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Hox11 paralogous genes are required for formation of wrist and ankle joints and articular surface organization

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Limb skeletal elements are connected by distinct synovial joints, but the mechanisms regulating joint formation, diversity, and organization remain unclear. Previous studies showed that *Hox11* mouse mutants have severe developmental defects in radius and ulna and tibia and fibula, but wrist and ankle joint formation and characteristics were not examined in detail. We now find that E11.5 and E12.5 triple *Hox11aaccdd* mutants exhibit a significant reduction in prospective carpal and tarsal mesenchyme. Although the mesenchyme became segmented into individual carpal and tarsal skeletal elements with further development, the elements were ill defined and the more proximal elements (radiale, ulnare, talus, and calcaneous) actually underwent involution and/or fusion. Wild-type carpal and tarsal elements displayed a thick articulating superficial zone at their outer perimeter that expressed genes typical of developing joint interzones and articulating cells, including *Gdf5*, *Erg*, *Gli3*, collagen IIA, and lubricin, and defined each element anatomically. In mutant wrists and ankles, the superficial zone around each element was thin and ill defined, and expression of several of those genes was low and often interrupted. These and other data provide novel and clear evidence that *Hox11* paralogous genes regulate wrist and ankle joint organization and are essential for establishing carpal and tarsal element boundary and maintaining their articulating surface tissue.

Keywords: Hox genes; wrist and ankle joints; joint progenitor cells; limb skeletogenesis

Introduction

Formation of synovial joints involves a series of discrete and complex developmental steps during which joint primordia form at specific places and times in the developing embryo and eventually give rise to functional joints with characteristic organizational, morphologic, and mechanical properties.[1] In the limbs, the first sign of joint formation is the appearance of the so-called interzone at each prospective joint site.[2,3] The interzone consists of flat-shaped and closely associated mesenchymal cells that demarcate the boundary between adjacent cartilaginous skeletal elements, be it at an elbow, knee, or interphalageal site. Classic experimental embryogenesis studies carried out several decades ago provided tantalizing evidence that the interzone is very important for joint formation,[2] but its

specific roles remained unclear. To tackle this question, we previously carried out genetic cell tracing and tracking studies.[4,5] We mated *Rosa R26R* reporter (LacZ) mice[6] with mice expressing Cre-recombinase in incipient limb joint interzones under the control of growth and differentiation factor-5 (*Gdf5*) regulatory sequences[7] and monitored the behavior, fate, and roles of *LacZ*-positive interzone-associated cells over prenatal and postnatal time. We found that the cells produced most if not all joint tissues, including articular cartilage, synovial lining, outer capsule, and intrajoint ligaments, signifying that interzone cells represent a specialized cohort of progenitor cells exclusively devoted to, and programmed for, joint formation. The data correlated well with previous histologic evidence that interzone cells are responsible for articular chondrocyte development.[8] An interesting recent study

using gene ablation strategies showed that emergence and function of interzone cells and formation of limb joints are hampered in mouse embryos lacking *MyoD* and *Myf5* or bearing a *Pax3* mutation,[9] strengthening long-held evidence that joint formation involves movement and mechanical stimuli from the developing musculature.[10,11] Clearly, the above and related studies have provided novel and far-reaching information on several key cellular and molecular aspects of limb joint formation. However, what has remained far less understood is how each limb joint acquires its unique shape, organization, and structure.[1] This key question is particularly pressing with regard to development of wrist and ankle joints, which are exceedingly complex in organization, morphology, and skeletal element composition and vary greatly among species, including mammalian species.[12–14]

Hox genes encode highly conserved and homeodomain-containing nuclear proteins originally described in *Drosophila* that function as master regulators of development and provide essential overall cues for body plan, axis formation, and organ development.[15,16] Mammals have 39 *Hox* genes that reside in four separate linkage clusters, termed A, B, C, and D, that are divided into 13 paralogous groups each composed of two to four members. *Hox9* through *Hox13* group genes are expressed in specific spatiotemporal patterns during limb development, and many studies have demonstrated their fundamental importance in determination, patterning, and growth of the different limb skeletal elements.[17] In particularly interesting examples, double mouse mutants of *Hoxa11/Hoxd11* were found to exhibit drastic defects in radius and ulna development,[18,19] and triple mutants lacking all three Hox11 paralogous genes *(Hoxa11, Hoxc11,* and *Hoxd11)* displayed severe defects in tibia and fibula development, while neighboring elements are largely normal.[20] The affected long bone elements were severely hypomorphic and misshaped; their chondrocytes failed to organize typical growth plates, exhibited abnormal patterns of expression of genes required for their maturation and hypertrophy including collagen X, and displayed a severe retardation in terminal maturation and endochondral ossification.[18] In addition, certain carpal and tarsal elements were found to be abnormal or even absent,[19] but further details and insights into the possible roles of *Hox11* genes in joint formation were not provided.

Thus, we carried out the present study to address this question and specifically clarify whether and how lack of *Hox11* paralogous group genes affects joint formation, organization, articular characteristics and/or morphogenesis, focusing on wrist and ankle development.

