LIM homeobox transcription factors integrate signaling events that control three-dimensional limb patterning and growth

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Vertebrate limb development is controlled by three signaling centers that regulate limb patterning and growth along the proximodistal (PD), anteroposterior (AP) and dorsoventral (DV) limb axes. Coordination of limb development along these three axes is achieved by interactions and feedback loops involving the secreted signaling molecules that mediate the activities of these signaling centers. However, it is unknown how these signaling interactions are processed in the responding cells. We have found that distinct LIM homeodomain transcription factors, encoded by the LIM homeobox (LIM-HD) genes Lhx2, Lhx9 and Lmx1b, integrate the signaling events that link limb patterning and outgrowth along all three axes. Simultaneous loss of Lhx2 and Lhx9 function resulted in patterning and growth defects along the AP and the PD limb axes. Similar, but more severe, phenotypes were observed when the activities of all three factors, Lmx1b, Lhx2 and Lhx9, were significantly reduced by removing their obligatory co-factor Ldb1. This reveals that the dorsal limb-specific factor Lmx1b can partially compensate for the function of Lhx2 and Lhx9 in regulating AP and PD limb patterning and outgrowth. We further showed that Lhx2 and Lhx9 can fully substitute for each other, and that Lmx1b is partially redundant, in controlling the production of output signals in mesenchymal cells in response to Fgf8 and Shh signaling. Our results indicate that several distinct LIM-HD transcription factors in conjunction with their Ldb1 co-factor serve as common central integrators of distinct signaling interactions and feedback loops to coordinate limb patterning and outgrowth along the PD, AP and DV axes after limb bud formation.

KEY WORDS: Ldb1, Lhx9, Lhx2, Limb development, Lmx1b, Signaling, Mouse

INTRODUCTION

Vertebrate limb outgrowth and patterning are coordinately regulated by signaling centers that produce secreted factors to control the behavior of their neighboring cells. During this process, transcription factors regulate both signal reception and emission by controlling gene expression. Proximodistal (PD) limb bud outgrowth is regulated by a positive-feedback loop between fibroblast growth factor 10 (Fgf10) of the distal limb mesenchyme and Fgfs of the apical ectodermal ridge (AER) (Lewandoski et al., 2000; Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999; Sun et al., 2002). Fgf10 is required for PD limb outgrowth as the limb bud in the Fgf10−/− mouse embryo fails to grow (Min et al., 1998; Sekine et al., 1999). The AER, a thickened epithelial structure formed at the distal edge of the limb bud, serves as a signaling center to control PD limb outgrowth and patterning (Bell et al., 1959) by secreting multiple Fgfs (Martin, 1998). Among these, Fgf4 and Fgf8 play prominent roles as limb bud development fails in their absence (Sun et al., 2002). In addition to sustaining cell survival, AER-Fgfs regulate PD-patterning gene expression during early limb bud development to specify a distal domain (Mariani et al., 2008). T-box transcription factors Tbx5 and Tbx4 are required for forelimb and hindlimb initiation, respectively, by activating the expression of Fgf10 in the presumptive and early limb bud mesenchyme (Agarwal et al., 2003; Naiche and Papaioannou, 2003; Rallis et al., 2003). They are not required for subsequent limb outgrowth or Fgf10 expression once the limb bud has formed (Hasson et al., 2007; Naiche and Papaioannou, 2007). Thus, the transcription mechanism that regulates Fgf10 expression in response to Fgf8 after limb bud initiation remains to be elucidated.

Anteroposterior (AP) limb patterning is controlled by Sonic hedgehog (Shh) expressed in the zone of polarizing activity (ZPA) located at the posterior limb margin (Riddle et al., 1993). AP limb patterning and growth is intrinsically connected to PD limb outgrowth. Shh signaling is required for Fgf4 expression in the AER (Laufer et al., 1994; Niswander et al., 1994). It also maintains the AER structure itself by regulating grem1 (Grem1) expression (Khokha et al., 2003; Kuniga et al., 1999). Conversely, Fgf signaling from the AER controls Shh expression in the ZPA (Laufer et al., 1994; Niswander et al., 1994). However, little is known about the transcriptional control of Shh-Fgf signaling interactions.

