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# A group 13 homeodomain is neither necessary nor sufficient for posterior prevalence in the mouse limb

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**Abstract**

Posterior prevalence is the general property attributed to HOX proteins describing the dominant effect of more posterior HOX proteins over the function of anterior orthologs in common areas of expression. To explore the HOX group 13 protein domains required for this property, we used the mouse *Prx-1* promoter to drive transgenic expression of Hox constructs throughout the entire limb bud during development. This system allowed us to conclusively demonstrate a hierarchy of Hox function in developing limbs. Furthermore, by substituting the HOXD11 or HOXA9 homeodomain for that of HOXD13, we show that a HOXD13 homeodomain is not necessary for posterior prevalence. Proximal expression of these chimeric proteins unexpectedly caused defects consistent with wild-type HOXD13 mediated posterior prevalence. Moreover, group 13 non-homeodomain residues appear to confer the property as proximal expression of HOXA9 containing the HOXD13 homeodomain did not result in limb reductions characteristic of HOXD13. These data are most compatible with models of posterior prevalence based on protein–protein interactions and support examination of the N-terminal non-homeodomain regions of Hox group 13 proteins as necessary agents for posterior prevalence.


*Keywords:* Posterior prevalence; HOXD13; Hox; Homeodomain; Limb

## Introduction

Hox genes are crucial developmental regulators in animals. The 5′ members of the A and D clusters, corresponding to paralog groups 9–13, evolved from a single ancestral gene related to *Drosophila Abdominal B* (*AbdB*) and are expressed during limb outgrowth (Dolle et al., 1989; Haack and Gruss, 1993; Izpisua-Belmonte et al., 1991; Nelson et al., 1996; Yokouchi et al., 1991).

Initiation and maintenance of the precise expression domains of Hox genes are critical for normal patterning. Generally, it has been shown that each *AbdB*-like paralog group expressed in the limb, 9–13, contributes primarily to the growth of one selected segment (Davis et al., 1995; Fromental-Ramain et al., 1996; Rijli and Chambon, 1997; Small and Potter, 1993; Wellik and Capecchi, 2003). Group 13 genes are most important for the development of the autopod, group 11 for the zeugopod and groups 9 and 10 for the stylopod.

Alteration, misexpression or gain-of-function in the expression of distally restricted Hox genes in the limb results in shortening of skeletal elements usually in regions proximal to their normal expression boundary (Caronia et al., 2003; Goff and Tabin, 1997; Knezevic et al., 1997; Morgan et al., 1992; Yokouchi et al., 1995). This phenomenon acting along the distal to proximal axis of the limb is analogous to the dominance of posteriorly expressed genes over anterior genes in the axial skeleton (Balling et al., 1989; Kessel and Gruss, 1990; Lufkin et al., 1992; McGinnis and Krumlauf, 1992; van den Akker et al., 2001; Wellik and Capecchi, 2003). This property, referred to as posterior prevalence (Duboule, 1991), has been studied in chicks and mice and, with minor variations, this Hox functional hierarchy is conserved from flies to mammals (Bachiller et al., 1994; Duboule and Morata, 1994; Gonzalez-Reyes and Morata, 1990).

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There are several experimental and natural examples of the effects of posterior prevalence and, when examined together, suggest the likelihood of a functional hierarchy in the limb buds. Misexpression of either *Hoxd13* and *Hoxa13* in chick limbs results in shortening of bones in segments proximal to the autopod consistent with posterior prevalence (Goff and Tabin, 1997; Yokouchi et al., 1995). Misexpression of two N-terminally truncated forms of HOXD13, lacking the first 80 or 98 amino acids, leads to milder shortening of the skeletal elements of the zeugopod, suggesting that the N-terminal region plays a role in the activity of the protein but not the specificity. The spontaneous mutant *Ulnaless* is an example of posterior prevalence in the mouse (Herault et al., 1997; Peichel et al., 1997; Spitz et al., 2003). The limbs of this mutant partially resemble *Hoxa11*/*Hoxd11* double homozygous null mice in that the zeugopods are extremely reduced. This phenotype results from abnormal proximal (zeugopodal) expression of *Hoxd12* and *Hoxd13*, as well as downregulation of *Hoxd11* expression normally present within the zeugopod. Another study used the *Hoxb6* promoter to misexpress *Hoxd12* in posterior lateral plate mesoderm, the distal, posterior forelimb bud and the entire hindlimb bud in transgenic mice (Knezevic et al., 1997). Transgenic mice showed preaxial (anterior) posteriorization and/or digit duplication, and reductions were isolated to preaxial skeletal elements in the hindlimb (Knezevic et al., 1997). Similarly, Morgan et al. (1992) and Goff and Tabin (1997) used retroviral expression in chick limbs and showed that *Hoxd11* induced preaxial polydactyly in wings and, like *Hoxd12*, posteriorization of anterior digit I. However, Goff and Tabin also found shortening of the leg zeugopods and concluded that *Hoxd11* could exert effects early during mesenchymal condensation as well as later during bone growth.

The phenomenon of posterior prevalence is interrelated with how different Hox proteins gain specificity in vivo (Duboule and Morata, 1994; Gehring et al., 1994) and ultimately is attributable to the primary amino acid sequence. In *Drosophila*, the C-terminal region, including but not exclusive to the homeodomain, which is involved in DNA binding and some cofactor interactions, plays a critical role in specificity of function (Chan and Mann, 1993; Gibson et al., 1990; Kuziora and McGinnis, 1989; Lin and McGinnis, 1992; Mann and Hogness, 1990; Zeng et al., 1993). Studies in animal models also point to the Hox homeodomain, as an important determinant of Hox functional specificity (Chang et al., 1996; Furukubo-Tokunaga et al., 1993; Kuziora and McGinnis, 1989; Lin and McGinnis, 1992; Mann and Affolter, 1998; Zappavigna et al., 1994; Zeng et al., 1993; Zhao and Potter, 2001, 2002). For example, substitution of the HOXA11 homeodomain with that of HOXA13 in mice revealed that the homeodomain of HOXA13, though duplicated from and highly similar to other *AbdB*-like HOX homeodomains, is functionally different from that of HOXA11. While the HOXA13 homeodomain showed equivalent function to HOXA11 in the axial skeleton and the male reproductive tract, it acted dominantly or with different function in the female reproductive tract and in the developing limbs where it led to shortening of the zeugopod (Zhao and Potter, 2002). However, it is not known whether all functional differences, including posterior prevalence, can be attributed solely to the amino acid sequence of the homeodomain (Chauvet et al., 2000; Galant and Carroll, 2002; Ronshaugen et al., 2002; Sreenath et al., 1996). For example, gain-of-function studies in transgenic mice that replaced the HOXA4 homeodomain with that of HOXC8 showed that the functional specificity of HOXA4 in the axial skeleton is determined by non-homeodomain residues (Sreenath et al., 1996). Moreover, non-homeodomain regions of HOX protein paralogs diverge significantly, contain cofactor interaction motifs and harbor activation and repression domains that offer numerous opportunities for functional differences and specificity to be achieved (Krumlauf, 1994; Mann and Morata, 2000; Merabet et al., 2003; Vigano et al., 1998; Williams et al., 2005; Yaron et al., 2001).

In this work, we systematically test the contribution of amino acid sequence to HOX group 13 posterior prevalence in the mouse limb using one experimental system. The mouse *Prx-1* promoter was used to drive mesenchymal transgenic expression of Hox constructs (Martin and Olson, 2000) at early stages during fore- and hindlimb development. We document expression of the transgenic RNA and protein and describe the phenotypes of transgenic founders and offspring. We show that, as expected, a HOX functional hierarchy exists in the developing limb. However, the non-homeodomain region of the group 13 HOXD protein was capable of exerting posterior prevalence with either a homeodomain from HOXD11 or HOXA9. Moreover, despite our expectation that the HOXD13 homeodomain would be the primary functional determinant of posterior prevalence, a HOXA9 protein with a HOXD13 homeodomain failed to exert posterior prevalence. These studies support efforts to identify the non-homeodomain protein motifs responsible for posterior prevalence.

## Materials and methods

### *Transgene creation and injection*

*Hoxd13* (1–2814 bp, Genbank NM_008275), *Hoxa13* (1–3101 bp, NM_008264), *Hoxd11* (−58–2231 bp, NM_008273) and *Hoxa9* (1–3001 bp, NM_010456) genomic DNA sequences were ligated within the *Bgl*II site of a 4.5 kb fragment containing the *Prx-1* promoter (Martin and Olson, 2000; Post et al., 2000). All Hox transgenes except *Hoxd11* have a consensus Kozak sequence (cccgccgccacc) engineered adjacent to the genuine start codon. The *Hoxd11* transgene includes 58 base pairs of endogenous 5′UTR sequence upstream of the start codon. All genes use their respective introns, 3′ UTRs and polyadenylation signals. $Hoxd13^{IQN>AAA}$ was created using PCR SOEing with primers containing the sequence alterations (Horton et al., 1989). All constructs were DNA sequence verified.