Results

Analysis of wrist joint development

Triple mouse embryo mutants lacking *Hox11* paralogous group genes were generated by *in vitro* fertilization followed by transfer to foster mothers as described.[20] Mutant embryos (designated as *Hox11aaccdd*) were harvested at E18.5 of development along with wild-type (WT) littermates. Embryos were processed for whole-mount staining with alcian blue and alizarin red to reveal their limb skeletal elements and cartilaginous and osseous portions. Compared to WT forelimbs (Fig. 1A), triple *Hox11* mutant limbs contained severely hypomorphic and misshaped radius and ulna (Fig. 1D, arrows), which were flanked by fairly normal humerus and digit elements. Closer inspection of WT specimens showed that all the characteristic wrist skeletal elements were readily recognizable by overall shape and anatomic location, were entirely cartilaginous at this stage, and stained strongly with alcian blue or safranin O (Fig. 1B–C). Thus, the radiale (*r*) and ulnare (*u*) abutted the distal end of radius (*rad*) and ulna (*ul*) (Fig. 1B–C) and were followed by a row of cartilaginous carpal bone elements (designated as 1, 2, 3, and 4/5) and a characteristic central (*c*) element flanking the metacarpals (designated I to V) (Fig. 1B–C).[21] (Note that the nomenclature for mouse wrist and ankle bones can vary in different studies).[22] The bulk of chondrocytes within wrist elements were not organized in growth plates (Fig. 1C) and did not express genes typical of maturing/hypertrophic chondrocytes and endochondral bone, including Indian hedgehog (*Ihh*), collagen X (*Col X*), and Osterix (*Osx*), which were expressed in the growth plates of neighboring long-bone anlaga (Fig. 1G–J).

When we examined companion E18.5 triple *Hox11* mutants, we found that several wrist elements were severely affected (Fig. 1E–F). The radiale and ulnare appeared to be missing, putative carpal bone element 3 was elongated and almost reached radius and ulna, and the central element appeared misplaced (Fig. 1E–F). Interestingly, a thick compact
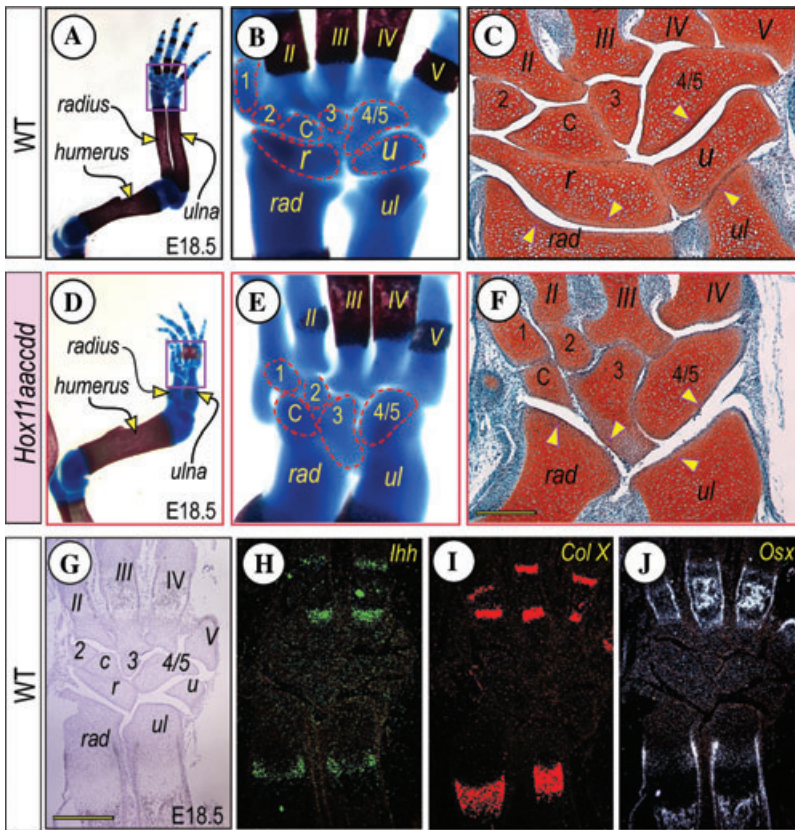
Figure 1. Anatomic, histochemical, and *in situ* hybridization analyses of forelimbs from E18.5 wild-type (WT) and triple *Hox11aaccdd* mutant embryos. (**A**) WT forelimb stained with alcian blue and alizarin red. (**B**) Higher-magnification image of boxed area in (A) showing dorsal view of wrist elements, including radiale (*r*), ulnare (*u*), carpal elements 1 to 4/5, and central element (*c*). Note that the more ventrally located pisiform is not visible. (**C**) Safranin O-stained section revealing the cartilaginous nature of carpal elements and presence of a compact superficial zone surrounding each element (*arrowheads*). (**D–F**) Whole-mount and histochemical analysis of companion E18.5 triple *Hox11* mutant specimens. Note in (**E**) the severe defects in carpal elements, including putative involution/absence of ulnare and radiale, repositioning of central element, and apparent elongation of carpal element 3 (that could also be due to fusion with pisiform). (**G–J**) Serial WT wrist sections processed for H&E staining (**G**) or *in situ* hybridization for (**H**) Indian hedgehog (*Ihh*); (**I**) collagen X (*Col X*); and (**J**) osterix (*Osx*). Note that none of the carpal elements expresses these gene markers of chondrocyte maturation and osteogenesis. *rad*, radius; *ul*, ulna; and *I* through *V*, metacarpals. Bar for C and F, 450 μm; bar for G–J, 700 μm.