Dorsoventral (DV) limb patterning is controlled by Wnt7a in the dorsal ectoderm and by engrailed 1 (En1) in the ventral ectoderm (Loomis et al., 1996; Parr and McMahon, 1995). Wnt7a activates the expression of a LIM homeodomain (LIM-HD) transcription factor Lmx1b in the dorsal limb mesenchyme (Riddle et al., 1995), and Lmx1b determines dorsal cell fates (Chen et al., 1998; Dreyer et al., 1998; Vogel et al., 1995). DV limb polarity also indirectly affects AP limb patterning because Wnt7a is required to regulate Shh expression in the ZPA (Parr and
McMahon, 1995; Yang and Niswander, 1995). Again, the transcription factors that regulate Shh expression and link DV and AP limb patterning are still unknown.

The LIM-HD regulators of transcription are evolutionarily conserved. In the developing Drosophila wing, the LIM-HD transcription factor apertus (ap) is expressed in dorsal cells and is required to specify dorsal cell fates. ap also directs limb outgrowth by establishing a signaling center at the boundary between dorsal and ventral cells (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Ng et al., 1996). In the developing mouse limb, Lhx2 and Lhx9, two functional homologs of ap, are expressed in the distal limb bud, whereas Lmx1b, another homolog of ap, is expressed in the dorsal limb mesenchyme (Chen et al., 1998; Rincon-Limas et al., 1999; Rodriguez-Esteban et al., 1998). However, neither Lhx2 nor Lhx9 mouse mutants show limb defects (Birk et al., 2000; Porter et al., 1997).

Here, we have taken a multifaceted loss-of-function approach to test whether three of the LIM-HD genes, Lmx1b, Lhx2 and Lhx9, act together to control mouse limb development in a way that resembles that of ap in Drosophila wing development. We have determined that Lhx2, Lhx9 and Lmx1b are major LIM-HD family members expressed in the developing limb bud. The limbs of the Lhx2+/−;Lhx9+/− double mutant embryos were significantly shorter with fewer digits. In addition, the function of Lhx2, Lhx9 and Lmx1b was reduced simultaneously in the pre-limb mesenchyme by Cre-mediated inactivation of Ldb1, which encodes an essential cofactor of LIM-HD factors (Agulnick et al., 1996; Mukhopadhyay et al., 2003; Zhao et al., 2007). In the Ldb1 mutant embryos, the limbs were ventralized and more severely shortened. Our analysis demonstrates that Ldb1 is required to maintain the expression of Fgf10 and Grem1 in the limb mesenchyme in response to Fgf8 and Shh, respectively. We conclude that a transcriptional apparatus encompassing LIM-HD factors and their co-factor Ldb1 acts as a central signaling integrator in distal limb mesenchymal cells to link limb patterning and growth along all three axes.

MATERIALS AND METHODS
Generation and genotyping the mouse lines
The Lhx2+/−, Lhx9+/− and Ldb1+/− mice have been described previously (Birk et al., 2000; Porter et al., 1997; Zhao et al., 2007). Genotyping was performed by PCR using genomic DNA prepared from tail snips or extra-embryonic membranes. The oligonucleotides used to genotype T-Cre mice were: Cre forward, 5′-CCATGAGTGACAAACCTGG-3′; Cre reverse, 5′-GGGACCCATTTTTCTCTCC-3′. Conditional Ldb1 mice were generated by using these oligos: Ldb1 forward, 5′-CAGCAAGCCGAGGAAACGGAGGAAACGGAGTAGTGAGGGAAGCAGT; Ldb1 reverse, 5′-GTGGTTGAGGTCTTCCCTCTCT-3′. To genotype the Ldb1 null allele, two oligos were used: NEO 3R forward, 5′-CAGCTTCCTTTGAGGGAATGCT-3′; Ldb1 reverse, 5′-TGCCACACACAGAATCTGCGTCTGGTGCAGTC-3′.

Skeletal analysis
Embryos at 15.5 dpc or 18.5 dpc were dissected in PBS. The embryos were skinned, eviscerated and fixed in 95% ethanol. Skeletal preparations were performed as described previously (McLeod, 1980).

Histology, in situ hybridization and immunohistochemistry
For in situ hybridization, embryos were fixed in 4% paraformaldehyde at 4°C overnight. Some fixed samples were embedded in paraffin and sectioned at 5 μm. Histological analysis, immunohistochemistry, whole-mount and radioactive 5′S RNA in situ hybridization were performed as described previously (Yang et al., 2003). The following primary antibodies were used: anti-Ldb1 polyclonal anti-Ldb1 (prepared in the lab 1:4000); anti-phospho-histone H3 antibody (1:500, Millipore); anti-BrdU (1: 20, Chemicon); and anti-cleaved caspase 3 (Aasp 175) (1:400, Cell Signaling Technology). Signals were detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The slides were counterstained with Methyl Green or Toluidine Blue.

