



ELSEVIER

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: [www.elsevier.com/locate/developmentalbiology](http://www.elsevier.com/locate/developmentalbiology)

## Autopodial development is selectively impaired by misexpression of chordin-like 1 in the chick limb

Justin M. Allen<sup>a,b,\*</sup>, Edwina McGlinn<sup>b,c,1</sup>, Adele Hill<sup>a,b</sup>, Matthew L. Warman<sup>a,b,d,\*</sup><sup>a</sup> Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, Boston Children's Hospital, Boston, MA 02115, USA<sup>b</sup> Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA<sup>c</sup> EMBL Australia, Australian Regenerative Medicine Institute, Monash University, Wellington Road, Clayton, VIC 3800, Australia<sup>d</sup> Howard Hughes Medical Institute, Boston Children's Hospital, Boston, MA 02115, USA

### ARTICLE INFO

#### Article history:

Received 28 February 2013

Received in revised form

22 May 2013

Accepted 3 June 2013

Available online 10 June 2013

#### Keywords:

Chordin-like 1

Chrdl1

BMP

Limb development

Chicken

### ABSTRACT

Chordin-like 1 (CHRD1) is a secreted bone morphogenetic protein (BMP) antagonist expressed in mesenchymal tissues whose function in development of the skeleton has not been examined in detail. Here we show *Chrdl1* is dynamically expressed in the early distal limb bud mesenchyme, with expression becoming downregulated as development proceeds. *Chrdl1* expression is largely excluded from the critical signaling center of the posterior limb bud, the Zone of Polarizing Activity (ZPA), as has been described for the BMP antagonist Gremlin (GREM1) (Scherz et al., 2004, Science, 305, 396–399). Unlike *Grem1*, *Chrdl1* is expressed in the hindlimb by a small subset of ZPA cells and their descendants suggesting divergent regulation and function between the various BMP antagonists. Ectopic expression of *Chrdl1* throughout the avian limb bud using viral misexpression resulted in an oligodactyly phenotype with loss of digits from the anterior limb, although the development of more proximal elements of the zeugopod and stylopod were unaffected. Overgrowths of soft tissue and syndactyly were also observed, resulting from impaired apoptosis and failure of the anterior mesenchyme to undergo SOX9-dependent chondrogenesis, instead persisting as an interdigital-like soft tissue phenotype. Sonic hedgehog (SHH) and fibroblast growth factor (FGF) signaling were upregulated and persisted later in development, however these changes were only detected late in limb development at timepoints when endogenous *Grem1* would normally be downregulated and increasing BMP signaling would cause termination of *Shh* and *Fgf* expression. Our results suggest that the early stages of the GREM1–SHH–FGF signaling network are resistant to *Chrdl1*-overexpression, leading to normal formation of proximal limb structures, but that later *Bmp* expression, impaired by ectopic CHRD1, is essential for formation of the correct complement of digits.

Published by Elsevier Inc.

### Introduction

Bone morphogenetic proteins (BMPs) are a family of signaling molecules with important functions in development and organogenesis. In developing skeletal tissues, BMPs regulate diverse processes such as differentiation of cartilage, muscle and bone, programmed cell death, and fracture repair (reviewed by Wu et al., 2007). The potent functions of BMPs are precisely regulated by extracellular antagonists and agonists, which alter BMPs

bioavailability, as well as by receptor subtypes and intracellular mediators (reviewed by Ramel and Hill, 2012).

Studies of human genetic disorders and model organisms have demonstrated that extracellular antagonists of BMP signaling have critical roles in skeletal development. Noggin-deficient mice have many developmental abnormalities including joint fusion, cartilage overgrowth and impaired somite formation (Brunet et al., 1998; McMahon et al., 1998; Wijgerde et al., 2005), and mutations in the *NOG* gene have been found in several human syndromes affecting skeletal development including proximal symphalangism and multiple synostosis (Gong et al., 1999) and brachydactyly type B2 (Lehmann et al., 2007).

Analysis of mice with impaired GREM1 signaling, either through targeted inactivation of the *Grem1* gene (Khokha et al., 2003) or mutant *limb deformity* mouse strains affecting *Grem1* expression (Zuniga et al., 2004), have demonstrated that GREM1-mediated inhibition of BMP signaling is critical for correct maintenance and eventual termination of signaling feedback loops

\* Corresponding authors at: Boston Children's Hospital, Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, Enders Building, Room 270, 300 Longwood Avenue, MA 02115, USA. Fax: +1 617 919 2371.

E-mail addresses: [justin.allen@childrens.harvard.edu](mailto:justin.allen@childrens.harvard.edu) (J.M. Allen),

[edwina.mcglinn@monash.edu](mailto:edwina.mcglinn@monash.edu) (E. McGlinn),

[adele.hill@childrens.harvard.edu](mailto:adele.hill@childrens.harvard.edu) (A. Hill),

[matthew.warman@childrens.harvard.edu](mailto:matthew.warman@childrens.harvard.edu) (M.L. Warman).

<sup>1</sup> Present address: EMBL Australia, Australian Regenerative Medicine Institute, Monash University, Wellington Road, Clayton, VIC 3800, Australia

between fibroblast growth factors (FGFs) and Sonic Hedgehog (SHH), which have essential roles in the outgrowth and patterning of the limb (reviewed by Bénazet and Zeller, 2009). Limb development defects arising from GREM1 deficiency can be rescued by reducing the expression of *Bmp4*, as demonstrated by crossing *Grem1*-null mice onto an allelic series of mutant strains expressing various levels of *Bmp4* (Bénazet et al., 2009). Chordin-null mice mostly die during embryogenesis or perinatally, exhibiting defects in the axial and craniofacial skeleton as well as tracheal cartilage (Bachiller et al., 2003).

Chordin-like 1 (CHRDL1), also known as neuralin-1 (Coffinier et al., 2001), ventroptin (Sakuta et al., 2001) and neurogenesis-1 (Ueki et al., 2003), is a secreted BMP-binding protein that contains three von Willebrand type C repeats with homology to chordin (Nakayama et al., 2001). Direct interaction of CHRDL1 with BMP4 has been demonstrated by several groups, and weaker interactions with other BMPs and TGF-beta superfamily members have also been described (Nakayama et al., 2001; Sakuta et al., 2001; Chandra et al., 2006; Kane et al., 2008). Inhibition of BMP4 activity by CHRDL1 has been demonstrated *in vivo* by the ability of exogenous CHRDL1 to induce secondary axis formation in *Xenopus* embryos, which was rescued by co-injection by BMP4 (Nakayama et al., 2001; Sakuta et al., 2001), and *in vitro* by antagonizing the inhibitory effect of BMP4 in angiogenesis assays (Kane et al., 2008). The modulation of BMP signaling by CHRDL1 has also been suggested to be dependent on the formation of a ternary complex with the co-factor Twisted gastrulation, which may influence the function of Chrd1 as either an agonist or antagonist of BMP signaling (Larman et al., 2009).

CHRDL1 has been well characterized in eye tissues where it is expressed in a specific graded pattern along axes of the retina, and misexpression studies have revealed functions for CHRDL1 in regional specification of retinotectal projection patterns (Sakuta et al., 2001). Furthermore, mutations or deletion of the *CHRDL1* gene in humans have recently been found in patients with X-linked megalocornea, an abnormality of the anterior segment of the eye which presents as a deep anterior chamber and a thin cornea of increased diameter which can lead to mosaic corneal degeneration and early cataract formation (Webb et al., 2012).

In addition to the retina, *Chrd1* expression has been described in a diverse range of tissues including limb bud mesenchymal cells and chondrocytes (Nakayama et al., 2001), however the function of CHRDL1 in developing skeletal tissues has not been analyzed in detail. We chose to use the chick limb as a model system for investigating the function of CHRDL1 since it is readily accessible to manipulation during embryonic development and has been used for decades as a model tissue for investigating signaling pathways important for patterning and development. We report that *Chrd1* is expressed in limb mesenchyme and in structures within somites, and that misexpression of *Chrd1* in limbs causes severe and specific defects in the development of digits but not in the development of proximal skeletal structures.

## Materials and methods

### Whole mount *in situ* hybridization

Probe templates were PCR-amplified and cloned into the pCR4-TOPO (Life Technologies) which contains T3 and T7 polymerase binding sites for transcription of anti-sense and control sense probes from linearized plasmids. Two independent non-overlapping riboprobes for *Chrd1* were amplified using the following primer pairs (cChrd1-1F CTGCCCTTGGGATGATGTCT with cChrd1-1R GAAGCTTGGCTGAAACCTG, and cChrd1-2F TGGCATCC-TAATCTCCGGCCT with cChrd1-2R CCCCAGCAGTAACACAGACC)

and both gave identical staining patterns. Labeled riboprobes were transcribed using the DIG or FITC RNA labeling mix (Roche) and purified using illustra ProbeQuant G-50 micro columns (GE Healthcare). Riboprobes were also generated using constructs for chick *Shh* (Riddle et al., 1993), *Fgf4* (Niswander et al., 1994), *Fgf8* (Crossley et al., 1996), *Grem1* (Capdevila et al., 1999), *Msx1* and *Msx2* (Robert et al., 1991), *Bmp2* and *Bmp4* (Francis et al., 1994), *Sox9* (Healy et al., 1999) and *Scleraxis* (*Scx*) (Schweitzer et al., 2001).

