Journal of Anatomy

J. Anat. (2010) **216**, pp683–691 doi: 10.1111/j.1469-7580.2010.01233.x

Comparison of *Iroquois* gene expression in limbs/fins of vertebrate embryos

Laura A. McDonald,[1] Dianne Gerrelli,[2] Yvonne Fok,[2] Laurence D. Hurst[1] and Cheryll Tickle[1]

[1]*Department of Biology & Biochemistry, University of Bath, Somerset, UK*
[2]*Human Developmental Biology Resource, Neural Development Unit, UCL Institute of Child Health, London, UK*

Abstract

In *Drosophila*, *Iroquois* (*Irx*) genes have various functions including the specification of the identity of wing veins. Vertebrate *Iroquois* (*Irx*) genes have been reported to be expressed in the developing digits of mouse limbs. Here we carry out a phylogenetic analysis of vertebrate *Irx* genes and compare expression in developing limbs of mouse, chick and human embryos and in zebrafish pectoral fin buds. We confirm that the six *Irx* gene families in vertebrates are well defined and that Clusters A and B are duplicates; in contrast, *Irx1* and *3*, *Irx2* and *5*, and *Irx4* and *6* are paralogs. All *Irx* genes in mouse and chick are expressed in developing limbs. Detailed comparison of the expression patterns in mouse and chick shows that expression patterns of genes in the same cluster are generally similar but paralogous genes have different expression patterns. Mouse and chick *Irx1* are expressed in digit condensations, whereas mouse and chick *Irx6* are expressed interdigitally. The timing of *Irx1* expression in individual digits in mouse and chick is different. *Irx1* is also expressed in digit condensations in developing human limbs, thus showing conservation of expression of this gene in higher vertebrates. In zebrafish, *Irx* genes of all but six of the families are expressed in early stage pectoral fin buds but not at later stages, suggesting that these genes are not involved in patterning distal structures in zebrafish fins.

Key words chick limb; human embryo; *Iroquois*; mouse limb; zebrafish pectoral fin bud.

Introduction

Iroquois (Irx) genes encode homeodomain-containing proteins belonging to the TALE (three amino acid loop extension) family. In *Drosophila*, where *Irx* genes were first discovered, there is a cluster of three closely related genes, *Araucan*, *Caupolican* and *Mirror*. Among the several roles now uncovered for these *Drosophila* genes are specification of the dorsal-most region of the wing imaginal disc to form notum (Diez del Corral et al., 1999; Cavodeassi et al., 2001) and later specification of the longitudinal wing veins L1, L3 and L5 (Gomez-Skarmeta et al., 1996). Vertebrate *Irx* genes have been identified and shown to be expressed in developing digits in mouse limbs (Houweling et al., 2001). A number of other genes encoding transcription factors involved in vein specification in *Drosophila* wings are also expressed in vertebrate limbs.

In most vertebrates, an ancestral *Irx* gene cluster of three genes appears to have been duplicated (Kerner et al., 2009). Thus there are six *Irx* genes in mouse and human (*Irx1–6*) in two clusters, the *IrxA* cluster containing *Irx1, 2* and *4,* and the *IrxB* cluster *Irx3 5,* and *6* (Peters et al., 2000). *Xenopus* also has six *Irx* genes (De la Calle-Mustienes et al., 2005). Ogura et al. (2001) described five chick *Irx* genes, *Irx1, 2 and 4,* in cluster A and *Irx3 and 5* in cluster B, but recently a fragment of a sixth chicken *Iroquois* gene has been identified (Kerner et al., 2009). Eleven *Irx* genes have been found in the zebrafish genome, where they are organized into four clusters apart from one isolated gene, *zIrx7*. The four zebrafish clusters appear to have originated by duplication of ancestors of the two mammalian clusters and have therefore been named *IrxAa* (*Irx1a, 2a, 4a*), *IrxAb* (*Irx1b, 4b*), *IrxBa* (*Irx3a, 5a, 6a*) and *IrxBb* (*Irx3b, 5b*) (Dildrop & Ruther, 2004; Feijoo et al., 2004; Lecaudey et al., 2005).

In developing mouse limbs, Zulch et al. (2001) have shown that *mIrx1* is expressed in the condensations of digits 2, 3 and 4 (the middle three digits of the paw) first, then *mIrx2* is later expressed strongly in digits 1 and 2 (the most anterior and most posterior digits, respectively) and digits 2, 3 and 4 express both *mIrx1* and *Irx2.* The expression of *Irx* genes in developing chick digits has not been described. In the chick wing and leg, three and four digits respectively develop, but their identity in relation to the pentadactyl limb plan is still debated (Vargas & Fallon, 2005; Xu et al., 2009). Therefore examination of *Irx* expression in the chick might not only reveal whether expression of these genes is

Correspondence
Cheryll Tickle, Department of Biology & Biochemistry, University of Bath, Somerset, BA2 7AY, UK. E: cat24@bath.ac.uk

Accepted for publication *5 March 2010*
Article published online *14 April 2010*

conserved in digit development in different vertebrates but also cast light on digit identity. We found that *Irx1* becomes expressed in all the digit condensations in both mouse and chick limbs, although the timing of expression differs in individual digits. We also examined *Irx1* expression in human limbs to see whether the pattern of expression of this gene is further conserved among higher vertebrates. We found in zebrafish that *Irx* genes are expressed in early pectoral fin buds but, unlike mouse, chick and human limbs are not expressed at later stages when distal structures are developing.

Materials and methods

Mouse embryos

Pregnant CD1 mice were obtained from either the Wellcome Trust Resource Centre (University of Dundee) or the Resource Centre (University of Bath). Embryos of different developmental stages (E11.5–E14.5) were dissected from amniotic sacs and all membranes were removed before fixing in 4% paraformaldehyde overnight at 4 °C. Embryos were then dehydrated, stored in 100% methanol at −20 °C and processed for *in situ* hybridization.

Chick embryos

Fertilized White Leghorn chicken eggs were obtained from Henry Stewart (Lincolnshire, UK). Eggs were incubated on their sides at 38 °C in a humidified incubator for various lengths of time until embryos had reached the required developmental stage (Hamburger & Hamilton, 1992). Embryos were dissected from eggs and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, then gradually dehydrated through a methanol series. Embryos were stored at −20 °C for periods of up to several months and then processed for *in situ* hybridization.

