hairy1A* ( $P < 0.0001$ ). Similarly, the mineralized region of the skeletal elements is much less affected by *c-hairy1B* virus than by *c-hairy1A* (Fig. 3D;  $P < 0.0001$ ). This confirms that the two forms of *c-hairy1* are indeed functionally different.

#### Effect of *c-hairy1A* misexpression on cartilage element development

The longitudinal growth of skeletal elements is associated with the proliferation and progressive differentiation of the chondrocytes. Chondrocytes of progressively older stages of differentiation are arranged linearly within the cartilage element (from the ends toward the center) and can be recognized by their morphology and by molecular markers (reviewed in Erlebacher et al., 1995; Vortkamp et al., 1996). The transitions between successive stages of differentiation are tightly controlled by negative feedback loops that involve long-range signaling between different cell populations (Hartmann and Tabin, 2000; Karsenty, 2001; Vortkamp et al., 1996; Wallis, 1996). Furthermore, Notch signaling has been implicated in cartilage development (Crowe et al., 1999). Thus, we addressed whether *c-hairy1* regulates a particular point in the differentiation of the chondrocytes by investigating the effect of ectopic *c-hairy1A* on chondrocyte morphology and gene expression.

Infected and uninfected wings of E10 embryos were sectioned and stained with hematoxylin and eosin. In both infected and control wings, the transitions from round to flat proliferating chondrocytes and from flat to large hypertrophic chondrocytes occurred at the same distance from the ends of the humerus, ulna and third digit metacarpal (Fig. 4A and E; data not shown). However the central region, consisting of hypertrophic chondrocytes, was shortened in each skeletal element of the infected side of the embryo.

To confirm and extend this finding, we performed in situ hybridization on sections of infected and control wings with probes for *Collagen II* (*Col II*; to mark the growth plate, the prehypertrophic chondrocytes and a subset of hypertrophic chondrocytes; Nah et al., 1988), *Indian Hedgehog* (*Ihh*;

prehypertrophic chondrocytes; Vortkamp et al., 1996), and *Collagen X* (*Col X*; hypertrophic chondrocytes located more centrally than *Ihh*-expressing chondrocytes; Linsenmayer et al., 1991; Vortkamp et al., 1996), and analyzed the humeri, ulnae, and third metacarpals (Fig. 4B–D, F–H; data not shown). We measured the distance between the termini of the cartilage elements and the boundaries of each of the domains of expression of these genes relative to the uninfected contralateral control. These measurements allowed us to conclude that, while the end regions of the bones appear normal, it is the central region of each long bone whose length is shortened in the virus-infected limbs. Since *Col X* is expressed most centrally, it best reveals the effect of ectopic *c-hairy1A*; in the case of the third metacarpal shown in Fig. 4H, on the infected side, the region intervening between the two *Col X* expression domains is entirely absent. In each skeletal element, the length by which this region is shortened accounts for the length by which the entire skeletal element is shortened.

Is the effect of *c-hairy1A* cell-type specific, or specific to the position within the bone, regardless of cell type? To address this, we investigated the *Ihh* and *Col X* expression patterns in the phalanges (Fig. 4I and J), since their development lags behind the more proximal elements (Saunders, 1948). In the proximal phalanx of the third digit of an E10 embryo, both *Ihh* and *Col X* are normally expressed in a single, central domain with no intervening, nonexpressing cells. In *c-hairy1A* virus-infected limbs, this *Ihh* domain is shorter than in the contralateral control (Fig. 4I) and *Col X* only starts to be expressed in a few cells, whereas in the contralateral phalanx, a significant expression domain has already been established (Fig. 4J). Therefore, ectopic *c-hairy1A* affects the central region of the cartilage element regardless of what type of chondrocytes occupy it.

Taken together, these data suggest that ectopic *c-hairy1A* does not affect chondrocytes at a specific step in their differentiation. Rather, the central-most cells characteristic of a particular stage of development are absent in infected limbs. This argues that ectopic *c-hairy1A* leaves intact the patterning machinery that regulates cartilage growth and differentiation. Instead, it causes a reduction in the cell number within the cartilage element, or a delay in cartilage development.

