CORRIGENDUM

An I47L substitution in the HOXD13 homeodomain causes a novel human limb malformation by producing a selective loss of function

Caronia, G., Goodman, F. R., McKeown, C. M. E., Scambler, P. J. and Zappavigna, V. *Development* **130**, 1701-1712.

While this article was in press, a phenotype similar to that described, caused by an identical mutation, was identified by Johnson et al. in two further families from the south-east of England (Johnson et al., 2003). Microsatellite genotyping showed that the affected individuals from all three families share the same haplotype across the HOXD cluster region, suggesting that the mutation arose in a common ancestor (F.R.G., unpublished).

Johnson, D., Kan, S., Oldridge, M., Trembath, R. C., Roche, P., Esnouf, R. M., Giele, H. and Wilkie, A. O. M. (2003). Missense mutations in the homeodomain of HOXD13 are associated with brachydactyly types D and E. *Am. J. Hum. Genet.* (in press).

The authors apologise to readers for this omission.

Development 130, 1701-1712

doi:10.1242/dev.00396

DEVELOPMENT AND DISEASE

An I47L substitution in the HOXD13 homeodomain causes a novel human limb malformation by producing a selective loss of function

Giuliana Caronia[1,*], Frances R. Goodman[2,*], Carole M. E. McKeown[3], Peter J. Scambler[2] and Vincenzo Zappavigna[1,4,†]

[1]Department of Molecular Biology and Functional Genomics, DIBIT-H San Raffaele, Via Olgettina 58, 20132 Milano, Italy
[2]Molecular Medicine Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK
[3]Clinical Genetics Unit, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK
[4]Department of Animal Biology, University of Modena and Reggio Emilia, Via Campi 213/d, Modena 41100, Italy
*These authors contributed equally to this work
[†]Author for correspondence (e-mail: zappavigna.vincenzo@hsr.it)

Accepted 13 January 2003

SUMMARY

The 5′ members of the Hoxa and Hoxd gene clusters play major roles in vertebrate limb development. One such gene, *HOXD13*, is mutated in the human limb malformation syndrome synpolydactyly. Both polyalanine tract expansions and frameshifting deletions in HOXD13 cause similar forms of this condition, but it remains unclear whether other kinds of *HOXD13* mutations could produce different phenotypes. We describe a six-generation family in which a novel combination of brachydactyly and central polydactyly co-segregates with a missense mutation that substitutes leucine for isoleucine at position 47 of the HOXD13 homeodomain. We compared the HOXD13(I47L) mutant protein both in vitro and in vivo to the wild-type protein and to an artificial HOXD13 mutant, HOXD13(IQN), which is completely unable to bind DNA. We found that the mutation causes neither a dominant-negative effect nor a gain of function, but instead impairs DNA binding at some sites bound by wild-type HOXD13. Using retrovirus-mediated misexpression in developing chick limbs, we showed that wild-type HOXD13 could upregulate chick *EphA7* in the autopod, but that HOXD13(I47L) could not. In the zeugopod, however, HOXD13(I47L) produced striking changes in tibial morphology and ectopic cartilages, which were never produced by HOXD13(IQN), consistent with a selective rather than generalised loss of function. Thus, a mutant HOX protein that recognises only a subset of sites recognised by the wild-type protein causes a novel human malformation, pointing to a hitherto undescribed mechanism by which missense mutations in transcription factors can generate unexpected phenotypes. Intriguingly, both HOXD13(I47L) and HOXD13(IQN) produced more severe shortening in proximal limb regions than did wild-type HOXD13, suggesting that functional suppression of anterior Hox genes by more posterior ones does not require DNA binding and is mediated by protein:protein interactions.

Key words: Hox genes, Limb malformations, Missense mutation, DNA binding, Posterior prevalence

INTRODUCTION

Hox genes encode a set of highly conserved transcription factors, which control cell fates and regional identities along the primary body axis in all metazoans. Most vertebrates, including humans, have 39 Hox genes arranged in four clusters (Hoxa-Hoxd) (Krumlauf, 1994). The 5′ Hoxa and Hoxd genes (paralogous groups 9-13), which are related to *Drosophila Abd-B*, are also important in patterning the limbs (Zakany and Duboule, 1999) and are expressed in a regionally restricted fashion during limb development (Duboule and Morata, 1994; Nelson et al., 1996). Targeted mutagenesis and overexpression of individual and multiple 5′ Hoxa and Hoxd genes in mouse (reviewed by Rijli and Chambon, 1997; Zakany and Duboule, 1999) and chick (Morgan et al., 1992; Yokouchi et al., 1995; Goff and Tabin, 1997) do not produce homeotic transformations in the limbs, but instead alter the size, shape and number of specific bones, and delay chondrification and ossification. These genes thus appear to determine region-specific growth and differentiation in the limb skeletal elements, acting combinatorially at both early and late stages of development (Morgan and Tabin, 1994; Zakany and Duboule, 1999). The molecular pathways in which they act, however, are currently poorly understood. Hox proteins bind specific DNA sequences via the homeodomain, a highly conserved DNA-binding motif, and are thought to regulate overlapping sets of target genes, but few such targets have yet been identified (Graba et al., 1997).

To date, only two Hox genes have been proven to be mutated in human malformation syndromes, *HOXD13* in synpolydactyly (SPD) and *HOXA13* in hand-foot-genital syndrome (reviewed by Goodman, 2002). SPD is a rare dominantly inherited limb malformation, which is characterised by syndactyly between the third and fourth fingers and between the fourth and fifth toes, with a partial or complete extra digit in the syndactylous web. Typical SPD is caused by expansions of a 15-residue polyalanine tract in the N-terminal region of HOXD13 (Muragaki et al., 1996; Akarsu et al., 1996; Goodman et al., 1997). The mutant protein is thought to act as a dominant negative, interfering functionally with wild-type HOXD13 and other 5′ HOXD proteins expressed in the autopod (Zakany and Duboule, 1996; Bruneau et al., 2001). An atypical form of SPD, which is characterised by a distinctive foot phenotype, has also been identified in four unrelated families. Three families harbour different frameshifting deletions in *HOXD13* (Goodman et al., 1998; Calabrese et al., 2000), which are predicted to result in truncated proteins unable to bind DNA, while the fourth family harbours a missense mutation in helix II of the HOXD13 homeodomain (R31W), which is predicted to destabilise the homeodomain (Debeer et al., 2002). All four mutations are thus likely to cause functional haploinsufficiency for HOXD13. The typical and atypical forms of SPD produced by these two classes of mutations are very similar, however, leaving open the possibility that other *HOXD13* mutations may produce unexpected phenotypes.

Although the identification of human limb malformations caused by *HOXD13* mutations provide a unique opportunity to gain insight into the role of HOXD13 in limb development, no previous studies have characterised the effects of these mutations at a molecular level. We describe a six-generation family in which a unique combination of brachydactyly and central polydactyly co-segregates with a missense mutation in helix III of the HOXD13 homeodomain (I47L). We have compared the functions of the HOXD13(I47L) mutant protein with those of wild-type HOXD13 and a HOXD13 mutant unable to bind DNA. We show that the I47L substitution does not produce a dominant-negative effect or a gain of function, but instead impairs DNA binding at a subset of sites recognised by wild-type HOXD13, causing a selective loss of function. Consistently, retrovirus-mediated overexpression of wild-type HOXD13 in the chick autopod upregulates chick *EphA7*, a putative downstream target of *Hoxa13*, but overexpression of HOXD13(I47L) does not. Interestingly, the two mutant proteins produce more severe phenotypes than the wild-type protein in proximal regions of the chick limb, suggesting that functional suppression of anterior Hox proteins by more posterior ones does not require DNA-binding activity and may be mediated solely by protein:protein interactions.

MATERIALS AND METHODS

Patient evaluation and mutation analysis

The family was identified following referral to the Clinical Genetics Unit, Birmingham Women's Hospital, UK. Nine affected individuals (indicated in Fig. 1) were examined clinically, and radiographs of both hands and both feet were obtained. Venous blood samples for DNA extraction were taken from affected and unaffected individuals with their informed consent and approval from the local ethical committee. To search for mutations in *HOXD13*, the coding region of the gene was amplified by PCR in four segments (Goodman et al., 1997). Amplified fragments were cycle sequenced either directly (Applied Biosystems Prism Dye Terminator Kit) or after cloning into pCR-Script (Stratagene) on an ABI 377 automated sequencer (Applied Biosystems). The presence of the mutation was confirmed by PCR amplification of a 100 bp region of exon 2 with forward primer D13-CF (5′-ACCTATCTGAGAGACAAGTGACCC-3′) and reverse primer D13-NR (5′-GGACCACATCAGGAGACAGTATC-3′), which yielded a product from the mutant but not the wild-type allele at an annealing temperature of 60°C.

Plasmid constructs

To generate open reading frames (ORFs) for wild-type and mutant *HOXD13*, wild-type exon 1 and wild-type and mutant exon 2 were amplified separately by PCR from patient genomic DNA and cloned into pCR-Script. Each exon was amplified from the cloned template using tailed primers, and exon 1 was spliced to either wild-type or mutant exon 2 by overlap extension (SOEing) PCR. The wild-type and mutant ORFs were cloned into pCR-Script and their sequences verified by DNA sequencing. To generate expression constructs for HOXD13 and HOXD13(I47L), the respective ORFs were cloned in frame with a hemagglutinin (HA) tag into the SV40-based vector pSG-5 (Stratagene). HOXD13(IQN) was generated by PCR mutagenesis of the wild-type construct, and the presence of the desired base changes was verified by DNA sequencing. The expression of all three proteins was tested by in vitro transcription-translation and SDS-PAGE. Expression constructs for glutathione-S-transferase (GST)-homeodomain fusion proteins were produced by cloning restriction fragments containing the homeodomains of HOXD13 (HOXD13HD), HOXD13(I47L) [HOXD13HD(I47L)] and HOXD13(IQN) [HOXD13HD(IQN)] into the bacterial expression vector pGEX4T-1 (Amersham Biosciences). The pTHCR reporter construct was described previously (Zappavigna et al., 1991). Avian retroviral expression constructs containing HOXD13, HOXD13(I47L) and HOXD13(IQN) were generated by cloning the appropriate restriction fragments into the shuttle vector pSLAX13 and then subcloning into the retroviral vector pRCAS(BP)A (Morgan and Fekete, 1996).