Whole mount *in situ* hybridization for single or dual mRNA detection was performed as previously described (McGlenn and Mansfield, 2011). Stained embryos were then photographed using a Leica MZ-FLIII microscope with a DFC420 camera and Firecam 3.1 software (Leica).

After photography, all virus-injected embryos used for *in situ* hybridization were then assayed for injection quality by immunostaining for the viral gag antigen using 3C2 monoclonal antibody-containing hybridoma supernatant (Developmental Studies Hybridoma Bank) at 1:5 dilution in DMEM medium containing 10% FCS and 0.2% Triton-X100 and developed with the Vectastain Elite ABC anti-mouse kit (Vector Labs) and SigmaFast DAB tablets (Sigma). Reported results were observed in at least four virus-infected limbs and never detected in contralateral uninfected limbs or control limbs.

### TUNEL

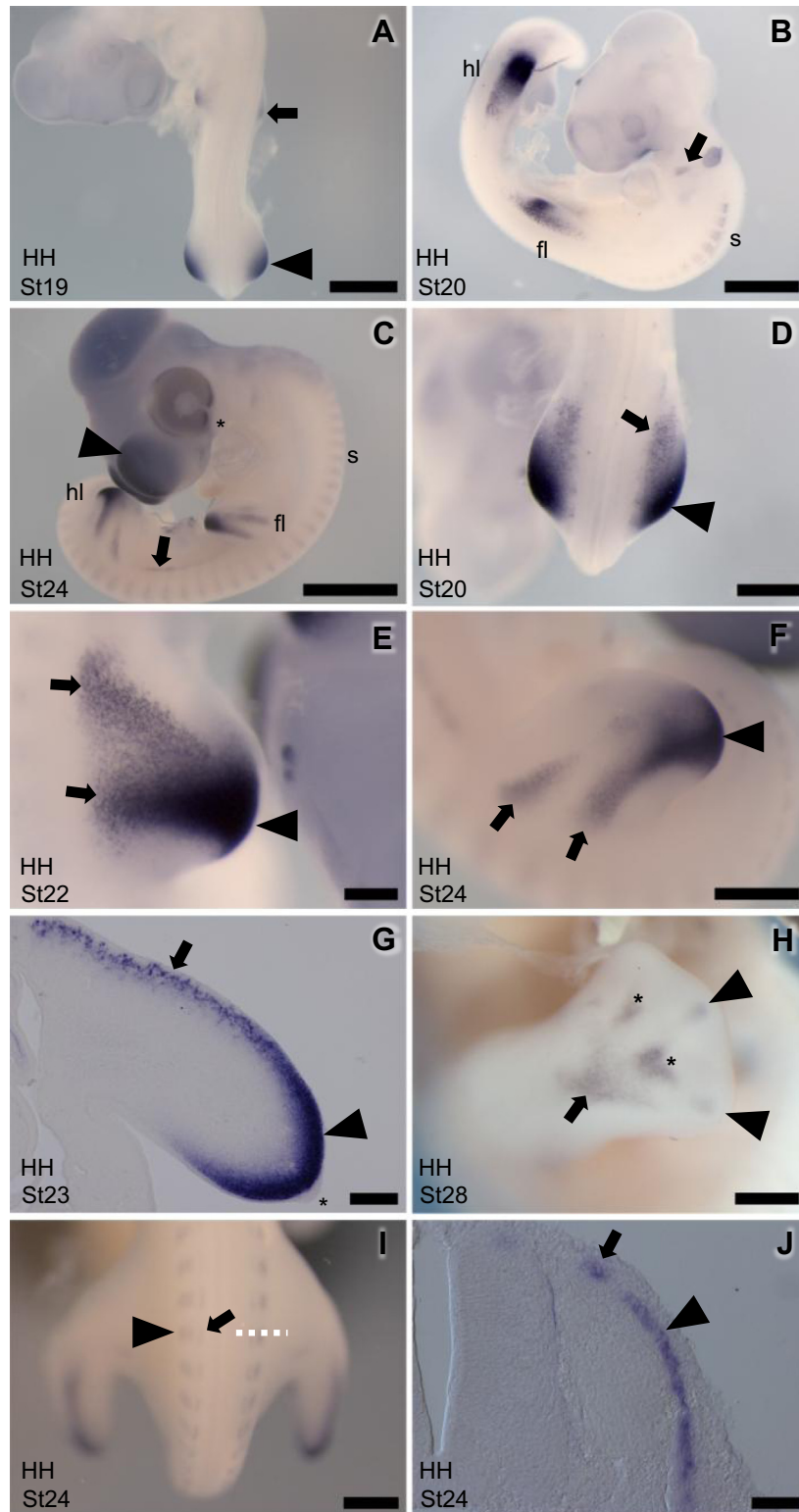
For whole mount detection of apoptosis, embryos were fixed, dehydrated in methanol, digested with proteinase K and then post-fixed with paraformaldehyde and glutaraldehyde as described for whole mount *in situ* hybridization. The *In situ* Cell Death Detection AP kit (Roche) reaction mixture was prepared by adding 1 volume of enzyme solution to 9 volumes of label solution. Negative control samples were incubated in label solution without enzyme added. Samples were incubated at 37 °C in the dark for 1 h, and then washed in PBS overnight at 4 °C. Labeled apoptotic cells were then detected with 1:5000 alkaline phosphatase-conjugated anti-FITC Fab fragments (Roche) in PBST with 1% heat-inactivated sheep serum overnight at 4 °C after blocking for 1 h in 10% sheep serum. Embryos were then washed in PBST for 24 h with several changes and detected with BM Purple (Roche) as described for whole mount *in situ* hybridization.

### RCAS construct preparation

The replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (RCAS) retroviral vector RCAS-*Chrd1* construct was generated by subcloning from an expression vector in which mouse *Chrd1* was amplified by RT-PCR using primers containing EcoRI and XhoI sites and cloned into a pcDNA3.1 vector (Life Technologies) modified with an immunoglobulin signal peptide followed by a haemagglutinin (HA) tag (Rhee et al., 2005). The modified *Chrd1* sequence containing the vector signal peptide and tag was amplified using primers containing SgrAI sites and a Kozak sequence 5' to the ATG and were subcloned into an RCAS-A vector modified by the insertion of a multiple cloning site (MCS) containing an SgrAI site (generously provided by Dr Jimmy Hu). The RCAS-Nog vector was made by RT-PCR from chick embryo cDNA using the following primers containing SgrAI and NotI sites, cNog-F TAGCCACCGGTGACCATGGATCATTCCAGTGCCTTG and cNog-R TCGAGCGGCCGCTAGCAGGAGCACTTGCCTC, and subcloned into the MCS-containing RCAS-A vector.

### RCAS injection of chick embryos

RCAS-*Chrd1*, RCAS-Nog and RCAS-GFP virus were produced as previously described (Logan and Tabin, 1998). Eggs were incubated



**Fig. 1. Expression of *Chrd11* in limbs and somites during chick embryonic development.** (A) At Hamburger–Hamilton (HH) stage 19, *Chrd11* expression is first detected in the developing forelimb (arrow) and hindlimb (arrowhead) buds. (B) At stage 20, *Chrd11* is strongly expressed in the forelimb (fl) and hindlimb (hl) buds, branchial arches (arrow) and in the somites (s). (C) Later at stage 24 *Chrd11* is also expressed in the forebrain (arrowhead), ventral retina (asterisk) and the somite ventrolateral lip (arrow). (D–F) Through stages 20–24, *Chrd11* is expressed in the distal mesenchyme of the limb bud (arrowhead) as well as superficial mesenchymal cells that form two stripes along the limb as development progresses (arrows). (G) Sections through the limb at stage 23 reveal that the domains of distal mesenchymal cells (arrowhead) and the more sparse mesenchymal cells along the limb (arrow) are located just below the ectoderm, and notably the distal mesenchymal expression is directly adjacent to, but excluded from, the apical ectodermal ridge (asterisk). (H) At stage 28, *Chrd11* is expressed in superficial mesenchyme (arrow), in the interdigital tissue adjacent to the digits (asterisk) as well as in the phalanx-forming region (arrowheads) at the tips of the digits. (I–J) In the somites, *Chrd11* is expressed in the myotome (arrowhead) as well as the dorsomedial lip (arrow). Dashed white line in I indicates the region sectioned in J. Bar: 1 mm (A, B), 2 mm (C), 0.5 mm (D, F, H, I), 50  $\mu$ m (E), 0.25 mm (G).

at 38 °C, and 3 ml of albumen was removed from the egg by aspiration before virus injection. Aliquots of RCAS virus, containing 0.025% sterile Fast Green as a tracking dye, were loaded into pulled glass capillary needles and injected into the right lateral plate mesoderm of Hamburger and Hamilton (1951) stage 9–11 embryos using a direct displacement apparatus and micromanipulator (Logan and Tabin, 1998). Control embryos were injected with RCAS-GFP to control for the injection procedure and to observe quality of infection.

