Original Article

Impairment of *Sox9* Expression in Limb Buds of Rats Homozygous for Hypodactyly Mutation

(limb development / hypodactyly / Sox9 / Bmp signaling / the Norway rat)

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Abstract. Rat hypodactyly *(hd)* is an autosomal recessive mutation manifesting in homozygotes as reduction or loss of digits II and III. We mapped the *hd* allele to a short segment of chromosome 10, containing 16 genes. None of these genes has been shown to influence limb development yet. *In situ* hybridization showed no changes in several important patterning genes (*Shh*, *Fgf8*, *Bmp2, 4, 7*). However, we found that expression of cartilage condensation marker *Sox9*, and Bmp receptor *Bmpr1b* (acting as an upstream activator of *Sox9* expression) is absent from the subepithelial mesenchyme of the digit condensations II and III. The failure of the chondrogenic condensations to extend towards the subepithelial mesenchyme may reduce the size of digit primordia and underlie the subsequent loss of phalanges and reduction of metacarpals/metatarsals in *hd* rats.

Introduction

The limb of vertebrates serves as a valuable model of embryonic development. Assignment as well as readout of the positional information and coordination of cell proliferation, differentiation, motility, and death must be coordinated for proper formation of the elaborately patterned limb (Niswander, 2003). One of the fundamental aspects of limb development is the formation of the skeleton. During development of the limb skeleton, the limb bud mesenchyme first assembles into precartilage condensations that precede cartilage formation and enchondral ossification (Fell, 1925). The position and shape of the mesenchyme condensations is determined by interplay of various modes of cell communication. Signalling through the major limb patterning centres, apical ectodermal ridge (AER) and zone of polarizing activity (ZPA), as well as segmental patterning induced by *Hox* genes are thought to play a substantial role in this process (Capdevila and Izpisua Belmonte, 2001; Zakany and Duboule, 2007); however, the details of the skeletal element patterning have not been elucidated yet.

Precartilaginous condensations are formed in a proximal-distal order and can be visualized by *Sox9* expression earliest by E9.5 in the mouse forelimb (Ng et al., 1997).

Sox9, a transcription factor of the high-mobility group (HMG) family, is considered to be a master regulator of chondrogenesis. Conditional inactivation of Sox9 at varying times during mouse limb development has revealed that it is involved in all important steps of the cartilage formation, i.e. in condensation, proliferation and maturation. Akiyama (Akiyama et al., 2002) generated mouse embryos in which Sox9 was removed from limb mesenchymal cells prior to the onset of condensation, resulting in total absence of condensations and subsequent development of extremely short limbs without any skeletal components. Inactivation of Sox9 after mesenchymal condensation led to severe chondrodysplasia, when most of condensed mesenchymal cells did not differentiate into chondrocytes and proliferation of chondrocytes was inhibited.

Differentiation of chondrocytes is accompanied by secretion of molecules such as collagen type II (Col2a1), type IX (Col9a1), and aggrecan, forming cartilage-spe-

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Abbreviations: AER – apical ectodermal ridge, BMP – bone morphogenetic protein, BN – brown Norway rat, dpc – days post coitum, *hd* – hypodactyly, HMG – high-mobility group, ISH – *in situ* hybridization, PBS – phosphate-buffered saline, *Shbg* – sex hormone-binding globulin, SHR – spontaneously hypertensive rat, WHD – Wistar hypodactylous rat, WT – wild type, ZPA – zone of polarizing activity.

cific extracellular matrix. Sox9 directly induces the expression of *Col2a1*, a molecular marker of chondrocytes (Bell et al., 1997). Sox9 works in concert with two other transcription factors from the HMG family, Sox5 and Sox6 (Smits et al., 2001).

Bone morphogenetic protein (BMP) signalling (namely Bmp2, Bmp4, and Bmp7) transduced by heterodimers of type I receptors (Bmpr1a, Bmpr1b, ActR1) with type II receptor (Bmpr2) is important for early events in chondrogenesis. Studies have shown that Bmpr1a and Bmpr1b have distinct roles during chondrogenesis. In chick, Bmp signal transduced by Bmpr1b appears to be particularly important for precartilaginous condensation, while Bmpr1a is active during later phases of chondrogenesis (Zou et al., 1997). Mice deficient in *Bmpr1b* (Yi et al., 2000) are viable, exhibiting defects confined to phalangeal elements and appendicular joints, when initial formation of the digital rays occurs normally, but proliferation of prechondrogenic cells and chondrocyte differentiation are markedly reduced. *Bmpr1a* conditional knockout mice (Yoon et al., 2005) exhibit generalized chondrodysplasia with long bones shortened and ossification delayed. In double ($Bmpr1a^{-/-}$ and $Bmpr1b^{-/-}$) mutants (Yoon et al., 2005), all bones that form through endochondral ossification are either absent or malformed, and differentiation of prechondrocytes into chondrocytes is severely affected. Importantly, double mutants do not express *Sox9*, indicating that BMP signalling acts as an upstream activator of *Sox9* expression.

The *hd* mutation in the rat was described in 1973 (Moutier et al., 1973). Hypodactyly is an autosomal recessive trait – *hd*/*hd* homozygotes are affected, +/*hd* heterozygotes are indistinguishable from wild-type animals at the phenotypic level. The phenotype consists of the reduction or loss of digits II and/or III, in both fore- and hindlimbs. Moreover, males are infertile due to the impairment of the latest step of male gametogenesis – spermiogenesis (Liška et al., 2009).