Cell culture and transfections

P19 mouse embryonic carcinoma cells were cultured in Minimum Essential Medium Alpha (Invitrogen) supplemented with 10% foetal calf serum (Celbio), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. Primary chick embryo fibroblasts (CEF cells) were cultured in Dulbecco's Medium (Life Technologies). Transfections were carried out by $CaPO_4$ precipitation (Di Nocera and Dawid, 1983). In a typical experiment, 10 μg of pTHCR reporter plasmid, 0.5-2.5 μg of expression construct and 0.25 μg of pCMV-βgal (Clontech) as internal control were used per 10 cm dish. Forty-eight hours after transfection, cells were washed, lysed and assayed for luciferase and β-galactosidase expression (Zappavigna et al., 1994). Immunoblots of extracts from transfected cells showed that the expression constructs produced identical amounts of HOXD13 and HOXD13(I47L) (data not shown).

Electrophoretic mobility shift assays (EMSAs)

Full-length HA-tagged HOXD13 and HOXD13(I47L) proteins were synthesised in vitro using the TNT-coupled transcription/translation system (Promega), diluted in 13 μl of δ-buffer (20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) and pre-incubated with 100 ng poly-(dI-dC) in a total volume of 20 μl 1× binding buffer (0.1 M KCl, 2 mM $MgCl_2$, 4 mM spermidine, 0.1 mg/ml BSA) for 15 minutes on ice. The amount of reticulocyte lysate used was adjusted to normalise for translated protein content. 20,000-50,000 cpm of ^{32}P-labelled probe were added and samples were incubated

for 30 minutes on ice. Reactions were separated on 6% polyacrylamide gels in 0.5× TBE, which were dried and exposed to Kodak X-OMAR film at –80°C. GST-HOXD13HD, GST-HOXD13HD(I47L) and GST-HOXD13HD(IQN) fusion proteins were expressed in *E. coli*, purified according to established methods and analysed by SDS-PAGE and Coomassie staining. Probe sequences are given in Figs 5 and 6. The SelD13 probe was generated by annealing the SelD13 oligonucleotide pool (Fig. 5C) with a 10-fold molar excess of the primer Sel3 (5′-GGCGAGATCTCTCGA-GGG-3′) and extending with Klenow polymerase in the presence of α-[^{32}P]-dCTP. Quantitative evaluation of the DNA-binding efficiencies was performed using an Amersham Bioscience Densitometer.

DNA binding site selection assays

Bacterially expressed, purified GST-HOXD13HD and GST-HOXD13HD(I47L) fusion proteins were loaded onto glutathione-Sepharose 4B resin (Amersham Biosciences) and incubated with the SelD13 oligonucleotide pool in 1× binding buffer for 30 minutes at 4°C. After five washes in 500 μl 1× binding buffer, beads were incubated at 95°C for 5 minutes to release the bound DNA. Aliquots of the heated binding reaction mixture were amplified by PCR for 25 cycles (95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute) with primers complementary to the non-random flanking arms of SelD13 (Sel3 as above; Sel5 5′-CCGCGGATCCGTCGACGG-3′), and the amplified DNA used in a further binding reaction. After five rounds of selection, the amplified DNA was cloned and cycle sequenced. Consensus sequences were aligned using the program CLUSTAL W (Thompson et al., 1994) as implemented in the Biology WorkBench Version 3.2 (http://workbench.sdsc.edu).

Avian retrovirus production, microinjection, cartilage staining and whole-mount in situ hybridisation

CEF cells were transfected by $CaPO_4$ precipitation with 20 μg of the RCAS-HOXD13, RCAS-HOXD13(I47L) or RCAS-HOXD13(IQN) retroviral constructs to generate virus stocks, which were harvested, concentrated and titrated on CEF cells (Morgan et al., 1992). A titre of approximately 1×10^8 cfu/ml was obtained for each virus. Fertilised eggs were incubated at 37°C for 1.5 days until stage 10 (Hamburger and Hamilton, 1992), when virus was introduced into the prospective right limb area by a series of five to ten closely spaced injections (Morgan et al., 1992). The eggs were returned to the incubator and harvested at stages 28-34. For cartilage staining, embryos were fixed in 96% ethanol for 3-5 days, stained in Alcian Blue (15 mg Alcian Blue 8GX (Sigma) in 80 ml 96% ethanol and 20 ml glacial acetic acid) for 24 hours, and rinsed twice in 96% ethanol for 2 days, before being cleared first in 1% KOH on ice and then in 2% KOH:glycerol (20:80). Injected and control limbs were dissected and photographed through a dissecting microscope. Cartilage lengths were measured using an ocular reticule. Whole-mount in situ hybridisation (Wilkinson and Nieto, 1993) was performed using a digoxigenin-labelled antisense mRNA probe to chick *EphA7* (Araujo and Nieto, 1997).

RESULTS

A new brachydactyly-polydactyly syndrome

We identified a new dominantly inherited distal limb malformation in a large Caucasian English family (Fig. 1). At least 18 individuals from six successive generations were affected, nine of whom were available for examination. All had bilateral, symmetrical brachydactyly in the hands and feet, with considerable variation in severity (Fig. 2A-N). Thus, all had hypoplasia or absence of the fifth distal phalanges, usually

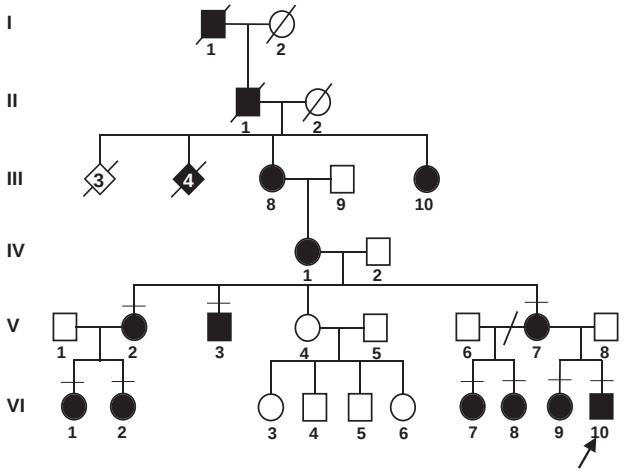

Fig. 1. Pedigree drawing of the family with brachydactyly-polydactyly. Black symbols represent affected individuals. The arrow indicates the proband. A horizontal line above a pedigree symbol indicates that the individual was examined clinically.

accompanied by hypoplasia or absence of the fifth nails, but in some the second, third and fourth distal phalanges and nails were hypoplastic as well. All also had hypoplasia or deformity of the middle phalanges of the toes (but not the fingers), and most had shortening of the third to fifth metacarpals and metatarsals, with the fourth metacarpals and metatarsals always being most severely affected. Moreover, two had bilateral hypoplasia of the styloid process of the ulna, and one had bilateral hypoplasia of the middle cuneiforms (distal tarsals), with compensating overgrowth of the second metatarsals. This pattern of digital shortening is distinct from that in all five recognised types of heritable brachydactyly (Temtamy and McKusick, 1978). In addition, two individuals (VI.7 and VI.10) had bilateral asymmetrical partial duplication of the fourth fingers (Fig. 2G-J). A third individual (IV.1), though unavailable for examination, was reported to have a double nail on the right fourth finger. This pattern of central polydactyly in the hands is similar to that in SPD, although no family member had polydactyly in the feet.

A new missense mutation (I47L) in the homeodomain of HOXD13

Direct sequencing of HOXD13 in the mother of the proband (V.7) revealed a heterozygous A-to-C transition in exon 2 at position 940 of the coding sequence. This base change, which converts amino acid 314 (residue 47 of the homeodomain) from isoleucine to leucine, does not alter a restriction site, but its presence was confirmed by cloning the PCR product and sequencing individual clones (Fig. 3A,B). The same base change was identified by direct sequencing in six other affected family members (V.2, V.3, VI.1, VI.2, VI.7 and VI.8), but not in one unaffected family member (V.4) or in 50 unrelated unaffected controls. The co-segregation of the base change with the phenotype was further demonstrated by PCR amplification of genomic DNA from affected and unaffected individuals with a forward primer specific for the mutant allele (Fig. 3C).

X-ray crystallographic and NMR studies of homeodomain/DNA complexes have shown that residue 47 is one of four