Human embryonic tissue

The Human Developmental Biology Resource (HDBR) is a collection of human embryonic and fetal material ranging from 4 to 12 weeks of development; tissue from this resource was used to study expression of our gene of interest (*Irx1*) in a project registered with the In-House Gene Expression Service at the Institute of Child Health (ICH), London. Human embryonic limb tissue from the HDBR was processed for *in situ* hybridization at the ICH. Limbs from Carnegie Stage 18 (CS18) and CS19 embryos were used for section *in situ* hybridization to detect *hIrx1* (IMAGE clone 1706829). Digital images of *Irx1* gene expression patterns produced from the study were provided by the HDBR.

Zebrafish embryos

Danio rerio (zebrafish) embryos were randomly bred naturally, and fertilized eggs were allowed to develop at 28 °C. Some embryos were treated with 1-phenyl-2-thiourea (PTU) to inhibit pigmentation and allow *in situ* patterns to be seen more clearly. Zebrafish embryos were staged according to Kimmel et al. (1995), and embryos at 30 h post fertilization (hpf), 36 or 48 hpf were then fixed in 4% PFA. Embryos were fixed overnight at 4 °C, dehydrated, stored in 100% methanol at −20 °C and then processed for *in situ* hybridization.

Whole mount *in situ* hybridization

Plasmid DNA for the synthesis of RNA probes for *in situ* hybridization were obtained as follows: cIrx1, cIrx2, cIrx3 (kind gift from Dr A. Munsterberg), cIrx4 (chEST360a5), cIrx5 (chEST413l15), mIrx1-6 (IMAGE clones 2615958, 5347048, 1255751, 40089986, 3469768, 4512181, respectively), zIrx1a-7 (kind gift from Dr S. Schnieder-Maunoury), hIrx1 (IMAGE clone 1706829). All plasmids used for *in situ* hybridization were sequenced using the DNA sequencing service at the Wellcome Trust Biocentre (University of Dundee). Whole mount *in situ* hybridization of chick, mouse and zebrafish embryos was performed as previously described (Wilkinson & Nieto, 1993; Lecaudey et al., 2004).

Section *in situ* hybridization

Limbs were isolated from HH27 and HH28 chicks and E12.5 and E13.5 mice which had been stored in 100% MeOH. The limbs were embedded in paraffin wax. Sections were cut at a thickness of 14 μm and mounted onto aminoalkylsilane-coated slides. Sections were de-waxed by baking the slides at 60 °C for 1 h and then allowing them to cool to room temperature. *In situ* hybridization was then carried out on sections as previously described (Moorman et al., 2001).

Photography

A dissection microscope with a Jenoptic C14 digital camera (Laser Optic System) was used to take photographs of whole mount specimens using OPENLAB software. *In situ* hybridization specimens were photographed in phosphate-buffered saline on 1% agarose plates using *Drosophila* pins to orientate embryos into the correct positions. Sections were photographed using a compound Leica DMR microscope with a Nikon D1X digital camera.

Phylogenetic analysis

Accession numbers of sequences belonging to the *Iroquois* gene families were extracted from Hovergen (Duret et al., 1994). Four families were employed: HBG006181, HBG006180, HBG073961 and HBG093209. These were supplemented with sequences identified as putative orthologs within the Hologene framework. Complete sequences were employed where possible. For a list of genes and accession numbers see Table S1. Translations of all sequences were extracted. Alignment was performed on the translated sequences using M-COFFEE (Wallace et al., 2006; Moretti et al., 2007).

Results

Phylogeny of vertebrate *Iroquois* genes

To study the phylogeny of vertebrate *Irx* genes including those from chick, mouse, human and zebrafish, accession numbers of sequences belonging to the *Irx* gene families

were extracted from Hovergen (a database of homologous vertebrate genes). These were supplemented with sequences identified as putative orthologues. Complete sequences were used where possible, and alignment was performed using M-COFFEE software.

Phylogeny was constructed by two methods. First, phylogeny was constructed using PAUP* beta 4 (Swofford, 2003). A distance metric was employed with the quartet puzzling method. Hundred replicates were performed to generate support values. This tree is shown in Fig. S1. The second method, a Bayesian approach (Huelsenbeck & Ronquist, 2001), employed MRBAYES (v 3.1.2) to perform two parallel runs of one million searches. A burn in of 5000 of the 10 000 resolved trees was employed to determine the 50% majority consensus rule unrooted tree. This and appropriate support values are shown in Fig. 1.

The main point of the phylogeny reconstruction was to ensure that genes were assigned correctly to their ortholog groups. Overall, the six *Irx* gene families define themselves very well, with both reconstruction methods agreeing. For example, the *Irx1* gene family is well defined with all genes in expected phylogenetic locations. A few genes appear to have been mis-named: chicken *Irx5* (XM_001234058) should be *Irx6*, human *Irx2a* (U90304) is a variety of *Irx5,* as is the sequence identified as a possible chicken *Irx2* [*Irx2p, p* for possible, (XM_001234100]; Figs 1, S1). We will refer to the previously named c*Irx5* as c*Irx6* in the following description. A complete sequence with a properly annotated protein for chicken *Irx5* was not found in the Hovergen database. The tree nicely shows the paralogous *Iroquois* genes in each cluster; *Irx1*and *Irx3*, *Irx2* and *Irx5* and *Irx4* and *Irx6* in clusters A and B, respectively (see also Kerner et al., 2009). The