We mapped *hd* previously to chromosome 10 (Křenová et al., 1999). Rat *hd* is therefore distinct from mouse *Hd*, caused by a 50 nucleotide deletion in exon 1 of the *Hoxa13* gene (Post et al., 2000). Besides the different mode of inheritance and different phenotype, its mapping excluded *Hoxa13* mutation as the molecular cause, since the *HoxA* cluster is located on rat chromosome 4. We previously investigated the possibility of *Shbg* (sex hormone-binding globulin) gene as the positional and molecular candidate because of the localization on the non-recombinant segment of chromosome 10 and the infertility seen in *hd/hd* males. *Shbg* was free of the coding sequence variation that could be associated with *hd*. The apparent expression of *Shbg* was stronger in *hd/hd* mutants, but this phenomenon might be due to a higher proportion of Shbg-producing Sertoli cells in the mutants (Liska et al., 2004).

We present here a fine mapping of *hd* to 464 kb, leading to *Shbg* exclusion. None of the 16 genes in the region has been implicated in limb development yet. Therefore, we screened limb buds of the mutants by whole-mount *in situ* hybridization (ISH) for expression of a panel of genes with known role in limb development. Many of the key patterning genes (including *Fgf8* and *Shh)* remain unchanged in the mutants; however, markers of chondrogenic condensations, *Sox9* and *Bmpr1b*, do not extend distally to the subepithelial layer in regions marking digit primordia II and III. This may be causative for the impairment of digit formation with subsequent reduction defects.

Material and Methods

Animals

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), which is in compliance with the European Community Council recommendations for the use of laboratory animals 86/609/ECC. All experiments were approved by The Charles University Animal Care Committee. *hd* is propagated as WHD (Wistar hypodactylous) strain by brother-sister mating of *hd/hd* females with (fertile) +/*hd* males. Congenic brown Norway rat (BN)-*hd* and spontaneously hypertensive rat (SHR)-*hd* rats were derived by cross-intercross mating of hypodactylous WHD females to BN/Cub or SHR/OlaIpcv males, respectively, using DNA-marker-assisted selection. Phenotype was scored visually.

Fine mapping (positional cloning)

Combined backcross (N = 497) and intercross (N = 353) between WHD and BN was employed. From 497 intercross animals, 50 were derived from BN-*hd* congenic production. The same is true for 33 out of the 353 backcross animals. Tail DNA was genotyped by PCR amplification of microsatellite markers selected from public databases or derived from the rat chromosome 10 sequence using Pompous (Fondon et al., 1998). Primer3 was used for primer design (Rozen and Skaletsky, 2000). The linkage map was constructed using MapManager QTX (Manly et al., 2001) separately for backcross, intercross, and congenic backcross- and intercross-like families and merged manually to form an integrated map.

Skeletal staining

Cartilage was stained by alcian blue, bone by alizarin according to Mundlos (2000).

Whole-mount in situ hybridization

Whole-mount ISH was performed as described in Stricker et al. (2006).

Results

Fine mapping of hd mutation

Using a combined backcross-intercross approach, we were able to refine the mapping of *hd* to 0.15 cM interval, which corresponds to a 463,782 bp segment of the

