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Survival of *Hoxa13* Homozygous Mutants Reveals a Novel Role in Digit Patterning and Appendicular Skeletal Development

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Abstract

The loss of HOXA13 function severely disrupts embryonic limb development. However, because embryos lacking HOXA13 die by mid-gestation, the defects present in the mutant limb could arise as a secondary consequence of failing embryonic health. In our analysis of the mutant *Hoxa13*^{GFP} allele, we identified a surviving cohort of homozygous mutants exhibiting severe limb defects including: missing phalanx elements, fusions of the carpal/tarsal elements, and significant reductions in metacarpal/metatarsal length. Characterization of prochondrogenic genes in the affected carpal/tarsal regions revealed significant reduction in *Gdf5* expression, whereas *Bmp2* expression was significantly elevated. Analysis of *Gdf5* mRNA localization also revealed diffuse expression in the carpal/tarsal anlagen, suggesting a role for HOXA13 in the organization of the cells necessary to delineate individual carpal/tarsal elements. Together these results identify *Gdf5* as a potential target gene of HOXA13 and confirm a specific role for HOXA13 during appendicular skeletal development.

Keywords

HOXA13; Sox9; Gdf5; mesenchymal condensation; skeletal development; adult phenotype

INTRODUCTION

Mutations in *HOXA13* cause Hand-Foot-Genital- (HFGS) and Guttmacher-(GS) Syndromes, autosomal dominant disorders that profoundly affect limb and genitourinary (GU) development (Stern et al., 1970; Mortlock and Innis, 1997; Innis et al., 2002). In the limb, the loss of HOXA13 function primarily affects autopod development causing syndactyly, preaxial hypodactyly, postaxial polydactyly, and brachydactyly (Burke et al., 1995; Goodman et al., 2000; Innis et al., 2002; Mitsubuchi and Endo, 2006). While gene targeting in mice has provided insight into the cellular and molecular basis for these malformations, these analyses have not been extended beyond embryonic day (E) 15.5, as embryos lacking HOXA13 die due to a required function for HOXA13 in the developing placenta (Fromental-Ramain et al., 1996;

Stadler et al., 2001; Shaut et al., 2008). Prior to embryonic death, HOXA13 functions in the limb to regulate *Bmp2*, *Bmp7*, *EphA7*, and *Sostdc1* to control inter-digital programmed cell death and cell sorting (Stadler et al., 2001; Knosp et al., 2004, 2007; Salsi and Zappavigna, 2006). While it is clear that the perturbation of target genes necessary for apoptosis and cell sorting contributes to the defects in the *Hoxa13* homozygous mutant autopod, it is also possible that a portion of these defects arise as a consequence of the mid-gestational lethality, particularly in the hindlimb which exhibits arrested chondrogenesis and is more severely affected in *Hoxa13* mutants (Fromental-Ramain et al., 1996; Stadler et al., 2001; Knosp et al., 2004, 2007; Salsi and Zappavigna, 2006).

Interestingly, while breeding the *Hoxa13^{GFP}* mutant allele onto a C57 BL/6J genetic background, we identified a small percentage of *Hoxa13^{GFP}* homozygous mutants that survived to adulthood. The survival of *Hoxa13^{GFP}* homozygous mutant adults provided the opportunity to establish whether gestational lethality or the loss of HOXA13 function in the limb caused the defects present in the mutant limb. Analysis of the homozygous adult mutant limbs revealed extensive malformations of the autopod skeleton including reductions in digit length, loss of the second phalanx segments (P2), and fusion of the first and third phalanx segments (P1, P3) in the hindlimb. Characterization of HOXA13 protein in the developing autopod revealed localization in the outer edges of all the carpal/tarsal elements of heterozygous control limbs, whereas homozygous mutant littermates exhibited diffuse expression in the carpal/tarsal regions. *Gdf-5*, which is essential for joint formation, exhibited diffuse expression in the carpal/tarsal regions of *Hoxa13^{GFP}* homozygous mutants. Analysis of the undifferentiated limb mesenchyme revealed small changes in programmed cell death in the hindlimb whereas no differences in proliferation in the cells expressing HOXA13 were detected in the autopod. Taken together, these findings indicate that the loss of HOXA13 function in the limb, rather than mid-gestational lethality, underlies the majority of the brachydactyly and hypodactyly defects. Finally, the expression of HOXA13 in the periphery of each carpal/tarsal skeletal element as well as the fusion of many of these skeletal elements in *Hoxa13^{GFP}* homozygous mutant adults reveals a novel role for HOXA13 in regulating joint development by establishing the boundaries between autopod skeletal elements, a function previously unrecognized in *Hoxa13* homozygous mutants (Fromental-Ramain et al., 1996; Stadler et al., 2001; Knosp et al., 2004, 2007).

RESULTS

Identification of Surviving *Hoxa13^{GFP}* Homozygous Mutant Mice

Male *Hoxa13^{GFP}* heterozygous mutant mice were maintained on a mixed 129SVJ × C57BL/6J x Swiss Webster background. On this mixed genetic background, no surviving *Hoxa13^{GFP}* homozygous mutant mice were detected from more than 200 heterozygous mutant intercrosses (data not shown). This result is consistent with our initial characterization of the *Hoxa13^{GFP}* mutant allele (Stadler et al., 2001) as well as studies of two additional *Hoxa13* mutant alleles first described by Fromental-Ramain and colleagues (1996) and subsequently assessed for gestational lethality by Warot et al. (1997).