#### Bead implantation

Affygel beads (BioRad) were soaked in 0.1 mg/ml recombinant mouse BMP4 (R&D Systems) overnight at 4 °C. Eggs incubated to HH stage 22 were windowed to expose the embryo and a BMP4 bead or PBS control was implanted into the limb mesenchyme. The eggs were re-sealed with tape and incubated at 38 °C for a further 6 h before dissection and processing for whole mount *in situ* hybridization.

#### Skeletal staining

Staining of skeletal tissues with Alcian Blue and Alizarin Red was performed as previously described (McLeod, 1980). Skeletal preparations in 100% glycerol were photographed using a Leica MZ-FLIII microscope with a DFC420 camera and Firecam 3.1 software (Leica).

## Results

#### *Chrdl1* is expressed in early limb and somite development

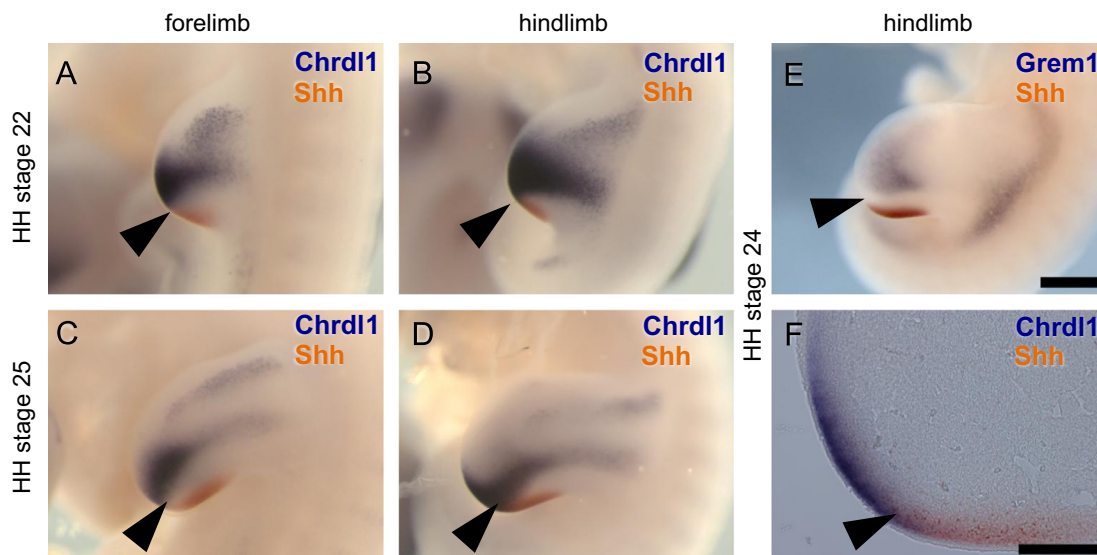
The expression of *Chrdl1* in the developing chick embryo was analyzed by *in situ* hybridization. From HH stage 19, *Chrdl1* is strongly expressed in the limb bud mesenchyme (Fig. 1A–G), with expression also in the somites, eye, and branchial arches (Fig. 1B and C). In the limb bud, *Chrdl1* is expressed in two distinct domains visualized from stages 20–24 (Fig. 1D–G). A broad domain is first observed throughout the limb mesenchyme extending to the distal tip, predominantly posteriorly biased, but largely excluded from the posterior ZPA. In addition, from stage 20 *Chrdl1*

is expressed by sparse cells in the superficial mesenchyme that form two stripes along the anterior and posterior limb as development progresses (Fig. 1D–F). These cells co-localize with a subset of *Pax3*-expressing cells (Supplementary Fig. 1), suggesting that *Chrdl1* may also be expressed by myogenic progenitors. Consistent with this, sections through somites reveal that *Chrdl1* is expressed in the myotome and at the sites of the dorsomedial and ventrolateral lips of the maturing somite (Fig. 1I–J). At later developmental stages (Fig. 1H), expression of *Chrdl1* in the limb is significantly downregulated, though expression is detected in the interdigital mesenchyme and at the digit tips in the phalanx-forming region, a region where quantitative regulation of BMP activity has been suggested to dictate digit identity (Suzuki et al., 2008).

#### Regulation of *Chrdl1* expression

The strong expression of *Chrdl1* in the posterior distal limb bud mesenchyme and its general exclusion from the ZPA looks broadly similar to that of *Grem1* (Merino et al., 1999), a BMP antagonist critical for limb development (Khokha et al., 2003). Exclusion of *Grem1* from the ZPA and importantly from all cells descendant from this signaling center (Fig. 2E), is critical for the timely downregulation of the SHH–FGF feedback loop and ultimately size regulation of the autopod (Scherz et al., 2004). To determine if *Chrdl1* expression is regulated by similar mechanisms as *Grem1*, we performed dual-color *in situ* hybridization for *Shh* and *Chrdl1* across various stages of limb development (Fig. 2A–D, F). In the forelimb, non-overlapping expression is observed for *Shh* and *Chrdl1* at all stages, with a zone of exclusion between the ZPA and *Chrdl1*-expressing cells widening over time (Fig. 2C compared to 2A). While this also holds true at stage 22 in the hindlimb (Fig. 2B), there is limited separation of the two expression domains over time, and moreover, at later stages of development a population of cells express both *Chrdl1* and *Shh* (Fig. 2D and F) indicating that, in contrast to *Grem1*, some hindlimb ZPA cells and their descendants are able to express *Chrdl1*.

BMPs have been demonstrated to be necessary and sufficient to induce expression of *Grem1* and *Nog*, a mechanism whereby induction of an antagonist can regulate the levels of BMP activity (Capdevila et al., 1999; Gazzero et al., 1998; Nissim et al., 2006). To investigate whether *Chrdl1* undergoes similar regulation, we



**Fig. 2.** *Chrdl1* expression in the posterior hindlimb bud overlaps with the ZPA. *Chrdl1* expression in the forelimb bud does not overlap with that of *Shh* in the forelimb bud (A, C) and the distance between these domains increases during development (compare A with C; arrowhead), suggesting similar regulation as *Grem1* (E). However in the hindlimb (B, D, F; arrowhead), there is overlap of the *Chrdl1*- and *Shh*-expressing domains. Bar; 0.5 mm (A–E), 100  $\mu$ m (F).

implanted beads soaked in BMP4 into a region in the HH stage 22 proximal limb bud normally devoid of *Chrdl1* expression and could demonstrate that *Chrdl1* is induced by BMP4 (Fig. 3A and B;  $n=4/5$ ). Furthermore, we observed that *Chrdl1* expression was strongly downregulated in chick limbs in which BMP signaling was reduced by infection with RCAS-Nog virus (Fig. 3C and D;  $n=3/3$ ), providing further evidence that *Chrdl1* expression is induced by BMPs.

*Chrdl1*-overexpression induces abnormalities of the autopod through inhibition of programmed cell death and overgrowth of soft tissues

To elucidate the *in vivo* activity of CHRDL1 during limb development, we ectopically expressed *Chrdl1* in developing chick embryos using an RCAS viral expression system. For each microinjection, the right side lateral plate mesoderm of the embryo was selectively targeted, such that the contralateral uninjected side could serve as an internal negative control. Additional embryos were injected with RCAS-GFP for control for any effects of the microinjection procedure and viral infection (not shown).

The autopod of RCAS-*Chrdl1* injected limbs were expanded along the anterior-posterior axis (Fig. 4A–D). Ectopic soft tissue outgrowths were also detected projecting from *Chrdl1*-overexpressing limbs (arrowhead in Fig. 4A). Apoptosis was decreased in RCAS-*Chrdl1* injected limbs along the apical ectodermal ridge (AER) and in the anterior and posterior necrotic zones (Fig. 4C and D) which have important roles in shaping the developing limb (Fernández-Terán et al., 2006). RCAS-*Chrdl1* injected limbs also displayed syndactyly, the abnormal fusion of digits by soft tissue (Fig. 4E), as well as impairments in digit development (see below). In contrast to previous models of BMP perturbation displaying dorsal-ventral patterning defects (reviewed by Robert, 2007), expression of the dorsalizing factor *Lmx1b* remained restricted to the dorsal mesoderm in RCAS-*Chrdl1* infected limbs and its domain of expression was not altered (data not shown).

