Hoxa11 and Hoxd11 Regulate Chondrocyte Differentiation Upstream of Runx2 and Shox2 in Mice

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Abstract

During limb development, posterior Hox genes of the Hoxa- and Hoxd cluster provide positional information along the limb axis. Here we report a new function for Hoxa11 and Hoxd11 in regulating the early steps of chondrocyte differentiation. We analyzed forelimbs of Hoxa11^−/−,d11^−/− and Ulnaless mice, which are characterized by specifically shortened zeugopods. By detailed morphological and molecular analyses, we show that loss of Hoxa11 and Hoxd11 in the ulna of both mutants leads to an arrest of chondrocyte differentiation at a step before the separation into round and columnar cells takes place. Furthermore, we demonstrate that Hoxa11 and Hoxd11 act upstream of Runx2 and Shox2, two key regulators of chondrocyte differentiation. We hypothesize that Runx2 activates Shox2 in early chondrocytes, which at later stages induces Runx2 expression to regulate hypertrophic differentiation. These results give insight into mechanisms by which positional information might be translated into a specific bone pattern.

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Introduction

The long bones of vertebrates are formed by the process of endochondral ossification, during which a cartilage intermediate is established and subsequently replaced by bone [1–3]. Endochondral ossification is initiated by mesenchymal cells that condense and differentiate into chondrocytes, which form the cartilage anlagen of the future skeletal elements. The cartilage anlagen are surrounded by a layer of fibroblast-like cells, the perichondrium. In the center of the cartilage anlagen, the cells start to successively differentiate into several types of chondrocytes, each showing a characteristic cell shape. Initially, the proliferating chondrocytes separate into two cell types: round, low-proliferating chondrocytes at the distal end of the skeletal element and columnar, high-proliferating chondrocytes in the center [4,5]. The columnar chondrocytes are flattened and arranged in columns along the longitudinal axis. Once the chondrocytes stop proliferating, they differentiate into hypertrophic chondrocytes, which finally undergo apoptosis. During hypertrophic differentiation the chondrocytes increase their volume and produce a mineralized extracellular matrix. In parallel with hypertrophic differentiation, cells of the perichondrium differentiate into osteoblasts forming the peristeme. From the peristeme, blood vessels invade the hypertrophic region providing osteoblasts and osteoclasts to replace the cartilage by bone [1–3].

Hox genes belong to the highly conserved family of homeobox transcription factors that play a critical role in defining regional identity along the primary body and limb axis during embryonic development [6,7]. In mice, 39 Hox genes are organized in 4 clusters (Hoxa-Hoxd), each of which is located on a different chromosome. Based on sequence similarities and the relative position in the cluster, Hox genes are divided into 13 paralogous groups [8,9]. The formation of limbs is mainly regulated by posterior genes of the Hoxa- and Hoxd-cluster (Hoxa9-a13, Hoxd9-d13).

The expression of Hox genes is tightly regulated in time and space. Along the anterior-posterior body and limb axis the order of Hox gene expression reflects their arrangement on the chromosome with 3' genes being expressed earlier and more anteriorly than 5' genes [10,11]. During limb development, the expression of Hoxd genes is under the control of two enhancers that are located on either side of the Hoxd cluster [12–16]. In a first phase of Hoxd gene expression, the colinear activation is regulated by the “Early Limb Control Region” (ELCR), which is located at the 3' end of the cluster. During this phase, the Hoxd genes are successively expressed, starting with Hoxd1 being expressed throughout the early limb bud and ending with the expression of posterior Hox genes progressively restricted to the posterior region of the limb bud. In a second phase of Hoxd gene expression, only the posterior Hoxd genes (Hoxd9-d13) are expressed in reverse colinearity. This phase is regulated by the “Global Control Region” (GCR) that is located at the 5' side of the Hoxd cluster [13,15,16]. This nested expression pattern of Hoxd genes in combination with genes of the Hoxa cluster generates a “Hox code” that specifies the identity of the limb skeletal elements [17].