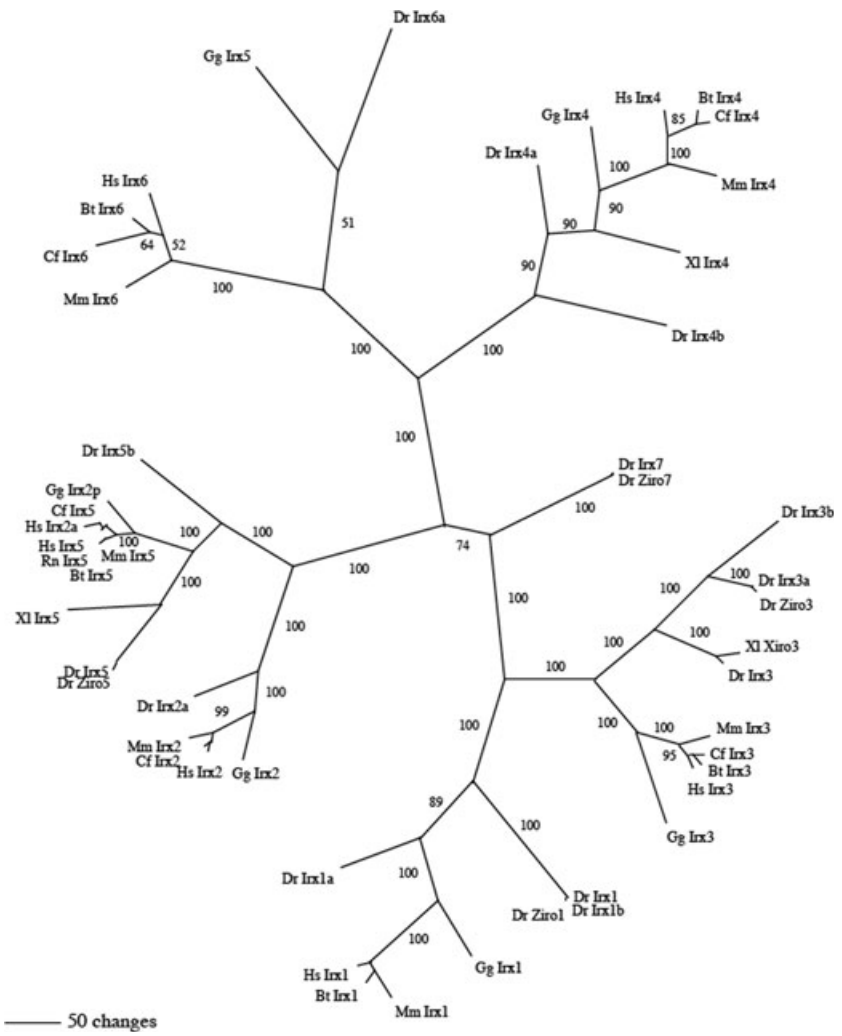

Fig. 1 An unrooted Bayesian phylogram of *Iroquois* genes. Numbers indicate support values. Note that each of the six *Irx* genes form well defined groupings, e.g. *Irx1* gene family is well defined with all genes in expected phylogenetic locations. Chick *Irx5* is misidentified, as it belongs to *Irx6* groupings. Species are as follows: Hs, *Homo sapiens*; Mm, *Mus musculus*; Bt, *Bos taurus*; Cf, *Canis familiaris*; Gg, *Gallus gallus*; Dr, *Danio rerio*; Xl, *Xenopus laevis*; Rn, *Rattus norvegicus.*

branch lengths from the root to each duplication are about the same, suggesting that the ancestral set of three genes all duplicated at one time.

Mouse limb *Iroquois* expression

Expression patterns of *mIrx1-6* were studied in the limbs of mouse embryos from E11.5 to E14.5. *mIrx1* and *mIrx2* have similar patterns of expression in limbs throughout development (although *mIrx2* expression is much weaker than *mIrx1*), consistent with previous reports (Houweling et al., 2001; Zulch et al., 2001). At E11.5, *mIrx1* and *mIrx2* are expressed in the proximal regions of the forelimb and hindlimb (Fig. 2A,E). At E12.5, expression is stronger in the developing digit condensations. Expression of both genes is seen first and is strongest in digits 2–4, with weaker expression in digits 1 and 5 (Fig. 2B,F). At later stages of digit development (E13.5), expression of *mIrx1* and *mIrx2* is stronger in the joint-forming regions of digits 2, 3 and 4 (Fig. 2C,G). By E14.5, expression is seen in joint-forming regions in all digits in both forelimb and hindlimb (Fig. 2D,H).

Like *mIrx1* and *mIrx2*, *mIrx6* is also expressed proximally during early stages of limb development (Fig. 2U), whereas *mIrx3*, *4* and *5* appear to be expressed distally at E11.5 (Fig. 2I–J, M–N and Q–R, respectively). *mIrx3* and *mIrx4* show similar expression patterns at later stages of digit formation (E13.5 and E14.5). *mIrx3* is expressed distally in interdigital spaces and then around the tips of the digits (Fig. 2K,L), and *mIrx4* shows a similar pattern (Fig. 2O,P). *mIrx5* and *mIrx6* are both expressed in the proximal interdigital spaces of the forelimb and hindlimb at late stages of limb development (Fig. 2T; W–X, respectively).

Chick limb *Iroquois* expression

In situ hybridization was carried out to determine the expression pattern of the five chicken *Irx* genes described