*Chrdl1* misexpression causes defects in digit development

Alcian blue and Alizarin red-stained skeletal preparations of RCAS-*Chrdl1*-infected chick embryos revealed a severe oligodactyly

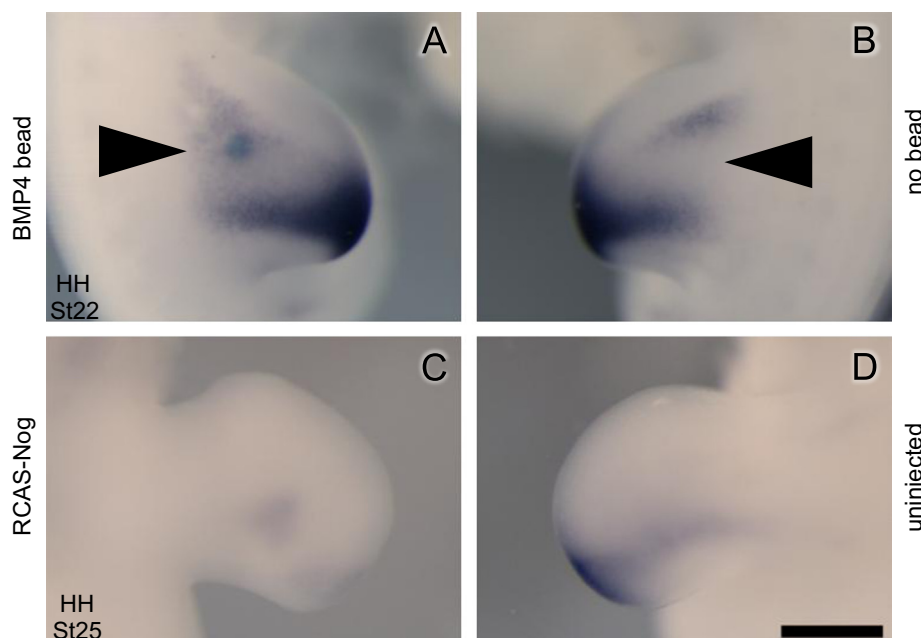
phenotype primarily affecting the anterior digits (Fig. 5B and D). Notably, the more proximal limb structures of the stylopod (humerus, femur) and zeugopod (radius, ulna, tibia, fibula) developed normally and were indistinguishable between infected and uninfected tissues (Fig. 5A–D). The severity of the oligodactyly varied between embryos but most commonly included formation of a single posterior forelimb digit and, in the hindlimb, formation of only one or two posterior digits with reduced numbers of phalanges and truncation of one or two anterior tarsometatarsals at the diaphysis. Analysis of the reduction in skeletal elements (Table 1) was performed on the hindlimb, which has greater numbers of digits and phalanges than the forelimb in chick, giving a better quantitative readout of the phenotype.

*Chrdl1*-misexpression upregulates the SHH-FGF feedback loop

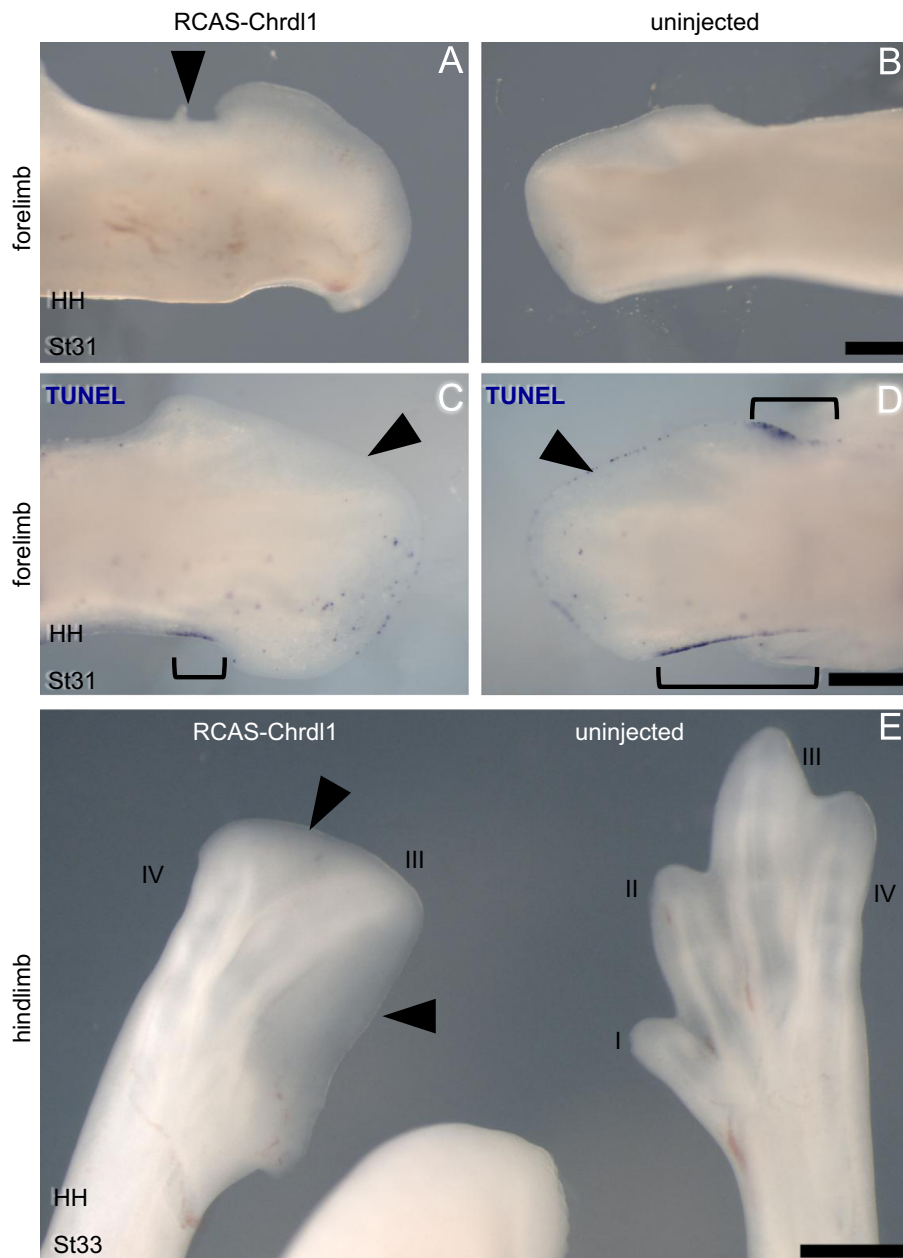
The expression of key components of the SHH-FGF feedback loop critical for growth and patterning of the developing limb were analyzed in RCAS-*Chrdl1* injected limbs. No changes in the intensity or domain of expression of *Shh*, *Fgf4*, *Fgf8* or *Grem1* were detected prior to stage 25 (data not shown), which is in contrast to what has been described for RCAS-mediated misexpression of *Grem1* (Capdevila et al., 1999) or *Nog* (Pizette and Niswander, 1999; Pizette et al., 2001). However, the expression of *Fgf4* ( $n=10/14$ ) and *Fgf8* ( $n=6/10$ ) was upregulated in *Chrdl1*-overexpressing limbs at stage 25 (Fig. 6A and C; blue arrows;) compared to contralateral uninjected control limbs (Fig. 6B and C; black arrows), and the domain of expression was expanded anteriorly. *Shh* expression was also increased in RCAS-*Chrdl1* injected limbs (Fig. 6D; blue asterisk;  $n=10/14$ ), whereas no consistent changes in *Grem1* expression were detected ( $n=6$ ). Examination of later timepoints demonstrated that *Fgfs* continue to be expressed by AER cells in *Chrdl1*-overexpressing limbs at stage 28, whereas their expression is downregulated and barely detectable in uninjected limbs (Fig. 6E compared to F;  $n=6/6$ ).

*Chrdl1*-overexpression alters expression of genes downstream of BMPs

A potential early anterior patterning defect underlying the digit loss in *Chrdl1*-misexpressing limbs was examined by *in situ*



**Fig. 3.** *Chrdl1* expression is induced by BMP signaling. Implantation of a bead soaked in BMP4 (100  $\mu\text{g}/\text{ml}$ ) induced ectopic *Chrdl1* expression A; arrowhead) in a region negative for expression in contralateral control limbs with no bead (B; arrowhead). Infection of limbs with RCAS-Nog caused a significant downregulation of *Chrdl1* expression (C, D). Bar; 0.5 mm (A–D).

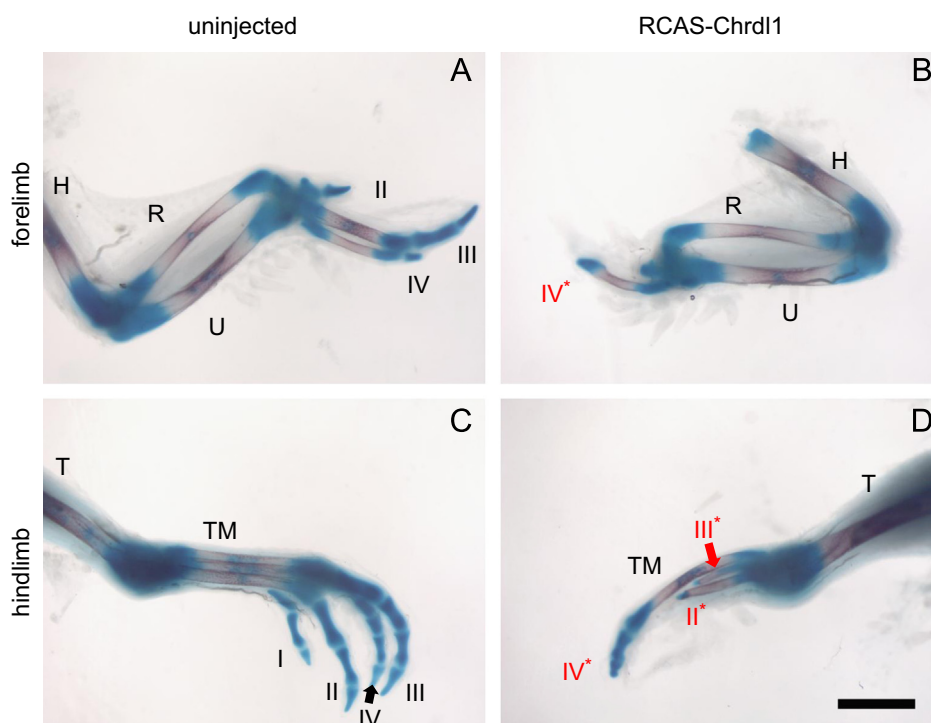


**Fig. 4. Abnormalities of autopodial development induced by viral misexpression of *Chrdl1*.** Misexpression of *Chrdl1* caused anterior-posterior overgrowth of the autopod and abnormal soft tissue outgrowths (A; arrowhead). Reduced levels of apoptosis, detected by TUNEL assay (blue) were detected in the AER (C,D; arrowheads) as well as in the anterior and posterior necrotic zones (C, D; brackets). Interdigital webbing persisted in RCAS-*Chrdl1* infected limbs (E; arrowheads) and anterior digits were truncated or absent (E; digits labeled I–IV). Bar: 0.5 mm (A–D), 1 mm (E).