multilayer of flat-shaped cells constituted the outer articulating perimeter of each WT element, stained relatively weakly with safranin O, and clearly demarcated and defined each element's outer boundary (Fig. 1C, arrowheads). This zone, however, was significantly less evident in mutants (Fig. 1F, arrowheads).

To further characterize these joint changes, we examined earlier developmental stages and determined the expression of genes associated with joint formation and function, including *Gdf5*, a signaling growth factor protein that is expressed in mesenchymal interzone and joints;[23] *Erg*, an *ets*-containing transcription factor expressed by interzone and linked to articular cartilage development;[24] and matrilin-1, a matrix macromolecule whose expression pattern distinguishes growth plate from joint-associated chondrocytes.[25] In E15.5 WT specimens, the genes displayed typical and clear-cut expression patterns. *Gdf5* and *Erg* were strongly
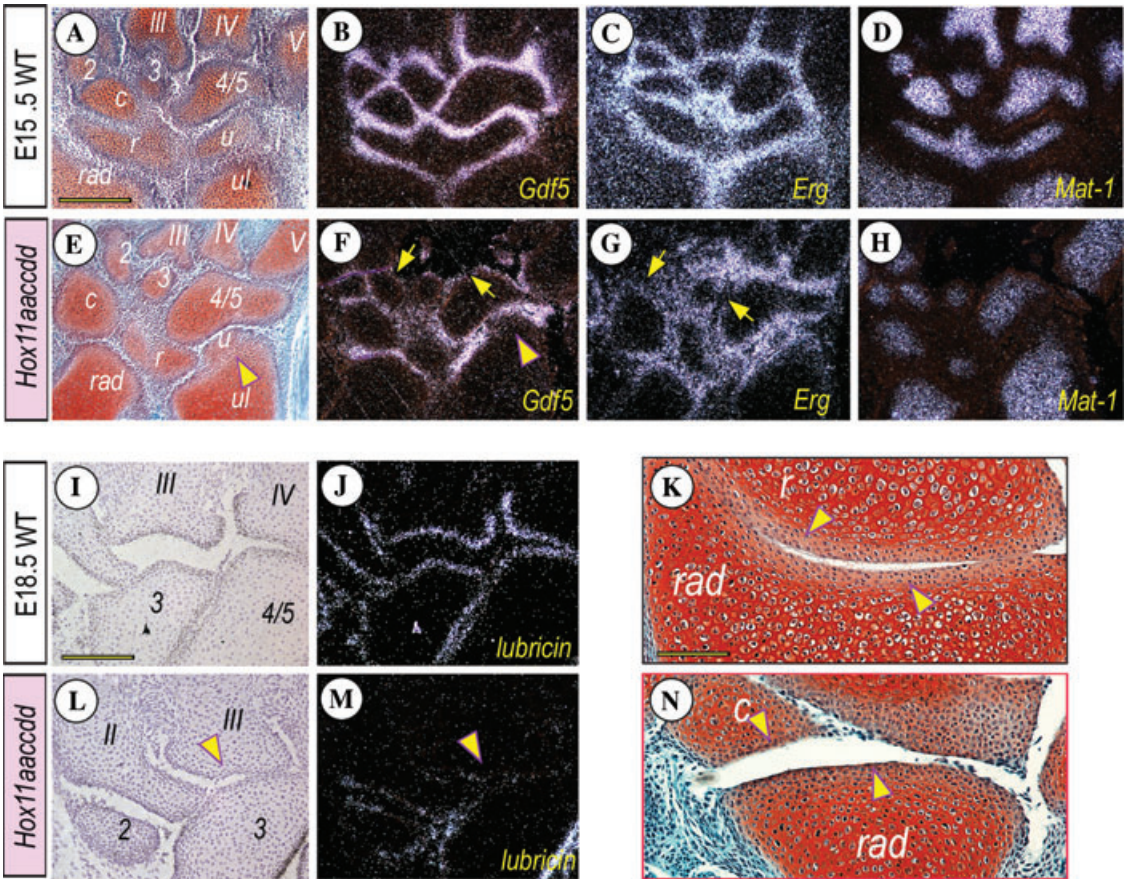
Figure 2. Histochemical and *in situ* hybridization analyses of wrist joint development in wild-type (WT) and triple *Hox11* mutant embryos. (**A–D**) Serial E15.5 WT wrist sections processed for safranin O staining (**A**) or *in situ* hybridization for (**B**) *Gdf5*; (**C**) *Erg*; and (**D**) matrilin-1. Note the presence of characteristic carpal elements in (**A**) and the strong and continuous *Gdf5* and *Erg* expression all around each carpal element in (**B–C**). (**E–H**) Serial sections from E15.5 triple *Hox11* mutants processed as above. Note the hypomorphic nature of putative radiale (*r*) and ulnare (*u*) and misplacement of putative central (*c*) element in (**E**); and reduced and interrupted expression of *Gdf5* and *Erg* (*arrows*) in (**F–G**). As pointed out in Figure 1, absolute identification of mutant carpal element was difficult because of the severe morphologic and topographic changes. (**I–N**) Sections of E18.5 WT (**I–J**) and triple *Hox11* mutant (**L–M**) wrists processed for H&E staining and lubricin expression; note the marked decrease in lubricin expression in mutants (*arrowhead* in **L–M**). Companion sections were stained with safranin O; note the presence of a normal thick articulating multilayer in WT (*arrowheads* in **K**) staining weakly with safranin O and the severe reduction of the multilayer in mutants (*arrowheads* in **N**). Bar for A–H, 275 μm; bar for I–J and L–M, 150 μm; and bar for K and N, 100 μm.