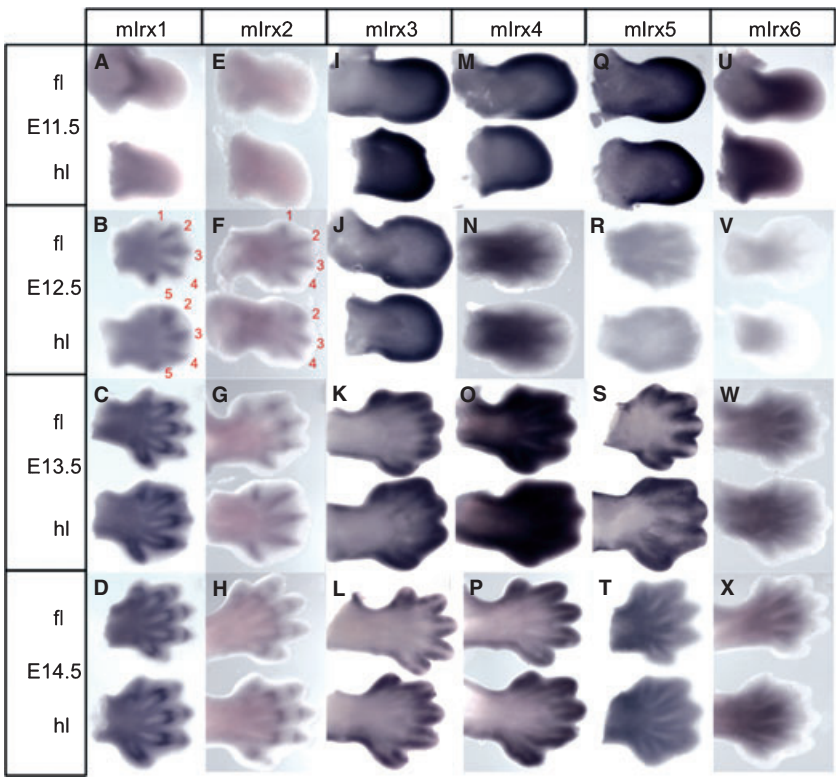

Fig. 2 *mIrx1-6* expression in E11.5–E14.5 mouse limbs. *mIrx1* and *mIrx2* have the same pattern of expression in limbs throughout development, although *mIrx2* expression is much weaker. (A,E) *mIrx1* and *mIrx2* are proximally expressed in forelimb and hindlimb at E11.5. (B,F) Expression of *mIrx1* and *mIrx2* is restricted to condensing digits at E12.5, with stronger expression in digits 2–4. (C,G,D,H) *mIrx1* and *mIrx2* are expressed in joint-forming regions at E13.5 and E14.5. (I,J) *mIrx3* is expressed at the distal edge of both forelimb and hindlimb at E11.5 and E12.5. (K,L) Strong expression of *mIrx3* at distal edges of digits at E13.5 and E14.5 and also interdigitally at E13.5. (M) *mIrx4* distally expressed at E11.5. (N) At E12.5, *mIrx4* expression is reduced; expression does not reach the tip and appears to be weakly expressed in proximal interdigital regions. (O) *mIrx4* strong in interdigital regions at E13.5, and then at the distal edges of the digits at E14.5 (P). (Q) *mIrx5* stronger in distal limb regions at E11.5. (R) At E12.5, *mIrx5* expression very weak, but faint expression can be seen in forelimb interdigits. (S) *mIrx5* restricted to interdigital regions at E13.5, with stronger expression around digit tips in the forelimb. (T) At E14.5, *mIrx5* expressed between and around the edges of the digits. (U) *mIrx6* expressed in the middle of forelimb and proximally in hindlimb at E11.5. (V) Weak *mIrx6* expression seen at E12.5 in region just proximal to digital plate. (W,X) *mIrx6* expression in proximal interdigital regions at E13.5 and E14.5.

by Ogura et al. (2001), which according to our phylogenetic analysis are *clrx1, clrx2, clrx3, clrx4* and *clrx6.* Limbs at stages of development HH24^{-}29^{+} were studied.

The gene with the strongest, most specific expression in the digit-forming region was found to be *clrx1*. *clrx1* is expressed at low levels in the proximal region of both wing and leg at HH24 (Fig. 3A). By HH25/26, weak proximal expression remains, but this is now accompanied by a strong, posterior distal domain of expression in the leg (Fig. 3B,C arrowed). By HH27, *clrx1* is also detected in the posterior region of the hand plate in the wing (Fig. 3D). As the limbs develop, *clrx1* expression spreads across the digital plate and comes to be expressed in two distinct spots in the wing and three in the leg; the spots appearing to correspond to developing digit condensations (Fig. 3E). These spots of expression then extend across the limb anteriorly, with expression later becoming stronger and more restricted to the developing digits (Fig. 3F). At later stages of development, *clrx1* expression is reduced posteriorly and becomes restricted to anterior digits, particularly digits 2 and 3 in both the wing and leg (Fig. 3G,H). It should be noted that *clrx1* expression never extends right up to the apical ectodermal ridge, the thickened epithelium at the tip of the wing bud.

clrx2 and *clrx3* are both expressed distally at HH24-25 in both wing and leg buds (Fig. 3I,J; Fig. 3O,P, respectively) but there is no detectable expression of *clrx4* and *clrx6* (Fig. 3U–W; Fig. 3a–c, respectively). *clrx2*, *clrx3*, *clrx4* and *clrx6* are then all expressed distally from HH26/27 onwards. *clrx2* is highly expressed throughout the digital plate (Fig. 3K–N), whilst *clrx3* expression is strongest around the distal rim of the limbs (Fig. 3Q–T). *clrx4* is also strongly expressed distally during later digit-forming stages (Fig. 3X–Z). *clrx6* can be seen in anterior and posterior proximal regions in the wing, and in posterior interdigital areas in the leg at HH27 (Fig. 3d). By HH28, *clrx6* expression is clearly visible in the interdigital spaces, with expression stronger in the interdigital spaces of the leg (Fig. 3e arrows).

Chick, mouse and human Irx1 section *in situ*

As our expression studies showed *Irx1* to be strongly expressed in the digit condensations of both chick and mouse embryos, we examined expression of this gene in