#### Ectopic *c-hairy1A* expression delays erosion of cartilage and vascular invasion of skeletal elements

The formation of the bone marrow cavity is initiated during stage 35 (E9) in the tibia and at a later stage in the ulna (Pechak et al., 1986). This occurs within the mid-diaphyseal region of each cartilage element as the vasculature and associated cell types invade the cartilage core. During this process, chondrocytes are removed and the cartilage matrix digested (Tyler, 1991), eroding the cartilage. Since marrow cavity formation initiates at the center of each cartilage element, we asked whether ectopic *c-hairy1A*

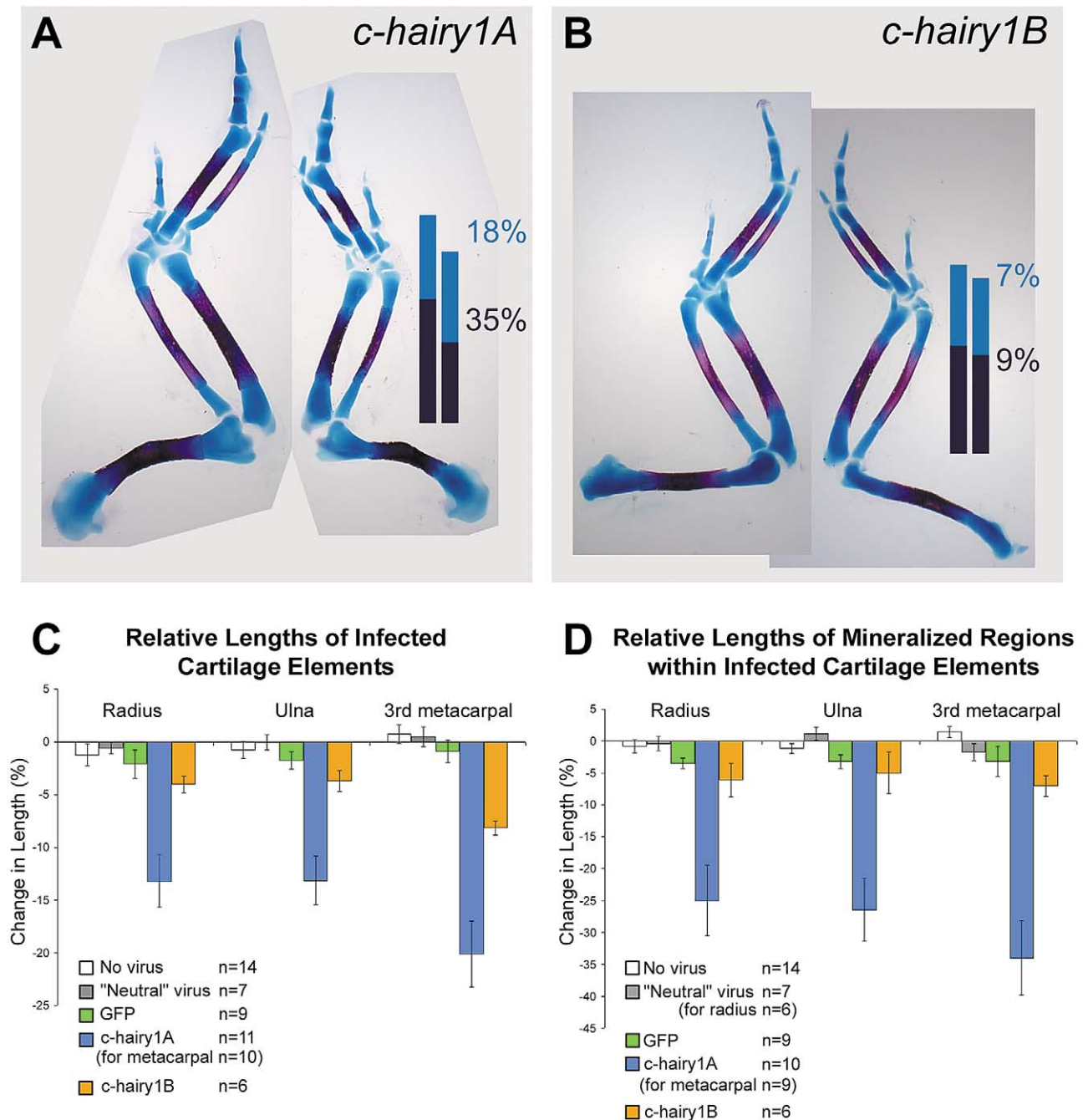


Fig. 3. Misexpression of *c-hairy1A* (A) results in shortening of the skeletal elements and a disproportionately greater reduction in the length of the mineralized regions. Misexpression of *c-hairy1B* (B) has a more subtle effect. (A, B) Pairs of E10 wings; the right wing buds were infected with *c-hairy1A* (A) or *c-hairy1B* (B); left wings are uninfected controls. Staining with Alcian Blue (blue) for cartilage and Alizarin Red (burgundy) for mineralized tissue. The pairs of bars in each panel represent the lengths of the ulnae of the control (left bar) and the infected (right bar) wings. The burgundy portion of each bar represents the length of the mineralized region of the corresponding ulna (numbers indicate % reduction—the blue for the entire ulna, burgundy for ossified region). (C, D) Histogram showing the mean change in whole skeletal element (C) or mineralized region (D) length following infection with various retroviruses. Bars represent the percentage by which the total length of the skeletal element (C) or its mineralized region (D) was reduced relative to the corresponding measurement in the contralateral, uninfected, skeletal element. Values are means  $\pm 1.96 \times$  standard error (this encompasses 95% of the distribution). For each skeletal element (indicated above each group of bars), the effect of five viruses is shown. The legends under each chart indicate the color code for each virus, as well as the number of specimens analyzed (*n*) in each experiment.

expression affects this process, and if so, whether it delays or completely blocks it.

The replacement of chondrocytes with noncartilaginous

tissue was visualized in whole mount by Alcian Blue staining (Fig. 5A–D). Regions of cartilage erosion are visible as clear cavities in the continuous blue staining. In the stage 36