expressed by the superficial zone and neighboring cells all along the perimeter of each element (Fig. 2A–C), while matrilin-1 was expressed by underlying chondrocytes constituting the bulk of each element (Fig. 2D). In E15.5 triple *Hox11* mutant wrists, expression levels of all these genes appeared muted (Fig. 2E–G) and the patterns were ill-defined and at times discontinuous (Fig. 2F–G, arrows). The

same discontinuity was seen at E18.5 when we examined expression of another typical and critical joint-associated macromolecule: lubricin. Its transcripts were uniformly strong all along the articulating superficial zone in WT elements (Fig. 2I–J), but were at times totally missing from the surface of mutant elements (Fig. 2L–M, arrowhead). It was again quite clear that the superficial zone was thick
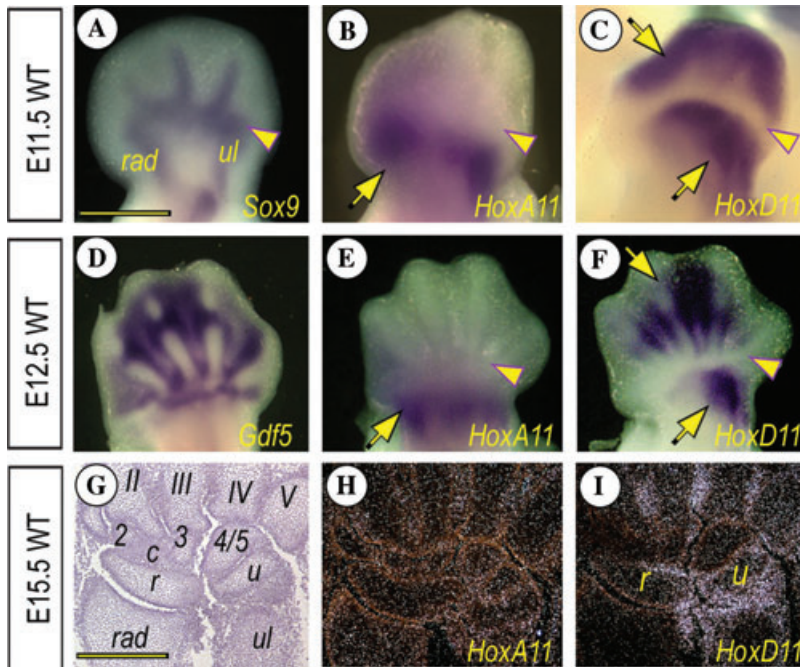
Figure 3. Whole-mount and *in situ* hybridization analyses. (**A–C**) WT E11.5 forelimbs processed for expression analysis of (**A**) *Sox9*; (**B**) *HoxA11*; and (**C**) *HoxD11*. Note the low *Hox11* but significant *Sox9* expression in prospective wrist area (*arrowhead*). (**D–F**) WT E12.5 forelimbs analyzed for expression of (**D**) *Gdf5*; (**E**) *HoxA11*; and (**F**) *HoxD11*. Note the persistent low *Hox11* expression over prospective wrist area (*arrowhead*). See text for further details. (**G–I**) Serial sections through E15.5 WT wrist processed for H&E staining (**G**) and expression of (**H**) *HoxA11*; and (**I**) *HoxD11*. Note the significant *HoxD11* expression over prospective ulnare (*u*) and radiale (*r*). *rad*, radius; *ul*, ulna; and *II* through *V*, metacarpals. Bar for A–F, 650 μm; and bar for G–I, 300 μm.

and well defined in wild types (Fig. 2K, arrowheads), but was ill defined and thinner in mutants (Fig. 2N, arrowheads).