Prx1-Hox transgenes were digested with *Sal*I to remove vector sequences, gel purified and injected into fertilized B6/D2 F2 or B6/SJL F2 mouse eggs with the help of the University of Michigan Transgenic Core Facility (www.med.umich.edu/tamc/). Transgenic founders were identified by PCR using primers specific to *Prx-1* and the appropriate Hox gene.

### *Chimeric and mutant transgene construction*

For the chimeric Hox constructs Prx1-$Hoxd13^{d11HD}$ and Prx1-$Hoxd13^{a9HD}$, the *Hoxd11* or *Hoxa9* homeobox replaces the *Hoxd13* homeobox; all other sequences are the same as Prx1-Hoxd13. Prx1-$Hoxa9^{d13HD}$ contains the *Hoxd13* homeobox replacing the *Hoxa9* homeobox; all other sequences are the same as that for Prx1-Hoxa9. Chimeric and mutant constructs were generated using PCR

SOEing (Horton et al., 1989) and completely sequenced to ensure the absence of PCR-induced mutations. *Prx-1* is expressed as early as E9.5 in the lateral plate mesoderm in the prospective limb fields before the limb bud is visualized (Leussink et al., 1995). Expression continues throughout the undifferentiated mesenchyme of developing limbs and is subsequently downregulated as cells begin chondrogenic differentiation. Expression is maintained in the limb through E17.5 within the perichondrium, dermis and developing tendons (Leussink et al., 1995; Martin and Olson, 2000). Immunocytochemistry confirmed expression of Prx1-Hox transgene constructs and proper nuclear localization of the protein. *Prx-1* driven HOXD13, HOXA13 and HOXA9 proteins were localized to the nucleus in transfected Cos-7 cells (data not shown). HOXD11 expression could not be evaluated as there is no antibody currently available.

### *Skeletal preparation and analysis*

Transgenic founders were evaluated for limb skeletal malformations by staining with Alcian blue and Alizarin red at selected stages (Kimmel and Trammell, 1981). Transgenic progeny were generated for skeletal analysis by crossing founders, when possible, with C57BL/6J mice. Images of skeletons were captured using a DEI750 Optronics digital camera and Adobe Photoshop software. Bone lengths were analyzed using the public domain NIH ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

### *Whole-mount in situ hybridization*

In situ hybridization using transgenic embryos of various stages was completed and confirmed that the *Prx-1* promoter drives transgene RNA expression throughout the limb from E10.5 to E12.5 as previously reported (Martin and Olson, 2000) (data not shown). All expression analysis was performed using embryo offspring collected from matings of C57BL/6J mice with transgenic founders at E10.5, E11.5, E12.5 or E13.5. Embryos were staged by assigning noon of the day of vaginal plug as E0.5. 1114 base pairs of 3′UTR from *Hoxa9* were amplified from C57BL/6J RNA and ligated into the pCR4-TOPO vector (Invitrogen). *Hoxd13* and *Hoxd11*, and *Shh* (Echelard et al., 1993) were kindly provided by D. Duboule and A. McMahon, respectively. Single-stranded RNA probes were synthesized from these DNA templates. Whole-mount in situ hybridization with a single digoxygenin-labeled RNA probe was performed as previously described (Bober et al., 1994), except that BM purple (Roche) was used as the substrate for alkaline phosphatase.

### *Whole-mount immunohistochemistry*

Immunohistochemistry was performed as described (Yokouchi et al., 1995) on wild-type and transgenic embryos collected at E11.5 or E12.5. The anti-HOXA13 (Post et al., 2000), anti-HOXD13 (Williams et al., 2005) and rabbit pre-immune serum were used at a dilution of 1:10,000. The secondary antibody donkey anti-rabbit HRP-conjugate (Amersham) was used at a dilution of 1:500.

### *Preparation of limb bud lysates and western analysis*

Embryos were collected at E12.5 from matings between transgenic founder 42 (Prx1-Hoxa9$^{d13HD}$) and wild-type C57BL/6J females in cold PBS, pH 7.4. Extra-embryonic membranes were removed and saved for DNA isolation and genotyping. Distal and proximal segments of each limb were dissected, and all distal and proximal segments from each embryo were pooled separately, snap frozen in liquid nitrogen and stored at −80°C prior to genotyping. Equal numbers of transgenic and wild-type limbs were pooled. Lysates were prepared by homogenizing limb tissue in lysis buffer [10 mM HEPES (pH 7.5), 1 mM EDTA, 250 mM NaCl, 0.5% NP-40, protease inhibitors (Roche)] and incubating on ice for 25 min. After adding 5 M NaCl to a final concentration of 420 mM, lysates were homogenized and incubated for 5 min on ice. Two microliters β-mercaptoethanol was added to each lysate, and lysates were heated to 100°C for 5 min. Lysates were centrifuged at 5000 rpm for 5 min and supernatants removed to fresh tubes and stored at −20°C (Chen et al., 2004). HOXA9 positive control lysate was created similarly using tissue culture cells transfected with a pCMV5 driven *Hoxa9* expression construct.

Equal protein for each sample was separated by electrophoresis using 12% SDS-polyacrylamide gels and subsequently electrotransferred to nitrocellulose. Membranes were blocked by incubation with 5% Carnation milk for 1 h at room temperature. Primary antibodies were used at 1:1000 for goat anti-HOXA9 (Santa Cruz Biotechnology, Inc) and 1:10,000 for rabbit anti-HOXD13 (Williams et al., 2005). The secondary antibodies, anti-goat HRP (Santa Cruz Biotechnology, Inc) and anti-rabbit HRP-conjugated (Amersham), were used at 1:2000 and 1:10,000 respectively. Proteins were visualized using Supersignal chemiluminescent substrate (Pierce).

## Results

### *HOXA13 and HOXD13 misexpression results in severe skeletal reduction in proximal regions of mouse limbs*

*Hoxa13* and *Hoxd13* are the most 5′ Hox genes within the A and D clusters. These genes are the most distally restricted of the Hox genes expressed in the developing limb bud and promote growth of the autopod elements (Fromental-Ramain et al., 1996). Based on prior experiments, we hypothesized that these genes would pattern dominantly over other normally expressed anterior *AbdB*-like Hox genes in the zeugopod and stylopod upon whole limb transgenic expression (Goff and Tabin, 1997; van der Hoeven et al., 1996; Yokouchi et al., 1995).

### *Founder analysis*

Five Prx1-Hoxd13 and five Prx1-Hoxa13 transgenic founders were collected at E16.5 and prepared for skeletal staining. Representative results of the phenotypic effects of transgenic expression of *Hoxd13* or *Hoxa13* throughout the mesenchyme of developing mouse limbs are shown in Fig. 1A, B and described in Supplementary Tables 1 and 2. Every founder analyzed at E16.5 had severe reduction of zeugopodal bones in both the fore- and hindlimbs. Average zeugopod reductions in E16.5 Prx1-Hoxd13 founders were: radius 92%, ulna 85%, tibia 89% and fibula 89%. In E16.5 Prx1-Hoxa13 founders, the average skeletal reductions were: radius 72%, ulna 80%, tibia 76% and fibula 77%. Five of the ten Prx1-Hoxd13 and Prx1-Hoxa13 founders also had severe reduction/malformation of the stylopods and scapulas; the other five founders had minimal defects in these regions. Transgene expression also resulted in variable preaxial polydactyly, of both fore- and hindlimbs, or oligodactyly of the forelimbs in founders 712.1, 702.4 and 702.1. In non-limb regions, negative effects on the growth of the skull and mandibular bones were also observed presumably due to additional sites of *Prx-1* promoter expression (Martin and Olson, 2000), and such defects were associated with lethality soon after birth.

### *Transgenic progeny*

Two additional male Prx1-Hoxd13 founders, 706.6 and 713.7, were viable at birth, survived to reproductive age and were mated to produce transgenic progeny. 713.7 was unable to reproduce. Progeny from 706.6 were collected for phenotypic and transgene expression analysis. RNA and protein expression from the Prx1-Hoxd13 transgene was confirmed in progeny from 706.6 by in situ hybridization and immunohistochemistry (Figs. 1C–G). The transgenic offspring from 706.6 had a more