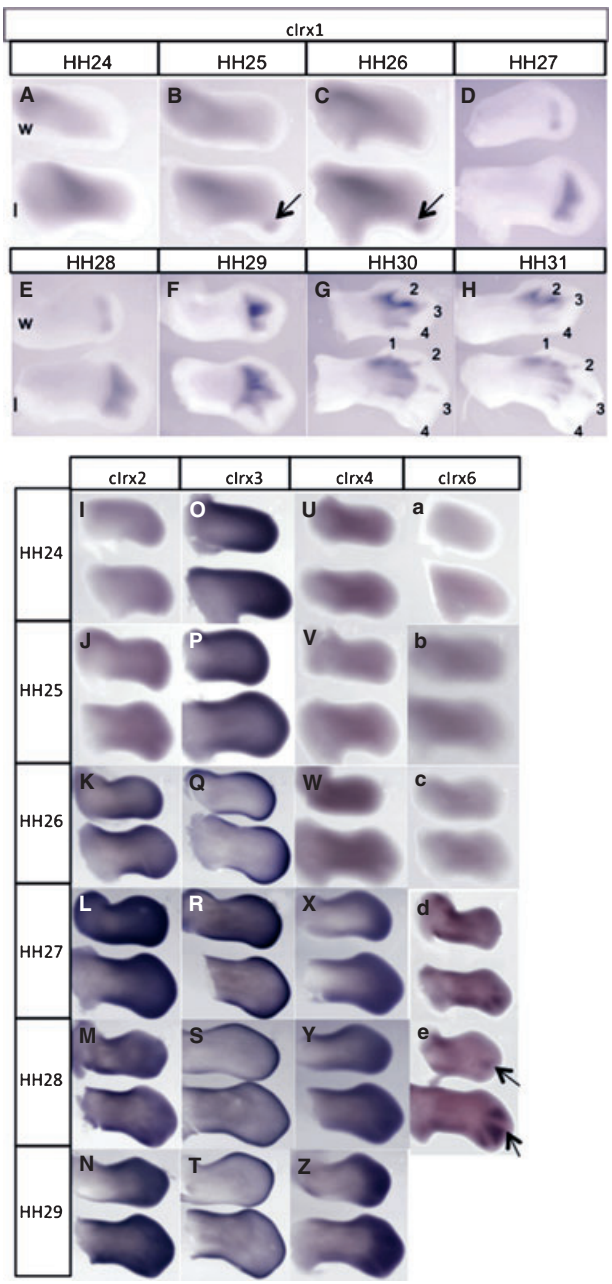

Fig. 3 *clrx1-6* expression in chick limbs. (A–C) *clrx1* expressed proximally in HH24–26 limbs, with distal expression initiated posteriorly in hindlimbs at HH25. (D) *clrx1* is initiated in distal wing at HH27 posteriorly, whilst *lrx1* in leg has extended anteriorly at this stage to become expressed in two spots. (E,F) *clrx1* expression extends anteriorly in wings and legs, becoming more strongly expressed in developing digits at HH29. (G,H) At late stages of digit development (HH30/31), expression of *clrx1* is restricted to anterior digits. (I,J) *clrx2* is expressed distally in both wing and leg at HH24/25, with high expression developing at anterior and posterior edges of the limbs between HH26 and 28 (K–M). (N) *clrx2* is strongly expressed in distal digit-forming regions at HH29. (O) *clrx3* is highly expressed in distal limb at early stages before becoming restricted to the outside edges of the limbs at HH25 (P). (Q–T) From HH26 to 29, *clrx3* expression is confined to the distal rim of wing and leg. (U–W) *clrx4* expression is not detected in limbs from HH24 to 26. (X,Y) Expression of *clrx4* is detected in the distal limb at HH27 and HH28. (Z) At HH29, strong expression of *clrx4* is seen distally in both wing and leg. (a–c) Like *clrx4*, *clrx6* expression is not detected in early (HH24–26) limb buds. (d) At HH27, anterior and posterior regions of expression in wing and in posterior interdigital regions in leg. (e) *clrx6* strongly expressed in leg interdigital regions, with weaker expression in wing interdigital regions at HH28.

more detail by carrying out section *in situ* hybridization. We also compared *Irx1* expression in sections of human embryonic limbs to investigate whether the pattern of expression of this gene is conserved amongst higher vertebrates. Comparable chick and mouse developmental stages were chosen (Martin, 1990). The appropriate human developmental stages were chosen with reference to the University of New South Wales human embryology website (http://embryology.med.unsw.edu.au).

In the chick at HH27, *clrx1* is expressed in the posterior digit condensation in the wing and restricted to posterior digit-condensations in the leg (Fig. 4A). This expression in condensing cells at early digit-forming stages is consistent with patterns shown in *in situ* whole mounts for *clrx1,* and confirms that the spots of *clrx1* expression correspond to the developing digits. At HH28, *in situ* sections show that *clrx1* is expressed more strongly around the edges of the digit condensations and in presumptive joint regions but more weakly in the centre of the condensations, details not apparent from the whole mount *in situ* (Fig. 4B).

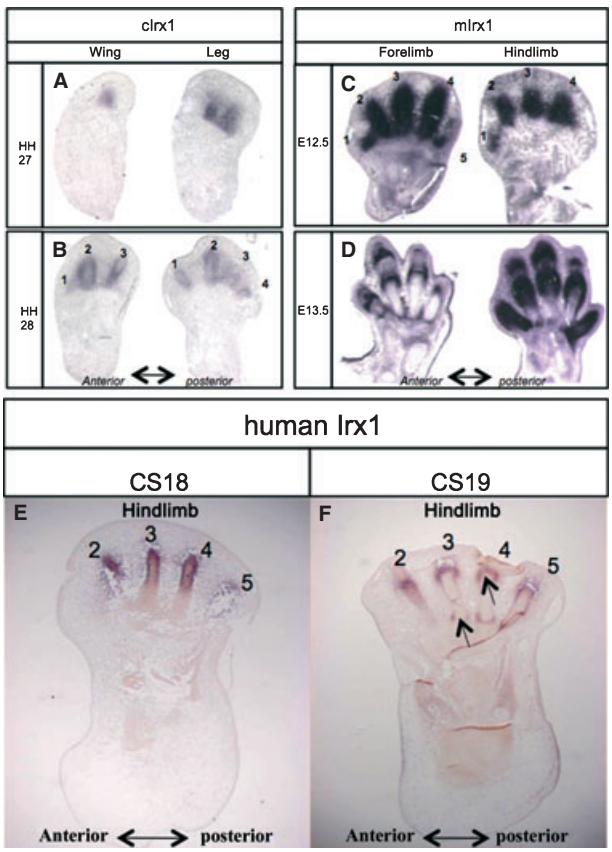

Fig. 4 Section *in situ* hybridization of *lrx1* in chick, mouse and human limbs. (A) At HH27, *clrx1* is expressed in the posterior digit condensation(s) in wing and leg. (B) At HH28, *clrx1* is not expressed throughout the cartilage digit rudiments but is expressed at the edges in both wing and leg and in developing joints. (C) *mlrx1* strongly expressed in all digit condensations of forelimb and hindlimb at E12.5. (D) At E13.5, expression in hindlimb throughout the skeleton of digits 1 and 5 and in joint-forming regions of digits 2, 3 and 4; in the more developmentally advanced forelimb, expression in joint-forming regions. Absence of digit 5 in hindlimb at E12.5 and digit 5 in forelimb at E13.5 is due to plane of section; additional serial sections showed *lrx1* expression in these digits. (E) *hlrx1* expressed in digits 2–5 of the hindlimb at CS18. (F) At CS19, expression in joint-forming regions of digits (arrows). Absence of digit 1 in E and F due to plane of section; additional serial sections showed expression in digit 1.