Surviving *Hoxa13^{GFP}* homozygous mutants were first detected from heterozygous intercrosses using mice that were bred for three successive generations onto a C57BL/6J genetic background (87.5% C57BL/6J). From these heterozygous mutant intercrosses, we observed several offspring that exhibited hypodactyly of digit I, syndactyly, and stiffness during walking (Fig. 1). Genotyping of these offspring confirmed that all the affected mice were homozygous for the *Hoxa13^{GFP}* mutant allele (Fig. 1). Of the 1,250 offspring produced by heterozygous mutant intercrosses, 11 homozygous mutants (10 female, 1 male) were recovered, and all exhibited hypodactyly of digit I as well as some degree of syndactyly between the remaining digits (data not shown). No autopod skeletal defects were detected in any heterozygous mutant

or wildtype offspring and, for this reason, both heterozygous mutant and wildtype mice were used as controls. Two additional crosses onto the C57BL/6J background (96.8% C57BL/6J) did not increase the frequency of surviving homozygous mutants or the severity of the autopod defects. Both male and female *Hoxa13^{GFP}* adult homozygous mutants were infertile (data not shown).

Analysis of the Adult *Hoxa13^{GFP}* Mutant Skeleton

Alcian blue and alizarin red staining of the surviving mutant forelimbs (22 total) and hindlimbs (22 total) revealed a complex series of autopod skeletal defects including absent skeletal elements, skeletal element fusion, and reduced skeletal element size. In the forelimb, the digit I phalanx element was absent in all limbs examined (22/22) whereas the metacarpal element I was absent in 10 of the 22 mutant forelimbs examined (Fig. 2). Characterization of metacarpal lengths of 3-month-old adults ($n = 3$ control and 3 homozygous mutant) revealed significant reductions in metacarpal elements II–V ($P \leq 0.01$) whereas metacarpal I did not exhibit a significant reduction in length ($P \leq 0.12$) (Fig. 2I and Supp. Table 1, which is available online). In the proximal forelimb autopod, the radiale and ulnare were reduced in size as were the distal carpal bones 2–5 in all mutant forelimbs (Fig. 2K and L). The central carpal bone was fused to carpal bone 2 in more than half of the mutant forelimbs (12/22) (Fig. 2L). No differences in the frequency of these defects were detected between the right and left forelimbs.

In the hindlimb, malformations of the digits and tarsal elements were more extensive compared to the homozygous mutant forelimb. Notably, the P2 phalanx segment was absent in all digits and metatarsal elements I–V were significantly shorter ($P \leq 0.01$) in all mutant hindlimbs (Fig. 2E–H, J and Supp. Table 2). Interestingly, in two homozygous mutant left hindlimbs, the P1 and P3 phalanx segments for digits III and IV were fused, creating a single proportionally larger P1 and P3 skeletal element (Fig. 2G). In the proximal autopod, the central tarsal elements were fused and undifferentiated, creating a mass of alcian blue–stained tissue (Fig. 2N). Fusions of the navicular and tarsal element 1 were present in all homozygous mutants, whereas fusions of the calcaneus and cuboideum were present in 17 of the 22 mutant hindlimbs examined (Fig. 2N). No defects were detected in the lumbar, sacral, or caudal vertebral elements in homozygous mutants compared to wild type or heterozygous mutant controls (data not shown).

HOXA13 Expression in the Carpal/Tarsal Regions

While previous studies of HOXA13 function have focused on its roles in limb mesenchyme condensation, interdigital programmed cell death, and zeugopod muscle development, less is known about the expression and function of *Hoxa13* in the developing carpal/tarsal regions (Fromental-Ramain et al., 1996; Stadler et al., 2001; Yamamoto and Kuroiwa, 2003; Knosp et al., 2004, 2007). Indeed, while previous studies of *Hoxa13* have detected expression in the digits and interdigital tissues of mid-gestation limbs (E13.5–14.5), no expression was seen in the carpal/tarsal regions using whole mount in situ hybridization (Knosp et al., 2004). Here we hypothesized that poor riboprobe penetration into the thicker carpal/tarsal regions underlies the absence of detected *Hoxa13* transcripts. To test this hypothesis, we used section in situ hybridization to characterize the expression of *Hoxa13* at E 14.5 in the developing carpal/tarsal regions (Fig. 3). By this approach, strong *Hoxa13* expression was detected in the distal digit tips as well as in the cells surrounding the developing metacarpal/metatarsal and carpal/tarsal skeletal elements of wildtype embryos (Fig. 3A and C). In homozygous mutants, *Hoxa13* was expressed similar locations in the digit tips and interdigital tissues; however, more diffuse expression was seen throughout carpal/tarsal regions, which lacked many of the developing skeletal elements (Fig. 3B and D). Finally, *Hoxa13* transcripts were also detected in the developing zeugopod musculature in both wildtype and homozygous mutant limbs, a finding consistent with HOXA13's previously reported role as a regulator of *MyoD* expression in the zeugopod musculature (Fig. 3A–D black arrowheads) (Yamamoto and Kuroiwa, 2003).