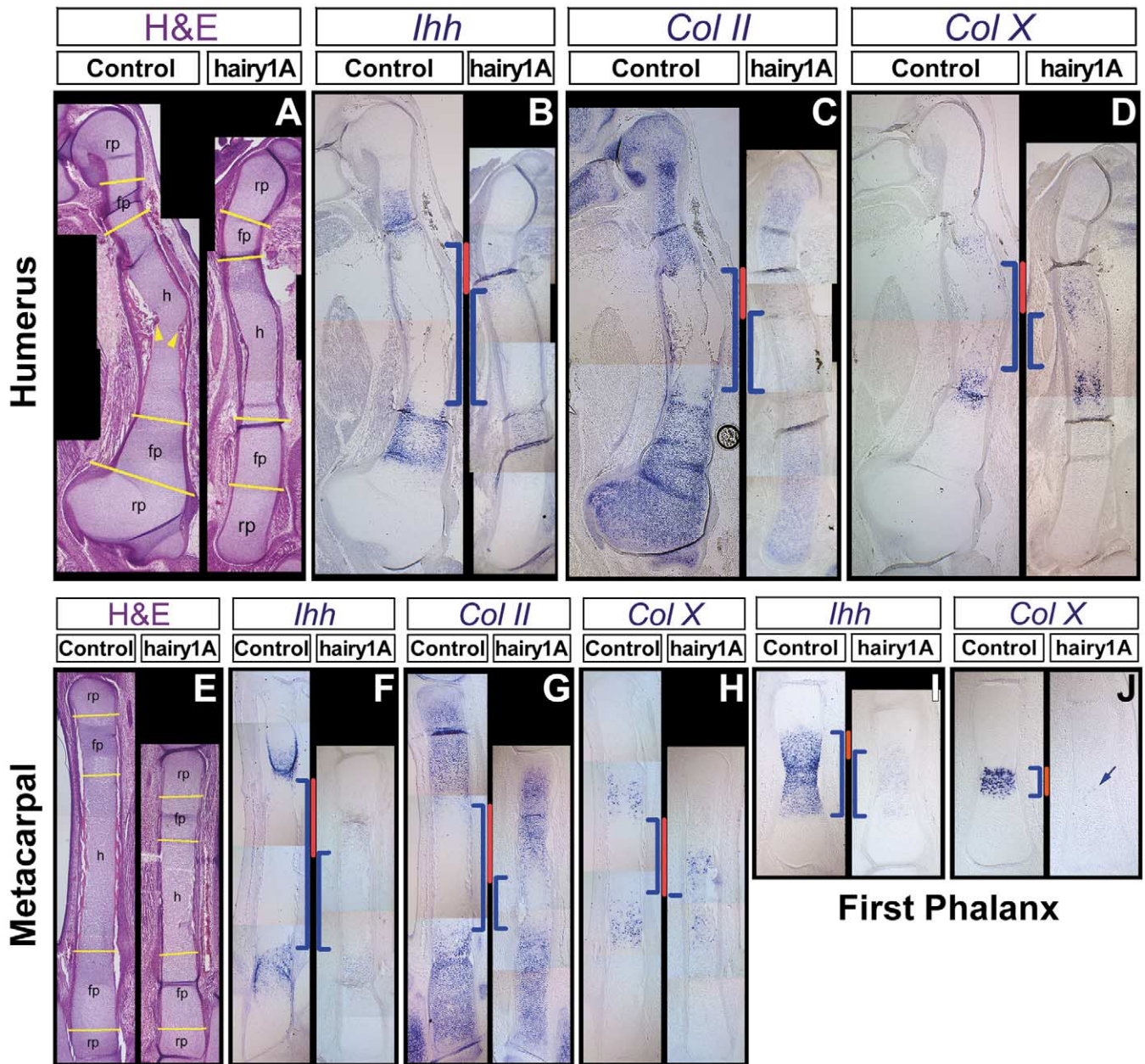


Fig. 4. Effect of *c-hairy1A* misexpression on chondrocyte differentiation. Pairs of longitudinal sections of control and *c-hairy1A* virus-infected (as indicated) E10 humeri. (A–D) 3rd metacarpals (E–H) and phalanges (I, J) aligned at the proximal ends (bottom). (A, E) Hematoxylin and eosin staining reveals (under higher magnification than shown) that regions of the hypertrophic chondrocytes (h) are shorter in the *c-hairy1A* virus-infected bones than in the control side, while the lengths of other regions (marked by yellow lines; rp, round proliferating; fp, flat proliferating) are not affected. In a control humerus (left in A), in contrast to the infected counterpart (right in A), the vasculature and the associated cell types have started to invade the cartilage core (yellow arrowheads). (B–D, F–J) Analysis of *Ihh*, *ColIII*, and *ColX* expression by in situ hybridization (probes indicated above each panel) reveals that misexpression of *c-hairy1A* affects only the central region of each cartilage element. In (B–D, F–H), blue brackets mark the regions between the two expression domains of each gene, and in (I, J), they mark the region expressing *Ihh* and *ColX* [in the infected phalanx only a few cells (blue arrow) express *ColX*]. In all cases, shortening of this region in the infected side is approximately equal to the difference (red bar) in the total length of the infected and control bones (i.e., more terminal regions of