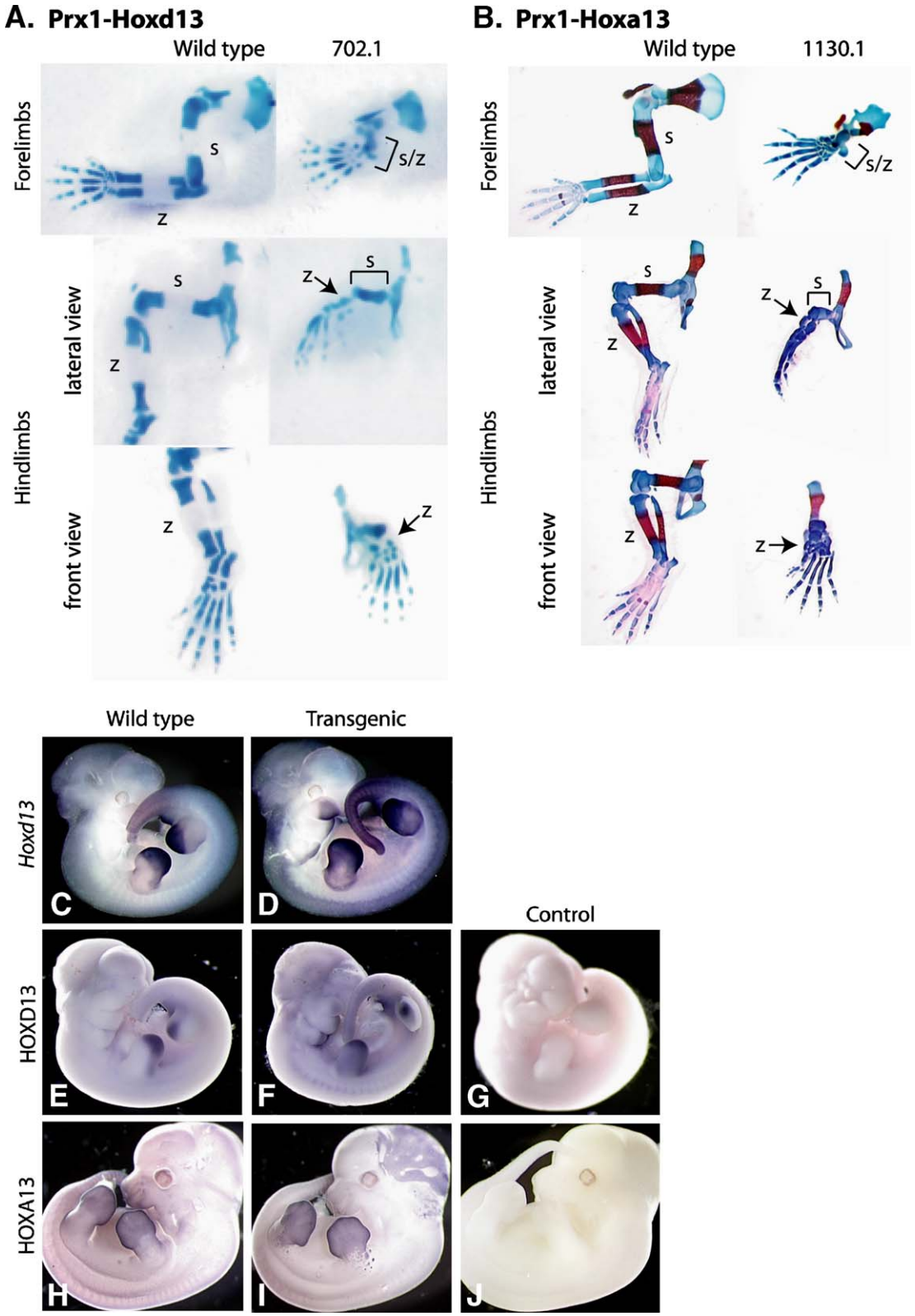


Fig. 1. Limb skeletal phenotype of representative (A) Prx1-Hoxd13 and (B) Prx1-Hoxa13 transgenic founders. Alcian blue stained left limbs from E16.5 transgenic founder Prx1-Hoxd13 702.1 and Alcian blue and Alizarin red stained left limbs from E16.5 founder Prx1-Hoxa13 1130.1 compared to E16.5 wild-type littermates. s, stylopod (humerus or femur); z, zeugopod (radius and ulna or tibia and fibula). (C–J) Transgene expression in progeny of Prx1-Hoxd13 706.6 and Prx1-Hoxa13 84. *Hoxd13* in situ hybridization on E11.5 (C) wild-type and (D) 706.6 transgenic progeny. HOXD13 whole-mount immunohistochemistry on E11.5 (E) wild-type and (F) 706.6 transgenic progeny. HOXA13 whole-mount immunohistochemistry on E12.5 (H) wild-type and (I) 84 transgenic progeny. (G, J) Pre-immune controls.

severe skeletal phenotype than the founder and died soon after birth. Overall, their limb phenotype was less severe than many founders, illustrating the variability as expected for this strategy.

Similarly, a single female Prx1-Hoxa13 founder survived to adulthood and produced progeny with a more severe phenotype that were also not viable. The lethality in these offspring precluded extensive analysis of this line. A single transgenic embryo was collected and showed proximal limb transgenic HOXA13 protein expression at E12.5 by whole-mount immunohistochemistry (Figs. 1H–J).

These observations demonstrate that proximal expression of HOXA13 or D13 in mouse forelimbs and hindlimbs causes severe skeletal reductions in both the zeugopod and stylopod and occasionally more proximal skeletal elements. Although overall the effects of HOXD13 and HOXA13 were similar, the average measures in the founders indicate that HOXD13 had a slightly greater effect on limb reduction of proximal structures. Furthermore, these effects establish a baseline for comparison for subsequent protein alterations.

### *Hoxd11 expression throughout the developing limb causes size reduction of stylopod bones*

Hox group 11 genes are necessary for the development of the zeugopodal elements of the limb (Davis et al., 1995; Wellik and Capecchi, 2003). Morgan et al. (1992) and Goff and Tabin (1997) showed that early retroviral misexpression of *Hoxd11* in the developing chick limb led to digit I to digit II conversion in the leg. Preaxial polydactyly (digit II morphology) was frequently observed in the wing, and a decrease in the lengths of the tibia and fibula was found due to reduction in bone growth. We anticipated that *Prx-1* driven expression of a group 11 gene should lead to negative effects primarily in the stylopod and potentially to zeugopod length, anterior digital posteriorization and/or preaxial polydactyly. To test this, three Prx1-Hoxd11 transgenic founders were generated.

#### *Founder analysis*

Founders 911.1 and 868.2 were prepared for skeletal analysis on their day of birth (P0). These two founders had no proximal skeletal reductions but exhibited preaxial polydactyly in the forelimbs (not shown; Supplementary Table 3). The third founder, 30, was viable and able to produce some viable transgenic progeny. The visible phenotype of founder 30 consisted of a single extra preaxial digit on the left forelimb autopod, malocclusion and pigmentation defects on the top of the head and along the body flank.

#### *Transgenic progeny*

Offspring from line 30 were analyzed for limb skeletal phenotypes and RNA expression. P0 progeny from founder 30 show reductions in stylopod bones of both the fore- and hindlimbs (Fig. 2A, Supplementary Table 3). These progeny had no reductions in the zeugopod of the hindlimb and normal digits; however, variable phenotypes were observed in the anterior forelimb zeugopod and in the autopod. These additional forelimb effects consisted of distal radius reduction with conversion of digit I to a triphalangeal digit or complete loss of the radius with oligodactyly. Transgene RNA expression was confirmed in transgenic embryos at E11.5 (Figs. 2B, C).

We hypothesized that the defects in our mice might be secondary to the induction of anterior Shh activity and subsequent activation of a more posterior Hox gene. It is well known that upon ectopic *Shh* expression posterior *Hoxd* genes can be induced on the anterior side of the developing limb bud where they are not normally expressed (Laufer et al., 1994; Niswander et al., 1994). Thus, we hypothesized a series of events leading to ectopic activation of a Hox gene more posterior than *Hoxd11* in proximal forelimb regions of our transgenic mice.

To test this hypothesis, we analyzed *Shh* and *Hoxd13* expression in developing limbs of transgenic embryos from line 30 at E11.5. *Shh* was found to be ectopically expressed at the anterior margin of the forelimb bud (Figs. 2D, E) or observed weakly along the entire anterior–posterior axis of the forelimb bud associated with limb bud narrowing (photo not shown). Moreover, *Hoxd13* was expressed far more anterior and proximal than normal in the forelimbs of these offspring (Figs. 2F, G). These results suggest that the reduction defects observed in the distal, anterior forelimb zeugopods of Prx1-Hoxd11 transgenic progeny likely result from induction of ectopic *Shh* and *Hoxd13* and consequently impart negative effects on anterior, distal zeugopod growth (Fig. 1). Variation in the results by comparison with Knezevic et al. (1997), Morgan et al. (1992) or Goff and Tabin (1997) may result from the timing and/or overall level of expression obtained in these two different systems.

In summary, expression of *Hoxd11* throughout the developing limbs results in reduction of stylopodal elements of both the fore- and hindlimbs in transgenic progeny. We also observed ectopic *Shh* and *Hoxd13* expression on the anterior margin of the forelimb, resulting in confined preaxial skeletal alterations. The characteristics of the preaxial-specific defects are distinct from those observed with Prx1-Hoxa13 or Prx1-Hoxd13 transgenic expression, which show reduction of the entire zeugopod and stylopod.

### *Prx-1 driven expression of Hoxa9 does not result in zeugopod reduction, but alters patterning in the forelimb*

The group 9 Hox genes are the most 3′ AbdB-like members of the A and D clusters. We hypothesized that 3′ members of the *AbdB*-related group, such as *Hoxa9*, would not cause skeletal reductions within the limb upon ectopic expression. If this hypothesis is correct, HOXA9 would provide a reference from which to evaluate more posterior Hox protein domains important for posterior prevalence. Thus, *Hoxa9* was expressed in the *Prx-1* transgenic system.

#### *Founder analysis*

Six Prx1-Hoxa9 founders were generated, and four were viable and able to reproduce. The single transgenic male founder, 20, did not transmit the Prx1-Hoxa9 transgene. The three remaining female founders, 23, 55, and 69 were bred to collect transgenic progeny for further analysis, and then each female founder was subsequently prepared for skeletal analysis.