Limbs explant culture
Limbs were removed from 10.5 dpc and 11.25 dpc embryos and cultured on nucleopore filters (0.1 μm pore size, Whatman) at the interface of air and medium (BGJb, Invitrogen) with antibiotics/antimycotic solution (Invitrogen). Beads were soaked in 1 μg/ml of either BSA, Shh, Fgf10 or Fgf8 protein (R&D Systems) for 1 hour and then inserted into the limbs. Limbs were cultured for 20-24 hours, fixed with 4% paraformaldehyde in PBS and then processed for whole-mount in situ hybridization.

Cell proliferation and apoptosis assays
Paraffin wax-embedded sections were stained with the rabbit anti-phospho-histone H3 antibody. Signals were detected using biotin-conjugated secondary antibodies using ABC kit (Vectorstain). Cell death was detected as described previously (Barrow et al., 2003; Reis and Edgar, 2004).

Primers used for examining the expression of LIM-HD genes by RT-PCR
Primers used were: Lhx1 F, 5′-AGACTGGCTCAACATCGTCTGTTA; Lhx1 R, 5′-GTGGCACAGTGTCCAATACCTGCT; Lhx2 F, 5′-AGCACTTTAACCAGCCGACGCT; Lhx2 R, 5′-ATGTTGCCAAGCCTGTTGCTCTT; Lhx3 F, 5′-ACGCCATGGCGACACAAAGT; Lhx3 R, 5′-TCGGTGTCGGGTGTCTTTACGTA; Lhx4 F, 5′-ACCTTGGACCTCA; Lmx1b F, 5′-CTCACGAGGAAGAGCAACCTGAA; Lmx1b R, 5′-GAGGGTCAGTCTGAGTTGCTCTT; Lmx1c F, 5′-CCTGCTGGTCGCGTT; Lmx1c R, 5′-GTTCTGGGCTGTT; Ldb2 F, 5′-TCCTGGGCTGTT; Ldb2 R, 5′-GGACCCGTGGAGTGCCAGCTA; Ldb3 F, 5′-GAGGGTCAGTCTGAGTTGCTCTT; Ldb3 R, 5′-CCTGCTGGTCGCGTT; Ldb4 F, 5′-GTCAGTGACCCAGGAAGGAAGGCAGAGT; Ldb4 R, 5′-GCTACACAGTGACCCAGGAAGGAAGGCAGAGT.

Preparation of tissues for scanning electron microscopy (SEM)
Samples were prepared for SEM according to the manufacturer (Ted Pella, Redding, CA). Briefly, limb samples were rinsed in 0.2 M sodium cacodylate buffer, postfixed in sodium cacodylate buffer (4% formaldehyde, 2% glutaraldehyde and 0.1 M sodium cacodylate) and stored at 4°C. The samples were dehydrated in graded ethanol solutions (e.g., 35, 50, 70, 95 and 100%), infiltrated in a 1:1 mixture of absolute ethanol and Peldri and 100% Peldri for 1 hour at 37°C. Following a sublimation was completed in a vacuum chamber at 4°C overnight. The samples were dehydrated in graded ethanol solutions (e.g., 35, 50, 70, 95 and 100%), infiltrated in a 1:1 mixture of absolute ethanol and Peldri and 100% Peldri for 1 hour at 37°C. Following a sublimation was completed in a vacuum chamber at 4°C. The samples were dehydrated in graded ethanol solutions (e.g., 35, 50, 70, 95 and 100%), infiltrated in a 1:1 mixture of absolute ethanol and Peldri and 100% Peldri for 1 hour at 37°C.

RESULTS
Lhx2, Lhx9 and their co-factor Ldb1 are required for limb development
Lhx2, Lhx9 and Lmx1b are homologous to the Drosophila ap gene, a major regulator of wing development. To test whether Lhx2, Lhx9 and Lmx1b play redundant roles in regulating limb development, we first compared their expression patterns in the early mouse limb bud (Fig. 1A). At 9.5 days post coitum (dpc), when the forelimb just forms, both Lhx2 and Lhx9 were expressed throughout the limb mesenchyme.