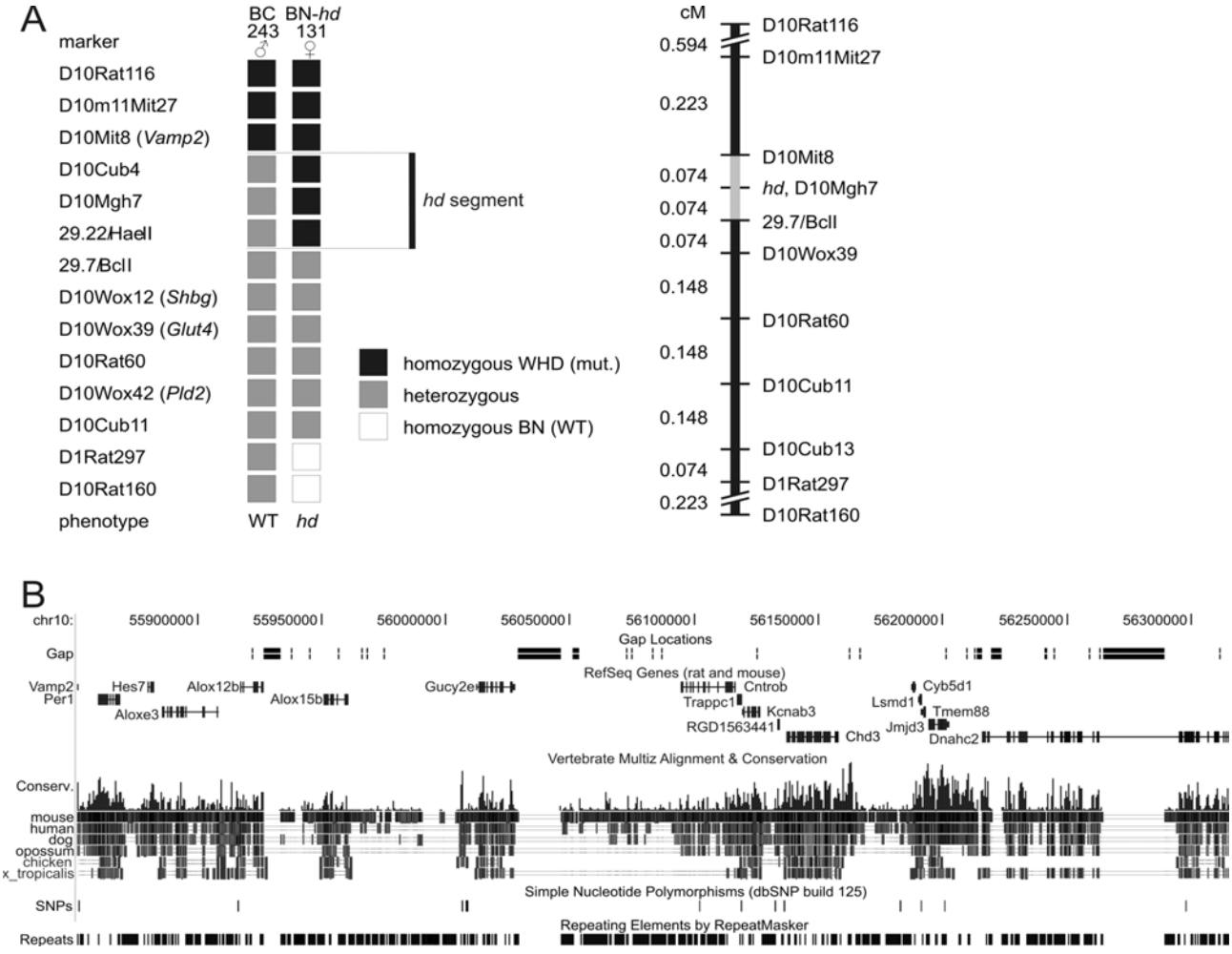

Fig. 1. Fine mapping of hd

(A) Key recombinants defining the *hd* region on the rat chromosome 10 (left) and the complete linkage map (right). Distances are shown in cM (Kosambi function). The map was derived from intercross (N = 497) and backcross (N = 353) progeny. (B) Visualization of the non-recombinant segment. Annotation tracts from top: chromosome position; gaps; genes from RefSeq database (Pruitt et al., 2007) – only those from rat and homologous genes from the mouse are shown; multiple vertebrate conservation by Multiz (Blanchette et al., 2004) + pairwise conservation with organism indicated; SNPs; and repetitive sequences. Detailed view of the 464 kb segment with additional annotation tracks can be obtained from UCSC Genome Browser internet page (www.genome.ucsc.edu). The respective coordinates are chr10:55851703-56315484, rat genome assembly version November 2004 (version 3.4 of the Rat Genome Sequencing Consortium). The interval contains 16 protein-coding genes (*Vamp2* is not counted, its coding sequence is outside the non-recombinant segment). There is no RNA-only coding gene in the segment.

rat genome, assembly 3.4 (Fig. 1A). This segment contains 16 genes and gene predictions (Fig. 1B). *Shbg* is localized 116,992 bp downstream of the 3' end of the non-recombinant segment (not shown), thus excluding the *Shbg* gene as a candidate.

Mutants show reduction or loss of the distal portion of digits II and III

hd mutation afflicts only the autopod of both fore- and hindlimbs (Fig. 2); stylopod and zeugopod of *hd*/*hd* mutants are comparable with wild-type controls (data not shown). Carpal and tarsal parts of the autopod are likewise unchanged in the mutants. Further, we found normal morphology of thumb and fingers and toes IV and V. However, digit II was usually nearly missing, with reduced or rudimentary metacarpal/metatarsal bone remaining. Digit III was more variably affected – it was often relatively preserved, or shortened and malformed or missing, like digit II, although relatively normal metacarpals/tarsals were formed. Notably, reduced digit III often displayed missing or greatly reduced ossification centres in phalanx 2 and 3. The latter forms earlier, by a different mechanism (Casanova and Sanz-Ezquerro, 2007; Montero and Hurle, 2007), at the tip of the distal phalanges.

Expression of known patterning genes is not changed in mutants

Using whole-mount ISH, we assessed the expression of a panel of genes known to be important for limb pat-