Mutations of posterior Hoxa and Hoxd genes cause distinct limb deformities. Loss of single Hox genes leads to mild alterations of individual skeletal elements, whereas the deletion of additional paralogous Hox genes enhances the severity of the phenotype. For example, loss of Hoxa11 or Hoxd11 in mice leads to mild
alterations of ulna and radius [18,19], whereas compound Hoxa11/D11 mice display a severe shortening of ulna and radius. Other skeletal elements develop more or less normally [20,21]. A similar forelimb phenotype has been observed in the dominant mouse mutant Ulnaless, which carries a radiation induced inversion of the complete Hoxd cluster [13,22–26]. Due to this inversion, the GCR is separated from the Hoxd cluster [13] leading to an altered temporal and spatial activation of posterior Hoxd genes. As a result, Hoxd12 and Hoxd13 are ectopically expressed in the zeugopod region, whereas the expression of Hoxa11 and Hoxd11 is significantly reduced [24,25]. Based on the similarities of the forelimb phenotype and the loss of Hoxa11 and Hoxd11 expression, Ulnaless mice have been hypothesized to mimic the Hoxa11/D11 mutation.

In this study, we investigated the molecular mechanisms acting downstream of Hox genes by analyzing chondrocyte differentiation in ulna and radius of Hoxa11−/−;D11−/− and Ulnaless mutant mice. Since the Ulnaless mutation is dominant (50% mutant embryos), this mouse strain facilitates the analysis of the phenotype.

Figure 1. Lack of hypertrophic differentiation in the ulna of Hoxa11−/−;D11−/− and Ulnaless forelimbs. Safranin-Weigert staining of E14.5 (A–F) and E16.5 (G–L) control (A, D, G, J), Hoxa11−/−;D11−/− (B, E, H, K) and Ulnaless (C, F, I, L) forelimb sections reveals disturbed chondrocyte differentiation. Hypertrophic chondrocytes are absent in ulna (E, K) and radius (B, H) of Hoxa11−/−;D11−/− mice and in the ulna (F, L) of Ulnaless mice. Only in the curve of the radius of E14.5 Ulnaless mice hypertrophic chondrocytes are detectable (C, arrow). At E16.5, mineralized matrix is produced in the radius of Ulnaless mice (I, arrow), while in the radius of Hoxa11−/−;D11−/− mice columnar chondrocytes are formed (H). These cells are not organized in columns along the longitudinal axis as in the control, but in anterior-posterior direction (G, H, see encircled columns in higher magnification of boxed regions). 160x magnification; P = proximal, D = Distal.

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compared to the double homozygous Hoxa11−/−;d11−/− embryos (6.25% mutant embryos).

By detailed morphological and molecular analyses, we show that the ulna is similarly affected in both mutants. Although chondrogenesis in the cartilage anlagen is initiated similar as in wild type mice at E12.5, the differentiation of chondrocytes is inhibited at an early step of the differentiation program at least until E16.5, when wild type limbs clearly have undergone hypertrophic differentiation. Molecular analyses indicate that the differentiation into round and columnar chondrocytes is disturbed. Furthermore, Shox2 and Runx2 expression cannot be detected in the mutant ulna, indicating that they act downstream of posterior Hox genes during chondrocyte differentiation.

Results and Discussion

The Ulna of Ulnaless Mice Serves as a Model for the Hoxa11−/−;d11−/− Mutation

Previously, it has been reported that Hoxa11−/−;d11−/− mice display a defect in hypertrophic chondrocyte differentiation in ulna and radius [20]. To gain insight into the molecular mechanisms acting downstream of Hox genes in chondrocytes, we investigated the process of chondrocyte differentiation in Hoxa11−/−;d11−/− mice at different developmental stages in detail and compared it to that of Ulnaless mutants. To confirm the similarity of the Hoxa11−/−;d11−/− and Ulnaless forelimb phenotypes, we analyzed forelimb sections of both mutants on a morphological level after Safranin-Weigert staining. At E14.5 and E16.5, ulna and radius of both mutants are significantly shortened compared to wild type limbs (Fig. 1). In addition, the radius is bent towards the anterior side (Fig. 1B, C, H, I). At both stages, the wild type cartilage elements of both zeugopod bones show well organized zones of chondrocytes: round chondrocytes located at the distal end of the cartilage element are followed by columnar and hypertrophic chondrocytes towards the center (Fig. 1A, D, G, J). In contrast, in the ulna of Hoxa11−/−;d11−/− mice no columnar or hypertrophic chondrocytes are detectable at E14.5 or E16.5 (Fig. 1E, K). In the radius hypertrophic differentiation is delayed at both stages, but a few columnar chondrocytes can be detected in the anterior curve at E16.5 (Fig. 1H). Interestingly, while columns in the radius of the control limb are aligned along the longitudinal axis, they are arranged in anterior-posterior direction in the Hoxa11−/−;d11−/− mutant radius (Fig. 1G, H, higher magnification of boxed regions). In the ulna of Ulnaless mutants hypertrophic differentiation is similarly blocked as in the ulna of Hoxa11−/−;d11−/− mutants (Fig. 1E, F, K, L). However, chondrocyte differentiation in the radius of Ulnaless mutants is less severely affected. Few cells with hypertrophic appearance can be detected at E14.5 and a distinct hypertrophic region is found at E16.5 (Fig. 1C arrow, I).