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expressed in the limb bud. See Fig. S1 in the supplementary material for immunohistochemistry on cryosections (9.5 dpc and 10.5 dpc) and (Fig. 1A). Expression of the Ldb1 protein in the hindlimb, as shown by immunohistochemistry on cryosections (9.5 dpc and 10.5 dpc) and paraffin sections (11.5 dpc and 12.5 dpc). Ldb1 was ubiquitously expressed in the limb bud. See Fig. S1 in the supplementary material for controls of the Ldb1 antibodies.

whereas Lmx1b expression was restricted to the dorsal limb mesenchyme. At later stages (i.e. at 11.0 dpc), when there is substantial PD outgrowth of the limb bud, Lhx2 and Lhx9 were similarly expressed in the distal limb mesenchyme. Lmx1b expression, although still restricted to the dorsal mesenchyme, showed no PD bias. At 12.5 dpc, when chondrogenesis starts in the limb, Lhx2 and Lhx9 expression was still distally restricted and found in the interdigital area. Lmx1b expression was weaker in the interdigital area, possibly owing to reduced cell numbers. These results suggest that Lhx2 and Lhx9 may play redundant roles in controlling limb development, and that Lmx1b may compensate for the function of Lhx2 and Lhx9 when they are missing in the dorsal limb bud mesenchyme. We also examined the expression of all LIM-HD genes in the hindlimb bud by reverse transcriptase-polymerase chain reaction (RT-PCR) (see Table S1 in the supplementary material) and found that Islet1 and Islet2 were expressed in the limb bud. Islet1 expression appeared to be confined to the early stages of hindlimb bud development (see Table S1 in the supplementary material), in agreement with the published expression patterns of Islet1 and Islet2 in the early limb bud (MGI:3508865, MGI:3509558). Expression of Islet1 was no longer detected in the hindlimb after 10.5 dpc by whole-mount in situ hybridization. Ldb1 is an obligatory co-factor of LIM-HD factors, as null mutants of Ldb1 phenocopy individual or combined mutants of LIM-HD genes in other contexts (Suleiman et al., 2007; Zhao et al., 2007). Ldb1 protein was ubiquitously expressed in the limb bud at all stages that we analyzed (Fig. 1B).

We generated Lhx2<sup>+/–</sup>;Lhx9<sup>+/–</sup> double mutant embryos to test their redundant functions in limb development. Most of the double mutant embryos died around 15.5 dpc with defects in both the forelimbs and the hindlimbs, and such limb defects were progressively more severe distally. The zeugopod (radius and ulna or tibia and fibula) and autopod (carpals/tarsals, metacarpals/metatarsals and digits) were severely shortened and only two or three digits had formed (Fig. 2A). These digits, especially in the forelimb, did not have clear identities, as carpal/tarsal bones were fused and the number of phalanges was reduced. The Lhx2<sup>+/–</sup>;Lhx9<sup>+/–</sup> and Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> mutants developed largely normal limbs (Fig. 2A; data not shown) except that distal phalanges were slightly shorter. These results indicate that Lhx2 and Lhx9 play equivalent and redundant roles in mouse limb development and that one gene copy of either Lhx2 or Lhx9 is sufficient to regulate PD limb outgrowth and AP limb patterning.

To further test whether Lmx1b, and possibly other LIM-HD genes, can function like Lhx2 and Lhx9 in the dorsal limb mesenchyme, we reduced the activities of all LIM-HD genes in the developing limb bud by inactivating Ldb1 specifically in the developing limb bud. We generated the Ldb1<sup>+/–</sup>;T-Cre mice by crossing mice carrying a floxed Ldb1 allele (Zhao et al., 2007) with T-Cre mice. T-Cre activity results in recombination in the mesoderm of the primitive streak and has been useful to bypass gastrulation defects, thereby resulting in embryos with mesodermal lineages that carry specific Cre-induced gene inactivation (Perantoni et al., 2005; Verheyden et al., 2005). As the limb bud mesenchyme is derived from mesoderm, Ldb1<sup>+/–</sup>;T-Cre mice should lack Ldb1 activity in this tissue. At 11.5 dpc, Ldb1 expression in the Ldb1<sup>+/–</sup>;T-Cre limb bud was absent in most cells of the hindlimb mesenchyme, whereas expression in the ectoderm was not affected (see Fig. S1A in the supplementary material). The Ldb1<sup>+/–</sup>;T-Cre embryos exhibited similar, yet slightly more severe, limb defects compared with those in the Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> double mutant embryos (Fig. 2B). We focused subsequent studies on the hindlimb as the forelimb phenotype of the Ldb1<sup>+/–</sup>;T-Cre embryos was weaker (Fig. 2B) because more cells have escaped Cre-mediated Ldb1 inactivation.