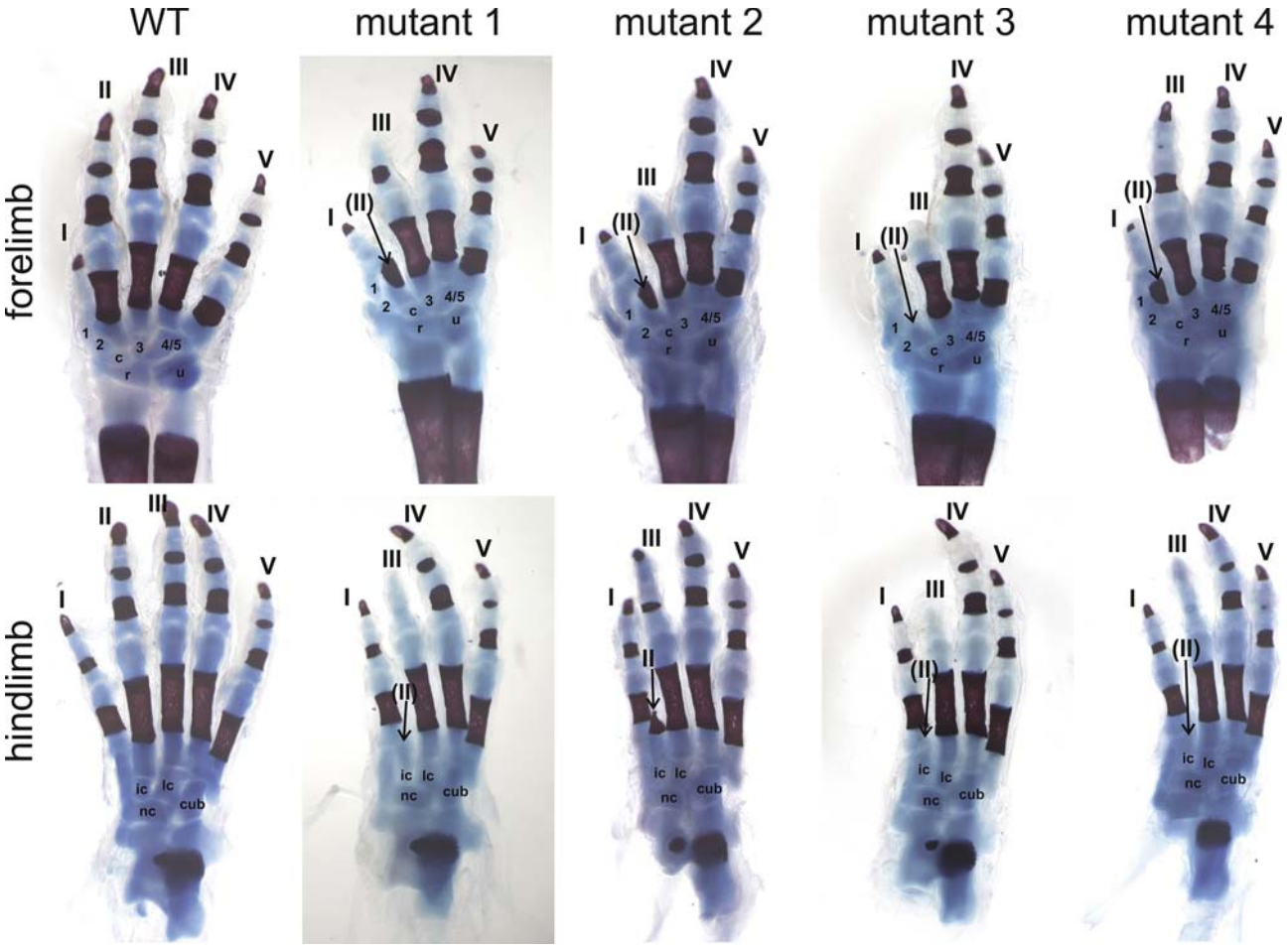

Fig. 2. Skeletal phenotype of hd mutants
Wild-type (WT) littermate on the left is compared to four mutants selected to represent the range of mutant phenotypes. Fingers are labelled by Roman numerals, carpal bones: 1 – *carpale I* (trapezium), 2 – *carpale II* (trapezoid), c – central carpal element, 3 – *carpale III* (*capitate*), 4/5 – *carpale IV* (*hamate*), r – *radiale*, u – *ulnare*; tarsal bones: ic – intermediate cuneiform, lc – lateral cuneiform, cub – cuboid, nc – navicular bone. In all mutants shown, digit II is reduced to a truncated or rudimentary metacarpal/metatarsal. Digit III has more variable affliction of the phalanges, which are shortened or missing. Note that ossification is also delayed (compare mutants 1, 3 and 4 with WT). Metacarpals and metatarsals of digit III are not significantly altered, as well as the carpal and tarsal elements. Samples were taken on first day after delivery (postnatal day 1, P1) and stained by alcian blue for cartilage and alizarin red for bone.

terning and morphogenesis. AER signalling was not altered in mutants, as can be demonstrated by expression of *Fgf8*, an established AER marker (Fig. 3A). ZPA signalling, conveyed by *Shh*, was also unchanged in the mutants, as inferred by *Shh* expression (Fig. 3B). Several members of the Bmp family are necessary for chondrogenic development of limb skeletal elements including digits. We therefore analysed the expression of *Bmp2, 4*, and *7*, which do not show apparent pattern alterations in *hd/hd* limb buds (Figs. 3C-E)

Mutants exhibit a distinct pattern of Bmpr1b and Sox9 expression

The initial *Sox9* expression pattern before distinct digit condensations are formed (13.5 dpc) is unchanged (Fig. 4A). Whole-mount ISH revealed differences in the expression of *Sox9*, a marker of chondrogenic condensation of the digit anlage. Normally, the expression at 14.5 dpc extends in the digit mesenchyme along the whole proximo-distal axis of the autopod, reaching the subepithelial layer (known as the progress zone). In *hd* mutants, this pattern is preserved for digits IV, V and digit I. However, for digit primordia II and III, there is a significant gap between the distal tip of the *Sox9*-positive primordium and the limb margin (AER) (Figs. 4B,C). Identical finding is replicated by *Bmpr1b* expression at 14.5 dpc, which does not reach the normal distal boundary with the progress zone (Fig. 4D,E). This altered expression pattern is concomitant with the formation of *Sox9*-positive digit primordium. The gross limb phenotype can already be recognized in mutants at this stage (14.5 dpc) in forelimbs.