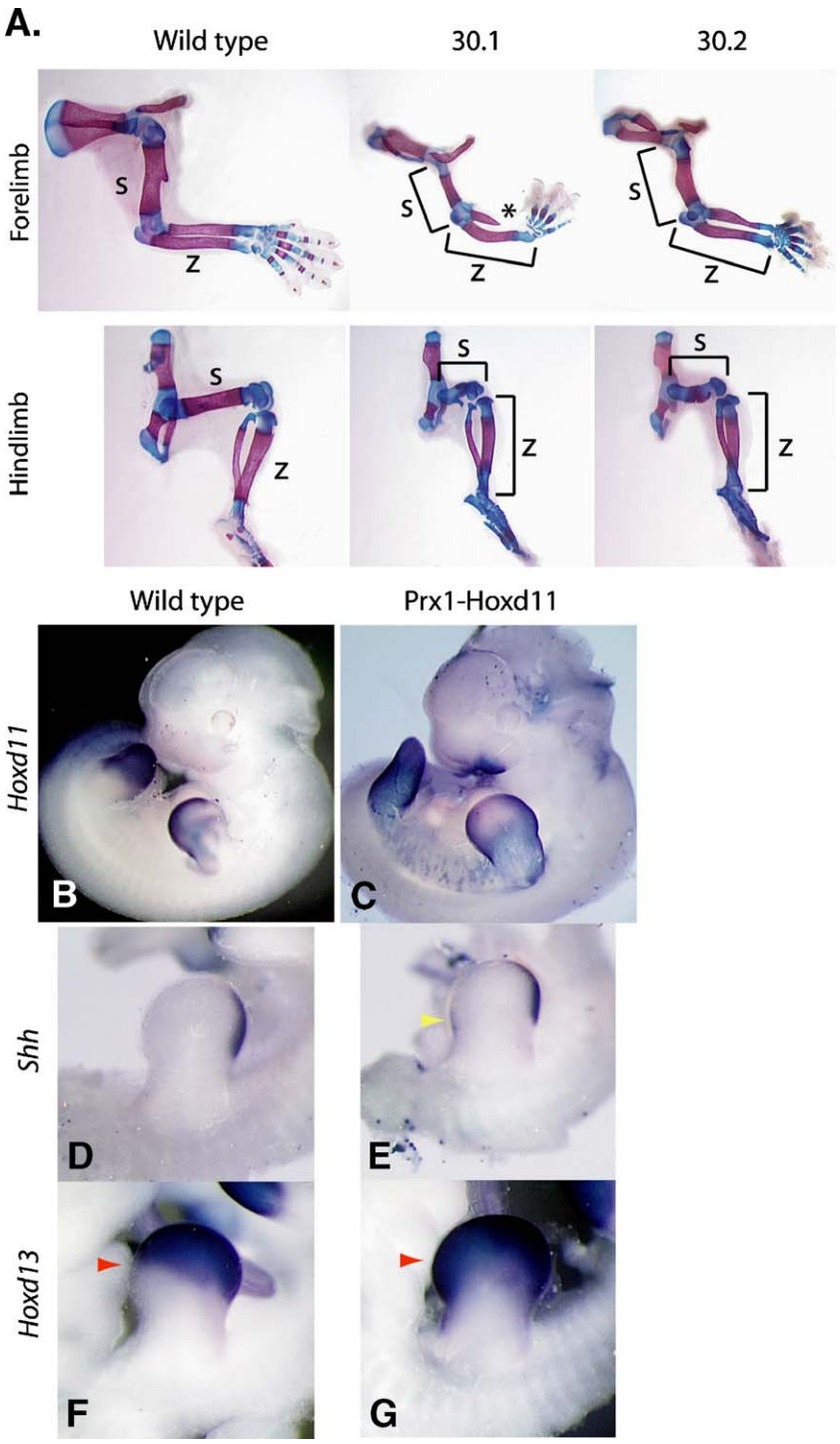


Fig. 2. Limb skeletal phenotype and transgene expression for Prx1-Hoxd11 progeny. (A) Alcian blue and Alizarin red stained limbs from two P0 progeny from founder 30, 30.1 and 30.2, compared to wild-type (the wild-type littermate was cannibalized, therefore a non-littermate P0 wild type was used for comparison). s, stylopod (humerus or femur); z, zeugopod (radius and ulna or tibia and fibula). Asterisk denotes distal truncation of the radius associated with ectopic ZPA activity. *Hoxd11* in situ hybridization on E11.5 (B) wild-type and (C) line 30 Prx1-Hoxd11 transgenic embryos. *Shh* RNA expression at E11.5 in left forelimbs of (D) wild-type and (E) line 30 Prx1-Hoxd11 transgenic embryos. Yellow arrow marks ectopic *Shh* expression. *Hoxd13* RNA expression at E11.5 in right forelimbs of (F) wild-type and (G) line 30 Prx1-Hoxd11 transgenic embryos. Red arrows indicate normal *Hoxd13* expression boundary.

The skeletal phenotypes of the six founders were variable, but all abnormalities were limited to the forelimbs (Fig. 3A, Supplementary Table 4); all hindlimbs were completely normal. Preaxial polydactyly, mirror image ulnar zeugopod and autopod duplications, absence of the humerus deltoid crest, and distal reduction of the anterior zeugopod element were observed. The forelimb phenotype of the HOXB8 transgenic mice (see Fig. 2 of Charite et al., 1994) appears similar to those of our HOXA9