hybridization analysis of the anterior limb markers *Alx4* and *Pax9*, and the digit patterning factors *Gli3*, *Hoxd12* or *Hoxd13* but no differences from uninfected limbs were detected (data not shown). Since *CHRDL1* is a putative BMP antagonist, we examined the expression of the BMP target genes *Msx1* and *Msx2* in response to *Chrdl1*-overexpression. In RCAS-*Chrdl1* injected limbs, *Msx1* was ectopically expressed at sites where anterior digits would normally develop (Fig. 7A; bracket;  $n=8/10$ ), whereas in control tissues *Msx1* was predominantly expressed in the interdigital mesenchyme at HH stage 31 and excluded from sites of digit formation (Fig. 7A'). However, the intensity of *Msx1* expression detected by *in situ* hybridization was not affected by *Chrdl1*-overexpression. *Msx2* was also found to be expressed ectopically in mesenchymal regions that have failed to differentiate to form

digits (Fig. 7B; bracket and arrowhead;  $n=9/12$ ), and levels of *Msx2* were downregulated compared to contralateral untreated limbs (Fig. 7B and B').

The expanded autopod of RCAS-*Chrdl1* injected limbs fails to form several skeletal elements, therefore we examined the expression of the transcription factor *Sox9*, a master regulator of chondrogenesis. Consistent with the loss of anterior digits observed in skeletal preparations, *Sox9* was not detected at the expected sites of the digital rays in the anterior limb after infection of the limb with RCAS-*Chrdl1* virus (Fig. 7C and C';  $n=7/12$ ). However, a dispersed ectopic *Sox9* hybridization signal was detected in the distal limb mesenchyme that has not condensed to form a digit (Fig. 7C; asterisk), consistent with previous studies whereby inhibition of *Bmp* signaling by *Nog*-misexpression



**Fig. 5. Misexpression of *Chrdl1* causes loss or truncation of anterior digits.** Alcian Blue (cartilage) and Alizarin Red (bone)-stained skeletal preparations demonstrate loss of digits, primarily affecting the anterior limb, in RCAS-*Chrdl1* infected limbs (B, D) compared to contralateral uninjected control limbs (A, C). Truncated digits are labeled with an asterisk. In these representative examples, digits II and III of the forelimb fail to develop whereas the distal phalanx of digit IV is missing (B compared to A). In the hindlimb, digit I is absent, digits II and III are truncated at mid-tarsometatarsus (TM) and digit IV has formed only two rather than five phalanges (D compared to C). The radius (R), ulna (U), humerus (H), tibia (T), fibula and femur (not shown) were morphologically indistinguishable between infected and non-infected limbs. Bar; 2 mm A–D.

**Table 1**

Quantitation of hindlimb defects in chick embryos whose right lateral plate mesoderm was infected with RCAS-*Chrdl1* or RCAS-GFP control virus.

Hindlimb phenotype scoring		RCAS- <i>Chrdl1</i>		RCAS-GFP	
		Right	Left	Right	Left
Unaffected		3/35	31/35	5/5	5/5
Mild	Loss of 1–2 distal phalanges	6/35	2/35	0/5	0/5
Moderate	Loss of > 2 phalanges, tarsometatarsals unaffected	6/35	1/35	0/5	0/5
Severe	Loss of multiple skeletal elements including loss/truncation of tarsometatarsal(s)	20/35	1/35	0/5	0/5

(Pizette and Niswander, 2000) or *Smad4* disruption (Bénazet et al., 2012) also resulted in diffuse, ectopic expression of *Sox9* and *Col2a1* but, since cell aggregation and condensation were inhibited, digit formation could not proceed.

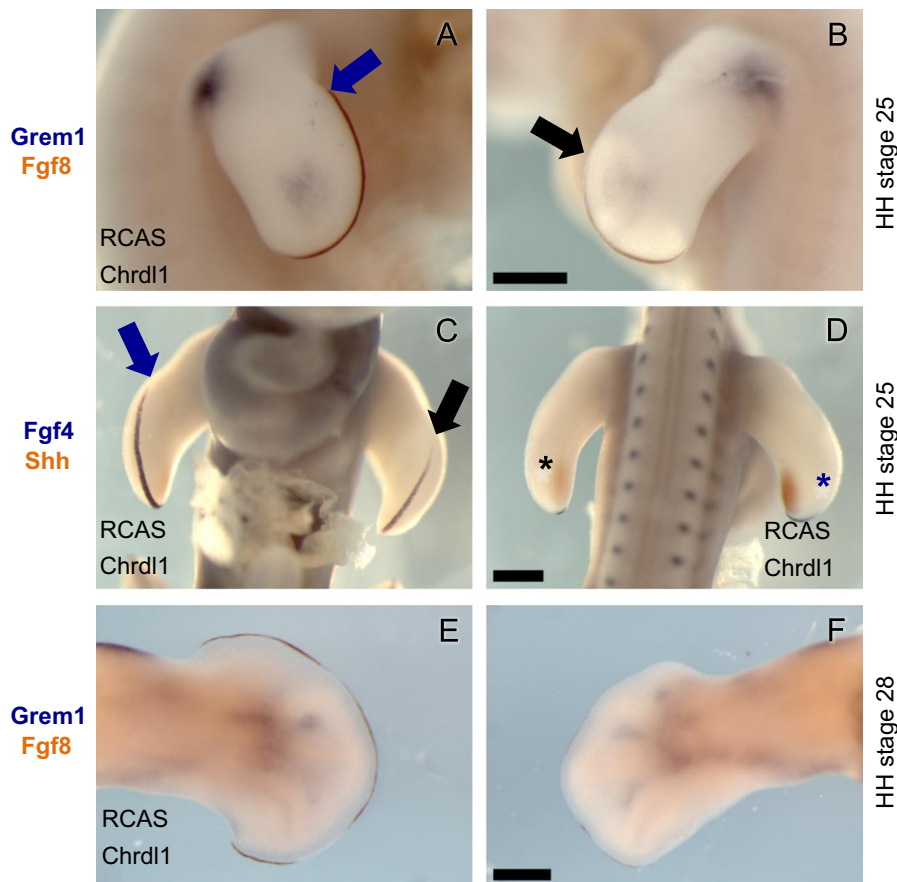
Expression of the tendon progenitor marker *Scleraxis* (*Scx*) has been shown to be impaired by BMPs and upregulated by antagonism of *Bmp* signaling (Schweitzer et al., 2001). In *Chrdl1*-overexpressing limbs, the loss or truncation of digit rays is accompanied by a loss of associated *Scx* expression (Fig. 7D and D';  $n=4/6$ ). However a broad increase in *Scx* expression was detected in the distal uncondensed limb mesenchyme (Fig. 7D; asterisk), consistent with previous results that BMP antagonism by *Nog* can induce *Scx* expression (Schweitzer et al., 2001). The pattern of *Scx* expression in the zeugopod appeared normal, suggesting that development of tendons proximal to the autopod was unaffected by *Chrdl1* misexpression.

BMP proteins have been shown to negatively regulate the expression of *Bmp* genes through an auto-regulatory loop (Bastida et al., 2009). We examined if inhibiting BMP signaling by overexpressing *Chrdl1* would impair the ability of BMPs to auto-regulate their own expression. *Bmp2* ( $n=9/10$ ) and *Bmp4* ( $n=5/8$ ) were both upregulated at the mRNA level in RCAS-*Chrdl1* injected limbs (Fig. 7E–F'), *Bmp2* expression was increased particularly in the

posterior limb (Fig. 7E; dashed bracket) whereas there was a region of strong *Bmp4* expression (Fig. 7F; black bracket) underlying the AER in infected limbs only. These data suggest that the ability of BMP proteins to negatively regulate the expression of *Bmp* genes has been impaired.