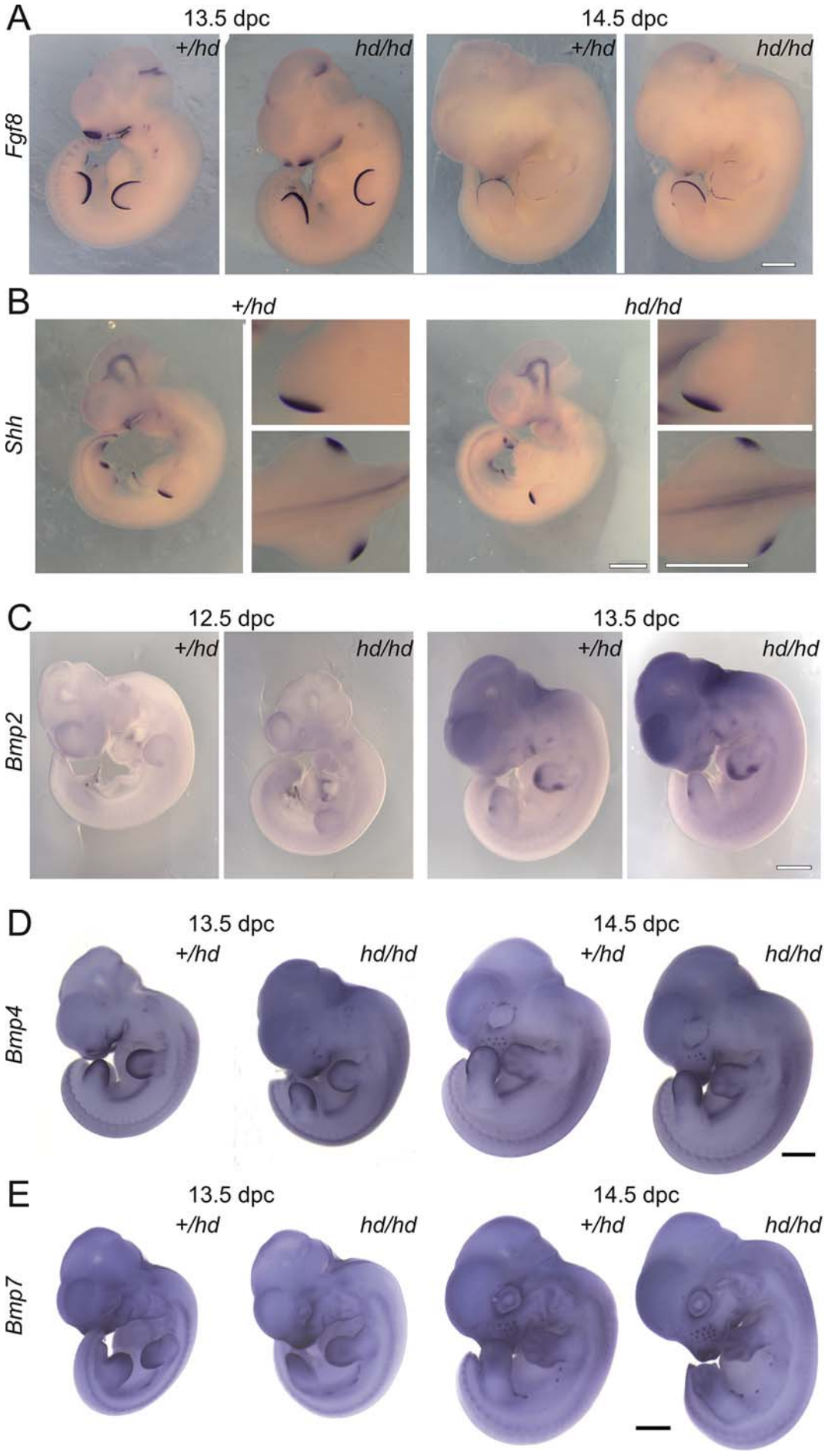
A
13.5 dpc
14.5 dpc
+/hd
hd/hd
+/hd
hd/hd
Fgf8
B
+/hd
hd/hd
Shh
C
12.5 dpc
13.5 dpc
+/hd
hd/hd
+/hd
hd/hd
Bmp2
D
13.5 dpc
14.5 dpc
+/hd
hd/hd
+/hd
hd/hd
Bmp4
E
13.5 dpc
14.5 dpc
+/hd
hd/hd
+/hd
hd/hd
Bmp7

Fig. 3. Expression of patterning genes in hd limb buds
(A) At 13.5 dpc, *Fgf8* is expressed in the AER of the limb buds and other regions (midbrain, nasal placode, branchial arches, otic vesicle). At 14.5 dpc, *Fgf8* signal in the AER is fading out. (B) *Shh* expression in the ZPA at 12.5 dpc. (C) *Bmp2* expression at 12.5 and 13.5 dpc. At 12.5 dpc, *Bmp2* is expressed at the periphery of the limb bud, presumably in both the AER and in subepithelial mesenchyme (sub-AER). Expression is also visible in the atrioventricular canal region of the heart. At 13.5 dpc, AER and sub-AER limb bud expression is more prominent, with distinct enlargement of the expression domain in its anterior and posterior aspects. There is clear expression in branchial arches and weak positivity in ventromedial parts of somites. Stronger signal in the brain of *hd/hd* embryos at 13.5 dpc is probably an artifact of the staining. (D) *Bmp4* expression at 13.5 and 14.5 dpc. In both stages there is strong AER and sub-AER expression. At 14.5 dpc, the expression extends into the interdigital mesenchyme, avoiding the precartilaginous condensations. In addition to the limb buds, *Bmp4* is expressed in dorsal parts of somites, branchial arches, nasal placode, whiskers, and gut. (E) *Bmp7* expression at 13.5 and 14.5 dpc. At 13.5 dpc, *Bmp7* is expressed throughout the limb bud, albeit with stronger signal at the periphery. At 14.5 dpc, *Bmp7* is expressed in interdigital mesenchyme, similarly to *Bmp4*. Besides limb buds there is positive signal in portions of the branchial arches, otic vesicles, throughout the dorsal somites, in whiskers, and around the eyes. Scale bars in all panels correspond to 1 mm.