Characterization of HOXA13-GFP protein in E 14.5 limbs revealed similar sites of expression and localization as detected for *Hoxa13* transcripts (Fig. 4). In heterozygous control limbs, low levels of HOXA13 protein were consistently detected in maturing skeletal element condensations whereas homozygous mutants appeared to maintain HOXA13-GFP expression throughout the carpal and tarsal regions with less restriction of HOXA13 to the cells surrounding the skeletal elements (Fig. 4A–D). The maintenance of HOXA13-GFP expression in the maturing mutant skeletal element condensations suggests that HOXA13 may down-regulate its own expression during skeletal element maturation. This finding is consistent with our previous analysis of *Hoxa13* mRNA localization in E13–14.5 homozygous mutant limbs, which detected elevated levels of *Hoxa13* transcripts in the maturing digit skeletal condensations of homozygous mutants (Knosp et al., 2004). Finally, HOXA13-GFP protein was also present in the mutant and control zeugopod where expression was detected in the cells surrounding the tibia and in the developing musculature (Fig. 4E–L). This finding is consistent with the detection of *Hoxa13* mRNA in the zeugopod (Fig. 3) as well as previously described immunohistochemical detection of HOXA13 in this same region (Fig. 4E–L) (Yamamoto and Kuroiwa, 2003).

Expression of *Sox9* and *Gdf5* in the Homozygous Mutant Carpal/Tarsal Region

Recognizing that SOX9 plays an essential role in chondrogenesis, we hypothesized that its expression may be disrupted in *Hoxa13^{GFP}* homozygous mutant autopods (Wright et al., 1995; Uchida et al., 1996; Wheatley et al., 1996; Ng et al., 1997; Akiyama et al., 2002, 2005; Akiyama, 2008). Characterization of *Sox9* mRNA localization by in situ hybridization revealed strong expression in all autopod cartilage condensations, which as early as E 13.5 showed a clear delineation of the cartilage condensations comprising the phalanges (I–V), metacarpals, metatarsals, and carpal/tarsal elements including the radiale, ulnare, calcaneous, and talus (Fig. 5A and C). In *Hoxa13^{GFP}* homozygous mutant forelimbs, strong *Sox9* expression was detected in the phalanges and the metacarpal elements corresponding to digits II–V (Fig. 5B). However, in the digit I region, *Sox9* expression was not localized to a defined cartilage condensation but exhibited diffuse expression throughout the digit I phalanx and metacarpal regions (Fig. 5B). The homozygous mutant forelimb also exhibited diffuse *Sox9* expression in the carpal region with poor delineation of developing carpal elements including the radiale and ulnare (Fig. 5B). In the mutant hindlimb, *Sox9* was diffusely expressed in the digit I region as well as in the tarsals and proximal metatarsals for digits II–V (Fig. 5D).

Next, because the loss of GDF signaling results in carpal/tarsal element fusions similar to those present in *Hoxa13^{GFP}* homozygous mutants, we examined whether *Gdf5* mRNA localization was perturbed in homozygous mutant limbs (Storm et al., 1994; Storm and Kingsley, 1999; Settle et al., 2003). Analysis of *Gdf5* expression in wild type forelimbs revealed consistent expression in cells flanking the phalanx and metacarpal elements as well as in the cells surrounding the developing carpal elements (Fig. 5E). In contrast, homozygous mutant forelimbs exhibited diffuse *Gdf5* expression in the digit condensations with nearly a complete absence of *Gdf5* transcripts in the digit I region (Fig. 5F). Diffuse *Gdf5* expression was seen in the mutant carpal elements compared to wild type controls (Fig. 5E and F). Wild type hindlimbs also exhibited strong *Gdf5* expression in the phalanx, metatarsal, and tarsal joint fields, whereas homozygous mutant littermates exhibited diffuse or low levels of *Gdf5* expression in the corresponding phalanx, metatarsal, and tarsal elements (Fig. 5G and H). Quantitation of prochondrogenic gene expression in the affected carpal/tarsal regions using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed a significant decrease in the expression of *Gdf5* ($P \leq 0.001$) and a significant increase in the expression of *Bmp2* ($P \leq 0.001$). No differences in the expression of *Bmp4*, *Bmp6*, *Bmp7*, or *Sox9* were detected between *Hoxa13^{GFP}* homozygous mutants and wild type controls ($P \leq 0.52$) (Fig. 6).

Analysis of Programmed Cell Death and Cell Proliferation in Limb Progenitors

Recognizing that *Hoxa13* is expressed in the autopod progenitor cell population, and that perturbations in the number of progenitor cells could cause the hypodactyly or brachydactyly exhibited by *Hoxa13^{GFP}* homozygous mutants, we examined whether cell proliferation and programmed cell death were affected in the undifferentiated mesenchyme of homozygous mutant limbs (Barna and Niswander, 2007; Lu et al., 2008). TUNEL analysis of forelimb and hindlimb autopods at E 11.5 revealed programmed cell death in the apical ectodermal ridge (AER) and anterior limb bud (Fig. 7). In the forelimb, no differences in TUNEL-positive tissues were detected in either the AER or the anterior limb bud (digit I region) between wild type (not shown), heterozygous mutant, or homozygous mutant littermates (4 of 4 mutant forelimbs examined (Fig. 7A and B). Interestingly, a larger region of TUNEL-positive cells was consistently present in the anterior hindlimb mesenchyme of the homozygous mutant (4 of 4 mutant hindlimbs examined) (Fig. 7D). Finally, using the GFP-tagged HOXA13 protein in conjunction with the mitosis antibody, antiphosphohistone H3 (APH3), we examined cell proliferation in the HOXA13 expression domain (green signal). Quantitation of the mitotic cells (red signal) present within the HOXA13GFP expression domain revealed no significant differences in mitotic cell number between homozygous mutant and heterozygous control forelimbs (4 of 4 mutant forelimbs examined) ($P \leq 0.28$) (Fig. 7E–L). Similarly, a comparison of mitotic cells present in the HOXA13 expression domain of homozygous mutant and heterozygous control hindlimbs also revealed no significant difference in mitotic cell number ($P \leq 0.13$).