Closer histologic examination of the E15.5 mutant wrists provided additional and interesting data and specifically the presence of primordia of radiale and ulnare (Fig. 2E). Both were hypomorphic and irregular compared to those in the wild type (Fig. 2A); the ulnare was nearly fused with ulna and in fact, the ulnare-ulna border lacked *Gdf5* transcripts (Fig. 2E–F, arrowhead). Since radiale and ulnare were actually undetectable in older mutant wrists such as at E18.5 (Fig. 1E–F), it is likely that the E15.5 primordia underwent involution or fused with neighboring elements over time.

To uncover possible mechanisms underlying the above changes, we examined the patterns of *Hox11* group gene expression in the wrist region over developmental time. In E11.5 and 12.5 embryos, *HoxA11* was strongly expressed in the prospective zeugo-pod (radius/ulna) region (Fig. 3B and E, arrow) and *HoxD11* was strongly expressed over zeugopod and autopod (carpal/digit) regions (Fig. 3C and F, arrows). However, the intervening prospective wrist area exhibited low *HoxA11* and *HoxD11* expression (Fig. 3B–C and E–F, arrowhead). By E15.5, however, *HoxD11* was conspicuously expressed and particularly so over the prospective ulnare and radiale areas (Fig. 3I), while *HoxA11* remained mildly expressed (Fig. 3G–H). Closer histologic and *in situ* hybridization analyses revealed that the prospective wrist region at E12.5 was still composed of a seemingly homogenous population of mesenchymal cells that were not segmented into discrete skeletal condensations (Fig. 4A, circled area); the cells, however, already expressed the chondrogenic master gene *Sox9* (Fig. 3A, arrowhead), indicating their commitment to chondrogenesis. Absence of discrete wrist cell condensations was at variance with processes occurring in adjacent radius, ulna,
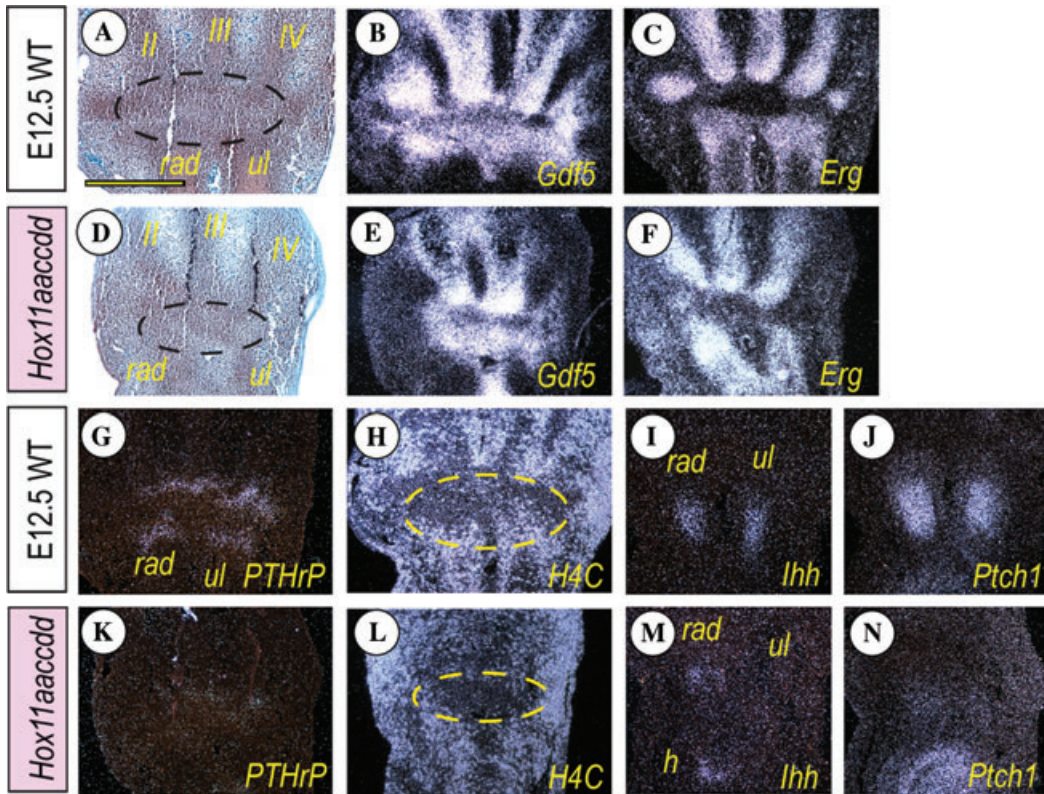
Figure 4. Development of prospective wrist mesenchyme. (A–F) Serial forelimb sections from E12.5 WT embryos (A–C) and triple *Hox11* mutants (D–F) processed for histologic study (A and D) and expression of (B and E) *Gdf5*; and (C and F) *Erg*. Note the reduction of prospective wrist mesenchyme in mutants (*circled area* in D) compared to controls (*circled area* in A). *rad*, radius; *ul*, ulna; and *II* through *IV*, metacarpals. (G–N) Sections from E12.5 WT embryos (G–J) and triple *Hox11* mutants (K–N) processed for expression of (G and K) *PTHrP*; (H and L) histone 4C; (I and M) Indian hedgehog (*Ihh*); and (J and N) Patched-1 (*Ptch1*). Note in mutants the considerable decreases in *PTHrP*, *Ihh,* and *Ptch1* expression (K, M and N) and proliferation (*circled area* in L) compared to controls. *II* through *IV*, metacarpals. Bar for A–N, 650 μm.