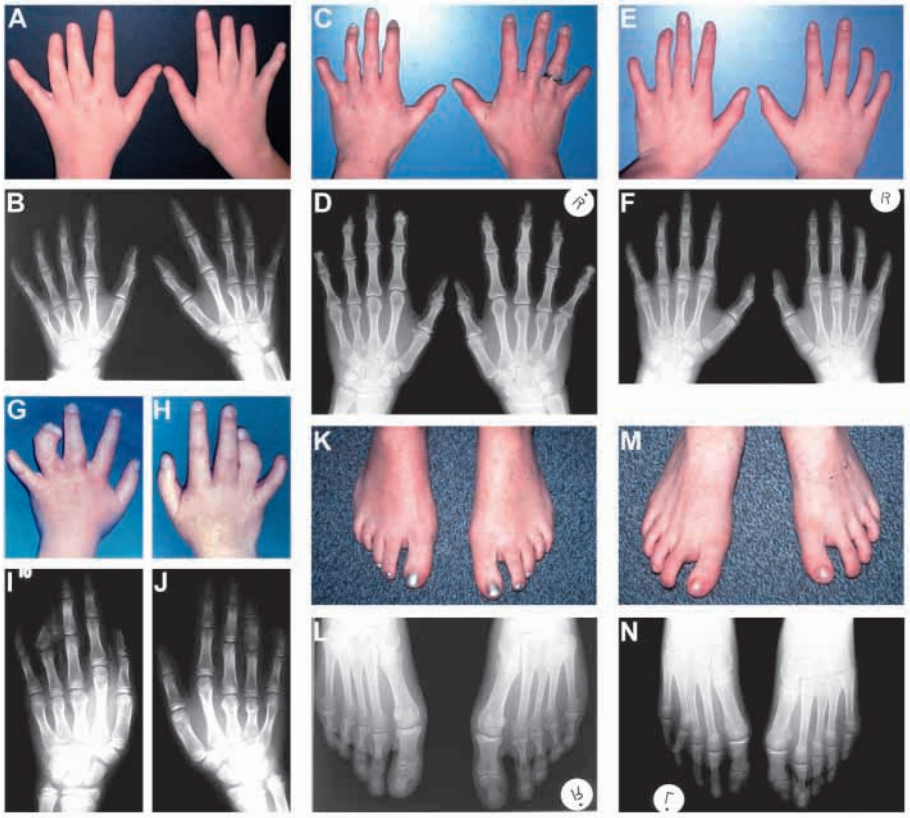

Fig. 2. The brachydactyly-polydactyly phenotype. (A,B) Hands of IV.8, showing small fifth distal phalanges and very short third to fifth metacarpals. (C,D) Hands of V.2, showing absent fifth distal phalanges and nails, small second and fourth distal phalanges, short third to fifth metacarpals, and hypoplasia of the styloid process of the ulna. (E,F) Hands of VI.2, showing very small fourth and fifth distal phalanges, absent or hypoplastic fourth and fifth nails, and short third to fifth metacarpals. (G-J) Hands of VI.7, showing partial fourth finger duplication (abnormally wide middle phalanges, with a bracket epiphysis on the left), small second, fourth and fifth distal phalanges, hypoplastic fifth nails, and very short third to fifth metacarpals. (K,L) Feet of V.2, showing absent fourth and fifth distal phalanges and nails, small second and third distal phalanges with hypoplastic nails, small middle phalanges, and short third to fifth metatarsals. (M,N) Feet of VI.2, showing absent fifth distal phalanges and nails, small first to fourth distal phalanges, hypoplastic second to fourth nails, small middle phalanges, short third to fifth metatarsals, and small middle cuneiforms with overgrowth of the second metatarsals.

highly conserved residues in the recognition helix of the homeodomain that make base-specific contacts with DNA (Gehring et al., 1994; Fraenkel and Pabo, 1998). A mutation at this position would therefore be expected to affect the DNA-binding capacity of HOXD13. The substitution replaces one non-polar branching amino acid with another, however, prompting us to investigate whether it might alter rather than abolish DNA binding.

The I47L mutation impairs HOXD13's ability to activate transcription at the HCR element

No naturally occurring HOXD13 DNA-binding sites have been characterised, as none of the target genes of HOXD13 have yet been identified. To examine whether the I47L substitution affects the ability of HOXD13 to control transcription, we used the HCR sequence (Fig. 4C), a highly conserved 92 bp regulatory element derived from the *HOXD9* promoter, which can mediate transcriptional activation by HOXD9 and HOXD10 (Zappavigna et al., 1991). To compare HOXD13(I47L) with a HOXD13 mutant that is completely unable to bind DNA, we also generated an artificial mutant, HOXD13(IQN), carrying alanine substitutions at positions 47(I), 50(Q) and 51(N) of the homeodomain. These three highly conserved residues in the recognition helix make crucial base-specific DNA contacts (Gehring et al., 1994), so we predicted that replacing them with alanine would abolish DNA binding altogether. We transiently co-transfected P19 cells with a luciferase reporter construct driven by the HCR sequence (pTHCR), together with increasing amounts of SV40-driven constructs expressing HOXD13, HOXD13(I47L) or HOXD13(IQN). Although HOXD13 increased basal reporter activity five- to sixfold, HOXD13(I47L) increased it only about

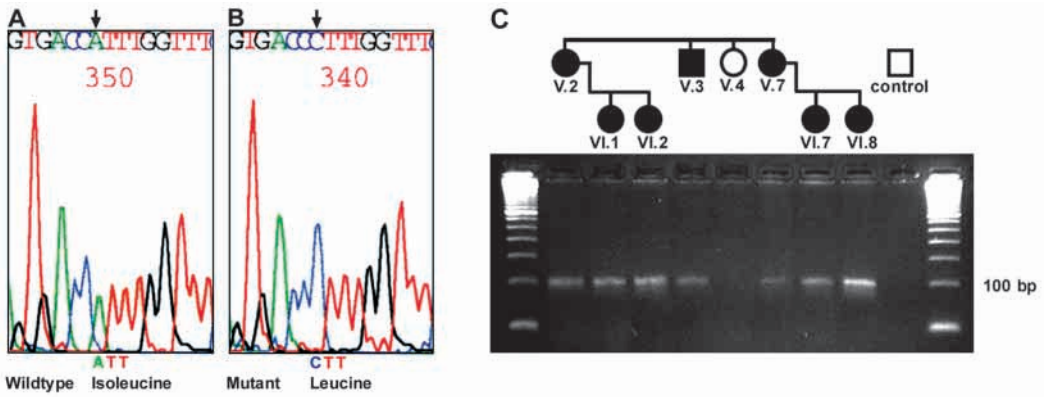

Fig. 3. The novel point mutation in *HOXD13*. (A,B) Electropherograms showing the wild-type (A) and mutant (B) alleles (subcloned) in V.7. (C) PCR amplification of genomic DNA using a forward primer specific for the mutant allele yielded a 100 bp product in seven affected family members but no product in an unaffected family member or a control individual.

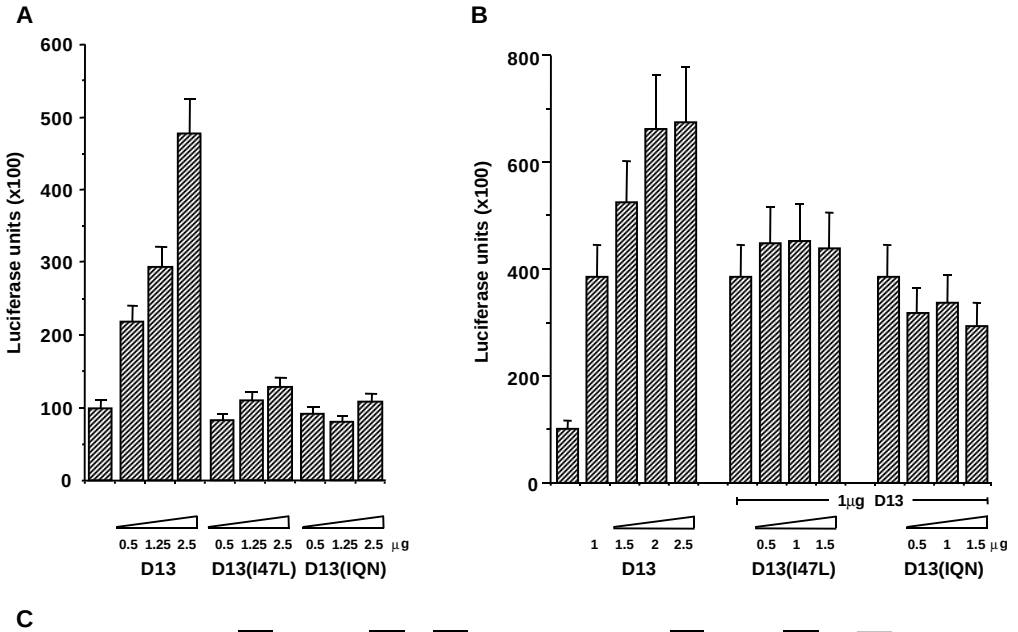

Fig. 4. Transcriptional activity of HOXD13, HOXD13(I47L) and HOXD13(IQN) at the HCR. P19 cells were transiently transfected with 10 μg of pTHCR reporter construct and the indicated quantities of expression constructs. Bars represent the mean luciferase activity±s.e.m. of at least six independent experiments. (A) Transcriptional activity mediated by increasing amounts of pSG-HOXD13, pSG-HOXD13(I47L) or pSG-HOXD13(IQN) assayed separately. (B) Transcriptional activity mediated by 1.0 μg pSG-HOXD13 in the presence of increasing amounts of pSG-HOXD13(I47L) or pSG-HOXD13(IQN), with the activity mediated by increasing amounts of pSG-HOXD13 shown for comparison. (C) Sequence of the HCR element, with potential Hox core binding motifs (5′-TAAT-3′ or 5′-TTAT-3′) on the forward strand and reverse strands over- and underlined respectively. The single 5′-TTAC-3′ motif is highlighted in grey.

1.5-fold, and HOXD13(IQN) had virtually no effect (Fig. 4A). Thus, the I47L substitution severely compromises the ability of HOXD13 to activate transcription through the HCR element, suggesting that it significantly impairs the capacity of the protein to bind DNA.

HOXD13(I47L) does not interfere with transcriptional activation by wild-type HOXD13

To examine whether HOXD13(I47L) can act as a dominant negative, we co-transfected P19 cells with fixed amounts of pTHCR and the HOXD13 expression construct, together with increasing amounts of the HOXD13(I47L) expression construct. As shown in Fig. 4B, the levels of reporter activity obtained with 1.0 μg of HOXD13 together with 0.5, 1.0 or 1.5 μg of HOXD13(I47L) were in each case slightly higher than that obtained with 1.0 μg of HOXD13 alone, although substantially lower than those obtained with 1.5, 2.0 or 2.5 μg of HOXD13 alone. In a similar experiment with HOXD13(IQN), the levels of transcriptional activation were marginally lower than that obtained with 1.0 μg of HOXD13 alone. Thus transcriptional activation by wild-type HOXD13 is not significantly inhibited by co-expression of either HOXD13(I47L) or HOXD13(IQN), suggesting that neither mutant acts as a dominant negative.