each bone are not affected by *c-hairy1* virus). Note that in the first phalanx (I, J) the regions of chondrocytes expressing *Ihh* and *ColX* are affected by *c-hairy1* virus. This is not the case in more proximal (humerus and metacarpal) bones (B, D, F, H) where the expression domains have normal lengths. Therefore, misexpression of *c-hairy1* affects the central region of each bone independently of the type of chondrocytes that populates it.

(E10) tibia, this process is much less pronounced in *c-hairy1A*-infected limbs when compared with the uninfected contralateral controls ( $n = 4$ ). The infected radius of the same stage embryo shows no signs of cartilage erosion,

while bone marrow formation has started in the contralateral control.

To confirm this finding by direct observation of the invading cells within the cartilage core, we examined sec-



tions stained with hematoxylin and eosin. In every case where the invasion of the cartilage core by the vasculature and associated cell types can be seen in the control side of the embryo, the invasion was less extensive in the infected side (Fig. 5E–H). For example in Fig. 5G and H, bone marrow formation has been initiated in the uninfected ulna, but not in its infected counterpart, while in a different ulna (Fig. 5E and F), the area of vascular invasion is smaller on the infected side. Thus, *c-hairy1A* misexpression results in a delay, but not a block, in the initiation of bone marrow formation within cartilage elements.