To further characterize the chondrocyte differentiation defect on a molecular level, we analyzed the expression of genes demarcating distinct chondrocyte subpopulations by *in situ* hybridization. The extracellular matrix protein *Type II collagen (Col2a1)* and the transcription factor *SRY-box containing gene 9 (Sox9)* are markers for cartilage condensations and proliferating chondrocytes [27–29] (Fig. 2A and data not shown). Although in Hoxa11−/−;d11−/− mice the condensations of ulna and radius are smaller than in wild type mice [20] (Fig. 1), Col2a1 and Sox9 are expressed in all chondrocytes at E14.5 (Fig. 2B and data not shown). A similar expression pattern of Col2a1 and Sox9 was detected in E14.5 Ulnaless mice (Fig. 2C and data not shown) indicating that cells condense and differentiate into chondrocytes. *Indian hedgehog (Ihh)*, a key regulator of endochondral ossification, is expressed in early hypertrophic chondrocytes of E14.5 and E16.5 wild type limbs [30,31] (Fig. 2D, arrow and data not shown). Corresponding to the morphological analysis (Fig. 1), no hypertrophic, Ihh-expressing cells are found in the ulna of Hoxa11−/−;d11−/− or Ulnaless mutants at E14.5 and E16.5 (Fig. 2E, F and data not shown) [20]. However, cells in the radius of Ulnaless mice that appear hypertrophic by morphology (Fig. 1C, arrow) express Ihh at E14.5 (Fig. 2F, arrow).

To exclude an early delay in chondrogenesis as the cause for the inhibition of hypertrophic differentiation we analyzed Col2a1 expression in E12.5 Ulnaless mice. Similar to data published for E12.5 Hoxa11−/−;d11−/− mice [20], we detected no significant difference in Col2a1 expression in the zeugopod of E12.5 Ulnaless mice (Fig S1) indicating that chondrocyte differentiation is properly initiated.

In summary, although the initial condensation of chondrocytes is not severely affected, we detected a severe inhibition of further chondrocyte differentiation in the zeugopod of Hoxa11−/−;d11−/− mutants that is maintained at least until E16.5. A comparable delay in chondrocyte differentiation was detected in the zeugopod of Ulnaless mice. Interestingly, chondrocyte differentiation seems to be more severely delayed in the ulna of both mutants, whereas the radius is less affected. To understand the role of posterior Hox genes in regulating chondrocyte differentiation, we thus concentrated our analysis on the ulna of both mutants.

Chondrocytes in the Ulna of E13.5 Ulnaless Mice are not Competent to Undergo Hypertrophic Differentiation