The I47L mutation impairs the ability of HOXD13 to bind DNA

To investigate whether the weak transcriptional activation mediated by HOXD13(I47L) at the HCR reflects defective DNA binding, we performed EMSAs using oligonucleotide probes derived from this element. The HCR contains several sites recognised in vitro by 5′ HOXD proteins and supports the formation of multiple retarded complexes with different stoichiometries in EMSAs with HOXD10 (Zappavigna et al., 1991). We first used bases 1-55 of the HCR (HCRα; Fig. 5A), which contain four sets of TAAT and/or TTAT motifs that could potentially be bound by HOX proteins. While HOXD13 bound HCRα efficiently, forming three different retarded complexes (Fig. 5A, lanes 3-5), HOXD13(I47L) bound more weakly, producing detectable complexes only at higher protein concentrations (Fig. 5A, lanes 7 to 9). We next used bases 3-25 of the HCR (HCR I; Fig. 5B), because they contain an 8 bp sequence (5′-TTTTATTA-3′) that is identical to the consensus binding site previously reported for Abd-B and Hoxa10 (5′-TTTTAT(T/G)(A/G)-3′) (Ekker et al., 1994; Benson et al., 1995) and differs at just two positions (underlined) from the consensus binding site previously reported for Hoxd13 (5′-TTTTACGA-3′) (Shen et al., 1997). HOXD13 formed a strong retarded complex with HCR I (Fig. 5B, lane 3), whereas HOXD13(I47L) bound only weakly (Fig. 5B, lane 4). We subsequently used bacterially expressed, purified GST fusions of the HOXD13 and HOXD13(I47L) homeodomains [GST-HOXD13HD and GST-HOXD13HD(I47L)] to determine their dissociation constants (K_d) at this site in EMSAs, obtaining K_d values of 5×10^{-8} M and 3×10^{-7} M respectively (data not shown). Thus, HOXD13(I47L) fails to recognise at least some sites bound by the wild-type protein. This suggests that the I47L mutation produces a loss of function, but leaves open the possibility that it produces a gain of function by changing binding site specificity.

To explore these alternatives, we designed an oligonucleotide probe (SelD13; Fig. 5C) with 12 central bases derived from HCR I (GTTTTATTAGGG), but with the ATTAG sequence replaced by five random bases (GTTTTNNNNNGG), thus generating a large pool of sequence variants. We expected that a straightforward switch in binding specificity would result in equally efficient binding by HOXD13 and HOXD13 (I47L), as the different optimal binding sequences would be equally represented in the pool. As shown in Fig. 5C, when the SelD13 probe was incubated

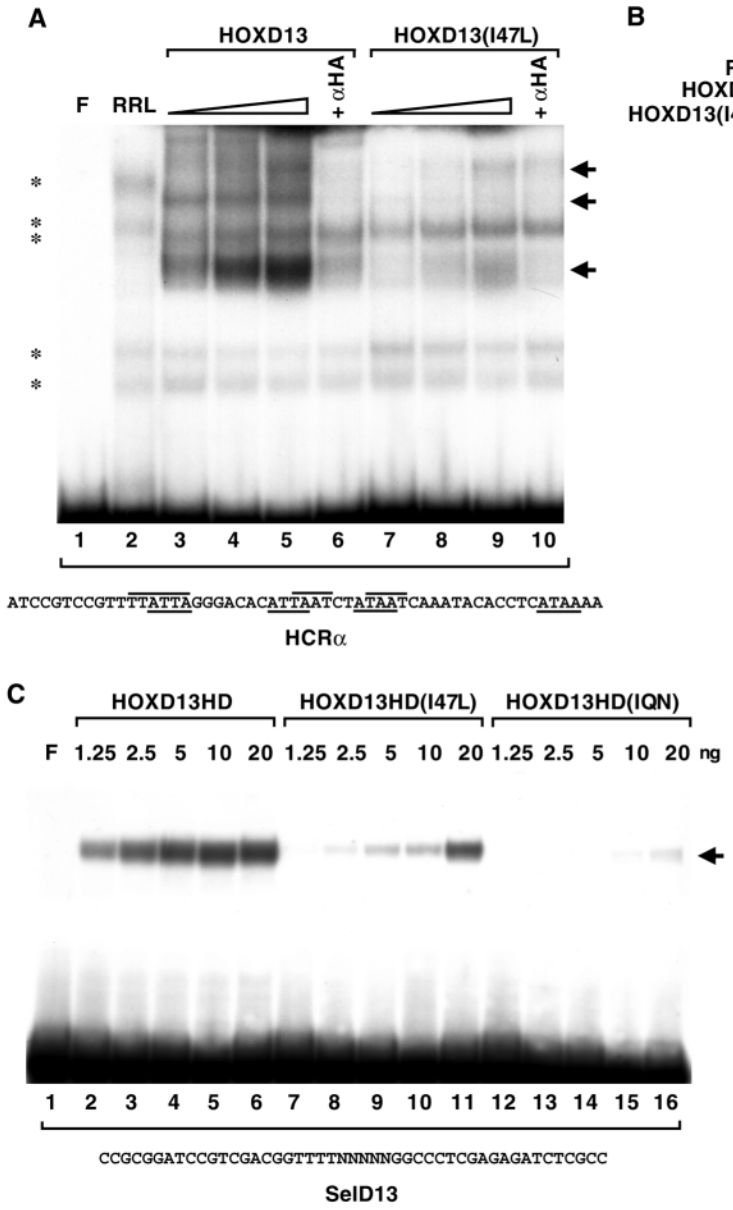

Fig. 5. HOXD13(I47L) displays impaired DNA-binding ability in EMSAs. Probe sequences are shown below the gels, with potential Hox core binding motifs (5′-TAAT-3′ or 5′-TTAT-3′) on the forward strand and reverse strands over- and underlined respectively. Arrows indicate bands corresponding to HOX protein:DNA complexes. Asterisks indicate nonspecific bands produced by the reticulocyte lysate. (A) Binding of in vitro translated HOXD13 (lanes 3-6) and HOXD13(I47L) (lanes 7-10) to ^{32}P-labelled HCRα (lane 1). (B) Binding of in vitro translated HOXD13 (lane 3) and HOXD13(I47L) (lane 4) to ^{32}P-labelled HCR I (lane 1). In A and B, to identify HOX protein:DNA complexes, 200 ng of anti-HA antibody (Santa Cruz Biotechnology) was added to the binding reactions (A, lanes 6 and 10; B, lanes 5 and 6). In B, to assess binding specificity, a 300-fold molar excess of cold competitor HCR I was added to the binding reactions (lanes 7 and 8). (C) Binding of bacterially expressed, purified GST-HOXD13HD (lanes 2-6), GST-HOXD13HD(I47L) (lanes 7-11) and GST-HOXD13HD(IQN) (lanes 12-16) to ^{32}P-labelled SelD13 (lane 1).

with increasing amounts of the purified GST-homeodomain fusion proteins, GST-HOXD13HD bound efficiently at all concentrations tested (lanes 2-6) whereas GST-HOXD13HD(I47L) bound only at higher concentrations (lanes 7-11). GST-HOXD13HD(IQN) bound marginally even at the highest concentration tested (lanes 12-16), indicating, as predicted, that HOXD13HD(IQN) does not recognise any site in the pool efficiently. The intermediate binding levels seen with HOXD13HD(I47L) are not consistent with a straightforward switch in binding specificity, but could have occurred either because the mutant protein binds more weakly than the wild-type protein at all sites in the pool, or because the mutant binds a smaller subset of sites in the pool.

HOXD13(I47L) binds a subset of the sites recognised by HOXD13

To distinguish between these possibilities, we performed binding site selection assays with purified GST-HOXD13HD and GST-HOXD13HD(I47L) using the SelD13 oligonucleotide pool. After five rounds of selection, the sequences of 100 oligonucleotides selected by each protein were examined. Of 100 sites selected by wild-type HOXD13 (Fig. 6A), 48 had a TTA**T** core, followed in 36 by TGG. Only 2 had a TAA**T** core. 32 had a TTA**C** core, followed in 25 by GAG. The remaining 18 had a TAA**C** core, followed in 17 by GAG. Wild-type HOXD13 therefore appears to have an equal preference for two distinct sites, one (site 1) with T in the fourth core position (TTTTA**T**TGG) and the other (site 2) with C in the fourth core position (TTT(T/A)A**C**GAG) (Fig. 6B). By contrast, HOXD13(I47L) recognised a more restricted set of sites. Of 100 sites selected (Fig. 6A), only three had a TTA**T** or TAA**T** core. 73 had a TTA**C** core, followed in 47 by GAG, while 20 had a TAA**C** core, followed in 11 by GAG. HOXD13(I47L) therefore appears to recognise one of the two sites selected by wild-type HOXD13 (site 2), while almost completely failing to recognise the other (site 1) (Fig. 6B).

To confirm these findings, we performed EMSAs using probes containing site 1 (with the TTAT core) and the two variants of site 2 (with TTAC and TAAC cores). As shown in Fig. 6C, HOXD13 bound all three probes with comparable efficiency (lanes 2-4, 9-11 and 16-18). HOXD13(I47L) bound the TTAT-containing probe much more weakly than the wild-