Discussion

During our fine linkage mapping experiments, we found that *Shbg*, our previously suggested candidate gene, is recombined from the *hd* locus. It is still possible that the non-recombinant segment contains a *cis*-acting (non-coding, regulatory) sequence capable of long-range activation/inhibition of *Shbg* expression. However, *Shbg* is situated with its 3' end towards the non-recombinant segment with four other genes in both orientations interfering (*Efnb3, Wrap53,Tp53* and *Atp1b2*). Such hypothetical long-range signalling sequence would therefore likely influence these genes, too, which would be demonstrated by appropriate phenotypic consequences. E.g. Ephrin B3 (encoded by *Efnb3*) is necessary for proper neuronal control of locomotor activity such as walking (Kullander et al., 2003). Such a phenotype was not observed in *hd* mutants. The same is true for *Tp53 – hd* mutants do not have increased tumour incidence.

We revealed 16 positional candidates for *hd* here. None of these genes was implicated in limb development, nor in any developmental process as far as we know, including cell proliferation and cell death. Our approach to dissect the *hd* phenotype was based on scanning the expression of known patterning genes. We believe this can get us closer to the mechanistic understanding of the *hd* phenotype pathogenesis.

Understanding *hd* may offer a unique insight into limb development. First, there is as yet an unanticipated gene involved in the pathogenesis of this phenotype, as none of the candidate genes was associated with limb development as far as we know. Second, the phenotype itself is unique. Although we can find many examples of digit or autopod reduction in many models as well as in the clinic (Post et al., 2000; Chiang et al., 2001; Schwabe and Mundlos, 2004; Ogino, 2007; Gao et al., 2009), none of them is, to the best of our knowledge, similar to *hd* in details.

Expression of Bmps is unchanged in *hd* mutants, so the *hd* mutation appears to impair autopod development downstream of Bmp. On the other hand, digit formation is affected upstream of *Sox9*, as *Bmpr1b*, upstream activator of *Sox9* expression (Yoon et al., 2005) shows an altered expression pattern parallel to that of *Sox9*. Therefore, despite normal Bmp levels, the absence of Bmpr1B in the apical portion of the putative digital rays II and III leads to the absence of *Sox9* expression and subsequent lack of cartilage and bone formation. Mutations of human *BMPR1B* cause brachydactyly type A2 (Lehmann et al., 2003). Interestingly, the index finger is shortened in patients with this syndrome, and digit II is reduced or missing in *hd* mutants. However, the question arises whether there is direct influence of *hd* upon *Bmpr1b* expression. If such influence existed, it would be spatially restricted to the distal portion of the digital ray. A plausible hypothesis may be formed in conjunction with the progress zone model (Summerbell et al., 1973; Tabin and Wolpert, 2007), where the digits grow distally by recruitment of cells from the mesenchyme layer just beneath the epithelium. This model was updated recently by introducing the concept of phalanx-forming region (Suzuki et al., 2008) or digit crescent (Montero et al., 2008), a specialized group of cells at the tip of each digit, expressing *Bmpr1b* and *Sox9*, that recruits the mesenchyme cells from the progress zone and promotes their differentiation into the digit condensations. The *Bmpr1b* and *Sox9* expression pattern in mutants suggest that possibly these cells are recruited from the progress zone, but they are not properly instructed to become part of the digit anlagen, remaining undifferentiated instead. Perhaps these cells later adopt the "interdigital fate" and are lost by apoptosis, like the normal interdigital mesenchyme.

In conclusion, we present here fine mapping of *hd* mutation with resulting 16 positional candidate genes. We propose a connection between impairment of limb development in *hd* homozygotes and defective formation of the prechondrogenic condensations in apical portion of digits II and III.

Acknowledgment

Gene abbreviations are used according to Entrez Gene Database (Maglott et al., 2007) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