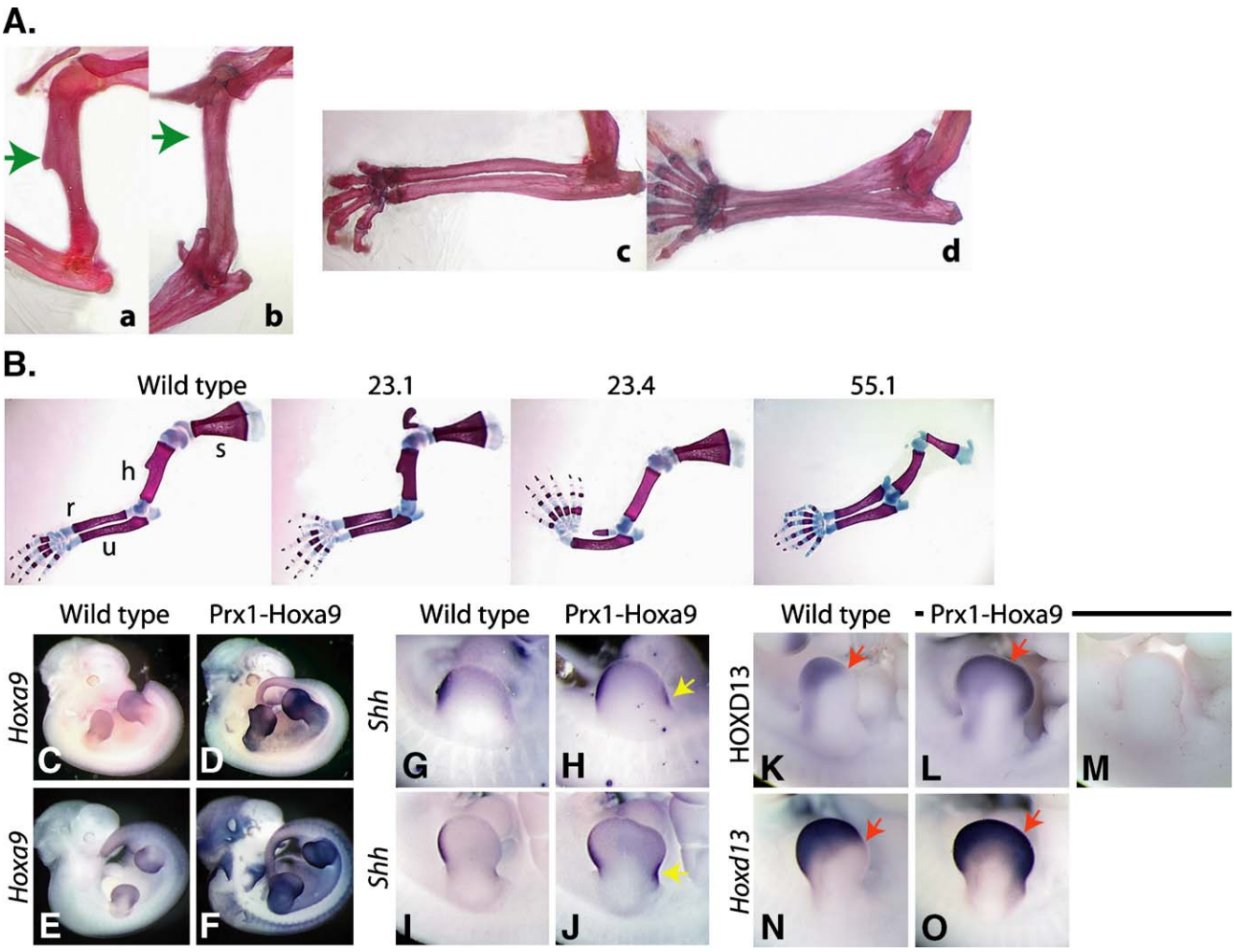


Fig. 3. Prx1-Hoxa9 founder and progeny skeletal phenotypes and expression analysis. (A) Representative Prx1-Hoxa9 founder forelimb phenotypes (a) wild-type stylopod and (b) transgenic founder 69 showing loss of the humerus deltoid crest (green arrow), (c) wild-type forelimb zeugopod and (d) transgenic founder 69 exhibiting mirror image duplication of the ulna. (B) Alcian blue and Alizarin red stained forelimbs from Prx1-Hoxa9 progeny from founders 23 and 55. s, scapula; h, humerus; r, radius; u, ulna. Expression analyses on Prx1-Hoxa9 progeny. (C–F) Transgenic *Hoxa9* expression by in situ hybridization at E11.5. (C) Wild-type, (D) transgenic embryo from line 23, (E) wild-type and (F) transgenic embryo from founder 55. *Shh* expression in line 23 transgenic embryo forelimbs at (H) E10 and (J) E11.5 compared to (G, I) wild-type. Yellow arrows mark ectopic *Shh* expression. HOXD13 protein expression in forelimbs from (K) wild-type and (L) line 23 Prx1-Hoxa9 transgenic embryos at E11.5. (M) Pre-immune control. *Hoxd13* RNA expression in forelimbs of (N) wild-type and (O) Prx1-Hoxa9 transgenic embryos from founder 55 at E11.5. Red arrows indicate the normal anterior Hoxd13 expression boundary (protein, K–M; RNA, N, O).

mice in inducing ulnar duplication, reduction in size of the deltoid crest and polydactyly. No Prx1-Hoxa9 founders we produced had zeugopod or stylopod shortening typical of posterior prevalence. Other phenotypic outcomes in non-limb sites were vertex skull defects, malocclusion and hair pigmentation variation consistent with other Hox transgene effects.

*Transgenic progeny*

Offspring were collected from founders 23, 55 and 69. Transgenic offspring were affected with the same types of defects seen in the founders. However, malformations in P0 progeny from founders 55 and 69 were more severe, in the limb and non-limb regions, resulting in lethality after birth. In these offspring, additional defects were observed affecting only forelimbs including loss of clavicles, partial to complete loss of the stylopod and scapula and, in one limb only, a single rudimentary cartilage element was present apparently where the joint of the stylopod and zeugopod would have formed (photo not shown). Viable progeny were obtained from line 23 and showed variable preaxial polydactyly, occasional presence of an extra zeugopodal element between the radius and ulna and variable distal reduction of the radius as seen for some Prx1-Hoxd11 mice (Fig. 3B). Line 23 progeny also displayed malformations most frequently in the left forelimb, and when both sides were affected, the left side was more severe.

Two transgenic embryos at E11.5 were recovered from founder 55 for RNA expression analysis. In addition, the viable progeny from line 23 were used to generate embryos for RNA and protein expression analyses. Transgenic *Hoxa9* RNA expression was very high, throughout all limb buds of lines 23 and 55 embryos (Figs. 3C–F). Similar to the radial defects in Prx1-Hoxd11 limbs, distal reduction of the radius and preaxial

polydactyly in line 23 were suggestive of *Shh* and posterior Hox gene induction. Variable levels of ectopic anterior *Shh* expression were observed in line 23 embryo forelimbs consistent with variable degrees of preaxial polydactyly (Figs. 3G–J). Of three transgenic embryos analyzed at E10, only 1 exhibited an anterior region of *Shh* expression in the left forelimb. This suggests that the time of ectopic *Shh* expression initiation is variable. In addition, two embryos examined at E11.5 showed different levels of anterior ectopic *Shh* expression in the forelimbs. These findings correlate well with the variation in skeletal defects observed in line 23 forelimbs. This ectopic expression was also most often seen on the left consistent with the more frequent preaxial polydactyly and distal radius truncations observed on that side. HOXD13 protein was also ectopically induced on the anterior side of the Prx1-Hoxa9 forelimb buds with preaxial polydactyly (Figs. 3K–M). The degree of transgenic HOXD13 expression correlated with the extent of polydactyly and reduction of the distal radius seen in line 23 progeny. *Hoxd13* expression was also assessed in one E11.5 embryo from line 55, which exhibited anterior, ectopic expression similar to line 23 (Figs. 3N, O).

Overall, other than reduction of the deltoid crest, *Prx-1* driven expression of *Hoxa9* resulted in no length reduction of skeletal elements in the stylopod or zeugopod consistent with posterior prevalence. Similar to *Hoxd11*, transgenic *Hoxa9* expression in the forelimb was able to induce anterior ectopic *Shh* and *Hoxd13* expression leading to secondary morphological alterations in limb patterning.

### *Prx1-Hoxd13$^{IQN>AAA}$ results in proximal skeletal reductions in mouse limbs*

Retroviral misexpression of a DNA binding mutant of HOXD13 (I47, Q50, N51 converted to alanine residues) in the chick causes shortening of the zeugopod consistent with posterior prevalence (Caronia et al., 2003). We sought to determine whether this is also true in mice, as well as whether more proximal elements are affected.

#### *Founder analysis*

Four transgenic Prx1-Hoxd13$^{IQN>AAA}$ founders were generated, each exhibited severe reductions within the proximal fore and hindlimbs (Fig. 4A, Supplementary Table 5). Average skeletal reductions in the forelimb were: humerus 41%, radius 68% and ulna 63%, and in the hindlimb were: femur 9.5%, tibia 70.1% and fibula 51%. While mild digit malformations were observed, preaxial polydactyly or conversion of digit I to a more posterior digit identity was not seen in any of the founders.

Our results in mice confirm the studies of Caronia et al. (2003) in chicks that I, Q, and N (amino acids 47, 50 and 51 of the homeodomain) are not necessary for posterior prevalence. Since residues I, Q and N make base contacts with the major groove of DNA and this mutant has severely reduced DNA binding capability in vitro, it appears that monomeric DNA binding capability is not necessary for HOXD13 to interfere with the growth of proximal limb skeletal elements (Caronia et al., 2003; Gehring et al., 1994). Moreover, if cofactor interaction is necessary for posterior prevalence effects, these residues of the homeodomain are not critical for that interaction.

### *HOXD13 proteins with proximal AbdB-like homeodomain substitutions cause posterior prevalence*

Even though monomeric group 13 HOX protein DNA binding capability is not necessary for posterior prevalence, this does not rule out a contribution of the homeodomain in posterior prevalence. We sought to further test the contribution of the homeodomain sequence using homeobox swaps. Two transgene constructs were generated: Prx1-Hoxd13$^{d11HD}$ and Prx1-Hoxd13$^{a9HD}$, with either the HOXD11 or the HOXA9 homeodomain sequences replacing those of HOXD13. We reasoned that, if posterior prevalence is controlled by the homeodomain, then these homeodomain swap experiments with d11HD and a9HD should mimic the phenotypes of Prx1-Hoxd11 and Prx1-Hoxa9 mice.

#### *Founder analysis*

The skeletal phenotypes of three of five P0 Prx1-Hoxd13$^{d11HD}$ founders (2, 3 and 11) were similar to those of Prx1-Hoxd13 founders (Fig. 4B, Supplementary Table 6). The forelimb zeugopod in founder 2 was absent, and in founders 1, 4 and 11 the zeugopods were severely reduced. More proximal defects included loss of the humerus deltoid crest and in founders 1, 2 and 11 mild malformation of the scapula. In the hindlimbs, founders 2, 3 and 11 exhibited severe reduction of zeugopodal skeletal elements, and founders 1 and 4 were mildly malformed. Hindlimb stylopods from all founders were normal.

Three founders at E16.5 and two adult founders were recovered from injection of Prx1-Hoxd13$^{a9HD}$ (Fig. 4B, Supplementary Table 7). All of the founders had forelimb malformations including reductions of the zeugopod and stylopod, reduction of the scapula, polydactyly and oligodactyly. Two of the five founders exhibited hindlimb zeugopod defects. Founder 22 had mild lengthening of the fibula, which was misarticulated with the femur. Founder 54 had mild reduction in the length of the zeugopod and stylopod bones.