It has previously been described that Ihh-deficient mice undergo accelerated hypertrophic differentiation [31]. Here we show that despite the lack of Ihh expression in the ulna of the Hox mutants at E14.5 and E16.5, no sign of hypertrophic differentiation can be detected (Fig. 1E, F, K, L; Fig. 2E, F and data not shown). To exclude that the level of Ihh expression is below the sensitivity of the *in situ* hybridization, we analyzed the expression of the Ihh receptor Patched homolog 1 (*Pch1*), which is highly expressed in cells receiving a Hedgehog signal. In E14.5 wild type skeletal elements, *Pch1* is expressed in all proliferating chondrocytes with highest expression level adjacent to the Ihh expression domain [31] (Fig. 2G, yellow arrow). However, in the ulna of both Hox mutants, *Pch1* expression is only detected at basal expression levels (Fig. 2H, I, red arrows). To further exclude residual levels of Ihh activity, we treated E13.5 forelimb explants of the dominant *Ulnaless* mutant with cyclopamine, which inhibits Ihh signaling thereby accelerating hypertrophic differentiation [32,33]. In cyclopamine treated wild type cultures, hypertrophic differentiation was accelerated, as indicated by the increased expression domain of the hypertrophic marker *Ihh* and the shortened zone of proliferating chondrocytes in ulna, radius and in the digits (Fig. 3A, C). In contrast, in cyclopamine treated explant cultures of *Ulnaless* mice, we did not observe any Ihh-expressing hypertrophic chondrocytes in the ulna (Fig. 3B, D, red arrows), although Ihh expression was induced in the digits (Fig. 3B, D, yellow arrows). To confirm that hypertrophic differentiation is blocked in the mutants, we analyzed the expression of Type X collagen (Col10a1), another marker specifically expressed in hypertrophic cells [34]. Similar to Ihh, we did not detect any Col10a1-expressing chondrocytes in the ulna of cyclopamine treated *Ulnaless* limbs (Fig. 3F, H, red arrows), but observed accelerated hypertrophic differentiation in the digits (Fig. 3F, H, yellow arrows).

We can thus conclude that chondrocytes in the ulna of E13.5 *Ulnaless* mice are not competent to initiate hypertrophic differentiation upon lack of Ihh signaling.
Furthermore, it is interesting to note that the differentiation defect is maintained in the serum free organ cultures, in which the surrounding tissue is rapidly lost. We thus hypothesize that information inhibiting chondrocyte differentiation in the ulna is contained in the skeletal element itself, either in the perichondrium or in the chondrocytes.

Hoxa11 and Hoxd11 Control Early Steps of Chondrocyte Differentiation

Since loss of Hoxa11 and Hoxd11 impairs chondrocyte differentiation, we wished to identify the specific step of the differentiation program affected in the Hox mutants. As described above, proliferating chondrocytes can be subdivided into round and columnar cells. To define the subpopulations of proliferating chondrocytes in the mutants, we analyzed the expression of Fibroblast growth factor receptor 3 (Fgfr3). In E14.5 wild type limbs, Fgfr3 is expressed at high levels in columnar chondrocytes (Fig. 4A, yellow arrow) and at lower levels in round cells (Fig. 4A, red arrow) [35]. In the ulna of both Hox mutants, we observed only low levels of Fgfr3 expression (Fig. 4B, C, red arrows) indicating that the separation into round and columnar chondrocytes had not taken place. This supports the morphological observation that no columnar chondrocytes are formed. To further test if the chondrocytes represent the round cell type, we analyzed the expression of Upper zone of growth plate and matrix associated protein (Ucma), which is strongly expressed in these cells at E16.5 in wild type skeletal elements [36] (Fig. 4D, arrow). Surprisingly, in both mutants the majority of chondrocytes in the ulna do not express Ucma. Instead, Ucma expression is restricted to the joint region in Hoxa11−/−;d11−/− and Ulnaless mice (G-I, red arrows). 80x magnification; R = radius, U = ulna.
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Hoxa11 and Hoxd11 Regulate Chondrocyte Differentiation

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Both show shortened limbs, an arrest in chondrocyte differentiation and the absence or severe reduction of \(\text{Ihh}\) expression \([37,38]\). Runx2 and Shox2 are thus good candidates to mediate the effect of \(\text{Hox}\) genes in chondrocyte differentiation. To test this hypothesis, we analyzed the expression of Runx2 and Shox2 in the ulna of the E14.5 \(\text{Hox}\) mutants by in situ hybridization. In E14.5 wild type limbs, Runx2 is expressed in early hypertrophic chondrocytes (Fig. 5C, yellow arrow) and in the perichondrium \([39]\) (Fig. 5C, red arrow), whereas Shox2 is expressed in proliferating chondrocytes at E14.5 and E16.5 \([40]\) (Fig. 6A, E, yellow arrows). In contrast, neither Runx2 nor Shox2 expression can be detected in chondrocytes of the ulna of either mutant (Fig. 5D, E; Fig. 6B, C, F, G, red arrows), although both genes are strongly expressed in other cartilage anlagen of the forelimbs (Fig. 5D, E, yellow arrows; Fig. 6B, C, G, yellow arrows).