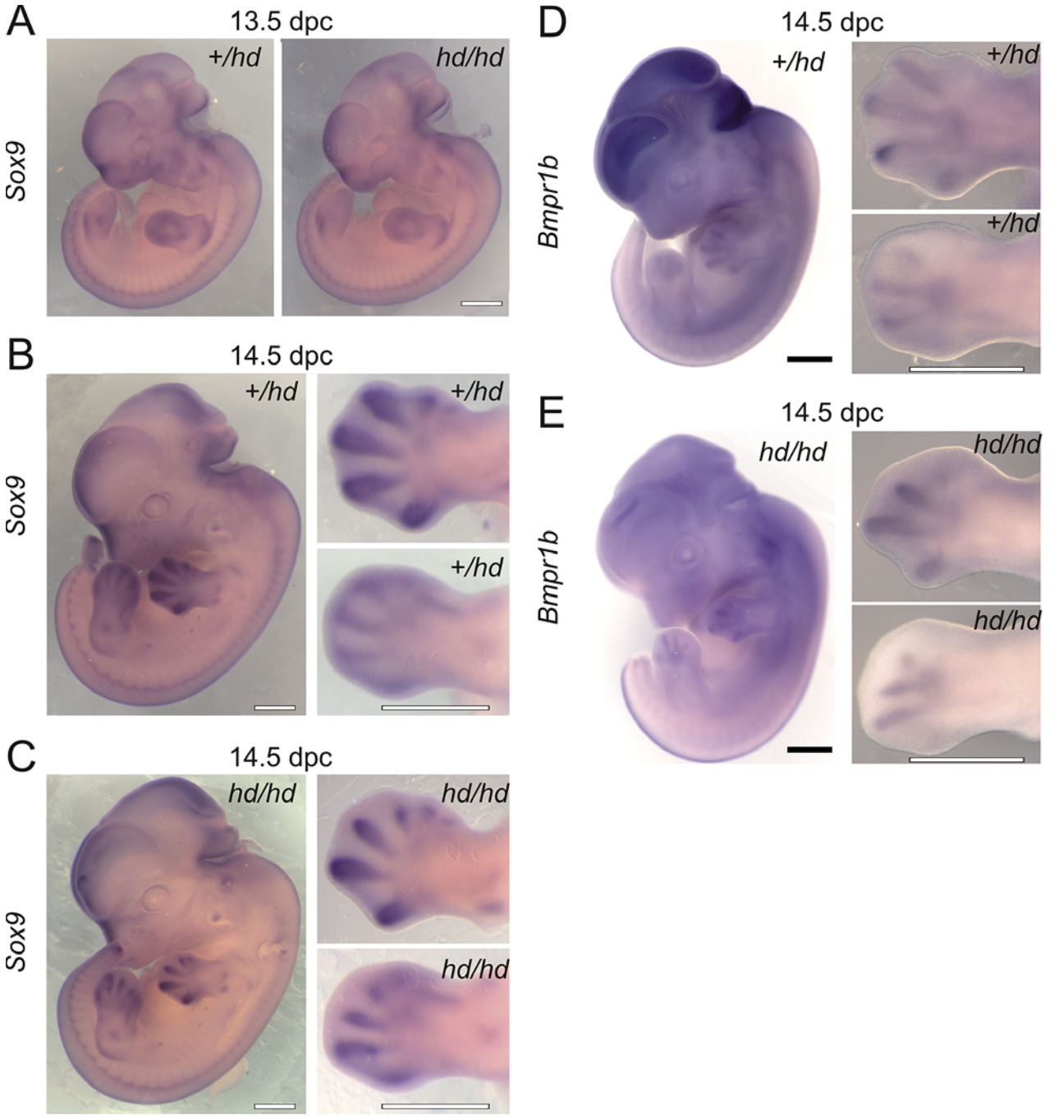

Fig. 4. Expression of Sox9 and Bmpr1b in hd limb buds
(A) *Sox9* is expressed in limb bud mesenchyme at 13.5 dpc. The expression is relatively weak (especially in hind limbs) and diffuse. In forelimbs, the signal is more concentrated towards the periphery. There is no difference between WT and *hd/hd* embryos. *Sox9* is also expressed in somites, neural tube and brain. (B) At 14.5 dpc *Sox9* clearly marks the precartilagenous condensations of the digital rays in forelimbs. The *Sox9* pattern of hind limbs is less developed, but shows the same tendency. (C) In both fore- and hindlimbs of *hd/hd* embryos, there is a gap between the AER and the tip of the *Sox9*-positive area for digits II and III, so the respective condensations are shorter than in WT. (D, E) *Bmpr1b* staining at 14.5 dpc reveals an expression pattern defect strikingly similar to *Sox9*. Scale bars in all panels correspond to 1 mm.

References

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., Crombrugghe, B. (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* **16,** 2813-2828.

Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P., Cheah, K. S. (1997) SOX9 directly regulates the type-II collagen gene. *Nat. Genet.* **16,** 174-178.

Blanchette, M., Kent, W. J., Riemer, C., Elnitski, L., Smit, A. F., Roskin, K. M., Baertsch, R., Rosenbloom, K., Clawson,

H., Green, E. D., Haussler, D., Miller, W. (2004) Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res.* **14,** 708-715.

Capdevila, J., Izpisua Belmonte, J. C. (2001) Patterning mechanisms controlling vertebrate limb development. *Annu Rev. Cell Dev. Biol*. **17,** 87-132.

Casanova, J. C., Sanz-Ezquerro, J. J. (2007) Digit morphogenesis: is the tip different? *Dev. Growth Differ.* **49,** 479-491.

Chiang, C., Litingtung, Y., Harris, M. P., Simandl, B. K., Li, Y., Beachy, P. A., Fallon, J. F. (2001) Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. *Dev. Biol.* **236,** 421-435.

Fell, H. B. (1925) The histogenesis of cartilage and bone in the long bones of the embryonic fowl. *J. Morphol.* **40,** 417-459.