DISCUSSION

Previous studies of murine HOXA13 function suggest an essential role in distal limb development, as homozygous mutant embryos exhibit a complete loss of digit I as well as many of the carpal and tarsal skeletal elements as late as E 15.5 (Fromental-Ramain et al., 1996; Stadler et al., 2001; Knosp et al., 2004, 2007). However, because *Hoxa13* homozygous mutants die by mid-gestation, the limb defects present in these embryos could reflect a secondary consequence of failing embryonic health. Our analysis of the autopod skeleton in surviving *Hoxa13^{GFP}* homozygous mutants provides evidence that skeletogenesis is delayed, as portions of the digit I metacarpus/metatarsus, which are missing in the mid-gestation mutant embryo, do eventually form, although they are often fused and reduced in size in the adult skeleton (Fromental-Ramain et al., 1996; Stadler et al., 2001). More importantly, the continued absence of several skeletal elements such as distal digit I and the P2 phalanx segments in the hindlimb also indicates that HOXA13 plays a specific and essential role in the formation of these structures.

Why the most distal structures in the autopod are more severely affected by the loss of HOXA13 function is unclear. One possible explanation is that decreased cell proliferation and/or increased programmed cell death in the distal autopod accounts for the loss of the structures. While we did not detect changes in cell proliferation, we did detect a small increase in programmed cell death in the anterior hindlimb of homozygous mutants. This small increase in programmed cell death could affect the formation of the anterior autopod skeletal elements. Indeed, recent studies of the autopod progenitor pools indicate that *Hoxa13* is strongly expressed in the distal autopod progenitors and that depletion of this progenitor pool causes both a delay in the formation of autopod skeletal elements as well as a loss in distal autopod structures (Lu et al., 2008).

Why Do *Hoxa13* Homozygous Mutants Survive?

While the survival of *Hoxa13^{GFP}* homozygous mutants demonstrates a specific role for HOXA13 in autopod skeletal development, it also prompts the question as to why only a small

percentage of these mice survive when previous characterizations of *Hoxa13* homozygous mutants failed to detect surviving mutants beyond E 15.5 (Fromental-Ramain et al., 1996; Stadler et al., 2001). One explanation for the survival of *Hoxa13^{GFP}* homozygous mutants could be variable penetrance of the placental vascular defect, which we previously established causes gestational lethality (Shaut et al., 2008). While we cannot exclude this possibility, our analysis of the embryonic placental vascular labyrinth in more than 24 homozygous mutants at both E 12.5 and E 13.5 consistently detected severe disruptions in the embryonic placental labyrinth vasculature, and suggests that other factors may be contributing to the survival of *Hoxa13^{GFP}* homozygous mutants (Shaut et al., 2008). An alternative possibility is that the introduction of strain-specific modifying loci from the C57BL/6J genetic background that were not enriched in the initial *Hoxa13^{GFP}* mouse colony may be contributing to homozygous mutant survival. The C57BL/6J background has been shown to exert a protective effect on gestational defects in neo-angiogenesis, a result attributed to a threefold overexpression of the VEGF receptor, FLK-1 (Hiratsuka et al., 2005; Gigante et al., 2006). More importantly, even a 50% C57BL/6J genetic background has been shown to rescue gestational lethality caused by vascular defects, particularly when combined with modifying loci present in the 129S2/SvHsd background (Tang et al., 2003). Because *Hoxa13^{GFP}* homozygous mutants also die from defects in the embryonic placental vascular labyrinth, it is possible that a protective effect may be derived from the C57BL/6J genetic background. Moreover, because HOXA13 regulates multiple vasculogenic genes during the formation of the placental vascular labyrinth, including *Tie2*, *Neuropilin-1*, *Enpp2*, and *Foxf1*, the C57BL/6J protective effect would not be completely penetrant, providing an explanation for the low number of surviving homozygous mutants (Shaut et al., 2008).

An Extended Role for HOXA13 in BMP-Regulated Skeletogenesis?

From this work, as well as our previous studies of syndactyly and the loss of digit I in *Hoxa13^{GFP}* mutant limbs, it appears that HOXA13 controls multiple aspects of autopod development through unique and tissue-specific combinations of BMP signaling molecules. Indeed, while the regulation of *Bmp2* and *Bmp7* by HOXA13 facilitates interdigital programmed cell death and provides a functional explanation for syndactyly present in *Hoxa13^{GFP}* mutant limbs, neither *Bmp2* nor *Bmp7* appear to be misexpressed in the digit I region of homozygous mutant limbs (Knosp et al., 2004, 2007). Instead, digit I defects may be caused by the loss of suppression of a BMP antagonist, *Sostdc1*, which is ectopically expressed in the digit I region of mutant limbs (Knosp et al., 2007). Additional BMP signaling components may also be affected in the digit I region, as SMAD 1,5,8 phosphorylation and BMP-regulated genes such as *Msx1*, *Ihh*, *Collagen II*, and *Collagen X* were also down-regulated in mutant digit I (Knosp et al., 2007). Moreover, the affected skeletal elements including digit I consistently exhibit a loss of mesenchymal cell condensation, a process that requires BMP signaling to facilitate the initial compaction of the pre-chondrogenic mesenchyme (Barna and Niswander, 2007). Finally, *Gdf5* represents yet another member of the BMP family whose expression in the carpal/tarsal regions may be regulated by HOXA13 as the loss of HOXA13 function causes a significant decrease in *Gdf5* expression and mice lacking GDF5 exhibit fusions of the carpal/tarsal elements similar to those present in *Hoxa13^{GFP}* homozygous mutants (Storm et al., 1994; Storm and Kingsley, 1999; Baur et al., 2000; Settle et al., 2003; Tylzanowski et al., 2006).