These results show that, within the context of the HOXD13 protein, a group 13 homeodomain, per se, is not necessary for posterior prevalence effects to be exerted in the forelimb or hindlimb zeugopod or stylopod. However, having a HOXD13 homeodomain, by comparison, appears to increase the severity of skeletal reductions. When comparing hindlimbs, which are not complicated by secondary effects caused by ectopic ZPA activity, a group 11 homeodomain is more potent operating in the context of upstream HOXD13 non-homeodomain residues than a group 9 homeodomain. Transgenic expression of Prx1-Hoxd13$^{d11HD}$ resulted in severe hindlimb zeugopod reduction in 3 of 5 founders (2, 3 and 11, Supplementary Table 6) and mild zeugopod reduction in the remaining 2 founders (1 and 4, Supplementary Table 6), whereas transgenic expression of Prx1-Hoxd13$^{a9HD}$ resulted in 1 of 5 founders exhibiting mild

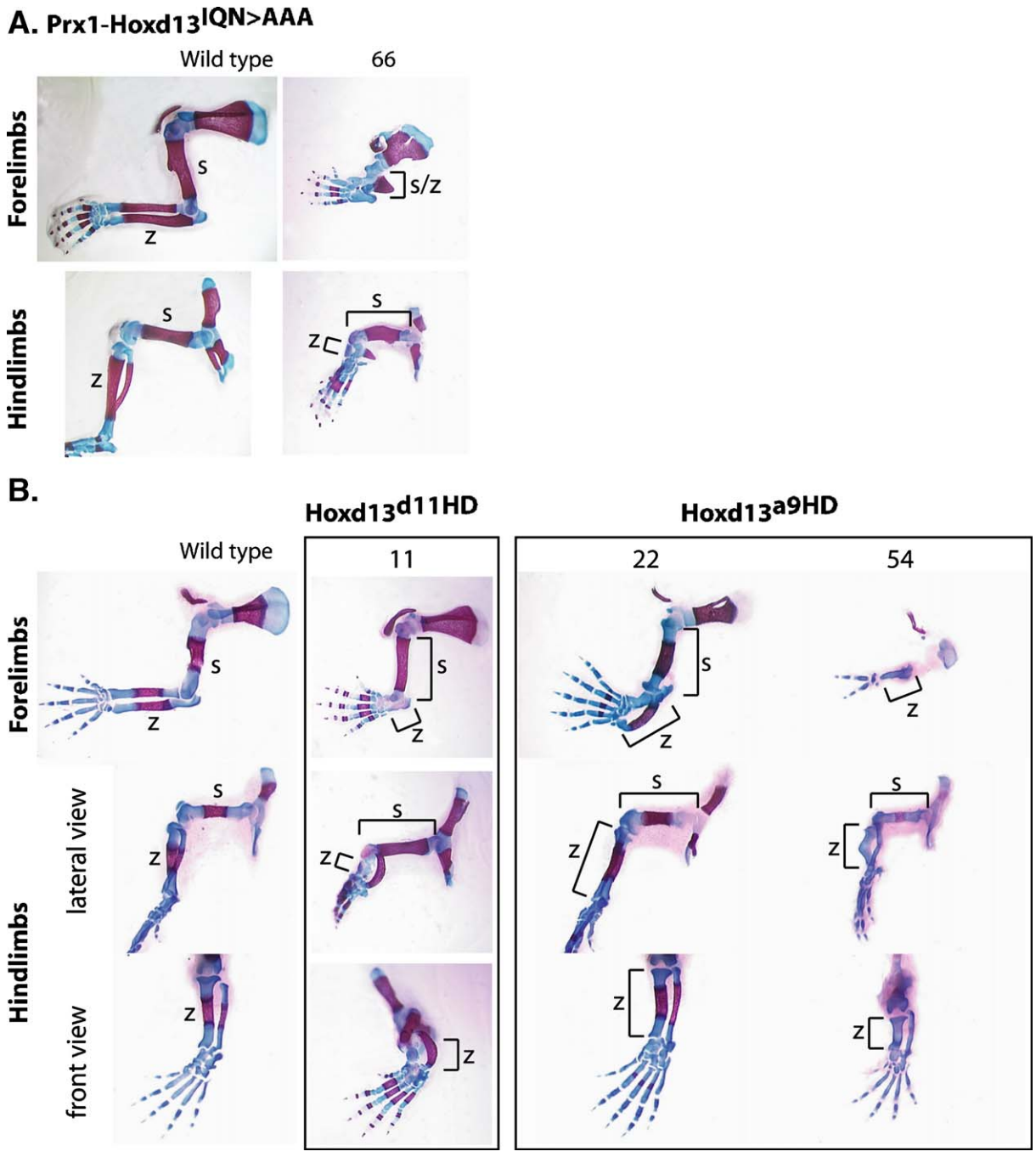


Fig. 4. Limb skeletal phenotype of representative transgenic founders. Alcian blue and Alizarin red stained left limbs from P0 founders: (A) Prx1-Hoxd13$^{IQN>AAA}$ founder 66 and (B) Prx1-Hoxd13$^{d11HD}$ founder 11 and Prx1-Hoxd13$^{a9HD}$ founders 22 and 54 compared to wild-type littermates. Prx1-Hoxd13$^{a9HD}$ 54 represents the most severe reduction observed with this transgene construct. s, stylopod (humerus or femur); z, zeugopod (radius and ulna or tibia and fibula).

hindlimb zeugopod shortening (founder 54, Supplementary Table 7).

*The HOXD13 homeodomain in the context of HOXA9 is not sufficient to cause posterior prevalence*

To test whether the HOXD13 homeodomain is sufficient to confer posterior prevalence capabilities to a more 3′ Hox protein, the transgene Prx1-Hoxa9$^{d13HD}$ was constructed. This construct consists of HOXA9 non-homeodomain regions together with the HOXD13 homeodomain.

*Founder analysis*

Three founders expressing the Prx1-Hoxa9$^{d13HD}$ transgene, 13, 20 and 42, were recovered and all were phenotypically normal (photos not shown).

*Transgenic progeny*

Progeny were collected from all three founders for skeletal and expression analyses. Transgenic offspring from founder 20 had variable forelimb phenotypes including no defects, mild scapula defects, loss of the humerus deltoid crest and preaxial polydactyly (Fig. 5A, and not shown). All progeny had

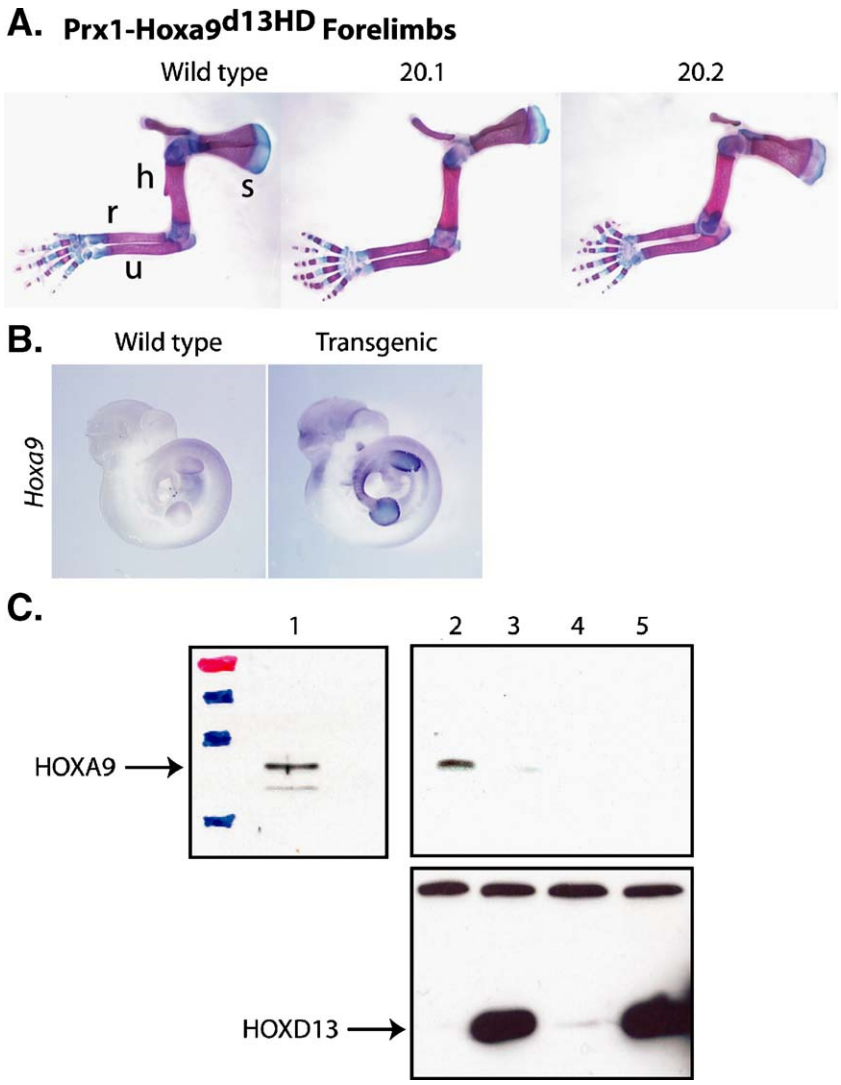


Fig. 5. Prx1-Hoxa9$^{d13HD}$ skeletal phenotype and expression analysis. (A) Alcian blue and Alizarin red stained limbs from two P0 progeny from founder 20 compared to a wild-type littermate. A P0 pup from founder 42 exhibited a similar phenotype of preaxial polydactyly and no apparent shortening but was subsequently cannibalized preventing complete skeletal analysis. s, scapula; h, humerus; r, radius; u, ulna. (B) In situ hybridization on E11.5 wild-type and transgenic embryos from line 42 with a *Hoxa9* RNA probe. Similar robust transgene expression for lines 20 and 13 was also confirmed by in situ hybridization (not shown). (C) Western analysis on (1) HOXA9 positive control lysate, (2) transgenic line 42 proximal limb bud lysate, (3) transgenic line 42 distal limb bud lysate, (4) wild-type proximal limb bud lysate, and (5) wild-type distal limb bud lysate. HOXA9 protein was not detected in the wild-type lysates at this antibody titer; in other blots using a higher antibody concentration, HOXA9 was always detected more robustly in transgenic samples as compared to wild-type. The HOXD13 panel is a positive control western using the same protein samples as used for the HOXA9 blot above and shows robust HOXD13 expression in the distal segments of both transgenic and wild-type limb bud lysates.

completely normal hindlimbs. All transgenic pups from founder 20 that had preaxial polydactyly died after birth. Since founder 20 was female, viable transgenic male offspring were used to generate embryos for expression analyses. A single transgenic pup from founder 42 appeared similar to progeny from founder 20 in that it had preaxial polydactyly and no obvious shortening of the limbs. However, this pup died and was cannibalized preventing thorough skeletal analysis. All transgenic progeny of founder 13 had normal limbs.