and carpal elements that were far more advanced in development and were cartilaginous and exhibited characteristic expression patterns for *Gdf5*, *Erg* (Fig. 4B–C) and parathyroid hormone-related protein (*PTHrP*) (Fig. 4G). In E12.5 triple *Hox11* mutants, however, the prospective wrist mesenchymal region was much reduced in size and prominence (Fig. 4D, circled area), reminiscent of the smaller mutant zeugopod condensations described previously.[18] The neighboring radius and ulna elements were also small and retained strong expression of *Gdf5* and *Erg* (Fig. 4E–F), but exhibited extremely low *PTHrP* expression (Fig. 4K). Because *Ihh* regulates *PTHrP* expression and PTHrP in turn regulates

proliferation,[26] we examined expression of *Ihh* and its receptor *Patched1* and examined proliferation as indicated by histone 4C gene expression. Both *Ihh* and *Patched1* were markedly downregulated in mutant radius and ulna elements (Fig. 4M–N) compared to wild types (Fig. 4I–J) and, unexpectedly, there was also a marked decrease in cell proliferation within the prospective wrist mesenchyme (Fig. 4L, circled area) compared to controls (Fig. 4H, circled area). The data suggest the interesting possibility that derangement of the PTHrP-Ihh loop within zeugopod elements in *Hox11* mutants had negative repercussions, and caused hypomorphic growth of wrist mesenchyme.

Analysis of ankle joint development

We carried out a final set of studies to determine whether ankle joint development is also affected by *Hox11* gene deficiency. In E15.5 WT specimens, the talus (*ta*) and calcaneus (*ca*) elements were large, distinct, and strongly positive for safranin O and were followed by a row of cartilaginous tarsal bone elements 1, 2, 3, 4/5, and a central (*c*) element flanking the metatarsals (I-V) (Fig. 5A).[21] Each ankle element displayed strong *Gdf5*, *Erg*, and *Gli3* expression all along its perimeter (Figs. 5B–D). In E15.5 triple *Hox11* mutants, distinct elements corresponding to talus and calcaneus were undetectable, as were tarsal elements 1 and 2 (Fig. 5E). In addition, an abnormally shaped element occupied a central location and might represent an elongated central (*c*) element or a fused central-talus element (Fig. 5E). Despite these changes, expression of *Gdf5*, *Erg* and *Gli3* remained strong (Fig. 5F–H), as also seen in mutant wrists above.

By E18.5, the WT ankle elements were quite well defined and remained entirely cartilaginous and very positive for alcian blue and safranin O and collagen IIB expression (Fig. 5I–K). The bulk of their chondrocytes strongly expressed matrilin-1 (Fig. 5L) and their peripheral superficial zone strongly expressed lubricin and collagen IIA (Fig. 5M–N). Mutant ankle elements did exhibit a similar cartilaginous character but were malformed and fused (Fig. 5O–R, arrowheads); their peripheral superficial zone displayed markedly reduced lubricin expression and uneven collagen IIA expression (Fig. 5S–T) and lack of these transcripts in fused areas (Fig. 5S–T, arrowheads).

Discussion

We provide novel evidence here that wrist and ankle joint development requires the function of *Hox11* paralogous genes during mouse limb skeletogenesis. Lack of these genes leads to reduced mass and proliferation of prospective carpal/tarsal mesenchymal cells; deficient segmentation and boundary formation; dis-morphogenesis, involution, or even absence of certain carpal and tarsal elements; thinning of peripheral articulating superficial zone; and defects in expression of joint-associated genes, including lubricin. The data significantly extend our previous analyses of *Hox11* gene roles in zeugopod development[20] and demonstrate that *Hox11* genes coordinate and orchestrate development of zeugopod long bones with that of wrist and ankle elements and joints.[19] This is in keeping with previous studies on autopod development showing that *Hox12* and *Hox13* genes, and *Hoxa13* in particular, play essential roles in digit and metacarpal/metatarsal development as well as carpal and tarsal development.[22,27,28]