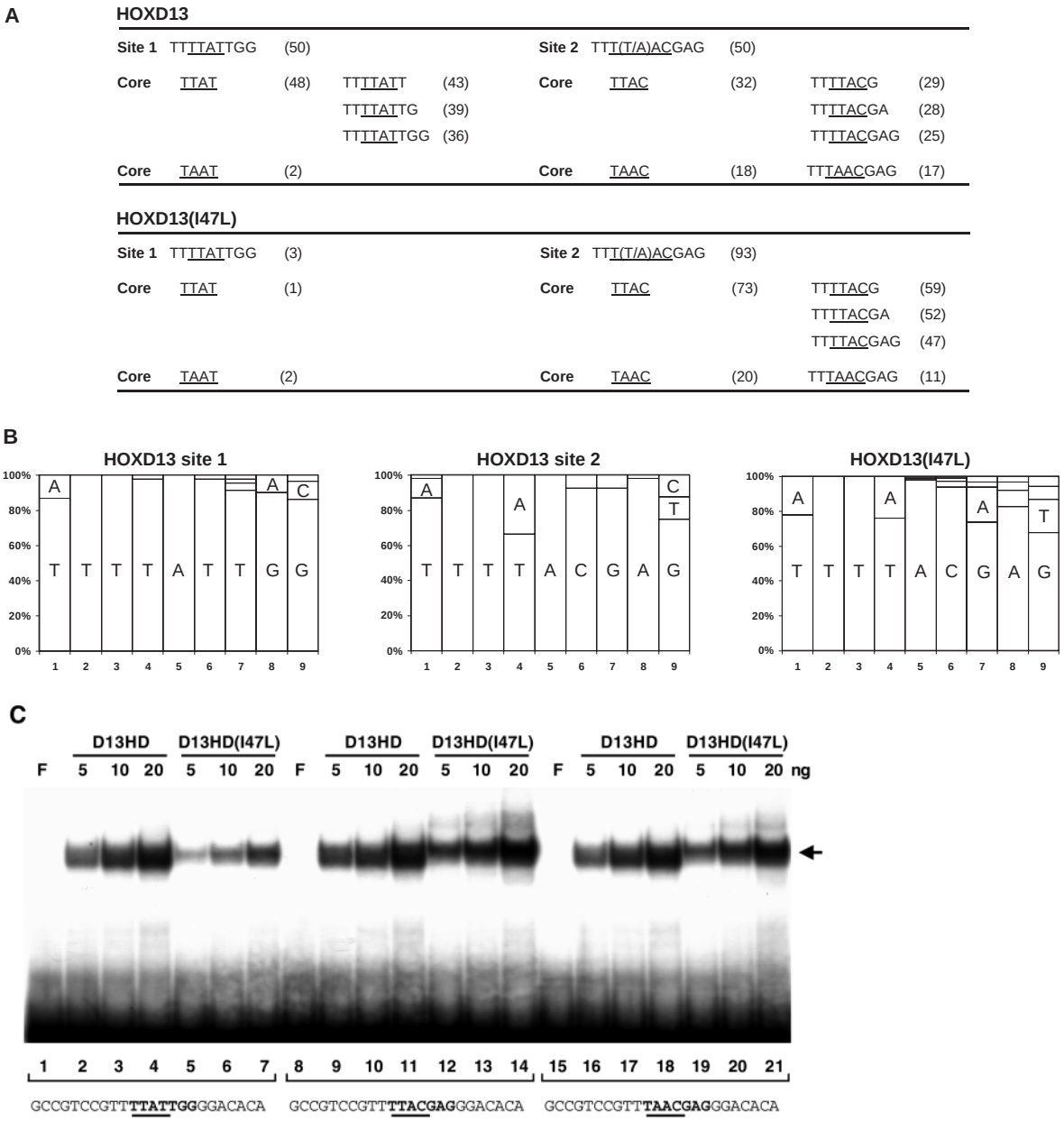

Fig. 6. HOXD13HD(I47L) binds a subset of the sites recognised by HOXD13HD. The sequences of 100 oligonucleotides selected by each homeodomain in site selection experiments were aligned using the program CLUSTAL W. Those selected by HOXD13HD fell into two equal-sized groups, one with a TTAT core (site 1) and the other with a T(T/A)AC core (site 2), whereas those selected by HOXD13HD(I47L) formed a single group with a T(T/A)AC core. (A) Numbers of oligonucleotides containing sites 1 and 2 selected by each homeodomain. (B) Results displayed as consensograms (Wilson et al., 1993). (C) Binding of GST-HOXD13HD and GST-HOXD13HD(I47L) to site 1 (TTATTGG, lanes 1-7) and the two variants of site 2 (TTACGAG, lanes 8-14, and TAACGAG, lanes 15-21). The arrow indicates bands corresponding to HOX protein:DNA complexes.

type protein (16- and ninefold less at 5 and 10 ng respectively, lanes 5-6), but bound the TTAC- and TAAC-containing probes with the same efficiency (0.9-fold less TTAC at 5 and 10 ng, lanes 12-13; 1.3- and 1.1-fold less TAAC at 5 and 10 ng respectively, lanes 19-20). Thus, the I47L mutation does not result in recognition of a novel DNA-binding sequence. Instead, it causes a selective impairment of DNA-binding ability, producing a marked reduction in affinity at one class of sites recognised by the wild-type protein, but no loss of affinity at the other.

Misexpression of HOXD13(I47L) in developing chick limbs does not affect the digits but produces more severe proximal abnormalities than HOXD13

To explore the effects of the I47L mutation further in vivo, we used the developing chick limb, a well-established model system for studying limb development in vertebrates. The availability of replication-competent retroviral vectors that permit gene transfer into avian cells has made it possible to manipulate chick limb development genetically in ovo (Morgan and Fekete, 1996). We therefore generated recombinant retroviral vectors expressing

full-length HOXD13, HOXD13(I47L) or HOXD13(IQN), and injected concentrated retroviral suspensions of comparable titres into the prospective right hindlimb field of stage 10 chick embryos in ovo. Control experiments using a retroviral construct expressing alkaline phosphatase (Fekete and Cepko, 1993) confirmed that the entire right leg bud was infected at high frequency (data not shown). Embryos were harvested at stage 32-33 (day 7.5-8) and stained with Alcian Blue to allow visualisation of cartilaginous skeletal elements. The left uninjected limbs were used as internal controls. The resulting phenotypes are summarised in Table 1 and illustrated in Fig. 7.

As reported previously (Goff and Tabin, 1997), embryos misexpressing wild-type HOXD13 (Fig. 7A) had mild shortening of the cartilages of the stylopod, zeugopod and proximal autopod, especially the femur and tibia. As a result, the fibula, which is normally shorter than the tibia, was usually about the same length, and often articulated with or was fused to the fibulare (Fig. 7A). No phalangeal abnormalities were ever observed, however, confirming that elevated levels of wild-type HOXD13 do not perturb normal development in the distal autopod.

Embryos misexpressing HOXD13(I47L) (Fig. 7B,C) likewise had no phalangeal abnormalities (except one embryo out of 38, which had an extra anterior digit). The cartilages of the stylopod, zeugopod and proximal autopod, however, were more severely shortened, especially the tibia, which on average was reduced to 54% of its normal length (Table 1). In 16% of embryos, the morphology of the tibia was also altered from a typical long bone cartilage to a rounded cartilage, with bowing of the fibula, sometimes producing a complete inversion of normal limb posture (Fig. 7C). In addition, 16% of embryos had extra cartilages in the zeugopod (Fig. 7B,C). Neither these tibial changes nor ectopic cartilages were ever observed in embryos misexpressing HOXD13 (Goff and Tabin, 1997) (this work).

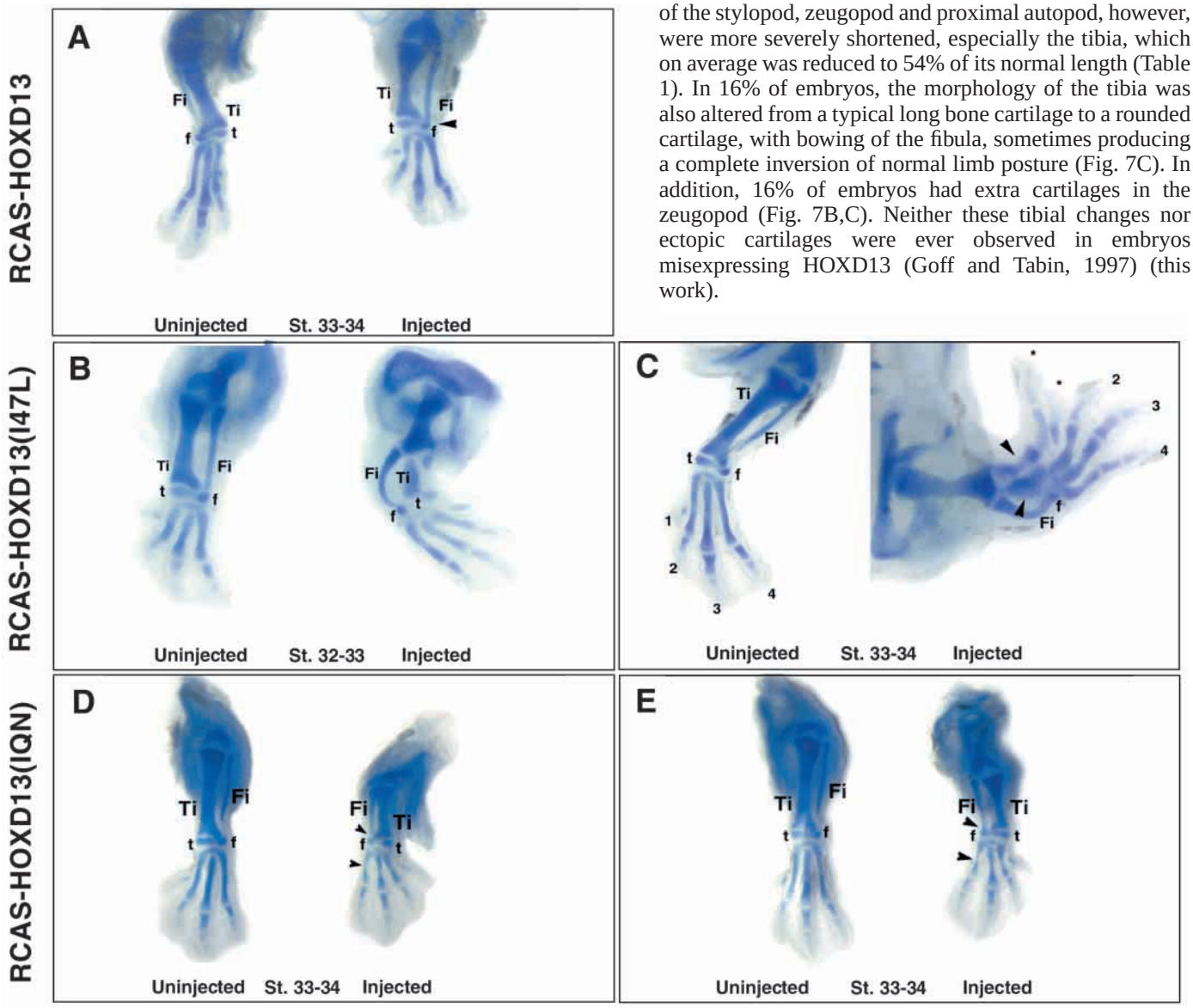

Fig. 7. Phenotypes produced by misexpressing HOXD13, HOXD13(I47L) and HOXD13(IQN) in developing chick limbs. Frontal views of stage 32-34 hindlimbs stained with Alcian Blue, showing the injected right limb on the right and the uninjected opposite limb on the left. Ti, tibia; Fi, fibula; t, tibiale; f, fibulare. (A) Embryo injected with RCAS-HOXD13, showing normal phalanges but mild shortening of the proximal cartilages, especially the femur and tibia, resulting in an abnormal articulation between the fibula and fibulare (arrowhead). (B,C) Embryos injected with RCAS-HOXD13(I47L), showing normal phalanges (the embryo in C was the only one out of 38 with a rudimentary extra digit (asterisked), but marked shortening of the proximal cartilages, particularly the tibia, which has a rounded morphology, and extra cartilages in the zeugopod (arrowheads). (D,E) Embryos injected with RCAS-HOXD13(IQN), showing normal phalanges but marked shortening of the proximal cartilages, especially the femur and tibia (again resulting in an abnormal articulation between the fibula and fibulare; upper arrowheads), and also the metatarsals (lower arrowheads).