Robust limb transgene RNA expression was observed in embryo offspring from all three founders, 13, 20 and 42 (Fig. 5B, and data not shown). To confirm transgenic protein expression, lysates from wild-type and transgenic limb buds were collected at E12.5 to reduce the chance of detecting wild-type HOXA9 and analyzed by western blotting with an anti-HOXA9 antibody. HOXA9 protein signal was much more robust than wild-type in both proximal and distal transgenic samples from lines 42 and 13, confirming the presence of the chimeric protein in these transgenic limbs (Fig. 5C and data not shown). The lack of skeletal reduction upon abundant ectopic limb expression of HOXA9 containing the HOXD13 homeodomain demonstrates that a group 13 homeodomain is not sufficient in all contexts to confer dominant function in the limb.

### *Hox sensitivity to genetic manipulation of coding sequence*

To precisely define protein regions that are necessary and sufficient for posterior prevalence, several deletion/substitution

constructs were made for Prx1-Hoxd13. These included: removal of the homeodomain, replacement of the homeodomain with a nuclear localization sequence and a series of N-terminal deletion constructs. N-terminally truncated Hox proteins have been reported to produce proximal skeletal reductions upon retroviral misexpression in chick (Goff and Tabin, 1997). In our experiments, deletion/substitution constructs produced normal protein expression and nuclear localization in tissue culture cell lines; however, in mice, the proteins could not be detected despite robust RNA expression (data not shown). We therefore altered our strategy to create a HOXD13 construct containing comparably small amino acid changes as opposed to large deletions.

*A highly conserved 6-residue motif N-terminal to the HOXD13 homeodomain is not necessary for posterior prevalence*

We sought to identify specific residues within the N-terminal, non-homeodomain region of HOXD13 that might be critical for posterior prevalence. Several highly conserved protein motifs exist upstream of the homeodomain (Mortlock et al., 2000). We selected six residues R, A, K, E, F and Y at positions 182–185, 188 and 189 because they are conserved among all group 13 proteins. Additionally, these residues were retained within an amino-terminally truncated HOXD13 protein that was sufficient to cause proximal limb reductions in chick retroviral experiments (Goff and Tabin, 1997). To determine if these residues are important to HOXD13 effects in the proximal limb, we created Prx1-Hoxd13$^{RAKEFY>GGGGGG}$ and evaluated its function in transgenic mice.

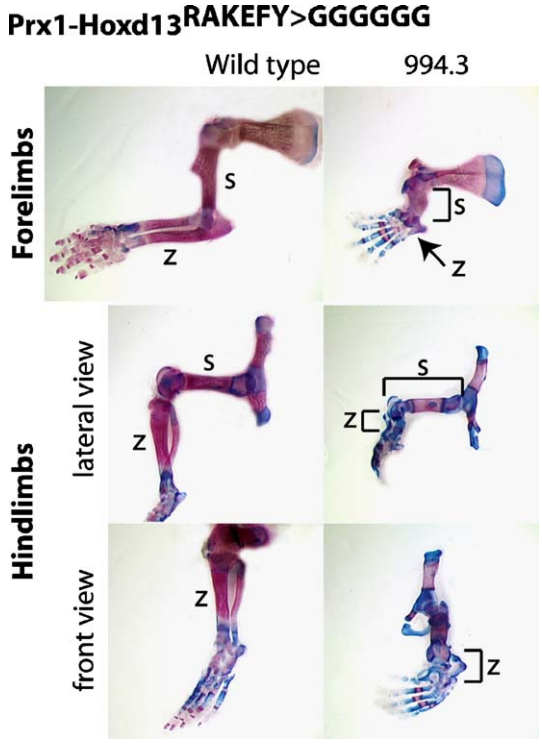


Fig. 6. Limb skeletal phenotype of a representative Prx1-Hoxd13$^{RAKEFY>GGGGGG}$ transgenic founder. Alcian blue and Alizarin red stained left limbs from P0 founder 994.3 compared to a wild-type littermate. s, stylopod (humerus or femur); z, zeugopod (radius and ulna or tibia and fibula).

*Founder analysis*

Five transgenic founders were obtained from injection of this construct, three at P0, founders 994.1, 994.3 and 996.1 and two adults, founders 22 and 51 (Fig. 6, Supplementary Table 8). Proximal regions of both the forelimbs and hindlimbs in founders 994.3 and 996.1 were severely shortened. Founders 22 and 994.1 exhibited mild zeugopod shortening and preaxial polydactyly in both the fore- and hindlimbs. Founder 22 did not transmit the transgene. The only detectable phenotype in founder 51 was a slight pigmentation defect around the nose. The transgene-specific PCR for this founder was extremely weak compared to a single copy transgene standard, suggesting possible mosaicism, which might explain the lack of a limb abnormality.

HOXD13$^{RAKEFY>GGGGGG}$ expressed throughout the entire developing limb resulted in severe skeletal reductions in the zeugopod and stylopod similar to those observed for wild-type HOX group 13 proteins. Therefore, this N-terminal motif, RAKE–FY, is not necessary for posterior prevalence.

## Discussion

*A hierarchy of Hox function in the developing mouse limb*

A transgenic approach using the murine *Prx-1* promoter for all experimental templates and comprehensive verification of transgene RNA and protein expression was utilized to systematically test the effects of misexpression of specific Hox genes in developing mouse limbs. The Prx1-Hox system used in our study produced generally a more severe range of reductions than those seen in previous studies using retroviral expression in chick limbs or transgenic mice. Like other reports, the Prx1-Hox transgenic mice also exhibited intra-construct variability in phenotype and these effects are likely due to alterations in level of expression due to transgene copy number variation and/or insertion site effects. One additional limitation is that we did not determine that the transgene was expressed in all founders; however, we analyzed mice that showed transgene effects in other areas of *Prx-1* expression. Furthermore, despite the fact that the *Prx-1* transgene promoter recapitulates normal *Prx-1* forelimb expression, there is a posterior hindlimb bud expression delay (Martin and Olson, 2000). Thus, absence of an effect in hindlimbs could represent the results of a delay in expression, which in retroviral experiments can cause an absence of a visible phenotype (Morgan et al., 1992). However, in the Prx1-Hoxa13 and Prx1-Hoxd13 transgenic founders, any effect of a delay in expression was not apparent, and therefore the delay is not likely a major cause of hindlimb phenotypic differences between transgenes. As with other misexpression studies, our approach can generate physiological as well as non-physiological effects due to these alterations in timing and expression level. Despite these limitations, we utilized multiple founders and offspring to derive clear comparative conclusions regarding the capabilities of the transgenic HOX proteins using this one system.

Whereas a functional hierarchy was suggested by other studies with individual genes, we definitively demonstrated that such a functional hierarchy exists in the developing mammalian limb. Proximal expression of *Hoxa13* or *Hoxd13* results in severe reductions of skeletal elements of the stylopod and zeugopod. No length reductions were observed in the autopods where *Hoxa13* and *Hoxd13* are normally expressed, as expected. Proximal misexpression of *Hoxd11* results in reduction of skeletal elements of the stylopod, but no direct negative effects in the normal domain of expression, the zeugopod or the more distal autopod. All hindlimbs of the Prx1-Hoxa9 transgenic mice were completely normal, as would be expected for the most 3′ (proximal) AbdB-like Hox group member expressed in the developing limb. The defects in the forelimbs are interesting in that they are similar to those documented for misexpression of *Hoxb8*, a gene for which individual loss of function mutations do not cause limb defects despite its expression early in the posterior limb bud mesenchyme (van den Akker et al., 1999). It is possible that the more proximal defects of the scapula and the humerus deltoid crest are due to prevalence over HOXC6 or HOXC8 which have been implicated in the development of these structures (Oliver et al., 1990; van den Akker et al., 2001). Overall, the results from transgenic expression of *Hoxd13*, *Hoxa13*, *Hoxd11* and *Hoxa9* throughout the developing mouse limb provide direct evidence that a functional hierarchy exists among Hox proteins in the context of normal limb development (Fig. 7). This system and the observations reported here provide a basis for future evaluation of sequence differences in the AbdB Hox family and the critical functional domains that are required for posterior prevalence.