The Loss of HOXA13 Function Impacts Multiple Aspects of Autopod Skeletogenesis

In the murine limb, skeletogenesis proceeds from a highly conserved series of branching, segmentation, and de novo mesenchymal condensations (Archer et al., 1984; Shubin and Alberch, 1986; Shubin, 1991). Our characterization of the skeletal defects in the adult mutant limb suggests that the loss of HOXA13 function impacts skeletogenesis during mesenchyme condensation and possibly boundary establishment. Where structures fail to form, such as the

P2 phalanx elements and distal digit I, defective mesenchymal condensation appears to be the precipitating event as cell proliferation was unaffected in the undifferentiated mesenchyme and only the anterior part of the mutant hindlimb exhibited any changes in programmed cell death. Moreover, although not condensed, the cells present in the affected digit I, carpal, and tarsal regions still express *Sox9*, suggesting that HOXA13-regulated mesenchymal condensation is an essential process for cartilage development that may precede SOX9 and/or function independently in the autopod chondrogenic program (Uchida et al., 1996; Wheatley et al., 1996; Ng et al., 1997; Akiyama et al., 2002; Akiyama, 2008). In the central axis of the hindlimb, the fusion of the digit III and IV phalanges suggests a failure in the delineation of the boundaries between digits III and IV rather than a branching defect of the axial autopod condensations, which occur in the more proximal tarsal-metatarsal boundary (Shubin and Alberch, 1986; Shubin, 1991). Finally, the fusion of the carpal and tarsal elements as well as the loss of the P2 element suggests that HOXA13 mediates a final stage of autopod skeletogenesis by defining the segmentation and boundaries of the individual skeletal elements. As discussed earlier, BMP signaling is clearly required for this final process; however, it is not known whether HOXA13 directly regulates components of the BMP signaling pathway to facilitate skeletal element segmentation and boundary establishment (Storm and Kingsley, 1999; Baur et al., 2000; Settle et al., 2003; Barna and Niswander, 2007; Lu et al., 2008).

In summary, the survival of *Hoxa13^{GFP}* homozygous mutants indicates that the defects present in the embryonic autopod reflect an autopod-specific loss of HOXA13 function rather than a secondary consequence of failing embryonic health. The scope and severity of the skeletal defects present in the mutant autopod suggest that HOXA13 is functioning at multiple stages of mesenchymal condensation, branching, and segmentation. Finally, the identification of *Sox9* expression in digit I and the carpal and tarsal regions that failed to condense in the homozygous mutant autopod suggests that mesenchymal condensation precedes SOX9 function and that HOXA13 plays an essential role in the formation and patterning of the pre-chondrogenic limb mesenchyme. Together, these findings provide new insight into transcriptional hierarchies required for mesenchymal condensation and skeletal development in the tetrapod limb.

EXPERIMENTAL PROCEDURES

Generation of the *Hoxa13^{GFP}* x C57BL/6J Mutant Mice and Genotyping

The production of the founder mice bearing the *Hoxa13^{GFP}* mutant allele was originally described (Stadler et al., 2001) and the allele was subsequently maintained on a mixed genetic background consisting of C57BL/6J; Swiss Webster, and 129SVJ. Genotyping of the mutant *Hoxa13^{GFP}* was performed as described (Stadler et al., 2001). The enrichment of the *Hoxa13^{GFP}* mutant allele onto a C57BL/6J genetic background was achieved by five successive rounds of mating male mice heterozygous for the mutant *Hoxa13^{GFP}* allele to C57BL/6J females. Briefly, female C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were bred to *Hoxa13^{GFP}* heterozygous mutant male mice. F1 male offspring derived from this initial cross were genotyped using PCR and primers specific for the mutant *Hoxa13^{GFP}* allele as previously described (Stadler et al., 2001). F1 male offspring positive for the *Hoxa13^{GFP}* mutant allele were allowed to mature and breed to a new cohort of C57BL/6J females to produce F2 male offspring heterozygous for the *Hoxa13^{GFP}* mutant allele. This process was repeated up to three additional times to produce a final F5 generation of male and female mice heterozygous for the *Hoxa13^{GFP}* mutant allele. Intercrosses between heterozygous mutants at the F3 or F5 level of enrichment onto the C57BL/6J background were used to produce the surviving *Hoxa13^{GFP}* homozygous mutant mice. All experimental procedures, handling, and housing of the *Hoxa13^{GFP}* mouse colony were done in accordance with an approved animal protocol (OHSU protocol number: IS00000226 to H.S.S.).

Skeletal Preparations and Analysis

Skeletal preparations of adult *Hoxa13*^{GFP} mice were produced using a modification of the procedure described by Taylor (1967). The skin and all the internal organs were removed from the adult mice. The bodies were directly fixed in 95% ethanol for 5 days, and then incubated in acetone for 2 days to remove lipids. The bodies were stained for 10 days at 37°C in 30 ml of a fresh alcian blue/alizarin red S stain solution (15 mg alcian blue 8GX, 5 mg alizarin red S dissolved in 5% acetic acid, 75 ml of 95% ethanol, 20 ml H₂O). After staining, the bodies were washed with distilled water for 2 hr, the tissue was cleared by placing the skeletal preparations in a 30% saturated sodium borate, 1% trypsin solution, and incubating at 37°C for 6 hr. After the bones were clearly visible, the skeletons were transferred to a 1% KOH, 20% glycerol solution and incubated at RT for 3 weeks, followed by 1% KOH, 50% glycerol, then 1% KOH, 80% glycerol, for 1 week each. The skeletons were permanently stored in 100% glycerol. The stained skeletons were placed in a glass dish filled with 100% glycerol and photographed using a Canon EOS 40D camera equipped with a 100-mm f2.8 ultrasonic motor macro lens. High-magnification images of carpal and tarsal bones were produced using the same camera attached to a Leica MZ FLIII stereoscope. The metatarsals and metacarpal elements from six control and six homozygous mutant adults at six months of age were measured for length using photographs taken at the same magnification and the measuring tool in Adobe Photoshop CS4 extended edition. The measured values were entered into a Microsoft Excel spreadsheet and assessed for statistical differences using a Student's *t*-test. Structures distal to the metacarpal/metatarsal elements were not measured due to excessive curvature of the individual skeletal digits.