Table 1. Cartilage shortening in chick hindlimbs injected with RCAS-HOXD13, RCAS-HOXD13(I47L) or RCAS-HOXD13(IQN)

		Zeugopod		Autopod	
Mutation	Stylopod	Tibia	Fibula	Tarsometatarsals	Phalanges
D13 (*n*=30)	0.78±0.09	0.70±0.14	0.83±0.28	0.87±0.24	1
D13(I47L) (*n*=38)	0.72±0.11	0.54±0.14	0.72±0.14	0.80±0.12	1
D13(IQN) (*n*=102)	0.70±0.10	0.63±0.13	0.78±0.22	0.72±0.14	1

Figures represent the ratio of cartilage lengths in the injected limb to the corresponding cartilage lengths in the uninjected opposite limb. Values are the mean±s.e.m. *n*, number of embryos analysed.

Embryos misexpressing HOXD13(IQN) (Fig. 7D,E) again had no phalangeal abnormalities, but the proximal cartilages, especially the femur and tibia, were more severely shortened than in embryos misexpressing HOXD13, and the metatarsals were shorter than in embryos misexpressing either HOXD13 or HOXD13(I47L) (Table 1). However, no instances of altered tibial morphology or ectopic cartilages were observed in over 100 embryos examined. Strikingly, the phenotype produced by a HOXD13 mutant incapable of binding DNA is thus qualitatively similar to but quantitatively more severe than that produced by HOXD13.

Overexpression of HOXD13 upregulates *EphA7*, while overexpression of HOXD13(I47L) does not

In *Hoxa13*$^{-/-}$ mice, *EphA7* expression is significantly reduced in the condensing mesenchyme of the digits, carpals and tarsals, but not completely absent, suggesting that this ephrin receptor is a downstream target not only of Hoxa13 but perhaps also of other Hox proteins expressed in the developing autopod, like Hoxd13 (Stadler et al., 2001). To investigate whether HOXD13 controls *EphA7* expression, and, if so, whether the I47L mutation affects this activity, we analysed chick *EphA7* expression in chick limbs overexpressing HOXD13, HOXD13(I47L) or HOXD13(IQN). In control stage 28 hindlimbs, *EphA7* was expressed in the perichondrium of the phalangeal mesenchymal condensations (Fig. 8A,C,E), as reported previously in E13.5 mice (Stadler et al., 2001). Overexpression of HOXD13 markedly increased *EphA7* expression in the perichondrium but produced no significant ectopic expression (Fig. 8B). Overexpression of HOXD13(I47L) or HOXD13(IQN), however, had no effect upon *EphA7* levels (Fig. 8D,F). Thus, while HOXD13, like Hoxa13, upregulates *EphA7* expression in the autopod, neither HOXD13(I47L) nor HOXD13(IQN) retains this activity.

DISCUSSION

Several different mutations in *HOXD13* have previously been shown to cause the congenital limb malformation SPD (reviewed by Goodman, 2002), but little is yet known about how these mutations perturb protein function. We have identified and analysed a novel amino acid substitution in HOXD13, which causes a unique brachydactyly-polydactyly syndrome. Although some features of this syndrome (central polydactyly in the hands, middle phalanx hypoplasia in the feet) occur in both typical and atypical SPD, others (hypoplastic or absent nails and distal phalanges, short third to fifth metacarpals and metatarsals, hypoplastic middle cuneiforms and ulnar styloids) do not. Moreover, several features of one or both forms of SPD (fifth finger clinodactyly, partial duplication of the second metatarsals) are absent in the new syndrome. The abnormalities are nevertheless confined to the expression domain of *HOXD13* in the developing limb, which comprises the entire anteroposterior extent of the autopod, as far proximally as the distal carpals/tarsals, and the posterior border of the zeugopod (Dolle et al., 1991; Nelson et al., 1996). Interestingly, the hypoplasia or absence of the nails and distal phalanges closely resembles that in Brachydactyly type B, which is caused by mutations in *ROR2* (Oldridge et al., 2000). This supports suggestions that *ROR2*, which plays an essential role in cartilage growth and differentiation, may lie in the same molecular pathway as the Hox genes (DeChiara et al., 2000).

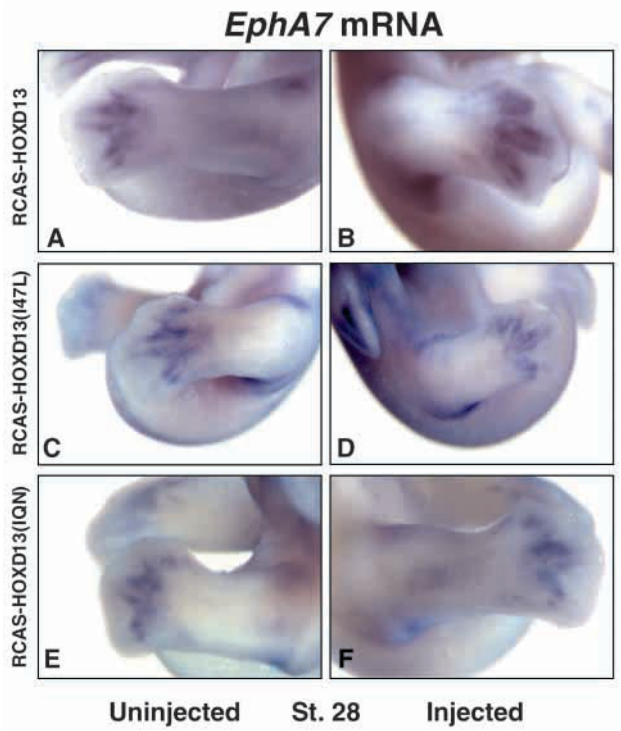

Fig. 8. Overexpression of HOXD13 upregulates expression of chick *EphA7*, but overexpression of HOXD13(I47L) does not. Wholemounts of stage 28 chick embryos expressing HOXD13, HOXD13(I47L) or HOXD13(IQN) in the right hindlimb. Embryos were hybridised in situ with an antisense chick *EphA7* probe. Chick *EphA7* is expressed in the perichondrium of the digital condensations. This expression is increased in the right limbs overexpressing HOXD13 (B), but unaltered in limbs overexpressing HOXD13(I47L) (D) or HOXD13(IQN) (F). In A,C,E, the respective contralateral uninjected limbs are shown.

The substitution in question alters the isoleucine residue at position 47 of the HOXD13 homeodomain to leucine. In the Antennapedia, engrailed and HOXB1 homeodomain/DNA complexes, I47 methyl groups make van der Waals contacts both with the C5 methyl group of the thymine at position 4 of the Hox core binding site (TAA**T**) and with C8 of the adenine at position 3 (TA**A**T) (Gehring et al., 1994; Fraenkel and Pabo, 1998; Piper et al., 1999). In keeping with this crucial role in DNA binding, residue 47 is highly conserved (Banerjee-Basu et al., 2001). Seven out of the eight *Drosophila* Hox proteins and all but two of the 39 vertebrate Hox proteins have isoleucine at this position, the exceptions all being group 2 paralogs (*Drosophila* proboscipedia, vertebrate Hoxa2 and Hoxb2), which have valine, another non-polar branching amino acid (Banerjee-Basu et al., 2001). Most other homeodomain proteins also have isoleucine or valine, although different amino acids occur in some atypical homeodomains (Banerjee-Basu and Baxevanis, 2001; Banerjee-Basu et al., 2001). The only two reported instances of leucine at position 47 are in highly divergent proteins, PaHB2 of the Norway spruce (*Picea abies*) and the bE1-bE7 mating type proteins of the smut fungus (*Ustiligo maydis*) (GenBank Accession Numbers AAL83725, AAA63553-6 and CAA38000-2).

No naturally occurring mutations affecting homeodomain residue 47 have previously been reported (D'Elia et al., 2001). An artificial I47A substitution in engrailed reduces DNA binding affinity 10- to 20-fold in vitro (Ades and Sauer, 1995), while an I47Q substitution in the Para-Hox protein IPF1 abolishes DNA binding in vitro and greatly reduces transcriptional activation in transfected cells (Lu et al., 1996). A V47L substitution in the POU homeodomain protein Oct2 also abolishes DNA binding in vitro (Stepchenko et al., 1997). No I47L substitution, however, has yet been analysed.

HOXD13(I47L) exhibits a selective loss of DNA-binding ability

Previous studies have shown that the most 5′ Hox protein in *Drosophila*, Abd-B, selects sites with a TTAT core (Ekker et al., 1994), whereas the 5′ vertebrate Hox proteins select sites with a TTAT or TTAC core (Benson et al., 1995; Shen et al., 1997). We found that HOXD13 displayed an equal preference for two distinct consensus sites, one containing a TTAT core (TTTTAT**T**TGG), and the other containing a TTAC or TAAC core (TTT(T/A)A**C**GAG). This second site is identical to the consensus Hoxd13 binding site obtained by Shen et al. (TTTTA**C**GAG) (Shen et al., 1997), except at position 4, where we observed a 64:36 T:A ratio whereas Shen et al. observed a strong preference for T. HOXD13(I47L) selected only the second of these sites (TTT(T/A)A**C**GAG), perhaps reflecting the inability of L47 to make the contacts normally made by I47 with a T residue in the fourth core position. Thus, the mutant protein, rather than recognising a novel binding site, appears to have lost the ability to recognise one class of site bound by the wild-type protein while retaining the ability to recognise the other.