In E12.5 mouse limbs, very strong *mlrx1* expression was seen in all developing digits of the forelimb and hindlimb (Fig. 4C and data from serial sections). At E13.5, expression is confined to the joint areas in all the digits in the section of the forelimb (Fig. 4D), whereas in the hindlimb, strong expression is seen throughout digits 1 and 5, and in the joint-forming regions of digits 2, 3 and 4, consistent with the patterns of expression observed in *in situ* whole mounts.

In CS18 human embryos, *hlrx1* is expressed in all the condensations of the toes in the hindlimb (Fig. 4E and data from serial sections). At CS19, *hlrx1* expression was clearly seen in the joint-forming regions of the digits (Fig. 4F; toe 1 is not present due to the plane of section). These *lrx1* expression patterns are almost identical to those seen in mouse limbs of equivalent stages (compare with Fig. 4C,D), showing that expression of this gene in the tissues of developing digits is highly conserved.

Zebrafish pectoral fin *Iroquois* expression

In situ hybridization was carried out to examine the expression of the 11 known zebrafish *Iroquois* genes in the pectoral fin buds of zebrafish embryos at different stages of development (30, 36 and 48 hpf). At 30 hpf, no *Iroquois* expression was detected in the pectoral fin buds (Fig. 5A–K). All probes were known to be working due to the strong expression of *Irx* genes seen in developing head and brain regions of embryos as previously described (Lecaudey et al., 2005). At 36 hpf, six of 11 genes were shown to be expressed in the pectoral fin buds. *zlrx1a* is faintly expressed throughout the pectoral fin, as shown in Fig. 5A′. *zlrx2a* shows clear expression throughout the pectoral fins (Fig. 5C′), and *zlrx3a* and *zlrx4a* are also expressed throughout the pectoral fins (Fig. 5D′, F′ respectively). *zlrx5a* shows the strongest expression in the pectoral fins (Fig. 5H′). Fig. 5I′′ shows that *zlrx5b* is also expressed in the pectoral fins. At 48 hpf, none of these genes was expressed in the pectoral fins, with the exception of *zlrx5a*, which is expressed very faintly (Fig. 5H′′).

Discussion

Phylogenetic analysis of vertebrate *Irx* genes, including those from chick, mouse, human and zebrafish showed that the six *Irx* gene families define themselves very well with all genes in expected phylogenetic locations. Chick *Irx5* has been previously misidentified, as it belongs to the *Irx6*

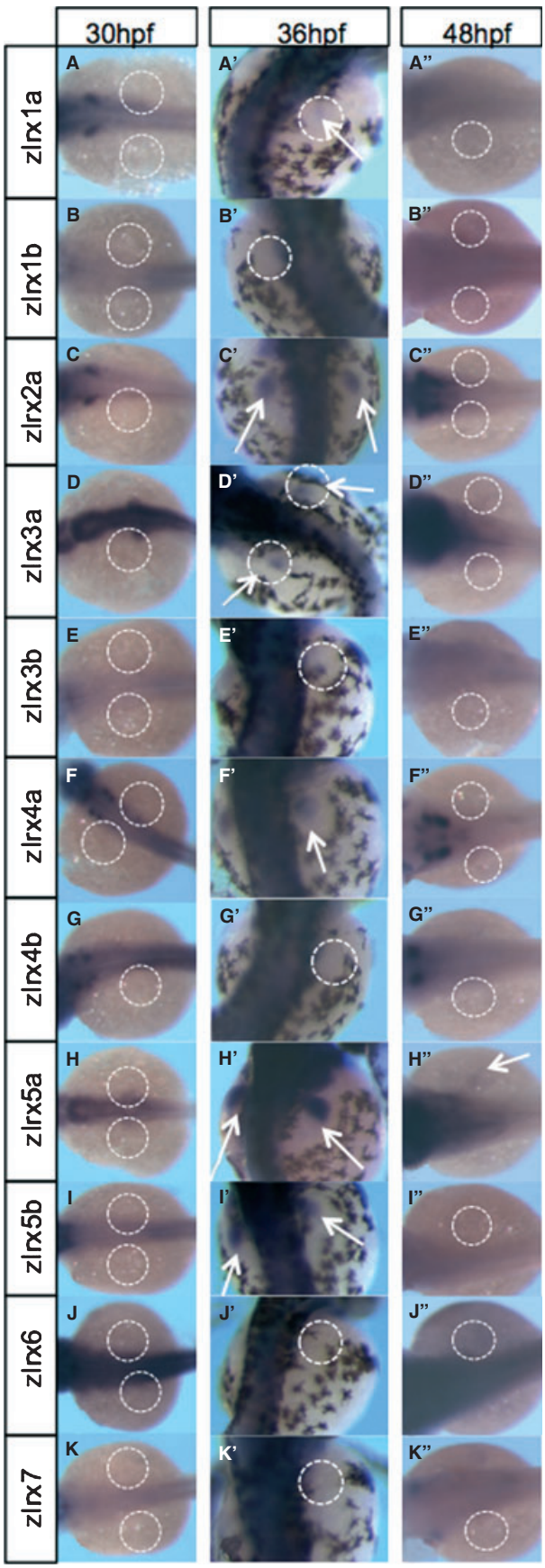

Fig. 5 Zebrafish *Iroquois* pectoral fin bud expression at 30, 36 and 48 hpf. Position of pectoral fin buds indicated by white-dashed circles; arrows indicate expression in pectoral fins. At 36 hpf, expression of six of 11 *zlrx* genes detected in pectoral fins. (A') *zlrx1a* faintly expressed in pectoral fins at 36 hpf. (C',D',F', respectively) *zlrx2a*, *zlrx3a* and *zlrx4a* expressed throughout developing pectoral fins (white arrows). (H') *zlrx5a* displays strongest pectoral fin expression (arrows in H'). (I') *zlrx5b* also expressed in pectoral fins, albeit with a weaker expression than *zlrx5a*. (H'') By 48 hpf, only *zlrx5a* shows pectoral fin expression (weak expression, arrow in H''). The 10 remaining *zlrx* genes are not expressed in pectoral fins at 48 hpf (A''–G'',I''–K'').

grouping; we used the name *clrx6* to refer to this gene. Our analysis also clearly showed that *lrx1 and 3, lrx2* and *5,* and *lrx4* and *6* are the nearest paralogs, confirming the results of Kerner et al. (2009).