### *Secondary patterning effects resulting from Hox overexpression*

Ectopic activation of polarizing activity or *Shh* in anterior limb domains associated with digital conversion to posterior identities and/or additional digits has been observed in chicks and mice overexpressing HOX proteins (Caronia et al., 2003; Charite et al., 1994; Chen et al., 2004; Goff and Tabin, 1997; Kmita et al., 2005; Knezevic et al., 1997; Morgan et al., 1992; Zakany et al., 2004). Therefore, the occurrence of some degree of preaxial polydactyly and/or mirror-image duplication in all of our Hox constructs, except for Hoxd13$^{IQN>AAA}$ and Hoxd13$^{d11HD}$, was not surprising. This result was most often observed in forelimbs in our studies. However, the frequency and involvement of forelimbs or hindlimbs varied among the mice.

However, distal radius shortening or absence observed in Prx1-Hoxd11 and Prx1-Hoxa9 forelimbs prompted us to determine whether this was also associated with ectopic, anterior activation of HOXD13. A similar distal truncation of the radius was observed in the studies of Knezevic et al. (1997) with Hoxd12, and Hoxd12 or Hoxd11 were shown to induce the expression of Shh in limbs and cultured cells. However, this effect was not observed in the experiments with Hoxd11 (see below) in chicks. We showed that in the Prx1-Hoxd11 and Prx1-Hoxa9 lines *Shh* and *Hoxd13* were ectopically activated in the anterior region of the forelimbs, which likely resulted in the distal radius reductions observed. In related work relative to DNA binding capabilities, we confirmed the findings of Caronia et al. (2003) in chicks showing that Prx1-Hoxd13$^{IQN>AAA}$ transgenic mice had severe proximal malformations yet normal digit number and identity. Thus, monomeric DNA binding ability is not critical for posterior prevalence. However, ectopic anterior activation of Shh activity, readily observed in Prx1-Hoxa13 and Prx1-Hoxd13 transgenic mice, appears to depend on this property.

Our results with *Hoxd11* are partially consistent with previous studies with *Hoxd11* chick retroviral expression (Morgan et al., 1992; Goff and Tabin, 1997). Both observed partial conversion of digit I to a digit II fate but no polydactyly in the leg, yet an extra digit in 46% of infected wings. Both studies infected chick limb fields at very early stages (10–12) of development, and complete infection of the limb fields with overexpression of the genes was documented. Moreover, a 12-hour delay in the timing of infection eliminated the effects observed illustrating the importance of the timing of HOX misexpression. None of our Prx1-Hoxd11 founders or offspring showed hindlimb preaxial polydactyly. On the other hand, we observed shortening of elements proximal to the normal domain of expression, mainly affecting the stylopod. Morgan

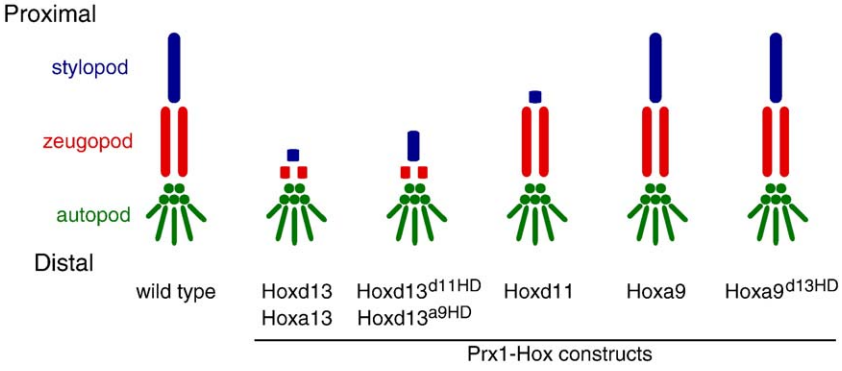


Fig. 7. A Hox group 13 homeodomain is neither necessary nor sufficient for posterior prevalence. Transgenic expression of Hoxd13$^{d11HD}$ or Hoxd13$^{a9HD}$ caused reduction in zeugopod skeletal elements. Transgenic expression of Hoxa9$^{d13HD}$ caused no reduction of skeletal elements. Segment reductions in the diagram represent regions exhibiting variable reduction. Effects of transgene expression on preaxial polydactyly are not depicted.

et al. (1992) observed shortening of the lower leg and Goff and Tabin (1997) also documented a shortening of the tibia and fibula in *Hoxd11* infected hindlimbs. In the latter study, this effect was related to a reduction in cell proliferation as a late effect of HOXD11 on bone growth, not at the stages of condensation. The reason for the variation in outcomes is not known.

It is also curious that HOXA9 had such a strong effect on the forelimb in our experiments, inducing not only extra digits but also ulnar duplication, yet absence of any observable effect in hindlimbs. This effect was observed not only in Prx1-Hoxa9 transgenic founders, but also in numerous offspring of two lines with striking forelimb involvement. HOXD9 chick limb bud infection (Morgan et al., 1992) resulted in no phenotype. It is intriguing that HOXD9 is expressed throughout the early fore- and hindlimb buds in chick, whereas HOXA9 starts out uniformly in both but develops early on a region of absent expression in the anterior/proximal forelimb bud (Nelson et al., 1996). Perhaps *Prx-1* transgenic expression throughout the forelimb domain, including this anterior/proximal region, more easily promotes ectopic anterior hedgehog activity and does not induce hindlimb patterning effects because HOXA9 is expressed throughout.

### *Non-homeodomain regions of HOXD13 are necessary for posterior prevalence*

Due to the highly conserved nature of the Hox homeodomain and its ability to bind DNA, the main role of Hox proteins during development is thought to be direct transcriptional regulation of target genes. This idea has been supported by many studies in *Drosophila*, both in vitro and in vivo, showing activation and repression of target gene transcription (Gehring, 1993). The importance of the homeodomain in Hox function during development has made it a major focus in studies exploring the mechanism(s) through which specificity is achieved by different Hox proteins. Evidence from *Drosophila* misexpression experiments largely supports a critical role of the homeodomain in determining unique Hox effects in vivo (Furukubo-Tokunaga et al., 1993; Gibson et al., 1990; Kuziora and McGinnis, 1989, 1990; Lin and McGinnis, 1992; Zeng et al., 1993). These studies support the conclusion that unique amino acids of the homeodomains of different Hox proteins alter DNA site recognition and hence target specificity. Other fly studies have demonstrated non-homeodomain contributions to unique function and a difference in the non-homeodomain protein regions required for specificity depending on the tissue of expression (Chauvet et al., 2000; Merabet et al., 2003). These observations support consideration of alternative mechanisms that have been proposed for Hox functional specificity such as cofactor interactions, inherent Hox activation or repression capabilities or activity regulation by the context of the DNA sequences bound (Capovilla and Botas, 1998; Chan et al., 1994; Chauvet et al., 2000; Li and McGinnis, 1999; Li et al., 1999).

In mammals, the involvement of different protein regions appears to depend on the tissue context as well (Sreenath et al., 1996; Zhao and Potter, 2001, 2002). These experiments, similar to those in *Drosophila*, support the notion that Hox proteins exploit multiple mechanisms to gain functional specificity.

Caronia et al. (2003) showed that monomeric DNA binding capability, per se, and HD residues I47, Q50 and N51, specifically, are not necessary for posterior prevalence in chicken retroviral misexpression experiments. We confirmed this result and extended the analysis with chimeric proteins to show that the HOXD13 homeodomain is neither necessary nor sufficient for posterior prevalence (Fig. 7). Both Hoxd13$^{d11HD}$ and Hoxd13$^{a9HD}$ proteins were sufficient to exert posterior prevalence effects. However, the homeodomain appears to affect the severity of overall skeletal shortening, as demonstrated by *Prx-1* driven expression of Hoxd13$^{a9HD}$ as compared to HOXD13. Proximal expression of the chimeric Hoxd13$^{a9HD}$ protein resulted in only one founder with hindlimb zeugopod reductions. In contrast, *Prx-1* driven HOXA9 never caused hindlimb defects, further supporting the conclusion that the HOXD13 non-homeodomain regions are able to cause posterior prevalence albeit less effectively. While the ability of the homeodomain to influence posterior prevalence was not unexpected, we did not anticipate that chimeric proteins without the HOXD13 HD would be capable of such effects based on the prior literature. To begin to determine the non-homeodomain regions of HOXD13 critical for the effects observed here, we made glycine substitutions for six conserved group 13 N-terminal residues (RAKE-FY) within HOXD13. These alterations to the HOXD13 protein did not inhibit its ability to exert posterior prevalence. We believe that creating point mutations to address the protein domain(s) necessary for posterior prevalence will be an effective method for identification of the region within the HOXD13 non-homeodomain portion that is critical for posterior prevalence in the future. The data support further efforts to define the critical non-homeodomain regions of group 13 proteins and the precise residues within the homeodomain that contribute to posterior prevalence.

This transgenic system will be useful in the future for identifying the molecular defects associated with proximal expression of distal HOX proteins. A systematic evaluation of genes suggested to play a role in a proximal limb program such as Gli3, Plzf and Meis may yield insight into the mechanism by which Hox proteins exert such selective interference upon misexpression (Barna et al., 2005; Mercader et al., 1999).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.05.027.

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