Consistent with these findings, HOXD13(I47L) bound the TTTTACGAG and TTTAACGAG sequences with the same affinity as wild-type HOXD13 in EMSAs, but displayed a significantly lower affinity for the TTTTATTGG sequence. The impaired capacity of HOXD13(I47L) to recognise TTAT- and TAAT-containing sites was further confirmed using the HCR element, which contains multiple TTAT and TAAT motifs bound by several 5′ HOXD proteins, but only one TTAC motif (Zappavigna et al., 1991). Although HOXD13 bound strongly and specifically to several of these sites in EMSAs, HOXD13(I47L) bound only weakly, with an affinity for one of them (HCR I, TTTTATTAG) about sixfold lower than that of the wild-type protein. Moreover, while HOXD13 activated transcription through the full-length HCR, HOXD13(I47L) did not. These observations suggest that HOXD13(I47L) is unable to regulate some of the genes regulated by wild-type HOXD13.

HOXD13 upregulates EphA7 expression but HOXD13(I47L) does not

Signalling between Eph receptors and their ephrin ligands plays a key role in regulating many developmental processes and is probably a downstream effector of several 3′ Hoxa and Hoxb genes [reviewed in (Frisen et al., 1999)]. Moreover, *Hoxa13* is necessary for normal *EphA7* expression levels in the autopod (Stadler et al., 2001). We found that misexpression of HOXD13 in chick limbs increased chick *EphA7* expression in the perichondrium of the digital condensations, showing that HOXD13, like Hoxa13, can upregulate *EphA7*. Interestingly, ectopic expression of chick *EphA7* was not induced, suggesting that additional factors are required for *EphA7* transcription. Misexpression of HOXD13(I47L), however, had no effect on chick *EphA7* levels, showing that the I47L mutation indeed impairs the capacity of HOXD13 to regulate one of its downstream targets. Although no other HOXD13 targets have yet been identified, our finding that HOXD13(I47L) retains the ability in vitro to bind some sites bound by HOXD13 suggests that it can still regulate at least some of these targets. It may thus partially or completely fail to regulate a subset of the genes normally controlled by HOXD13, such as *EphA7*, while correctly regulating the remainder, thus eliciting an unbalanced transcriptional response.

Misexpression of HOXD13(I47L) in chick limbs produces a phenotype both quantitatively and qualitatively different to that produced by HOXD13

To analyse the consequences of the I47L substitution in vivo, we used retrovirus-mediated expression in the developing chick limb. When overexpressed in the phalanges, where Hoxd13 is normally expressed, HOXD13(I47L), like wild-type HOXD13 (Goff and Tabin, 1997) (this work) and HOXD13(IQN), caused no defects. Thus, HOXD13(I47L) does not interfere with the functions of endogenous Hoxd13 in vivo, or with distal autopod development, indicating that it does not act by a dominant-negative or other gain-of-function mechanism. Consistently, HOXD13(I47L) did not interfere with transcriptional activation by HOXD13 at the HCR element in transiently transfected cells, and showed no gain or switch in DNA-binding activity in vitro.

In proximal limb regions, however, whereas HOXD13 caused only mild shortening of the long bone cartilages, HOXD13(I47L) produced severe shortening, as well as striking abnormalities of zeugopod morphology, including a change in the shape of the tibia from long to rounded cartilage and the formation of ectopic cartilages. Misexpression of HOXD13(IQN) likewise caused severe shortening of the proximal cartilages, but never produced the abnormal zeugopod morphology observed with HOXD13(I47L). The phenotype caused by HOXD13(I47L) in proximal limb regions

is thus qualitatively as well as quantitatively different from that produced by wild-type HOXD13, but is also qualitatively different from that produced by HOXD13(IQN), which is completely unable to bind DNA. This further strengthens the hypothesis that the I47L substitution results in a selective rather than a generalised loss of function. The additional zeugopod abnormalities caused by HOXD13(I47L) probably reflect its ability to control only a subset of the genes normally controlled by HOXD13, leading to an imbalance in the regulation of downstream targets. Interestingly, misexpression of wild-type Hoxa13 in chick limbs produces a similar zeugopod phenotype (Yokouchi et al., 1995), suggesting that some of the same targets may be involved.

A selective loss-of-function mechanism also accounts well for the novel brachydactyly-polydactyly syndrome produced by the mutation. Thus, the occasional central polydactyly in the hands, like that in typical and atypical SPD, probably reflects loss of transcriptional activity, while the impaired growth or absence of specific phalangeal and metaphalangeal bones, unaffected in both forms of SPD, probably reflects unbalanced regulation of the normal targets of HOXD13.

The ability of HOXD13 to interfere with proximal limb development does not require DNA binding

The shortening of proximal limb skeletal elements produced by ectopic expression of Hoxd13 in the chick (Goff and Tabin, 1997) (this work) has also been observed in the mouse. Thus, Hoxd13 expression in the prospective forearm causes shortening and bowing of the radius and ulna both in mice with a *Hoxd11/lacZ* transgene upstream of *Hoxd13* (van der Hoeven et al., 1996; Zakany and Duboule, 1999) and in *Ulnaless* mice (Herault et al., 1997; Peichel et al., 1997). Ectopic expression of Hoxa13 in the chick (Yokouchi et al., 1995) and of Hoxd12 in the mouse (Knezevic et al., 1997) also produce proximal limb shortening. Similarly, ectopic Hoxd gene expression resulting from mutations that perturb the regulation of the Hoxd cluster may underlie some types of mesomelic dysplasia in humans (Fujimoto et al., 1998; Spitz et al., 2002; Sugawara et al., 2002).

In all these cases, the zeugopod shortening closely resembles that in *Hoxa11*$^{-/-}$/*Hoxd11*$^{+/-}$ and *Hoxa11*$^{+/-}$/*Hoxd11*$^{-/-}$ mice (Davis et al., 1995), and appears to reflect functional interference with the endogenous group 11 Hox proteins by the ectopic group 12 and/or 13 Hox proteins (Zakany and Duboule, 1999). This is consistent with the well-established phenomenon known as phenotypic suppression in *Drosophila* and posterior prevalence in vertebrates, whereby the more posterior Hox proteins suppress the functions (but not the expression) of more anterior Hox proteins when co-expressed in same region (Bachiller et al., 1994; Duboule and Morata, 1994). The molecular basis for this functional hierarchy remains unclear, but competition for shared sets of target genes and for DNA-binding partners and/or transcriptional co-factors have been proposed (Duboule and Morata, 1994).

Strikingly, we found that the HOXD13(I47L) and HOXD13(IQN) mutants, the DNA-binding abilities of which are selectively and generally (respectively) impaired, caused more severe shortening of proximal limb cartilages than wild-type HOXD13. The capacity of HOXD13 to interfere with the growth-promoting functions of more anterior Hox proteins thus increases when its DNA-binding ability is impaired, suggesting that this interference is mediated by protein:protein interactions and is mitigated rather than exacerbated by the ability to regulate shared target genes. Our results therefore support a model in which posterior prevalence is based on competition for interacting partners and/or transcriptional co-factors, rather than on competition for targets.

Which region(s) of HOXD13 mediate these interactions? In the chick limb, misexpression of a Hoxd13 protein lacking the first 98 amino acids produced slightly less zeugopod shortening than full-length Hoxd13 (Goff and Tabin, 1997), suggesting a possible role for the poorly characterised N-terminal region. However, the zeugopod was severely shortened in mice homozygous for a ‘knock-in’ mutation in which the homeodomain of Hoxa11 was replaced with that of Hoxa13 (Zhao and Potter, 2001). Taken together with our findings, this result suggests that the homeodomain itself may be sufficient to mediate posterior prevalence by interacting with DNA-binding partners and/or co-factors without binding DNA. Further work will be required to characterise the protein motifs and factors involved.

We are most grateful to the family for their participation in our study. We also thank Christine Hall for help in assessing the radiographs, Valeria Marigo for advice on the manipulation of chick embryos and Angela Nieto for providing the chick *EphA7* cDNA clone. This work was supported by grants from the Italian Telethon to V.Z., the Italian Association for Cancer Research (AIRC) to V.Z., and from the UK Medical Research Council (Clinician Scientist Fellowship to F.R.G.).

REFERENCES

Ades, S. E. and Sauer, R. T. (1995). Specificity of minor-groove and major-groove interactions in a homeodomain-DNA complex. *Biochemistry* **34**, 14601-14608.

Akarsu, A. N., Stoilov, I., Yilmaz, E., Sayli, B. S. and Sarfarazi, M. (1996). Genomic structure of *HOXD13* gene: a nine polyalanine duplication causes synpolydactyly in two unrelated families. *Hum. Mol. Genet.* **5**, 945-952.

Araujo, M. and Nieto, M. A. (1997). The expression of chick *EphA7* during segmentation of the central and peripheral nervous system. *Mech. Dev.* **68**, 173-177.

Bachiller, D., Macìas, A., Duboule, D. and Morata, G. (1994). Conservation of a functional hierarchy between mammalian and insect *Hox/HOM* genes. *EMBO J.* **13**, 1930-1941.

Banerjee-Basu, S. and Baxevanis, A. D. (2001). Molecular evolution of the homeodomain family of transcription factors. *Nucleic Acids Res.* **29**, 3258-3269.

Banerjee-Basu, S., Sink, D. W. and Baxevanis, A. D. (2001). The Homeodomain Resource: sequences, structures, DNA binding sites and genomic information. *Nucleic Acids Res.* **29**, 291-293.

Benson, G. V., Nguyen, T. H. and Maas, R. L. (1995). The expression pattern of the murine *Hoxa-10* gene and the sequence recognition of its homeodomain reveal specific properties of *Abdominal B*-like genes. *Mol. Cell Biol.* **15**, 1591-1601.