We compared *lrx* expression patterns in the forming digits of mouse and chick limbs (Table 1). This comparison shows that genes in the same cluster are generally expressed in more similar patterns than are the paralogs (Table 1). In the mouse, for example, there are particularly striking similarities in expression between the Cluster A genes *mlrx1* and *mlrx2,* which are first expressed in digit condensations and then later very strongly in developing joints. But in contrast, for example, the expression of the paralogous B cluster gene to *mlr2, lrx5*, is at the edges of the digits and located interdigitally. In the chick, the similarity of the expression patterns of genes in the same clusters is not as striking. For example, the two cluster A genes, *clrx2* and *clrx4*, appear to be expressed in a broad distal region of the limb buds, whereas the cluster B genes, *clrx3* and *clrx6*, have very different expression patterns, with *clrx6* having marked interdigital expression. Furthermore there are no similarities in the expression patterns of paralogous chick genes. It therefore appears that duplication of the *lrx* cluster in vertebrates has led to a divergence in expression in the distal part of the limb which may then have allowed functional specialization.

Comparison of expression patterns of corresponding genes in mouse and chick revealed some similarities. Our results generally confirmed reported patterns of *lrx* gene expression in mouse limbs (Houweling et al., 2001; Mummenhoff et al., 2001; Zulch et al., 2001), except that we observed *lrx4* expression in the digit-forming region of the limb which had not previously been detected (Houweling et al., 2001). Cluster A genes, *mlrx1* and *mlrx2*, were expressed in digit condensations and Cluster B genes, *mlrx5* and *mlrx6*, interdigitally. Similarly, in the chick we found that *clrx1, 2, 3, 4* and *6* are all expressed in the digit-forming region of wing and leg buds. Also similar to the expression patterns in the mouse limb, *clrx1* is strongly expressed in digit condensations, whereas *clrx6* is expressed interdigitally.

A previous report on *lrx1* and *lrx2* gene expression in mouse limbs highlighted a difference in the timing of expression in different digits, with *mlrx1* being expressed

Table 1 Summary of expression of *Irx* genes in the developing digits of mouse and chick embryos.

	Cluster A			Cluster B		
	Irx1	*Irx2*	*Irx4*	*Irx3*	*Irx5*	*Irx6*
Mouse E12.5	Digit condensations	Digit condensations	Interdigital	Distal	Weak interdigital	Weak proximal
Mouse E13.5	Joints	Joints	Stronger interdigital	Distal edges of digits Interdigital	Edges of digits Interdigital	Interdigital
Mouse E14.5	Joints	Joints	Distal edges of digits	Distal edges of digits	Edges of digits Interdigital	Interdigital
Chick HH27	Digit condensations	Broad distal	Broad distal	Distal rim	N/A	Interdigital (leg)
Chick HH28	Digit condensations Joints	Broad distal	Broad distal	Distal rim	N/A	Interdigital (wing and leg)

more strongly initially in the middle digits 2, 3 and 4 and *mIrx2* more strongly initially in digits 1 (anterior) and 5 (posterior) (Zulch et al., 2001). However, in our analysis, *mIrx2* also appeared to be expressed first in the middle digits 2, 3 and 4. In the chick, the timing of *cIrx1* expression in the condensations of the digits in both the wing and leg is very different to that in the mouse and appears in sequence from posterior to anterior. *cIrx2* is broadly expressed in the distal region of the limb. In mouse, it has been suggested based on Alcian Green staining that the middle three digits form first (Martin, 1990; Zhu et al., 2008), whereas in the chick the condensations appear in a posterior to anterior sequence. Therefore our observations suggest that the timing of expression of *Irx* genes in both mouse and chick limbs is likely related to the order in which the digits form rather than to the identity of a particular digit.

We found that the pattern of *Irx1* expression is conserved in the tissues of developing digits of chick, mouse and human. In all cases, *Irx1* is expressed in digit condensations at equivalent stages, then, later, expression is reduced in the centre of the skeletal elements, remaining around the edges and also in the joints. Expression of *Irx1* in the joints is similar to genes such as *Gdf5* and *Wnt14* (Storm & Kingsley, 1999; Guo et al., 2004), although these genes are not initially expressed throughout the digit condensations. The similarity between mouse *Irx1* and human *Irx1* is striking. There is evidence that there are differences and similarities in the expression of other important developmental genes (such as *Wnt7a, Calpain3, Lhx3/4, Wnt8b*) in humans compared with model organisms such as the mouse (Lako et al., 1998; Fougerousse et al., 2000; Sobrier et al., 2004). It remains to be investigated whether the other human *Irx* genes are expressed in the same patterns as in the mouse.

The patterns of *Irx* gene expression in zebrafish pectoral fin buds differ from those in tetrapod limb buds. In zebrafish, representatives of all the *Irxa* genes (except *Irx6a*) together with *Irx5b* are expressed in early pectoral fin buds (36 hpf), but *Irx* gene expression is generally undetectable in later fin buds (48 hpf). This absence of *Irx* gene expression in later pectoral fin buds contrasts with the strong expression of most of the *Irx* genes in the distal regions of chick and mouse limb buds at comparable stages; chick stage 26 and mouse E11.5. These observations are intriguing in the context of previous work which compared *Hox* gene expression in zebrafish pectoral fin buds and tetrapod limb buds. Three stages of *Hox* gene expression have been recognized in tetrapod limbs with stage III associated with digit development (Nelson et al., 1996). Studies in zebrafish have shown that although similar patterns of phases II and III *Hox* gene expression can be recognized, the third phase (from 36 hpf onwards) may be absent (Sordino et al., 1995) or be somewhat different (Ahn & Ho, 2008). The fact that *Irx* genes are not detectably expressed in fin buds after 36 hpf is strikingly different to what is seen in chick and mouse limbs, in which *Irx* genes are expressed during phase III *Hox* gene expression. It remains to be seen whether these patterns of *Irx* genes are truly characteristic of fish fins or just a peculiarity of zebrafish.