In summary, our results show that two alternatively spliced variants of *c-hairy1* are expressed in developing limbs; ectopic expression of the two variants with a retroviral vector reveals that they are functionally distinct. Misexpression of *c-hairy1A* causes marked shortening of the central region of each skeletal element in the limb, which leads to a delay in vascular invasion and formation of the bone marrow.

## Discussion

### *Alternative splice isoforms of c-hairy1 differ within the basic domain*

Cloning of the full-length *c-hairy1* cDNA generated two products: one is identical to that previously published (Palmeirim et al., 1997) and the other isoform contained a 42-bp insertion. The genomic sequence revealed that the insertion occurred at an exon–exon junction and is therefore likely to result from alternative splicing. This was confirmed by RT-PCR on embryonic RNA with primers spanning this exon–exon junction: both splice forms are expressed. We named the new isoform *c-hairy1B*, and refer to the previously published isoform as *c-hairy1A*.

The 42 base pairs generate a 14-amino-acid insertion between the second and third amino acids of the basic region of the bHLH domain (Atchley and Fitch, 1997; Atchley et al., 1999) (Fig. 2A). As a result, the basic domains of *c-hairy1A* and *c-hairy1B* differ in the first two positions: RK (two basic amino acids) in *c-hairy1A*, and AE (a hydrophobic and an acidic amino acids) in *c-hairy1B*. Thus, the first two amino acid residues of the *c-hairy1B* basic domain have a net negative charge rather than a net positive charge. This makes it an unusual bHLH protein, since the vast majority of proteins in this family have basic amino acids in the first two positions of the basic domain (Atchley and Fitch, 1997; Atchley et al., 1999). Furthermore, while crystal structures have not been solved for any of the H/E(spl) transcription factors, a number of other bHLH proteins bound to their DNA targets were crystalized. In MyoD, Max, and PHO4 proteins, amino acids in the first

two positions of the basic domain can interact with the backbone and bases inside and outside the core hexamer of the binding site (Ferre-D'Amare et al., 1993; Ma et al., 1994; Shimizu et al., 1997). Thus, changing the net charge from negative to positive in these residues is likely to alter significantly the specificity or weaken the affinity of a transcription factor for DNA. Therefore, the two isoforms of *c-hairy1*, by forming homo- or heterodimers, could bind a range of DNA sites. Alternatively, *c-hairy1B* could antagonize the transcriptional repressor function of *c-hairy1A*.

### *c-hairy1A and c-hairy1B are functionally different*

Misexpression of the two isoforms of *c-hairy1* in chick limb buds revealed that, while *c-hairy1A* causes a strong reduction in the length of skeletal elements and the length of the mineralized region, the effects of *c-hairy1B* misexpression are barely distinguishable from controls, except in the more distal, metacarpal element (Fig. 3C and D). In this distal bone, both the *A* and *B* isoforms cause a shortening, but *c-hairy1A* still produces a significantly stronger phenotype. Importantly, infection with *c-hairy1B* caused this subtle phenotype even when limbs were injected with viral stock containing approximately 10-fold more infectious viral particles than *c-hairy1A* ( $4 \times 10^7$  and  $6 \times 10^8$  infectious particles per ml for *c-hairy1A* and *c-hairy1B* viruses, respectively), which infected nearly 100% of the cells (data not shown). Furthermore, the two viruses only differ by an additional 42 bases within the *c-hairy1B* coding region. This strongly suggests that the differences observed are due to differences in activity between the two forms of cHairy1 rather than to the efficiency of infection or the level of misexpressed protein.

Four possible interpretations could account for these results. First, *c-hairy1B* could bind the same promoters as *c-hairy1A*, but with a lesser affinity. Second, *c-hairy1B* may have lost the ability to bind DNA, but retained other functions not requiring DNA binding. This could appear as a quantitative effect if all of the functions affect the same pathway (for example, *Hes1* both inhibits *Mash1* transcription and interferes with the *Mash1* protein; Castella et al., 1999, 2000; Chen et al., 1997; Sasai et al., 1992). Opposite effects on cellular differentiation can lead to similar gross bone phenotypes (for example, *Wnt-5a* inhibits chondrocyte differentiation and *Wnt-4* promotes it; nevertheless, when misexpressed in limb buds, both *Wnt-5a* and *Wnt-4* cause shortening of skeletal elements; Hartmann and Tabin, 2000). Therefore a third possibility is that *c-hairy1B* could have a dominant negative function, and interfere with *c-hairy1A* or other dimerization partners (Leimeister et al., 2000). By the same argument, *c-hairy1A* and *c-hairy1B* could also bind different DNA targets and participate in different pathways. It is impossible to distinguish amongst these possibilities at present.