## Discussion

We investigated the expression and activity of CHRDL1 during skeletal development. *Chrdl1* is dynamically expressed during limb development, with broad expression in the limb bud mesenchyme at early embryonic stages, later becoming restricted to the interdigital mesenchyme and the distal tips of developing digits. *Chrdl1* expression was regulated by BMP levels in the limb bud. Reduction in BMP signaling by *Nog* misexpression strongly downregulated *Chrdl1*, and conversely implantation of beads soaked in BMP4 protein stimulated *Chrdl1* expression suggesting that, like *Nog* (Merino et al., 1998) and *Grem1* (Capdevila et al., 1999; Nissim et al., 2006), *Chrdl1* is induced by BMPs to fine tune the levels of BMP signaling. Increased transcription of *Bmps* were detected in *Chrdl1*-overexpressing limbs, consistent with inhibition of a negative auto-regulatory feedback loop whereby BMP proteins can



**Fig. 6. Upregulation of the SHH-FGF feedback loop in *Chrdl1*-overexpressing limbs.** *Fgf8* and *Fgf4* expression was more intense and extended further along the anterior limb in *Chrdl1*-overexpressing forelimbs (A, C; blue arrows) compared to control limbs (B, C; black arrows) at HH stage 25. *Shh* was also strongly upregulated in RCAS-*Chrdl1* injected limbs (D; blue asterisk) compared to controls (D; black asterisk). *Fgf8* expression also was detected at later timepoints in *Chrdl1*-overexpressing limbs (E) than in control limbs (F). No reproducible changes in *Grem1* expression were detected. Bar; 0.5 mm (A–D), 1 mm (E–F).

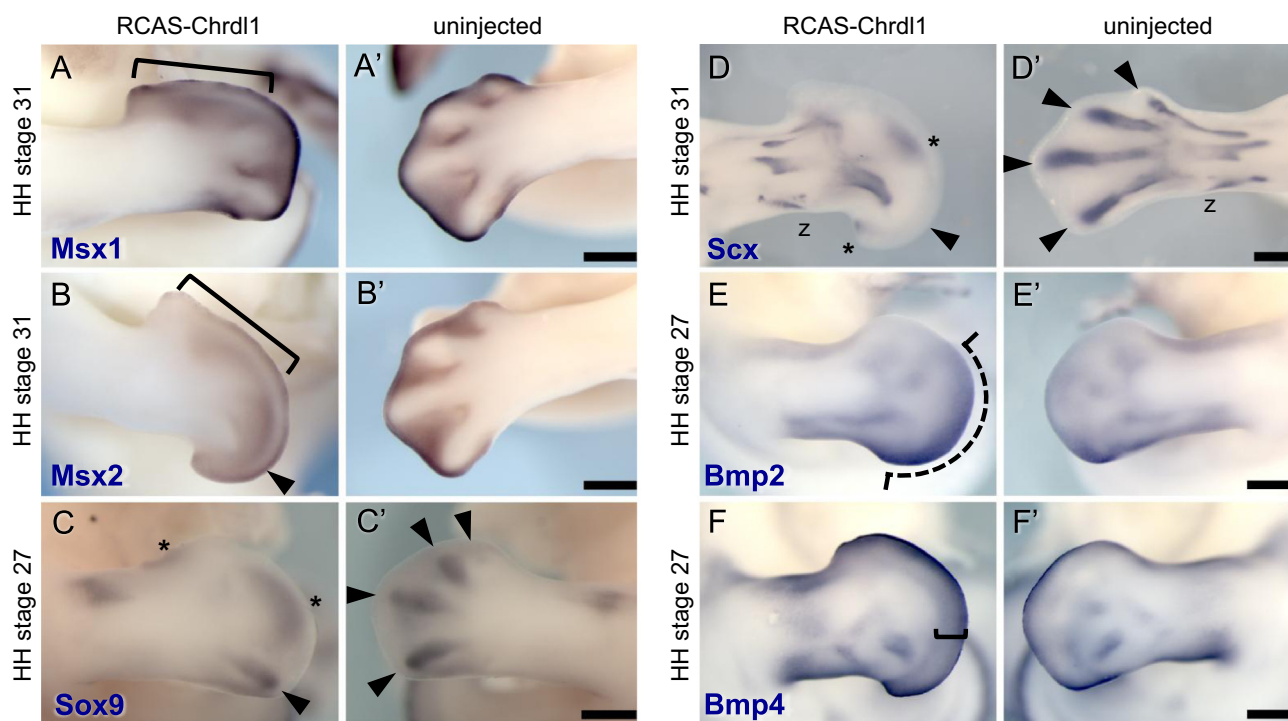
reduce transcription of *Bmp* genes (Bastida et al., 2009). *Fgfs* and *Shh* were also upregulated by *Chrdl1*-overexpression, which can also positively regulate *Bmp* expression (Bastida et al., 2009). *Chrdl1*-misexpression also resulted in downregulation of the BMP target gene *Msx2* demonstrating that although *Bmp* transcription was increased, overall BMP activity was reduced. No decrease in *Msx1* was detected, however several other models have also demonstrated that *Msx2* displays a greater sensitivity to manipulated BMP signaling than does *Msx1* (Khokha et al., 2003; Pizette and Niswander, 1999). *Chrdl1* expression was mostly absent from the ZPA signaling region of the posterior limb bud, which prompted us to examine whether, like the BMP antagonist *Grem1* (Scherz et al., 2004), the ZPA cells and their descendants are refractory to *Chrdl1* expression. We found that a subset of hindlimb ZPA cells express both *Shh* and *Chrdl1*, and that the ZPA descendants are therefore not refractory to *Chrdl1*, suggesting differences in regulation and function of BMP antagonists in limb development.

Ectopic overexpression of *Chrdl1* in developing chick limbs had a strikingly specific effect in impairing development of the autopod skeleton but with no detectable effect on the formation of more proximal skeletal elements. Additional phenotypes we observed, including digit truncations, syndactyly, soft tissue overgrowths, impaired apoptosis and AER expansion, have been reported in other models of BMP dysregulation, such as misexpression of *Nog* (Guha et al., 2002; Pizette and Niswander, 1999, 2000; Wang et al., 2004) or *Grem1* (Capdevila et al., 1999; Merino et al., 1999), overexpression of a dominant negative form of the

BMP receptor 1b (Zou and Niswander, 1996), or disruption of intracellular BMP signal transduction (Bénazet et al., 2012). Together, these data provide further confirmation that CHRDL1 also functions *in vivo* as a BMP antagonist and highlights the requirement for multiple BMP antagonists in exquisitely shaping limb formation.

In contrast to the autopod-specific phenotype observed with *Chrdl1*-misexpression, retroviral-mediated misexpression of *Grem1* (Capdevila et al., 1999) in chick limbs caused abnormal morphogenesis of zeugopodial as well as autopodial elements, whereas similar experiments with *Nog* (Pizette and Niswander, 2000) resulted in agenesis of limb skeletal elements. *Grem1* misexpression was described as having a biphasic effect in which early changes reinforcing AER and SHH signaling and limb outgrowth are aberrantly increased, along with the distally expressed homeobox transcription factor *Hoxd13*, and then prematurely downregulated, which may contribute to the severity of the dysmorphologies and distal truncations (Capdevila et al., 1999). In contrast, no changes in *Hoxd13* expression were detected in *Chrdl1*-overexpressing limbs (data not shown), and alterations to SHH-FGF signaling were not observed prior to HH stage 25, suggesting that high levels of *Chrdl1* misexpression at early timepoints are tolerated without altering SHH-FGF signaling, likely due to robust compensation by components of the signaling network at those stages (Bénazet et al., 2009; Lopez-Rios et al., 2012). It has been recently demonstrated in mice that disrupting BMP signal transduction in the developing autopod using *Hoxa13*-cre mediated *Smad4* deletion results specifically in loss of skeletal





**Fig. 7.** *Chrdl1*-overexpression alters expression of genes downstream of BMPs. *Msx1* and *Msx2* were strongly expressed in the interdigital mesenchyme in uninjected limbs at HH stage 31 (A', B') but in RCAS-*Chrdl1* infected tissues expression was also detected throughout the anterior autopod where digits would normally develop (bracket in A, B) or where digits appear truncated (arrowhead in B). *Msx2* was also expressed at lower levels in RCAS-*Chrdl1* infected limbs (compare B with B'). *Sox9* was expressed in the digit rays at HH stage 27 in control limbs (C'; arrowheads). In *Chrdl1*-misexpressing limbs, *Sox9* was only detected in the primordium of digit 4 (C; arrowhead), but diffuse expression was also present in the anterior and distal mesenchyme (C; asterisk). *Scx* expression strongly labeled the digits (D'; arrowheads) as well as developing tendons of the zeugopod (D'; z) in control limbs. Only a truncated digit 4 in the RCAS-*Chrdl1* infected autopod was labeled by *Scx* hybridization (D; arrowhead) and sites of ectopic expression were detected in the distal and posterior limb (D; asterisk), whereas the morphology of the zeugopodial tendons (D'; z) were unaffected. *Bmp2* and *Bmp4* were upregulated in the autopod of RCAS-*Chrdl1* infected limbs, with higher *Bmp2* in the posterior autopod (E; bracket compared to E') and *Bmp4* in the distal mesenchyme below the AER (F; bracket compared to F'). Bar; 0.5 mm (A–F').

elements in the autopod, and further that the anterior digits were more sensitive to either lower levels or earlier loss of *Smad4*-mediated BMP signal disruption (Bénazet et al., 2012). Endogenous *Chrdl1* expression is significantly downregulated in the distal limb mesenchyme at stage 28 as the SHH-FGF feedback loop is terminating and the process of chondrogenic differentiation is initiating in the autopod. *Chrdl1* expression was also absent from the sites of digit formation in stage 31 limbs suggesting that inhibition of BMP signaling by *CHRD1* must be relieved for digit development to occur.