Acknowledgements

The authors would like to thank Robert Kelsh for providing some of the zebrafish embryos, Dr S. Schneider-Maunoury for providing template DNA for zebrafish *Irx* genes and Matthew Towers for help with the images. Human embryonic and fetal material was provided by the MRC/Wellcome-funded Human Developmental Biology Resource. L.M. was sponsored by an ASGBI studentship, performed most of the experimental work and helped write the manuscript. C.T. is a Royal Society Professor and helped write the manuscript L.D.H. is a Royal Society Wolfson Research Merit Award holder and performed the bioinformatic analysis. D.G. and Y.F. carried out the work on the human material.

References

Ahn D, Ho RK (2008) Tri-phasic expression of posterior Hox genes during development of pectoral fins in zebrafish: implications for the evolution of vertebrate paired appendages. *Dev Biol* **322**, 220–233.

Cavodeassi F, Modolell J, Gomez-Skarmeta JL (2001) The Iroquois family of genes: from body building to neural patterning. *Development* **128**, 2847–2855.

De la Calle-Mustienes E, et al. (2005) A functional survey of the enhancer activity of conserved non-coding sequences from vertebrate Iroquois cluster gene deserts. *Genome Res* **15**, 1061–1072.

Diez del Corral R, et al. (1999) The Iroquois homeodomain proteins are required to specify body wall identity in Drosophila. *Genes Dev* **13**, 1754–1761.

Dildrop R, Ruther U (2004) Organization of Iroquois genes in fish. *Dev Genes Evol* **214**, 267–276.

Duret L, et al. (1994) HOVERGEN – a database of homologous vertebrate genes. *Nucleic Acids Res* **22**, 2360–2365.

Feijoo CG, et al. (2004) The Irx gene family in zebrafish: genomic structure, evolution and initial characterization of irx5b. *Dev Genes Evol* **214**, 277–284.

Fougerousse F, et al. (2000) Human-mouse differences in the embryonic expression patterns of developmental control genes and disease genes. *Hum Mol Genet* **9**, 165–173.

Gomez-Skarmeta JL, et al. (1996) Araucan and caupolican, two members of the novel iroquois complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85**, 95–105.

Guo X, et al. (2004) Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev* **18**, 2404–2417.

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

Houweling AC, et al. (2001) Gene and cluster-specific expression of the Iroquois family members during mouse development. *Mech Dev* **107**, 169–174.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Kerner P, et al. (2009) Evolutionary history of the iroquois/Irx genes in metazoans. *BMC Evol Biol* **9**, 74.

Kimmel CB, et al. (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253–310.

Lako M, et al. (1998) A novel mammalian wnt gene, WNT8B, shows brain-restricted expression in early development, with sharply delimited expression boundaries in the developing forebrain. *Hum Mol Genet* **7**, 813–822.

Lecaudey V, et al. (2004) The zebrafish Iroquois gene iro7 positions the r4/r5 boundary and controls neurogenesis in the rostral hindbrain. *Development* **131**, 3121–3131.

Lecaudey V, et al. (2005) Expression of the zebrafish Iroquois genes during early nervous system formation and patterning. *J Comp Neurol* **492**, 289–302.

Martin P (1990) Tissue patterning in the developing mouse limb. *Int J Dev Biol* **34**, 323–336.

Moorman AF, et al. (2001) Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount *in situ* hybridization protocol. *J Histochem Cytochem* **49**, 1–8.

Moretti S, et al. (2007) The M-Coffee web server: a meta-method for computing multiple sequence alignments by combining alternative alignment methods. *Nucleic Acids Res* **35**, W645–W648.

Mummenhoff J, et al. (2001) Expression of Irx6 during mouse morphogenesis. *Mech Dev* **103**, 193–195.

Nelson CE, et al. (1996) Analysis of Hox gene expression in the chick limb bud. *Development* **122**, 1449–1466.

Ogura K, et al. (2001) Cloning and chromosome mapping of human and chicken Iroquois (IRX) genes. *Cytogenet Cell Genet* **92**, 320–325.

Peters T, et al. (2000) Organization of mouse Iroquois homeobox genes in two clusters suggests a conserved regulation and function in vertebrate development. *Genome Res* **10**, 1453–1462.

Sobrier ML, et al. (2004) Pathophysiology of syndromic combined pituitary hormone deficiency due to a LHX3 defect in light of LHX3 and LHX4 expression during early human development. *Gene Expr Patterns* **5**, 279–284.

Sordino P, et al. (1995) Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature* **375**, 678–681.

Storm EE, Kingsley DM (1999) GDF5 coordinates bone and joint formation during digit development. *Dev Biol* **209**, 11–27.

Swofford DL 2003. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland, MA: Sinauer Associates.

Vargas AO, Fallon JF (2005) The digits of the wing of birds are 1, 2, and 3. A review. *J Exp Zool B Mol Dev Evol* **304**, 206–219.

Wallace IM, et al. (2006) M-Coffee: combining multiple sequence alignment methods with T-Coffee. *Nucleic Acids Res* **34**, 1692–1699.

Wilkinson DG, Nieto MA (1993) Detection of messenger RNA by *in situ* hybridization to tissue sections and whole mounts. *Methods Enzymol* **225**, 361–373.

Xu X, et al. (2009) A Jurassic ceratosaur from China helps clarify avian digital homologies. *Nature* **459**, 940–944.

Zhu J, et al. (2008) Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev Cell* **14**, 624–632.

Zulch A, et al. (2001) Expression pattern of Irx1 and Irx2 during mouse digit development. *Mech Dev* **106**, 159–162.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. An unrooted distance-based phylogram of *Irx* genes. Numbers indicate quartet puzzling support values.

Fig. S2. *mIrx1-6* expression in E11.5–E14.5 mouse limbs. These panels show unmanipulated images for comparison with images in Fig. 2.

Fig. S3. *cIrx1-6* expression in chick limbs. These panels show unmanipulated images for comparison with images in Fig. 3.

Table S1. List of genes and accession numbers used in construction of unrooted phylogram of vertebrate *Irx* genes.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.