Interestingly, in addition to being expressed in hypertrophic chondrocytes, Runx2 has been described to be expressed in the cartilage condensations \([41,42]\). To investigate if this early expression is regulated by \(\text{Hox}\) genes, we analyzed the expression of Runx2 at E12.5. In wild type limbs, strong Runx2 expression was detected in the condensing cartilage anlagen of the forelimb zeugopod (Fig. 5A). In contrast, already at this early stage, Runx2 expression is severely reduced in the condensing chondrocytes of the ulna of \text{Ulnaless} mice and could only be detected in the region of the future perichondrium (Fig. 5B, red arrow). This restriction of the Runx2 expression to the perichondrium was observed in the E14.5 ulna of both \text{Hox} mutants as well (Fig. 5D, E, red arrows).

To further investigate the epistatic relationship of Runx2 and Shox2, we analyzed the expression of Shox2 in E14.5 Runx2 mutants. As Runx2 and Runx3 have highly redundant functions...
**Figure 4. Hoxa11 and Hoxd11 control early steps of chondrocyte differentiation.** Sections of E14.5 (A–C) and E16.5 (D–F) forelimbs of control (A, D), Hoxa11^{-/-}; d11^{-/-} (B, E) and Ulnaless embryos (C, F) were hybridized with antisense riboprobes for Fgfr3 (A–C) and Ucma (D–F). High Fgfr3 expression (A–C, yellow arrows), which demarcates columnar chondrocytes in control limbs (A), is significantly reduced in the ulna of the Hox mutants (B, C). The observed Fgfr3 expression level is comparable with the expression in round chondrocytes of the control mice (A–C, red arrows).

Ucma, a marker for round cells (D, arrow), is not expressed in the majority of chondrocytes indicating that the differentiation is blocked before round and columnar chondrocytes are formed (E, F, arrows). 80x magnification; R = radius, U = ulna.

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**Figure 5. Runx2 acts downstream of Hoxa11 and Hoxd11.** Sections of E12.5 (A, B) and E14.5 (C–E) control (A, C), Hoxa11^{-/-}; d11^{-/-} (D) and Ulnaless (B, E) forelimbs were hybridized with Runx2 antisense riboprobe. In control forelimbs, Runx2 is expressed in the condensing cartilage anlagen of the zeugopod at E12.5 (A) and in early hypertrophic chondrocytes (C, yellow arrow) and the perichondrium at E14.5 (C, red arrow). In the ulna of both E14.5 Hox mutants, Runx2 expression cannot be detected in chondrocytes, but in the perichondrium (D, E, red arrows). In E12.5 Ulnaless mice, Runx2 expression is restricted to the region of the future, posterior perichondrium (B, red arrow). 80x magnification; R = radius, U = ulna.

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during chondrocyte differentiation and compound mutants display a similar arrest in chondrocyte differentiation as \textit{Hoxa11}\(^{-/-}\)/\textit{d11}\(^{-/-}\) and \textit{Ulnaless} mutants [37]. To exclude that the lack of \textit{Shox2} expression reflects a delay in development, we analyzed \textit{Runx2}\(^{-/-}\)/\textit{Runx3}\(^{-/-}\) double mutant forelimbs for this analysis. Although we detected strong expression of \textit{Shox2} in the surrounding limb tissue (Fig. 6D), its expression was severely reduced in chondrocytes at E14.5 (Fig. 6D, red arrow). To exclude that the lack of \textit{Shox2} expression in the ulna of \textit{Hoxa11}\(^{-/-}\)/\textit{d11}\(^{-/-}\) double mutant forelimbs revealed a lack of \textit{Shox2} expression upstream of \textit{Runx2} in the ulna of either mutant (Fig. 6F–H, red arrows). It is thus likely that early \textit{Runx2}/\textit{Runx3} expression acts upstream of \textit{Shox2}.

The role of \textit{Runx2} in inducing hypertrophic differentiation has been intensively studied [39]. The loss of \textit{Ihh}-expressing cells in the \textit{Hox} mutants is likely due to the absence of this inducer of hypertrophic differentiation. Furthermore, previous experiments indicated that \textit{Shox2}\(^{-/-}\) mutants show reduced \textit{Runx2} expression in the humerus [38,40] placing \textit{Shox2} upstream of \textit{Runx2}. Interestingly, our investigation of \textit{Runx2}\(^{-/-}\)/\textit{Runx3}\(^{-/-}\) double mutants revealed a lack of \textit{Shox2} expression upstream of \textit{Shox2}. This is supported by our expression analysis, in which we detect \textit{Shox2} expression in chondrocytes starting from E13.5 (data not shown), whereas the early expression of \textit{Runx2} can already be detected at E12.5 (Fig. 5A).