Bruneau, S., Johnson, K. R., Yamamoto, M., Kuroiwa, A. and Duboule, D. (2001). The mouse *Hoxd13*(spdh) mutation, a polyalanine expansion similar to human type II synpolydactyly (SPD), disrupts the function but not the expression of other *Hoxd* genes. *Dev. Biol.* **237**, 345-353.

Calabrese, O., Bigoni, S., Gualandi, F., Trabanelli, C., Camera, G. and Calzolari, E. (2000). A new mutation in *HOXD13* associated with foot pre-postaxial polydactyly. *Eur. J. Hum. Genet.* **8**, 140.

D’Elia, A. V., Tell, G., Paron, I., Pellizzari, L., Lonigro, R. and Damante, G. (2001). Missense mutations of human homeoboxes: A review. *Hum. Mutat.* **18**, 361-374.

Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. and Capecchi, M. R. (1995). Absence of radius and ulna in mice lacking *Hoxa-11* and *Hoxd-11*. *Nature* **375**, 791-795.

Debeer, P., Bacchelli, C., Scambler, P., de Smet, L., Fryns, J. and Goodman, F. (2002). Severe digital abnormalities in a patient heterozygous for both a novel missense mutation in HOXD13 and a polyalanine tract expansion in HOXA13. *J. Med. Genet.* **39**, 852-856.

DeChiara, T. M., Kimble, R. B., Poueymirou, W. T., Rojas, J., Masiakowski, P., Valenzuela, D. M. and Yancopoulos, G. D. (2000). *Ror2*, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. *Nat. Genet.* **24**, 271-274.

Di Nocera, P. P. and Dawid, I. B. (1983). Transient expression of genes introduced into cultured cells of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **80**, 7095-7098.

Dolle, P., Izpisua-Belmonte, J. C., Boncinelli, E. and Duboule, D. (1991). The *Hox-4.8* gene is localized at the 5′ extremity of the *Hox-4* complex and is expressed in the most posterior parts of the body during development. *Mech. Dev.* **36**, 3-13.

Duboule, D. and Morata, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.* **10**, 358-364.

Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A. (1994). The degree of variation in DNA sequence recognition among four Drosophila homeotic proteins. *EMBO J.* **13**, 3551-3560.

Fekete, D. M. and Cepko, C. L. (1993). Replication-competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo. *Mol. Cell. Biol.* **13**, 2604-2613.

Fraenkel, E. and Pabo, C. O. (1998). Comparison of X-ray and NMR structures for the Antennapedia homeodomain-DNA complex. *Nat. Struct. Biol.* **5**, 692-697.

Frisen, J., Holmberg, J. and Barbacid, M. (1999). Ephrins and their Eph receptors: multitalented directors of embryonic development. *EMBO J.* **18**, 5159-5165.

Fujimoto, M., Kantaputra, P. N., Ikegawa, S., Fukushima, Y., Sonta, S., Matsuo, M., Ishida, T., Matsumoto, T., Kondo, S., Tomita, H., Deng, H. X., D'Urso, M., Rinaldi, M. M., Ventruto, V., Takagi, T., Nakamura, Y. and Niikawa, N. (1998). The gene for mesomelic dysplasia Kantaputra type is mapped to chromosome 2q24-q32. *J. Hum. Genet.* **43**, 32-36.

Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. and Wuthrich, K. (1994). Homeodomain-DNA recognition. *Cell* **78**, 211-223.

Goff, D. J. and Tabin, C. J. (1997). Analysis of *Hoxd-13* and *Hoxd-11* misexpression in chick limb buds reveals that *Hox* genes affect both bone condensation and growth. *Development* **124**, 627-636.

Goodman, F., Giovannucci-Uzielli, M. L., Hall, C., Reardon, W., Winter, R. and Scambler, P. (1998). Deletions in *HOXD13* segregate with an identical, novel foot malformation in two unrelated families. *Am. J. Hum. Genet.* **63**, 992-1000.

Goodman, F. R. (2002). Limb malformations and the human *HOX* genes. *Am. J. Med. Genet.* **112**, 256-265.

Goodman, F. R., Mundlos, S., Muragaki, Y., Donnai, D., Giovannucci-Uzielli, M. L., Lapi, E., Majewski, F., McGaughran, J., McKeown, C., Reardon, W., Upton, J., Winter, R. M., Olsen, B. R. and Scambler, P. J. (1997). Synpolydactyly phenotypes correlate with size of expansions in HOXD13 polyalanine tract. *Proc. Natl. Acad. Sci. USA* **94**, 7458-7463.

Graba, Y., Aragnol, D. and Pradel, J. (1997). Drosophila *Hox* complex downstream targets and the function of homeotic genes. *BioEssays* **19**, 379-388.

Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. 1951. *Dev. Dyn.* **195**, 231-272.

Herault, Y., Fraudeau, N., Zakany, J. and Duboule, D. (1997). *Ulnaless* (*Ul*), a regulatory mutation inducing both loss-of-function and gain-of-function of posterior *Hoxd* genes. *Development* **124**, 3493-3500.

Knezevic, V., de Santo, R., Schughart, K., Huffstadt, U., Chiang, C., Mahon, K. A. and Mackem, S. (1997). *Hoxd-12* differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates *Sonic hedgehog* in a positive feedback loop. *Development* **124**, 4523-4536.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78**, 191-201.

Lu, M., Miller, C. and Habener, J. F. (1996). Functional regions of the homeodomain protein IDX-1 required for transactivation of the rat somatostatin gene. *Endocrinology* **137**, 2959-2967.

Morgan, B. A. and Tabin, C. (1994). *Hox* genes and growth: early and late roles in limb bud morphogenesis. *Develeopment Suppl.* 181-186.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol.* **51**, 185-218.

Morgan, B. A., Izpisua-Belmonte, J. C., Duboule, D. and Tabin, C. J. (1992). Targeted misexpression of *Hox 4.6* in the avian limb bud causes apparent homeotic transformations. *Nature* **358**, 236-239.

Muragaki, Y., Mundlos, S., Upton, J. and Olsen, B. R. (1996). Altered growth and branching patterns in synpolydactyly caused by mutations in *HOXD13*. *Science* **272**, 548-551.

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., Gonzales, E., Tessarollo, L., Parada, L. F. and Tabin, C. (1996). Analysis of *Hox* gene expression in the chick limb bud. *Development* **122**, 1449-1466.

Oldridge, M., Fortuna, A. M., Maringa, M., Propping, P., Mansour, S., Pollitt, C., DeChiara, T. M., Kimble, R. B., Valenzuela, D. M., Yancopoulos, G. D. and Wilkie, A. O. (2000). Dominant mutations in *ROR2*, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. *Nat. Genet.* **24**, 275-278.

Peichel, C. L., Prabhakaran, B. and Vogt, T. F. (1997). The mouse *Ulnaless* mutation deregulates posterior *HoxD* gene expression and alters appendicular patterning. *Development* **124**, 3481-3492.

Piper, D. E., Batchelor, A. H., Chang, C. P., Cleary, M. L. and Wolberger, C. (1999). Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. *Cell* **96**, 587-597.

Rijli, F. M. and Chambon, P. (1997). Genetic interactions of *Hox* genes in limb development: learning from compound mutants. *Curr Opin Genet Dev* **7**, 481-487.

Shen, W. F., Rozenfeld, S., Lawrence, H. J. and Largman, C. (1997). The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. *J. Biol. Chem.* **272**, 8198-8206.

Spitz, F., Montavon, T., Monso-Hinard, C., Morris, M., Ventruto, M. L., Antonarakis, S., Ventruto, V. and Duboule, D. (2002). A t(2;8) balanced translocation with breakpoints near the human *HOXD* complex causes mesomelic dysplasia and vertebral defects. *Genomics* **79**, 493-498.

Stadler, H. S., Higgins, K. M. and Capecchi, M. R. (2001). Loss of *Eph-receptor* expression correlates with loss of cell adhesion and chondrogenic capacity in *Hoxa13* mutant limbs. *Development* **128**, 4177-4188.

Stepchenko, A. G., Luchina, N. N. and Polanovsky, O. L. (1997). Conservative Val47 residue of POU homeodomain: role in DNA recognition. *FEBS Lett.* **412**, 5-8.

Sugawara, H., Egashira, M., Harada, N., Jakobs, T. C., Yoshiura, K., Kishino, T., Ohta, T., Niikawa, N. and Matsumoto, N. (2002). Breakpoint analysis of a familial balanced translocation t(2;8)(q31;p21) associated with mesomelic dysplasia. *J. Med. Genet.* **39**, E34.

Temtamy, S. A. and McKusick, V. A. (1978). Brachydactyly as an isolated malformation. In *The Genetics of Hand Malformations*, Vol. 14 (ed. D. Bergsma), pp. 187-225. New York: Alan R. Liss.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

van der Hoeven, F., Zakany, J. and Duboule, D. (1996). Gene transpositions in the *HoxD* complex reveal a hierarchy of regulatory controls. *Cell* **85**, 1025-1035.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Wilson, D., Sheng, G., Lecuit, T., Dostatni, N. and Desplan, C. (1993). Cooperative dimerization of paired class homeo domains on DNA. *Genes Dev.* **7**, 2120-2134.

Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H. and Kuroiwa, A. (1995). Misexpression of *Hoxa-13* induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev.* **9**, 2509-2522.

Zakany, J. and Duboule, D. (1996). Synpolydactyly in mice with a targeted deficiency in the *HoxD* complex. *Nature* **384**, 69-71.

Zakany, J. and Duboule, D. (1999). *Hox* genes in digit development and evolution. *Cell Tissue Res.* **296**, 19-25.

Zappavigna, V., Renucci, A., Izpisua-Belmonte, J. C., Urier, G., Peschle, C. and Duboule, D. (1991). *HOX4* genes encode transcription factors with potential auto- and cross-regulatory capacities. *EMBO J.* **10**, 4177-4187.

Zappavigna, V., Sartori, D. and Mavilio, F. (1994). Specificity of HOX protein function depends on DNA-protein and protein-protein interactions, both mediated by the homeo domain. *Genes Dev.* **8**, 732-744.

Zhao, Y. and Potter, S. S. (2001). Functional specificity of the Hoxa13 homeobox. *Development* **128**, 3197-207.