### *Alternative splicing of c-hairy1 is not conserved in all vertebrates*

Like in zebrafish and in *Xenopus*, a second closely related gene in the *c-hairy1* branch of the H/E(spl) family was identified in chick, *c-hairy2* (Jouve et al., 2000). Mouse *Hes1* is more closely related to *c-hairy2* than to *c-hairy1*. However, a comparison of the expression patterns in the neural tube pairs *Hes1* with *c-hairy1* (Jouve et al., 2000). We hoped that alternative splicing of *c-hairy1* would present an additional characteristic that could be used to analyze the evolutionary relationships among genes of this group. Sequence analysis (not shown) of the first intron of mouse *Hes1*, human *HES1* and *HES4*, zebrafish *Her9*, Japanese newt *HES1*, and *Drosophila hairy* and *deadpan* revealed no similar splice sites in any of these sequences. It is therefore likely that this type of alternative splicing is unique to birds.

### *c-hairy1 and Notch signaling in the distal mesenchyme of the limb*

*c-hairy1* is expressed in the limb mesenchyme starting early (stage 17) during limb bud development. This expression is maintained distally, in a narrow zone of mesenchyme abutting the AER until stage 28. *c-hairy1* is also expressed in the distal mesenchyme located further away from the AER. This domain of expression is restricted to the anterior two thirds of the limb bud until stage 24 and later evolves with the formation of the autopod. This spatiotemporal pattern of expression is very similar to that of chick *Serrate1*, a gene encoding a Notch ligand (see Fig. 5 in Myat et al., 1996). Since *c-hairy1* homologs mediate Notch signaling (Fisher and Caudy, 1998), close similarity between the expression patterns of *c-hairy1* and *Serrate1/Jagged1* is likely to reflect their participation in the same signaling pathway. Likewise, the domains of expression of *c-hairy1* and *Notch1* share some similarities, especially in the peridigital region and the central artery at later stages (see Vargesson et al., 1998).

Notch signaling has been implicated in development of the AER (Jiang et al., 1998; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Sidow et al., 1997). Contrary to previous reports (Jouve et al., 2000), we did not detect expression of *c-hairy1* in the AER itself. Our findings suggest that, in addition to the role of Notch signaling in the AER, this pathway (including *c-hairy1*) might regulate development of the distal limb mesenchyme.

### *c-hairy1 misexpression phenotype in skeletal element development*

Misexpression of *c-hairy1* leads to a reduction of limb size without affecting limb patterning. Perturbations that affect cartilage differentiation generally result in shortened skeletal elements. For example, this is one of the effects

observed after the misexpression of Notch ligand *Delta-1* (Crowe et al., 1999). This, and the fact that misexpression of *c-hairy1* results in a disproportionate reduction in the length of the mineralized region relative to the total length of a cartilage element, prompted us to address the possibility that *c-hairy1* affects chondrocyte differentiation.

The mesenchymal cells of the limb buds begin to condense into cartilage elements at stages 21–22 (E3) of chick development (Fell and Canti, 1934; Gould et al., 1972; Saunders, 1948; Searls et al., 1972). Later, the longitudinal growth of skeletal elements occurs in the terminally located proliferation zones of immature, dividing chondrocytes (Erlebacher et al., 1995). As chondrocytes leave this zone and are displaced centrally, they go through a number of maturation stages. Transitions from one stage of differentiation to the next occur at a specific distance from the ends of the cartilage elements. As *c-hairy1* is expressed in the perichondrium at later stages of chick limb development (not shown), one possibility is that it regulates cartilage development. However, we find that *c-hairy1A* misexpression does not affect the chondrocyte differentiation transition points, as defined by cell morphology as well as by the expression of *Ihh*, *Col II*, and *Col X*, all of which occur at a proper location relative to the ends of the skeletal elements. Rather, the effect is localized to the central region of the cartilage elements. This is confirmed by the observation that the initiation of bone marrow formation, which starts at the center of skeletal elements, is delayed in *c-hairy1A* infected limbs. Therefore, we propose that misexpression of *c-hairy1A* leads to a delay in skeletal element development. This delay could be caused by the reduced size of initial cartilage condensations. A further effect could be reduced chondrocyte proliferation or ectopic cell death during the longitudinal growth of skeletal elements. However, the machinery that patterns the growth plate of a skeletal element and thereby controls chondrocyte differentiation is not affected. This is shown by the fact that transitions between different stages of chondrocyte development occur at appropriate positions relative to the ends of skeletal elements. Thus, any reduction in the rate of proliferation of immature chondrocytes must be associated with the reduction in the rate of differentiation of the chondrocytes.