\textit{Runx2}/\textit{Runx3} and \textit{Shox2} seem thus to interact in a complex relationship downstream of \textit{Hox} genes in chondrocytes. At early stages \textit{Runx2}/\textit{Runx3} seem to act upstream of \textit{Shox2}, whereas at later stages \textit{Shox2} appears to act upstream of \textit{Runx} genes. If \textit{Runx2}/\textit{Runx3} induce the differentiation of round and columnar cells, which in turn express \textit{Shox2}, or if \textit{Shox2} together with \textit{Runx2}/\textit{Runx3} is required to drive the chondrocyte differentiation program has to be investigated in future experiments. In this respect it is interesting to note that loss of \textit{Runx2} affects hypertrophic differentiation strongest in the humerus [39], but loss of \textit{Runx2} and \textit{Runx3} inhibits differentiation in all skeletal elements of the limbs [37]. Similarly, the defects in \textit{Shox2}\(^{-/-}\) mice are strongest in humerus and femur [38]. Interestingly, humans express the two paralog genes, \textit{SHOX} and \textit{SHOX2}, whereas mice only contain \textit{Shox2}. Mutations in \textit{SHOX} are associated with Leri-

**Figure 6.** \textit{Shox2} acts downstream of both \textit{Hoxa11} and \textit{Hoxd11} as well as of \textit{Runx2}. \textit{Shox2} in situ hybridization on E14.5 (A–D) and E16.5 (E–H) control (A, E), \textit{Hoxa11}\(^{-/-}\)/\textit{d11}\(^{-/-}\) (B, F), \textit{Ulnaless} (C, G) and \textit{Runx2}\(^{-/-}\)/\textit{Runx3}\(^{-/-}\) (D, H) forelimbs revealed the absence of its expression in the ulna of both \textit{Hox} mutants (B, C, F, G, red arrows) and in ulna and radius of \textit{Runx2}\(^{-/-}\)/\textit{Runx3}\(^{-/-}\) mutant mice (D, H, red arrows). However, \textit{Shox2} expression was detected in other regions of the analyzed forelimbs (B, C, D, G, yellow arrows). 80x magnification (A–C, E–H), 100x magnification (D); R = radius, U = ulna. doi:10.1371/journal.pone.0043553.g006

**Conclusions**

Our analysis demonstrates that \textit{Hoxa11} and \textit{Hoxd11} regulate an early step of chondrocyte differentiation in the zeugopod of mice (Fig. 7). Although the differentiation of mesenchymal cells into \textit{Col2a1}-expressing chondrocytes is not significantly affected by loss of \textit{Hoxa11} and \textit{Hoxd11}, chondrocytes fail to undergo differentiation into \textit{Ihh}-expressing hypertrophic chondrocytes. Here we further define the stage at which chondrocyte differentiation is arrested. We show that, although the condensation of the skeletal elements is not obviously affected at E12.5, chondrocytes stay arrested at an early step in the differentiation program, before the differentiation into columnar and round chondrocytes takes place. In addition, our data identify a new chondrocyte subtype expressing low levels of \textit{Fgfr3} but no \textit{Ucma}, which is not competent to undergo hypertrophic differentiation in absence of \textit{Ihh} signaling.

We further demonstrate that \textit{Hox} genes act upstream of the transcription factors \textit{Runx2} and \textit{Shox2}, two key regulators of the chondrocyte differentiation program (Fig. 7), which are expressed in most cartilage anlagen of wild type long bones. The observation that their expression is specifically affected in the ulna of the \textit{Hox} mutants make them likely candidates to translate the positional code into a skeletal pattern. As individual skeletal elements seem to react with different sensitivity to changes in the expression of \textit{Runx2}, \textit{Runx3} and \textit{Shox2}, fine-tuning their expression by specific combinations of \textit{Hox} genes might provide a mechanism to modulate size and shape of a cartilage element.