Because of the multiplicity of skeletal elements involved, wrist and ankle development is indeed quite complex and obeys specific developmental rules. As our data reaffirm, the mesenchyme present in the prospective wrist area at E12.5 is still composed of cells that are not segmented into individual condensations corresponding to different wrist elements. This is in contrast to the more proximal zeugopod elements and the more distal metacarpal elements that are already distinguishable, distinct, and cartilaginous and exhibit prescribed elongated morphologies at those stages. Clearly, wrists and ankles do not obey the general proximal-to-distal rule of development in which proximal elements develop and mature prior to distal elements, and their mesenchyme is developmentally delayed compared to that in flanking long-bone elements. This retardation could actually be advantageous and provide the mesenchyme with additional time to receive and implement the complex patterning and determination cues that are likely to be needed to produce the multiple wrist and ankle skeletal elements with their intricate morphologies and topographic relationships. Such cues could include those from *Hox11* genes that do become more prominently expressed over time and particularly so over ulnare and radiale anlaga (Fig. 3I). Of interest, the latter data provide an explanation for our observation that radiale and ulnare (and talus and calcaneus) are strongly affected and are eventually undetectable as distinct entities in triple *Hox11* mutants; development of these more proximal elements could be strictly dependent on, and particularly affected by absence of, *Hox11* genes. This conclusion is reinforced by previous findings indicating that defects in proximal tarsal elements occur even in double *Hox11aadd* mutants, in which the hindlimb zeugopod elements are not severely affected.[19] Of importance to note, because experimental or congenital mutations in *Hox12* and *Hox13* genes can preferentially affect the more distal carpal or ankle elements,[28,29] it is possible that wrist and ankle joint development is mainly
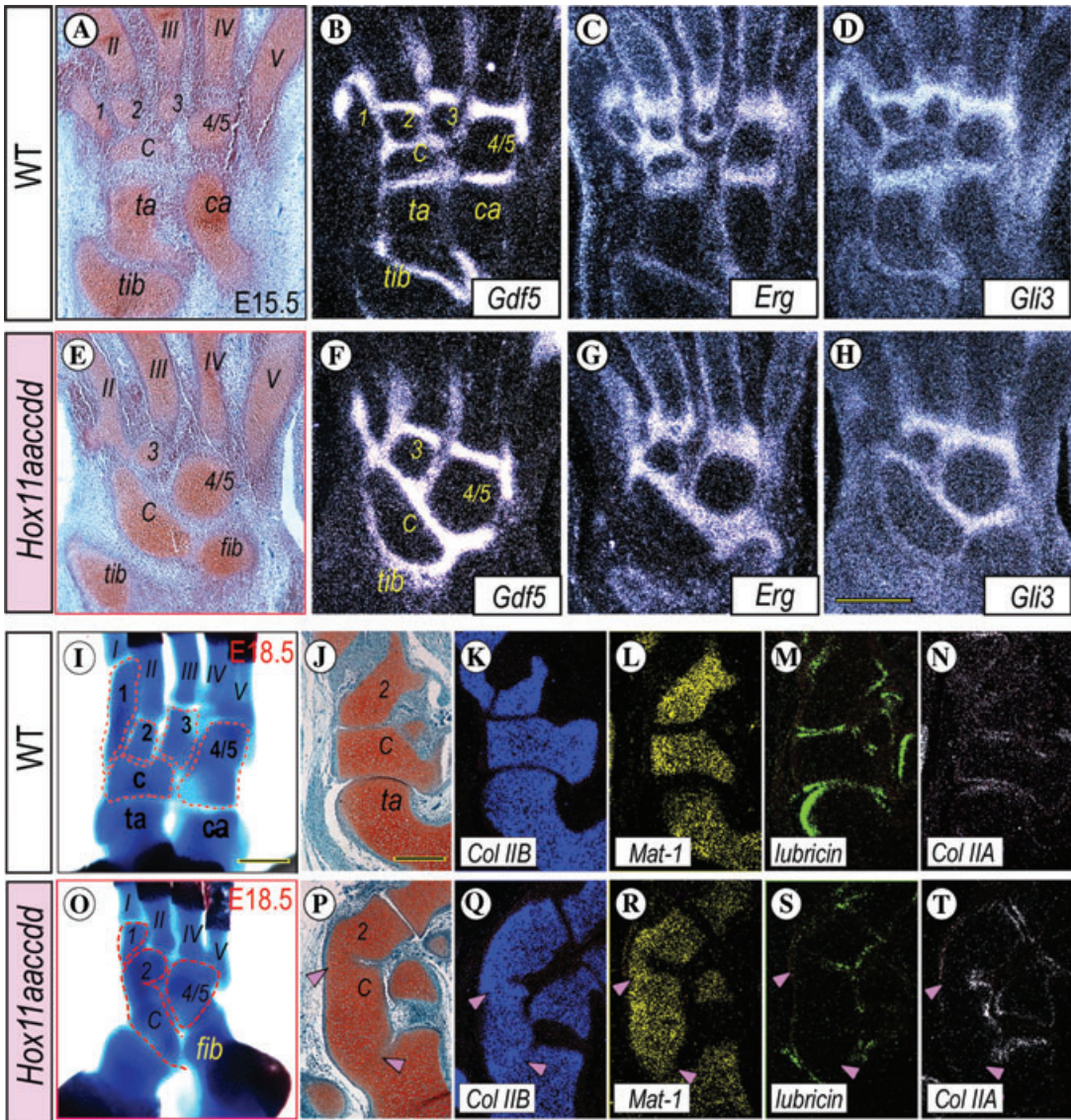
Figure 5. Histochemical and *in situ* hybridization analyses of ankle joint development. (A–D) Serial sections of E15.5 WT ankle processed for safranin O staining (A) or expression of (B) *Gdf5*; (C) *Erg*; and (D) *Gli3*. Note in (A) the presence of typical ankle skeletal elements including talus (*ta*) and calcaneus (*ca*) and strong expression of the three genes around each element (B–D). (E–H) Serial sections from companion E15.5 triple *Hox11* mutants processed as above. Note in (E) that distinct elements corresponding to talus, calcaneus, and tarsal elements 1 and 2 are not appreciable; an abnormally shaped element occupying a central location may represent an elongated central (*c*) element or a fused central-talus element. See text for further details. (I–N) Whole-mount dorsal view of alcian blue–stained E18.5 WT ankle with typical tarsal elements (I). Serial sections of WT ankle processed for safranin O staining (J) and gene expression of (K) collagen IIB (*Col IIB*); (L) matrilin-1 (*Mat-1*); (M) lubricin; and collagen IIA (*Col IIA*). (O–T) Whole-mount dorsal view of alcian blue–stained E18.5 triple *Hox11* mutant ankle with severely abnormal tarsal elements (O). Note that while the mutant elements were safranin O-positive (P) and expressed *Col IIB* and *Mat-1* (Q–R), they exhibited extensive fusion (P–Q, *arrowheads*) and lack of lubricin and *Col IIA* expression at fused sites (S–T, *arrowheads*). *II* through *V*, metatarsals. Bar for A–H, 275 μm; bar for I and O, 850 μm; and bar for J–N and P–T, 450 μm.