These observations are consistent with the possibility that establishing the transition points in the chondrocyte differentiation program is dependent more on the length of a skeletal element than on the developmental stage of the embryo. Thus, when assessing cartilage phenotypes, it might be important to compare wild type and mutant skeletal elements of the same length as well as of the same stage.

Since some H/E(spl) transcription factors can be effectors of Notch signaling, similarities could be expected in the phenotypes caused by misexpression of *c-hairy1A* and of other genes that activate this pathway. Misexpression of the ligand *Delta-1* in the limb bud leads to severe shortening of skeletal elements (Crowe et al., 1999). In contrast to our observations with *c-hairy1A*, however, this effect was at-

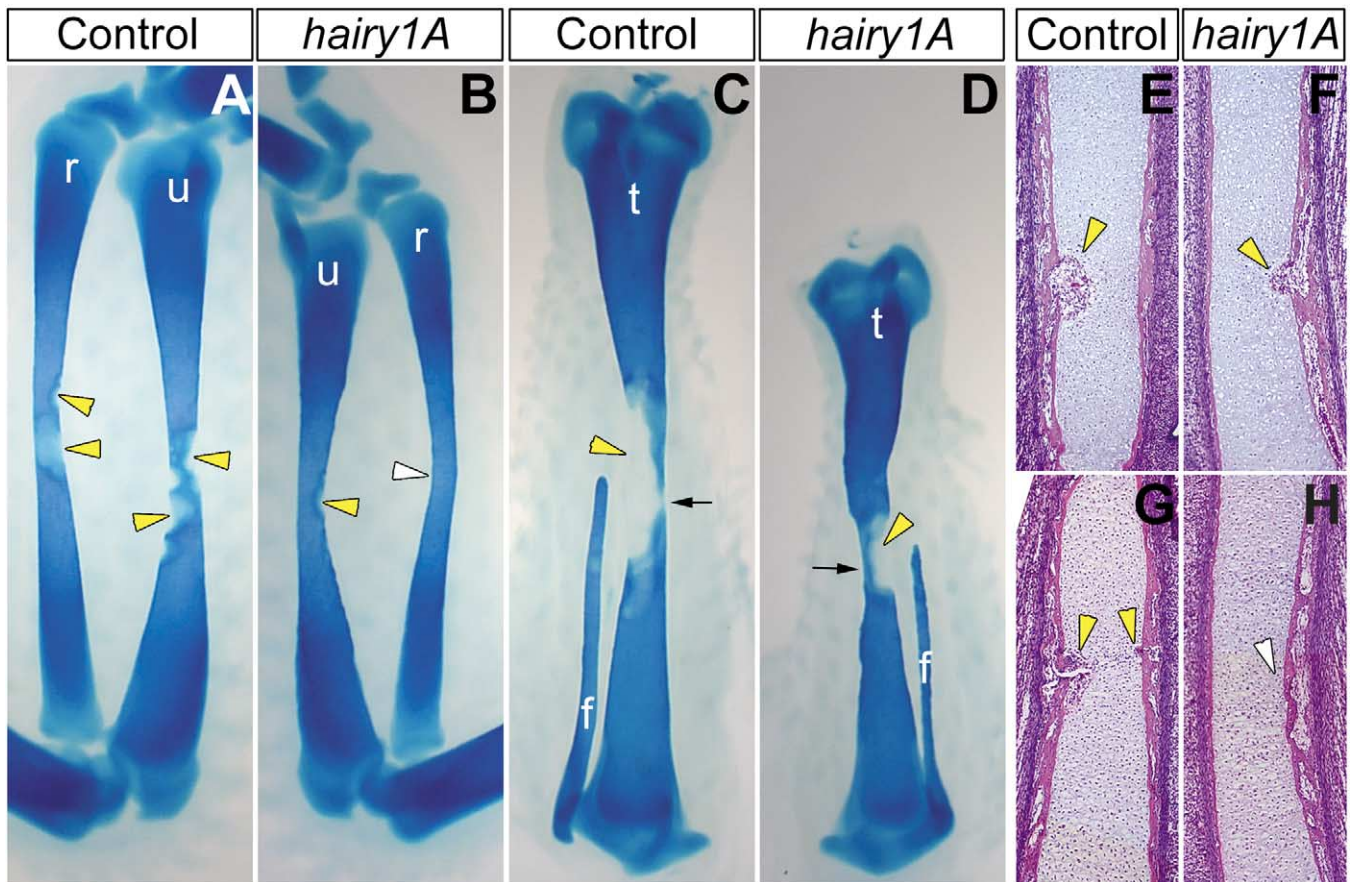


Fig. 5. Misexpression of *c-hairy1A* delays the initiation and progress of bone marrow formation in cartilage elements. (A–D) Whole-mount Alcian Blue staining of stage 37 embryos reveals excavation of cartilage as cavities within blue staining (yellow arrowheads) of zeugopod bones of wings (A, B) and legs (C, D). It is either absent (white arrowhead) or less extensive in the skeletal elements of the limb infected with *c-hairy1A* virus (B, D) relative to the uninfected contralateral bones (A, C). In the normal tibia (C), excavation has proceeded to the point where cartilage is no longer continuous along the length of the skeletal element (arrow indicates the breach), while in the infected side (D), the process has not penetrated through the entire diameter of the cartilage core (arrow). u, ulna; r, radius; t, tibia; f, fibula. (E–H) Hematoxylin and eosin staining of longitudinal sections through stage 36 ulnae showing the invasion of the cartilage core by vasculature and associated cell types (yellow arrowheads). The invasion is either less extensive in the *c-hairy1A* virus-infected ulna (F) than its uninfected contralateral counterpart (E) or completely absent (white arrowhead in H vs. G). In all panels, distal is to the top.