*Chrdl1* overexpression led to increased SHH and FGF signaling, detected as stronger *in situ* hybridization signals and broadened expression domains, as well as later persistence of FGF signaling from the AER. BMPs are known to negatively regulate expression of these signaling networks (Khokha et al., 2003; Zuniga et al., 1999) which, although critical for the growth and patterning of the limb, must be appropriately terminated to allow differentiation of skeletal tissues. In particular FGFs are essential for stimulating proliferation of limb bud mesenchymal cells and, along with Wnts, act to maintain them in an undifferentiated state (ten Berge et al., 2008). A potential function for FGF/Wnt signaling is to coordinate proliferation until the limb reaches the appropriate size and shape, such that cells within the limb bud outside the range of these 'differentiation-inhibiting' signals can then differentiate to form tissues such as cartilage (ten Berge et al., 2008). *Chrdl1* overexpression prevented the BMP-mediated inhibition of FGF and SHH signaling, and it is likely that the increased FGF signaling impairs the exit of mesenchymal cells from a proliferative state to undergo differentiation to form digits (ten Berge et al., 2008; Lopez-Rios et al., 2012).

Although anterior digits failed to form in *Chrdl1*-overexpressing limbs, ectopic broad domains of *Sox9* expression were detected in the anterior mesenchyme of infected limbs, but the *Sox9*-expressing cells failed to aggregate into condensations. This has previously been observed in *Smad4*-deficient autopods (Bénazet et al., 2012) and in *Nog*-overexpressing limbs (Pizette and Niswander, 2000) suggesting that although *Sox9* may be expressed by cells of the distal limb bud, this is not sufficient to drive the cell aggregation and mesenchymal condensation, which are required for chondrogenesis, in the absence of BMP signaling (Bénazet et al., 2012; Pizette and Niswander, 2000; Zeng et al., 2002). Ectopic *Scx* expression in the anterior mesenchyme of *Chrdl1*-overexpressing limbs was also diffuse rather than expressed by cells that form a developing digit structure. Tendon development and maintenance in the distal limb is highly dependent on the development of the digits (Hurle et al., 1990; Schweitzer et al., 2010), and *Scx* expression and therefore tendon formation is spatially restricted by BMP signaling from surrounding tissues such as the interdigital mesenchyme (Schweitzer et al., 2001). This provides a mechanism whereby RCAS-*Chrdl1* misexpression relieving this inhibition leads to ectopic *Scx* expression but not necessarily tendon formation which would require the presence of a digit.

The loss of digits in *Chrdl1*-misexpressing limbs seems contrary to studies in mice and humans where gene-deletion and/or putative loss-of-function mutations that reduce BMP signaling lead to a polydactyly phenotype (Bakrania et al., 2008; Choi et al., 2012; Luo et al., 1995; Selever et al., 2004). However, studies utilizing allelic series of *Bmp* mutant mice have demonstrated that reduction in BMP signaling can cause increased AER-FGF signaling, autopod enlargement and polydactyly, but that further inhibition of

BMPs beyond a certain threshold instead results in loss of skeletal elements by impairment of chondrogenesis (Bandyopadhyay et al., 2006; Bénazet et al., 2009). The skeletal malformations in *Chrdl1*-misexpressing limbs were restricted only to the autopod, and with anterior digits affected more severely than posterior digits. This anterior digit loss is unlikely to be a defect in patterning or anterior specification, since our *in situ* hybridization studies failed to show any abnormalities in expression of anterior markers such as *Alx4* or *Pax9* or genes involved in digit patterning such as *Gli3*, *Hoxd12* or *Hoxd13* (data not shown). The strong bias towards loss of anterior digits in *Chrdl1*-misexpressing limbs, also observed in mouse limbs deficient in Smad4-mediated BMP signal transduction (Bénazet et al., 2012), may reflect high levels of BMP activity in the posterior limb due to strong *Bmp2* and *Bmp7* expression (Geetha-Loganathan et al., 2006) as well as increasingly diminished *Grem1* expression (Scherz et al., 2004).

Ectopic *Grem1* and *Nog* have been demonstrated to cause severe limb malformations and truncations by early disruption of the SHH-FGF loop (Capdevila et al., 1999; Pizette and Niswander, 1999; Pizette et al., 2001; Zuniga et al., 1999). In contrast, alterations in SHH-FGF signaling in *Chrdl1*-misexpressing limbs were only detected later in limb development (HH stage 25 and later), at a timepoint when *Grem1* expression is normally being downregulated and increasing *Bmp* expression would contribute to termination of SHH-FGF signaling (Bastida et al., 2009; Bénazet et al., 2009; Scherz et al., 2004; Verheyden and Sun, 2008). These data suggest that the GREM1-SHH-FGF feedback system is resistant to *Chrdl1*-overexpression, but as *Grem1* expression is decreasing and the feedback loop begins terminating, ectopic CHRDL1 is sufficient to prevent BMPs from downregulating *Fgf* and *Shh* expression and from initiating SOX9-dependent cell condensation and chondrogenesis. The resistance of the GREM1-SHH-FGF network from *Chrdl1*-overexpression is unclear, but may result from different ligand specificity, binding affinity or protein activities of the highly divergent CHRDL1 and GREM1, or by the high buffering capacity of the signaling feedback system (Bénazet et al., 2009). These data illustrate that BMP levels must be mediated by antagonists for correct outgrowth and patterning to occur, through a process that is tolerant to *Chrdl1*-overexpression, but that later high levels of BMPs are crucial to formation of the correct complement of digits.

## Acknowledgements

This work was supported by the Howard Hughes Medical Institute. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. All chick experiments were performed in the Tabin laboratory. We thank Cliff Tabin for generous support and reagents, along with all members of the Tabin and Cepko labs who provided technical guidance and helpful discussions.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.06.003>.