In Situ Hybridization and Real-Time qRT-PCR

The mouse *Gdf5* and *Sox9* riboprobes were generated from plasmid expression constructs generously provided by Ronen Schweitzer (*Sox9*) and Cliff Tabin (*Gdf5*). Riboprobe synthesis and hybridization were performed on frozen sections using a modification of the in situ hybridization protocol described by Murtaugh et al. (1999). The *Hoxa13* riboprobe was synthesized as previously described (Knosp et al., 2004). Limbs were fixed overnight in 4% paraformaldehyde at 4°C. After fixation, the autopods were equilibrated in an increasing series of 1 × diethylpyrocarbonate-treated phosphate buffered saline (PBS) containing 15% and 30% sucrose at 4°C. After equilibrating in the 30% sucrose/PBS solution, the limbs were embedded in OCT (Tissue-Tek) and frozen. The OCT-embedded limbs were sectioned at a thickness of 20 µm using a Leitz Cryostat and placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and processed for in situ hybridization as described by Murtaugh et al. (1999). For all analyses, control and homozygous mutant limbs were treated identically using the same preparation of riboprobe, hybridization buffer, temperatures, and colorimetric detection times. After stopping the color reactions, the sectioned autopods were covered with 10% glycerol/PBS, coverslipped, and photographed using a Leica DMLB2 microscope equipped with DIC and a Q Imaging Micropublisher V digital camera. Individual photographs were assembled into limb photo montages using Adobe Photoshop CS4 extended edition.

Gene expression levels were quantitated using a real-time quantitative reverse transcriptase polymerase chain reaction method (qRT-PCR). Briefly, *Hoxa13* wild-type and homozygous mutant E13.5 carpal/tarsal regions were microdissected and pooled in DEPC-treated PBS and snap frozen on dry ice. After freezing, carpal/ tarsal total RNA was isolated using Trizol as described by the manufacturer (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using 1 µg total RNA according to the manufacturer's protocol (Super-Script III, Invitrogen). A minimum of three independent samples of each genotype (*Hoxa13* wild-type or homozygous mutant) were used for qRT-PCR analysis with the SYBR Green PCR Super Mix (BioRad, Hercules, CA) on a BioRad IQ5 real-time thermal cycler according to the manufacturer's instructions (BioRad). Fold change expression levels were determined after normalization of

the amplification products to *Gapdh* expression using the BioRad IQ5 software suite. The fold change differences were entered into a Microsoft Excel Spreadsheet and assessed for statistical differences using a Student's *t*-test. The following primer pairs were used for the qRT-PCR analysis: Bmp2F: GCTGTCTTCTAGTGTGCTGCT; Bmp2R: GCCTCAACTCAAATTCGC; Bmp4F: CCG AGCCAACACTGTGAGGA; Bmp4R: TGGGATGCTGCTGAGGTTGA; Bmp6F: CCCCACATCAACGACACCAC; Bmp6R: TCCCCACCACACAGTCCTTG; Bmp7F: GGAAGCATCTAAGGGTTCCA; Bmp7R: TTCCTGGCAGACATTTTTC; Gdf5F: GGGCAAGATGACCGAGGC; Gdf5R: ACTGGGGACCGCTGGCTT; Sox9F: AGGAAGCTGGCAGACCAGTA; Sox9R: TGTAATCGGGGTGGTCTTTC; GapdhF: CCACCCAGAAGACTGTGGAT; GapdhR: TTCAGCTCTGGGATGACCTT.

Immunohistochemistry and TUNEL Analysis

Cell proliferation in the autopod was assessed using an anti-phosphohistone H3 antibody (Millipore, Billerica, MA), which detects all cells progressing from G2 to M in the cell cycle (Hendzel et al., 1997). Quantitation of the mitotic cells within the HOXA13-GFP expression domain was performed as previously described (Morgan et al., 2003). Programmed cell death was assessed using a modification of the terminal UTP nick end labeling (TUNEL) technique as described (Maden et al., 1997). Briefly, limbs from E11.5–13.5 embryos were fixed at room temperature for 2–4 hr in 4% paraformaldehyde. After fixing, the limbs were washed for 3 hr at room temperature with several changes of 1× PBS containing 1% TritonX-100. The limbs were placed in 2.0-ml microfuge tubes and pre-incubated at 37°C for 30 min in PBS containing 1× terminal transferase buffer (Roche), 1% Triton X-100, and 2.5mM CoCl₂. The pre-incubation buffer was replaced with a 1× PBS solution containing 1× terminal transferase buffer, 10 mM dUTP (2:1 dUTP:-dUTP-biotin), 2.5 mM CoCl₂, 1% Triton X-100, and 5,000 U recombinant terminal transferase (Roche) per 1 ml of buffer, and incubated at 37°C for 3 hr. The limbs were then washed for 3 hr in PBS and incubated overnight at 4°C with streptavidin conjugated with Texas Red (Jackson Immunological). After washing for 1 hr in PBS, confocal analysis of the limbs was performed as described by Morgan et al. (2003).