coordinated by proximal *Hox11* action and distal *Hox12*/*Hox13* action.[19]

The superficial zone at the periphery of each wrist or ankle element is needed to initially delineate anatomically each element and subsequently provide a functional articulating surface producing key antiadhesive molecules, including lubricin. However, the zone is ill defined and thinner in *Hox11* mutant elements and exhibits uneven and interrupted expression of characteristic genes, including *Gdf5*, *Erg*, collagen IIA, and lubricin. Clearly, *Hox11* genes set in motion mechanisms by which each element is able to acquire and retain a functional superficial zone serving as a seamless segmentation boundary and articulating surface and able to express a plethora of unique gene products. Defects in such boundary definition may explain why primordia of radiale and ulnare were initially present in *Hox11* mutants at E15.5, but were no longer appreciable by E18.5; the primordia may have been unable to maintain their individuality and may have undergone involution or fusion with adjacent elements. This interpretation correlates quite well with conclusions reached in previous studies. In their analysis of Gdf protein roles in joint development, Settle *et al.*[21] found that *Gdf6*-null mouse embryos exhibit fusion of carpal and tarsal elements and this may be due to failure of the mesenchymal segmentation and boundary formation requiring *Gdf6*. Similar fusions among wrist and ankle skeletal elements were observed in *Gdf5*-null mouse embryos and were interpreted to arise as a secondary consequence of reduced skeletal mobility.[30] Carpal fusions were described also in *Wnt9a*- and *Wnt9a*/*Wnt4*-null embryos, and analysis of successive developmental stages led the authors to conclude that the carpal elements had initially formed and fusion occurred secondarily on account of defective joint/boundary formation in the absence of *Wnt9a* and *Wnt4*.[31] It is interesting to note that we observe not only fusions, but also eventual absence of distinct proximal carpal or tarsal elements, indicating that lack of *Hox11* function has far more deleterious effects and deranges not only the segmentation and boundary formation processes, but also survival and maintenance of the elements themselves.

The articulating superficial zone has a compact structure made of flat-shaped cells and stains weakly with alcian blue or safranin O, indicating that it has a fibrous or fibrocartilaginous charac-

ter. This is in contrast to the bulk of the carpal and tarsal elements that stain strongly with alcian blue or safranin O and are clearly composed of cartilaginous tissue with characteristic round-shaped chondrocytes. The compact fibrous character of the superficial zone is reminiscent of the mesenchymal interzone typical of nascent long-bone joints and of the thin layer of flat-shaped and lubricin-expressing cells that are present at the surface of articular cartilage in long bone joints.[5] It remains to be clarified whether the process of mesenchymal segmentation during carpal/tarsal development involves formation of stereotypic interzones similar to those in developing long-bone anlaga. This is a likely possibility since interzone cells and peripheral cells around carpal/tarsal elements share expression of several typical interzone genes including *Gdf5*, *Gli3*, and *Erg*. In our previous study of long-bone joint formation, we reported that deficiency in Wnt/β-catenin signaling causes severe reductions in superficial zone and lubricin gene expression in developing long-bone joints.[5] Thus, it is possible that *Hox11* genes could favor formation of a thick lubricin-expressing superficial zone around carpal and tarsal elements via direct or indirect stimulation of Wnt/β-catenin signaling and *Wnt9a* and *Wnt4* expression and action. Because Wnt/β-catenin signaling has anti-chondrogenic effects,[32,33] strong activity of this signaling pathway could also help to establish and maintain the fibrous or fibrocartilaginous character of the articulating multilayer.

Acknowledgments

Conflicts of interest

The authors declare no conflicts of interest.

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