## References

- Bachiller, D., Klingensmith, J., Shneyder, N., Tran, U., Anderson, R., Rossant, J., De Robertis, E.M., 2003. The role of chordin/Bmp signals in mammalian pharyngeal development and DiGeorge syndrome. *Development* 130, 3567–3578.
- Bakrania, P., Efthymiou, M., Klein, J.C., Salt, A., Bunyan, D.J., Wyatt, A., Ponting, C.P., Martin, A., Williams, S., Lindley, V., et al., 2008. Mutations in BMP4 cause eye, brain, and digit developmental anomalies: overlap between the BMP4 and hedgehog signaling pathways. *Am. J. Hum. Genet.* 82, 304–319.
- Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B.D., Rosen, V., Tabin, C.J., 2006. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* 2, e216.
- Bastida, M.F., Sheth, R., Ros, M.A., 2009. A BMP-Shh negative-feedback loop restricts Shh expression during limb development. *Development* 136, 3779–3789.
- Bénazet, J.-D., Zeller, R., 2009. Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system. *Cold Spring Harb Perspect. Biol.* 1, a001339.
- Bénazet, J.-D., Bischofberger, M., Tiecke, E., Gonçalves, A., Martin, J.F., Zuniga, A., Naef, F., Zeller, R., 2009. A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. *Science* 323, 1050–1053.
- Bénazet, J.-D., Pignatti, E., Nugent, A., Unal, E., Laurent, F., Zeller, R., 2012. Smad4 is required to induce digit ray primordia and to initiate the aggregation and differentiation of chondrogenic progenitors in mouse limb buds. *Development* 139, 4250–4260.
- ten Berge, D., Bruggmann, S.A., Helms, J.A., Nusse, R., 2008. Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. *Development* 135, 3247–3257.
- Brunet, L.J., McMahon, J.A., McMahon, A.P., Harland, R.M., 1998. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280, 1455–1457.
- Capdevila, J., Tsukui, T., Rodríguez Esteban, C., Zappavigna, V., Izpisua Belmonte, J.C., 1999. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* 4, 839–849.
- Chandra, A., Itakura, T., Yang, Z., Tamakoshi, T., Xue, X., Wang, B., Ueki, T., Sato, K., Uezato, T., Miura, N., 2006. Neurogenesis-1 differentially inhibits the osteoblastic differentiation by bone morphogenetic proteins in C2C12 cells. *Biochem. Biophys. Res. Commun.* 344, 786–791.
- Choi, K.-S., Lee, C., Maatouk, D.M., Harfe, B.D., 2012. Bmp2, Bmp4 and Bmp7 are co-required in the mouse AER for normal digit patterning but not limb outgrowth. *PLoS ONE* 7, e37826.
- Coffinier, C., Tran, U., Larraín, J., De Robertis, E.M., 2001. Neuralin-1 is a novel Chordin-related molecule expressed in the mouse neural plate. *Mech. Dev.* 100, 119–122.
- Crossley, P.H., Minowada, G., MacArthur, C.A., Martin, G.R., 1996. Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* 84, 127–136.
- Fernández-Terán, M.A., Hinchliffe, J.R., Ros, M.A., 2006. Birth and death of cells in limb development: a mapping study. *Dev. Dyn.* 235, 2521–2537.
- Francis, P.H., Richardson, M.K., Brickell, P.M., Tickle, C., 1994. Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. *Development* 120, 209–218.
- Gazzerro, E., Gangji, V., Canalis, E., 1998. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *J. Clin. Invest.* 102, 2106–2114.
- Geetha-Loganathan, P., Nimmagadda, S., Huang, R., Scaal, M., Christ, B., 2006. Expression pattern of BMPs during chick limb development. *Anat. Embryol.* 211 (Suppl 1), 87–93.
- Gong, Y., Krakow, D., Marcelino, J., Wilkin, D., Chitayat, D., Babul-Hirji, R., Hudgins, L., Cremers, C.W., Cremers, F.P., Brunner, H.G., et al., 1999. Heterozygous mutations in the gene encoding noggin affect human joint morphogenesis. *Nat. Genet.* 21, 302–304.
- Guha, U., Gomes, W.A., Kobayashi, T., Pestell, R.G., Kessler, J.A., 2002. *In vivo* evidence that BMP signaling is necessary for apoptosis in the mouse limb. *Dev. Biol.* 249, 108–120.
- Healy, C., Uwanogho, D., Sharpe, P.T., 1999. Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* 215, 69–78.
- Hurle, J.M., Ros, M.A., Gañan, Y., Macías, D., Critchlow, M., Hinchliffe, J.R., 1990. Experimental analysis of the role of ECM in the patterning of the distal tendons of the developing limb bud. *Cell Differ. Dev.* 30, 97–108.
- Kane, R., Godson, C., O'Brien, C., 2008. Chordin-like 1, a bone morphogenetic protein-4 antagonist, is upregulated by hypoxia in human retinal pericytes and plays a role in regulating angiogenesis. *Mol. Vis.* 14, 1138–1148.
- Khokha, M.K., Hsu, D., Brunet, L.J., Dionne, M.S., Harland, R.M., 2003. Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. *Nat. Genet.* 34, 303–307.
- Larman, B.W., Karolak, M.J., Adams, D.C., Oxburgh, L., 2009. Chordin-like 1 and twisted gastrulation 1 regulate BMP signaling following kidney injury. *J. Am. Soc. Nephrol.* 20, 1020–1031.
- Lehmann, K., Seemann, P., Silan, F., Goecke, T.O., Irgang, S., Kjaer, K.W., Kjaergaard, S., Mahoney, M.J., Morlot, S., Reissner, C., et al., 2007. A new subtype of brachydactyly type B caused by point mutations in the bone morphogenetic protein antagonist NOGGIN. *Am. J. Hum. Genet.* 81, 388–396.
- Logan, M., Tabin, C., 1998. Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses. *Methods* 14, 407–420.
- Lopez-Rios, J., Speziale, D., Robay, D., Scotti, M., Osterwalder, M., Nusspaumer, G., Galli, A., Holländer, G.A., Kmita, M., Zeller, R., 2012. GLI3 constrains digit number by controlling both progenitor proliferation and BMP-dependent exit to chondrogenesis. *Dev. Cell* 22, 837–848.
- Luo, G., Hofmann, C., Bronckers, A.L., Sohocki, M., Bradley, A., Karsenty, G., 1995. BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* 9, 2808–2820.
- McGlinn, E., Mansfield, J.H., 2011. Detection of gene expression in mouse embryos and tissue sections. *Methods Mol. Biol.* 770, 259–292.
- McLeod, M.J., 1980. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* 22, 299–301.

- McMahon, J.A., Takada, S., Zimmerman, L.B., Fan, C.M., Harland, R.M., McMahon, A.P., 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* 12, 1438–1452.
- Merino, R., Gañan, Y., Macias, D., Economides, A.N., Sampath, K.T., Hurlle, J.M., 1998. Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. *Dev. Biol.* 200, 35–45.
- Merino, R., Rodriguez-Leon, J., Macias, D., Gañan, Y., Economides, A.N., Hurlle, J.M., 1999. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development* 126, 5515–5522.
- Nakayama, N., Han, C.E., Scully, S., Nishinakamura, R., He, C., Zeni, L., Yamane, H., Chang, D., Yu, D., Yokota, T., et al., 2001. A novel chordin-like protein inhibitor for bone morphogenetic proteins expressed preferentially in mesenchymal cell lineages. *Dev. Biol.* 232, 372–387.
- Nissim, S., Hasso, S.M., Fallon, J.F., Tabin, C.J., 2006. Regulation of Gremlin expression in the posterior limb bud. *Dev. Biol.* 299, 12–21.
- Niswander, L., Jeffrey, S., Martin, G.R., Tickle, C., 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371, 609–612.
- Pizette, S., Niswander, L., 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development* 126, 883–894.
- Pizette, S., Niswander, L., 2000. BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev. Biol.* 219, 237–249.
- Pizette, S., Abate-Shen, C., Niswander, L., 2001. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* 128, 4463–4474.
- Ramel, M.-C., Hill, C.S., 2012. Spatial regulation of BMP activity. *FEBS Lett.* 586, 1929–1941.
- Rhee, D.K., Marcelino, J., Al-Mayouf, S., Schelling, D.K., Bartels, C.F., Cui, Y., Laxer, R., Goldbach-Mansky, R., Warman, M.L., 2005. Consequences of disease-causing mutations on lubricin protein synthesis, secretion, and post-translational processing. *J. Biol. Chem.* 280, 31325–31332.
- Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416.
- Robert, B., 2007. Bone morphogenetic protein signaling in limb outgrowth and patterning. *Dev. Growth Differ.* 49, 455–468.
- Robert, B., Lyons, G., Simandl, B.K., Kuroiwa, A., Buckingham, M., 1991. The apical ectodermal ridge regulates Hox-7 and Hox-8 gene expression in developing chick limb buds. *Genes Dev.* 5, 2363–2374.
- Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Si, Iemura, Yamamoto, T.S., Ueno, N., Noda, M., 2001. Ventroptin: a BMP-4 antagonist expressed in a double-gradient pattern in the retina. *Science* 293, 111–115.
- Scherz, P.J., Harfe, B.D., McMahon, A.P., Tabin, C.J., 2004. The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. *Science* 305, 396–399.
- Schweitzer, R., Chyung, J.H., Murtaugh, L.C., Brent, A.E., Rosen, V., Olson, E.N., Lassar, A., Tabin, C.J., 2001. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128, 3855–3866.
- Schweitzer, R., Zelzer, E., Volk, T., 2010. Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. *Development* 137, 2807–2817.
- Selever, J., Liu, W., Lu, M.-F., Behringer, R.R., Martin, J.F., 2004. Bmp4 in limb bud mesoderm regulates digit pattern by controlling AER development. *Dev. Biol.* 276, 268–279.
- Suzuki, T., Hasso, S.M., Fallon, J.F., 2008. Unique SMAD1/5/8 activity at the phalanx-forming region determines digit identity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4185–4190.
- Ueki, T., Tanaka, M., Yamashita, K., Mikawa, S., Qiu, Z., Maragakis, N.J., Hevner, R.F., Miura, N., Sugimura, H., Sato, K., 2003. A novel secretory factor, neurogenesis-1, provides neurogenic environmental cues for neural stem cells in the adult hippocampus. *J. Neurosci.* 23, 11732–11740.
- Verheyden, J.M., Sun, X., 2008. An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. *Nature* 454, 638–641.
- Wang, C.-K.L., Omi, M., Ferrari, D., Cheng, H.-C., Lizarraga, G., Chin, H.-J., Upholt, W.B., Dealy, C.N., Kosher, R.A., 2004. Function of BMPs in the apical ectoderm of the developing mouse limb. *Dev. Biol.* 269, 109–122.
- Webb, T.R., Matarin, M., Gardner, J.C., Kelberman, D., Hassan, H., Ang, W., Michaelides, M., Ruddle, J.B., Pennell, C.E., Yazar, S., et al., 2012. X-linked megalocornea caused by mutations in *CHRD1* identifies an essential role for ventroptin in anterior segment development. *Am. J. Hum. Genet.* 90, 247–259.
- Wijgerde, M., Karp, S., McMahon, J., McMahon, A.P., 2005. Noggin antagonism of BMP4 signaling controls development of the axial skeleton in the mouse. *Dev. Biol.* 286, 149–157.
- Wu, X., Shi, W., Cao, X., 2007. Multiplicity of BMP signaling in skeletal development. *Ann. N.Y. Acad. Sci.* 1116, 29–49.
- Zeng, L., Kempf, H., Murtaugh, L.C., Sato, M.E., Lassar, A.B., 2002. Shh establishes an *Nkx3.2/Sox9* autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. *Genes Dev.* 16, 1990–2005.
- Zou, H., Niswander, L., 1996. Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* 272, 738–741.
- Zuniga, A., Haramis, A.P., McMahon, A.P., Zeller, R., 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401, 598–602.
- Zuniga, A., Michos, O., Spitz, F., Haramis, A.-P.G., Panman, L., Galli, A., Vintersten, K., Klasen, C., Mansfield, W., Kuc, S., et al., 2004. Mouse limb deformity mutations disrupt a global control region within the large regulatory landscape required for Gremlin expression. *Genes Dev.* 18, 1553–1564.