Confocal Analysis of GFP Expression

Embryonic day-14.5 limbs were collected from *Hoxa13*^{GFP} heterozygous control and homozygous mutant embryos. The harvested limbs were processed for cryosectioning as described earlier. Limb sections ranging from 20 μm in thickness were placed on Superfrost Plus (Fisher Scientific) slides and allowed to rehydrate in 1× PBS. After rehydration, the cryosections were examined for HOXA13-GFP expression using Bio-Rad MRC 1024 confocal imaging system fitted with a Leica DMRB microscope, using filter sets provided by the manufacturer. Cryosections were imaged in single planes using the GFP-tagged nucleus as the primary focal plane. A Kalman digital averaging filter was used to reduce random noise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Surviving *Hoxa13^{GFP}* homozygous mutants exhibit malformations of the distal autopod including: hypodactyly, brachydactyly, and syndactyly. **A–D**: Comparison of wild type and homozygous mutant forelimb and hindlimb in 6-month-old littermates. Note the syndactyly and hypodactyly in the mutant forelimb and hindlimb. **E**: PCR genotyping of the 6-month-old *Hoxa13^{GFP}* mice confirmed that the mouse exhibiting autopod defects, sample A1, is a homozygous mutant. **F**: Representation of the *Hoxa13^{GFP}* targeted mutation and PCR genotyping strategy. Note that the GFP expression cassette replaces the last portion of *Hoxa13* exon 2, which encodes the third helix of the HOXA13 homeodomain.

Fig. 2.

Analysis of the appendicular skeleton in 3-month-old *Hoxa13^{GFP}* littermates. **A,B,E,F:** Analysis of wild type forelimbs and hindlimbs. Roman numerals I–V denote the digit skeletal elements. Phalanx segments are denoted by p1, p2, and p3. m, metatarsal skeletal element. **C,D,G,H:** Analysis of surviving homozygous mutant forelimbs and hindlimbs. Roman numerals I–V denote the digit skeletal elements. Shortened or missing digits are denoted by roman numerals flanked by an apostrophe. m, metatarsal; p1 and p3, phalanx segments. Note that the homozygous mutant hindlimbs are missing the p2 phalanx segment and that the p1 and p2 phalanx segments for digits III and IV are fused in the homozygous mutant left hindlimb. Blue staining, Alcian blue, which detects cartilage and connective tissue; red staining, alizarin

red, which detects mineralized bone. Bar represents 2 mm. **I**: Analysis of metacarpal lengths in control (black bars) and homozygous mutants (grey bars). Asterisks denote a significant difference in metacarpal length between control and homozygous mutant mice ($P \leq 0.01$). **J**: Analysis of metatarsal lengths in control (black bars) and homozygous mutants (grey bars). Asterisks denote a significant difference in metatarsal length between control and homozygous mutant mice ($P \leq 0.001$). **K,L**: Metacarpal and carpal elements present in the wild type and homozygous mutant left forelimbs. Roman numerals I–V, digit metacarpals; 1–5, distal carpal skeletal elements; U, ulna; R, radius; c, central carpal element; u, ulnare; r, radiale; pm, post-minimus bone. Malformed or fused skeletal elements are flanked by an apostrophe. **M,N**: Metatarsal and tarsal elements present in the wild type and homozygous mutant left hindlimb. Roman numerals I–V, digit metatarsals; 1–3, distal tarsal skeletal elements; ca, calcaneous; ta, talus; nav, naviculare; cub, cuboideum. Malformed or fused skeletal elements are flanked by an apostrophe. Blue staining, Alcian blue, which detects cartilage and connective tissue; red staining, alizarin red, which detects mineralized bone.

Fig. 3. Localization of *Hoxa13* mRNA in E 14.5 limbs using section in situ hybridization. **A:** *Hoxa13* expression in wildtype forelimbs. **B:** *Hoxa13* expression in homozygous mutant forelimbs. **C:** *Hoxa13* expression in wildtype hindlimbs. **D:** *Hoxa13* expression in homozygous mutant hindlimbs. Arrowheads denote expression in the zeugopod musculature. R, radius; U, ulna; T, tibia; F, fibula.

Fig. 4. Distribution of HOXA13-GFP protein in the carpal and tarsal skeletal elements of E 14.5 *Hoxa13^{GFP}* embryos. **A:** Heterozygous control forelimb. Note the localization of HOXA13-GFP to the periphery of each skeletal element. Roman numerals I–V denote the digit metacarpals. Distal carpal elements are denoted by the numbers 1–5. r, radiale; c, central carpal bone. **B:** HOXA13-GFP is diffusely distributed in the carpal region of homozygous mutants, which exhibit poor delineation of the individual carpal skeletal elements. Roman numerals I–V denote the digit metacarpals. Distal carpal elements are denoted by the numbers 2–4. Fused or malformed carpal elements are flanked by an apostrophe. c, central carpal bone. **C:** Localization of HOXA13-GFP protein to the periphery of each tarsal and metatarsal skeletal