Fondon, J. W., 3rd, Mele, G. M., Brezinschek, R. I., Cummings, D., Pande, A., Wren, J., O'Brien, K. M., Kupfer, K. C., Wei, M. H., Lerman, M., John D. Minna, J. D., Garner, H. R. (1998) Computerized polymorphic marker identification: experimental validation and a predicted human polymorphism catalog. *Proc. Natl. Acad. Sci. USA* **95,** 7514-7519.

Gao, B., Hu, J., Stricker, S., Cheung, M., Ma, G., Law, K. F., Witte, F., Briscoe, J., Mundlos, S., He, L., Cheah, K. S. E., Chan, D. (2009) A mutation in Ihh that causes digit abnormalities alters its signalling capacity and range. *Nature* **458,** 1196-1200.

Křenová, D., Pravenec, M., Housa, D., Liška, F., Bílá, V., Kašpárek, R., Křen, V. (1999) Linkage mapping of rat hypodactyly locus to chromosome 10. *Transplant. Proc.* **31,** 1620.

Kullander, K., Butt, S. J., Lebret, J. M., Lundfald, L., Restrepo, C. E., Rydstrom, A., Klein, R., Kiehn, O. (2003) Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. *Science* **299,** 1889-1892.

Lehmann, K., Seemann, P., Stricker, S., Sammar, M., Meyer, B., Suring, K., Majewski, F., Tinschert, S., Grzeschik, K. H., Muller, D., Knaus. P., Nürnberg, P., Mundlos, S. (2003) Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. *Proc. Natl. Acad. Sci. USA* **100,** 12277-12282.

Liska, F., Gosele, C., Kren, V., Hubner, N., Krenova, D. (2004) Molecular analysis of the sex hormone-binding globulin gene in the rat hypodactylous mutation (Hd). *Folia Biol. (Praha)* **50,** 63-68.

Liska, F., Gosele, C., Rivkin, E., Tres, L., Cardoso, M. C., Domaing, P., Krejci, E., Snajdr, P., Lee-Kirsch, M. A., de Rooij, D. G., Křen V., Křenová D., Kierszenbaum, A. L., Hubner, N. (2009) Rat hd mutation reveals an essential role of centrobin in spermatid head shaping and assembly of the head-tail coupling apparatus. *Biol. Reprod.* **81**, 1196-1205.

Maglott, D., Ostell, J., Pruitt, K. D., Tatusova, T. (2007) Entrez Gene: gene-centered information at NCBI. *Nucleic Acids Res.* **35,** D26-31.

Manly, K. F., Cudmore, R. H., Jr., and Meer, J. M. (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm. Genome* **12,** 930-932.

Montero, J. A., Hurle, J. M. (2007) Deconstructing digit chondrogenesis. *Bioessays* **29,** 725-737.

Montero, J. A., Lorda-Diez, C. I., Ganan, Y., Macias, D., Hurle, J. M. (2008) Activin/TGFβ and BMP crosstalk determines digit chondrogenesis. *Dev. Biol.* **321,** 343-356.

Moutier, R., Toyama, K., Charrier, M. F. (1973) Hypodactyly, a new recessive mutation in the Norway rat. *J. Hered.* **64,** 99-100.

Mundlos, S. (2000) Skeletal morphogenesis. *Methods Mol. Biol.* **136,** 61-70.

Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., Koopman, P. (1997) SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183,** 108-121.

Niswander, L. (2003) Pattern formation: old models out on a limb. *Nat. Rev.* **4,** 133-143.

Ogino, T. (2007) Clinical features and teratogenic mechanisms of congenital absence of digits. *Dev. Growth Differ.* **49,** 523-531.

Post, L. C., Margulies, E. H., Kuo, A., Innis, J. W. (2000) Severe limb defects in Hypodactyly mice result from the expression of a novel, mutant HOXA13 protein. *Dev. Biol.* **217,** 290-300.

Pruitt, K. D., Tatusova, T., Maglott, D. R. (2007) NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* **35,** D61-65.

Rozen, S., Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132,** 365-386.

Schwabe, G. C. Mundlos, S. (2004) Genetics of congenital hand anomalies. *Handchir. Mikrochir. Plast. Chir.* **36,** 85-97.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R. R., de Crombrugghe, B., Lefebvre, V. (2001) The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev. Cell* **1,** 277-290.

Stricker, S., Verhey van Wijk, N., Witte, F., Brieske, N., Seidel, K., Mundlos, S. (2006) Cloning and expression pattern of chicken Ror2 and functional characterization of truncating mutations in Brachydactyly type B and Robinow syndrome. *Dev. Dyn.* **235,** 3456-3465.

Summerbell, D., Lewis, J. H., Wolpert, L. (1973) Positional information in chick limb morphogenesis. *Nature* **244,** 492-496.

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. USA* **105,** 4185-4190.

Tabin, C., Wolpert, L. (2007) Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* **21,** 1433-1442.

Yi, S. E., Daluiski, A., Pederson, R., Rosen, V., Lyons, K. M. (2000) The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. *Development* **127,** 621-630.

Yoon, B. S., Ovchinnikov, D. A., Yoshii, I., Mishina, Y., Behringer, R. R., Lyons, K. M. (2005) Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. *Proc. Natl. Acad. Sci. USA* **102,** 5062-5067.

Zakany, J., Duboule, D. (2007) The role of Hox genes during vertebrate limb development. *Curr. Opin. Genet. Dev.* **17,** 359-366.

Zou, H., Wieser, R., Massague, J., Niswander, L. (1997) Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11,** 2191-2203.