**Materials and Methods**

**Ethics Statement**

Mice were kept and bred according to the institutional guidelines of the University Duisburg-Essen and the University...
typing was performed as previously described [37].

Differentiation steps; red arrows: gene regulation, dashed arrows: predicted epistatic relationship.

Figure 7. Model for the regulation of chondrocyte differentiation by posterior Hox genes. Hoxa11 and Hoxd11 regulate the differentiation into round and columnar chondrocytes upstream of the transcription factors Runx2 and Shox2. Black arrows: chondrocyte

Mice and Genotyping

For time pregnancies, noon of the day, when a vaginal plug was observed, was considered to be embryonic day (E) 0.5. Double mutant Hoxa11<sup>-/-</sup>;Hoxd11<sup>-/-</sup> embryos were generated by mating compound heterozygous parental mice as described before [20,21]. Embryos with at least one wild type allele for each gene were used as a control. Genotyping was performed by three primer-PCR with the following primers:

Runx2+ wt-for: 5’-gttgaaggccacgggcag-3’
Runx2- rev: 5’-cttgaaggccacgggcag-3’
Hoxa11+ Neo-for: 5’-cttgaaggccacgggcag-3’
Hoxa11-Neo: 5’-tctggattcatc-3’
Hoxd11+ Neo-for: 5’-cttgaaggccacgggcag-3’
Hoxd11-Neo: 5’-tctggattcatc-3’

Histological Analysis and in situ Hybridization

Embryos were dissected in phosphate buffered saline (PBS). Forelimbs were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, dehydrated and embedded in paraffin wax. Serial sections of 5 μm were stained or used for in situ hybridization. Safranin-Weigert staining was performed with a series of Weigert’s hematoxylin (Roth), 0.1% last green (Sigma) and 0.1% safranin O (Sigma) according to the staining procedure [46]. For radioactive in situ hybridization, antisense riboprobes were labeled with [P<sup>33</sup>]-UTP (Hartman Analytic). Hybridization was performed in 50% formamide at 70°C as described previously [30]. Developed slides were counterstained with 0.2% toluidine blue O (Sigma) in 1% sodium borate. The following probes were used for in situ hybridization: Ihh [47], Pchg1 [48], Fgfr3 [35], Ucn3 [36], Col2a1 [49], Col10a1 [50], Runx2 [42]. The Shox2 probe was amplified from cDNA of E15.5 wild type limbs using primers Shox2-for: 5’-ttgcaagtgacgcccttgte-3’, Shox2-rev: 5’-ggrctatc-cacttcactg-3’ and cloned into pCR4-TOPO (Invitrogen).

unless otherwise indicated, forelimbs of at least two individual mice were analyzed by Safranin-Weigert staining or hybridized with the same antisense probe showing the same result.

Limb Explant Cultures

Forelimbs of E13.5 Ulnaless embryos were dissected by removing skin and muscle. The limbs were cultured for 4 days in Biggers or BGJb medium (Invitrogen) supplemented with 1% antibiotic/antimycotic (Invitrogen), 0.1% bovine serum albumin (BSA) (Roth) and 1.4 mM glutamine (Invitrogen) in 50% formamide at 70°C, 5% CO₂. One forelimb of the embryo was treated with 10 μM cyclopamine (Calbiochem), whereas the other forelimb was used as untreated control. The medium was changed every day. The limbs were fixed for 3 hours in 4% PFA at 4°C and embedded in paraffin wax.
Imaging

Bright- and dark field pictures were taken with the camera SPOT 14.2 using the SPOT advanced software, version 4.5.7 (Diagnostic Instruments). In situ hybridization signals were visualized in either white or red using dark field microscopy with the illuminator Intralux 5000-1 (Volpi).

Supporting Information

Figure S1 Chondrocyte differentiation is initiated normally in the zeugopod of Ulnaless mice. In situ hybridization on E12.5 control (A) and Ulnaless (B) forelimbs with a Col2a1 antisense riboprobe reveals no difference in the Col2a1 expression in Ulnaless forelimbs compared to control (A, B; 80x magnification; R = radius, U = ulna). (TIF)

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Author Contributions

Conceived and designed the experiments: SG MV AV. Performed the experiments: SG YK. Analyzed the data: SG YK MV AV. Contributed reagents/materials/analysis tools: AV. Wrote the paper: SG MV AV.

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