tributed to a block in the transition in chondrocyte development from a prehypertrophic to a hypertrophic state: misexpression of *Delta-1* prevents *Col X* expression, while *Ihh* expression appears normal. In addition, misexpressed *Delta-1* can lead to breaks in the cartilage, to disorganization of the perichondrium, and to a missing second digit, none of which is observed in the *c-hairy1A* virus-infected limbs. Thus, while *Delta-1* and *c-hairy1* potentially could act in the same pathway, they do not appear to play related roles in limb development.

#### *Mechanisms of action of c-hairy1*

By what mechanisms could *c-hairy1* cause a reduction in cartilage condensations? One possibility is that *c-hairy1* might exert its effect by influencing AER formation or function. However, this is unlikely, as discussed above. Alternatively, as suggested by the early domain of expression of *c-hairy1* in the mesenchyme adjacent to the AER, misexpression of *c-hairy1*

in cells that move away from the AER might cause them to remain undifferentiated and also might have an effect on their proliferation. As a result, fewer cells would differentiate and form cartilage condensations. Interestingly, misexpression of *Msx1*, a homeobox transcription factor which, like H/E(spl) proteins, can maintain cells in an undifferentiated state in a number of systems (reviewed in Bendall and Abate-Shen, 2000), leads to shortened limb skeletal elements (Hu et al., 1998). The reduction in length is approximately the same as that caused by misexpression of *c-hairy1A* and appears to be accompanied by a delay in bone marrow formation. Interestingly, both *Msx1* and *c-hairy1* are expressed in the distal limb mesenchyme, although their expression domains are not identical (Hill et al., 1989; Robert et al., 1989; Suzuki et al., 1991).

It is tempting to speculate that *c-hairy1* regulates the size of the limb, most likely through an involvement of Notch signaling in the distal mesenchyme, controlling the rate at which cells leave this mesenchyme to begin their differentiation.

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