element in a heterozygous control hindlimb. Roman numerals III–V represent the metatarsal elements. 3, distal tarsal element associated with metatarsal III; cub, cuboideum; nav, naviculare; ca, calcaneous; ta, talus. **D**: HOXA13-GFP is diffusely distributed in the tarsal region of homozygous mutants, which exhibit poor delineation of metatarsal IV and the cuboidal (c) element as well as poor separation of metatarsal III from the third tarsal element (3). Roman numerals III–V denote the digit metatarsals. ca, calcaneus; ta, talus. Fused or malformed tarsal elements are flanked by an apostrophe. **E**: HOXA13-GFP expression in the forelimb zeugopod of a heterozygous control. Arrowhead denotes expression in the zeugopod musculature. **F**: Bright field image of E. **G**: HOXA13-GFP expression in the forelimb zeugopod of a homozygous mutant. Arrowheads denote expression in the zeugopod musculature. **H**: Bright field image of G. **I**: HOXA13-GFP expression in the hindlimb zeugopod of a heterozygous control. Arrow denotes expression in the cells surrounding the tibia. Arrowheads denote HOXA13-GFP expression in the zeugopod musculature. **J**: Bright-field image of I. **K**: HOXA13-GFP expression in the hindlimb zeugopod of a homozygous mutant. Arrow denotes expression in the cells surrounding the tibia. Arrowheads denote expression in the zeugopod musculature. **L**: Bright-field image of K. R, radius; U, ulna; T, tibia; F, fibula. Bars = 50 μ m.

Fig. 5.

Expression of *Sox9* and *Gdf5* in the E 13.5 autopod of wild type and *Hoxa13^{GFP}* homozygous mutants. **A:** *Sox9* expression is restricted to the condensing mesenchyme contributing to digits I–V as well as in the developing carpal in the wild type forelimb. r, radiale condensation; u, ulnare condensation. Arrow denotes the detection of *Sox9* expression in the condensing mesenchyme contributing to the digit I metacarpal region. **B:** Expression of *Sox9* in the homozygous mutant forelimb. Arrowhead denotes reduced condensation of the *Sox9*-expressing cells in the digit I region. Poor condensation was also detected in the *Sox9*-expressing cells in the radiale condensation (r). u, ulnare condensation. **C:** Expression of *Sox9* in the wild type hindlimb mesenchymal condensations contributing to digits I–V. White arrowheads denote the detection of *Sox9* expression in the condensations contributing to the digit metatarsal elements. Note the condensations forming the calcaneus (ca) and talus (ta) are already established in the control limb. **D:** Expression of *Sox9* in the homozygous mutant hindlimb. Note the diffuse expression in the regions contributing to the presumptive calcaneus (c) and talus (t). Black arrowheads denote the sites of metatarsal formation that exhibit missing or low levels of *Sox9* expression. A defined boundary is also absent between the talus and the digit I region, which contains a uniform layer of *Sox9*-expressing cells. **E:** *Gdf5* transcripts are localized to the joint fields of the digits as well as to the sites of carpal element delineation. Roman numerals denote the forelimb digits. Carpal skeletal elements 3 and 4/5 are also indicated. **F:** Homozygous mutant forelimbs exhibit reduced *Gdf5* expression in the digit I region as well as diffuse expression in the developing carpal elements. Roman numerals denote the forelimb digits. Carpal skeletal elements 3 and 4/5 are indicated and represent the only distinguishable carpal elements in the mutant forelimb. **G:** *Gdf5* expression in the wild type hindlimb corresponds to the sites of joint formation in the digits as well as the sites of tarsal element delineation. Roman numerals denote the developing digits. nav, naviculare tarsal element. **H:** Homozygous mutant hindlimb exhibits reduced and diffuse *Gdf5* expression in digits II–V. No *Gdf5* expression was detected in the digit I region or in the majority of the developing tarsal elements. nav, malformed naviculare tarsal element. Bar = 50 μ m.

Fig. 6. qRTPCR analysis of prochondrogenic gene expression in the carpal/tarsal region of *Hoxa13^{GFP}* homozygous mutants. Grey bars represent the fold-change differences in expression (Mutant/Wildtype). Asterisks denote a significant fold-change difference in gene expression. Inset depicts the region dissected for carpal/tarsal total RNA isolation.

Fig. 7.

Analysis of programmed cell death and proliferation in E 11.5 *Hoxa13^{GFP}* homozygous mutant and heterozygous control limbs. **A,C**: Fluorescent detection of programmed cell death using terminal UTP nick end labeling (TUNEL) in heterozygous control forelimbs and hindlimbs indicates a low level of programmed cell death (red signal) in the anterior autopod mesenchyme (white arrowhead) with higher levels of TUNEL-positive cells in the apical ectodermal ridge (aer; white arrow). **B,D**: Detection of TUNEL-positive cells in homozygous mutant forelimbs and hindlimbs reveals similar levels of programmed cell death in the aer compared to controls (white arrow) whereas the homozygous mutant hindlimb exhibited a greater amount of TUNEL-positive cells in the anterior limb bud (white arrowhead). Homozygous mutant forelimbs did not exhibit any increase in the number of TUNEL-positive cells in the anterior limb bud compared to heterozygous controls (white arrowhead). Bar = 100 μ m. **E,I,G,K**: Immunohistochemical detection (red signal) of mitotic cells using an anti-phosphohistone H3 antibody (APH3) in cryosections of heterozygous control forelimbs and hindlimbs expressing HOXA13-GFP (green signal). **F,J,H,L**: APH3 localization in the homozygous mutant autopod cryosections reveals similar levels of mitotic cells (red signal) in the regions expressing HOXA13-GFP (green). Yellow cells in I–L denote mitotic cells (APH3-positive) that are also expressing HOXA13-GFP. Bar = 100 μ m (A–D) and 50 μ m (E–L).