The hindlimb zeugopod and tarsal region of 15.5 dpc Ldb1<sup>+/–</sup>;T-Cre mutant embryos were more reduced compared with those in the Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> double mutant embryos (Fig. 2B). The autopod had only two or three digits, resembling the Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> double mutant limb (Fig. 2B). The tarsal region was fused and the digits left might be digit 1 and 4 according to their morphology and positions (Zhu et al., 2008). It appears that the phenotype in the Ldb1<sup>+/–</sup>;T-Cre limbs is somewhat less severe (see Fig. S2B and S3 in the supplementary material) because some cells had escaped recombination in the Ldb1<sup>+/–</sup>;T-Cre hindlimb buds and, as a result, a residual amount of Ldb1-positive (Ldb1<sup>+</sup>) cells formed patches in the distal limb bud at 12.5 dpc (see Fig. S1 in the supplementary material).

Importantly, expression of the dorsal limb marker Lmx1b was normal at 10.5 dpc but missing in patches of the dorsal limb bud at 12.5 dpc (see Fig. S2A in the supplementary material), suggesting partial ventralization of the Ldb1<sup>+/–</sup>;T-Cre limb. We reasoned that Ldb1 deficiency would lead to impaired Lmx1b function, ultimately resulting in loss of dorsal cell fate and then of Lmx1b expression. The DV patterning defect of the Ldb1<sup>+/–</sup>;T-Cre hindlimb bud and the similarities between its limb phenotype and that of the Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> embryos indicated that Lhx2, Lhx9 and Lmx1b are the major LIM-HD factors that act together with Ldb1 in regulating limb development.

**Lhx2, Lhx9 and their co-factor Ldb1 are required for AP limb patterning by controlling Shh expression**

To understand the molecular mechanism underlying the defects in the Lhx2<sup>+/–</sup>;Lhx9<sup>–/–</sup> and Ldb1<sup>+/–</sup>;T-Cre limbs, expression of key regulatory genes in early limb patterning and growth along the PD,
AP and DV axes were examined. As digit number is reduced when Shh activity is reduced or removed later in the limb bud (Lewis et al., 2001; Zhu et al., 2008), we examined Shh expression in the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> and Ldb1<sup>−/−</sup>;T-Cre embryos. In the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> mutant, there was a progressive loss of Shh expression in the ZPA (Fig. 3A). Shh expression was normal before 10.0 dpc (data not shown). Starting at 10.5 dpc, Shh expression was significantly reduced compared with the control in the AER, but failed to do so in the mutant hindlimb bud. Therefore, the more pronounced loss of Grem1 expression in the ventral limb is not a consequence of defects in DV limb patterning. These results raised an interesting possibility that the residual Grem1 expression in the dorsal limb mesenchyme is maintained by a dorsal-specific transcription factor that is partially redundant with Lhx2 and Lhx9, which can conceivably be Lmx1b. As Lmx1b function was also compromised in the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> limb bud, we reasoned that Grem1 expression should be lost to similar levels in both dorsal and ventral limb mesenchyme in the Ldb1<sup>−/−</sup>;T-Cre limb bud, and this was indeed the case (Fig. 3E). As Lmx1b is the only dorsal-specific LIM-HD factor in the developing mouse limb, our results suggest that Lmx1b can substitute for the function of Lhx2 and Lhx9 to a certain extent in the dorsal limb mesenchyme by regulating the expression of Grem1 and possibly other signaling factors.

To test whether reduced Grem1 expression in the Ldb1<sup>−/−</sup>;T-Cre and Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> mutant limb buds resulted from reduced Shh expression or Shh signaling activity, we inserted Shh-coated beads into the hindlimb buds of the Shh<sup>−/−</sup>, Ldb1<sup>−/−</sup>;T-Cre and Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> mutants, as well as wild-type embryos and compared Grem1 expression levels. Although Shh beads upregulated Grem1 expression in the Shh<sup>−/−</sup> limb bud, they failed to do so in the Ldb1<sup>−/−</sup>;T-Cre and Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> mutant hindlimb bud (Fig. 4A,B). In addition, Shh beads in the anterior limb bud of the wild-type and Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> embryos expanded Fgf4 expression in the AER, but failed to do so in the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> mutant limb.
bud (Fig. 4C). However, Ptch1 expression was similarly induced by the Shh beads in both wild-type and Ldb1c/–;T-Cre mutant hindlimb bud (Fig. 4D). These results indicate that Shh activation of Grem1 expression, rather than Shh signal transduction itself, requires LIM-HD/Ldb1 activities. Thus, Ldb1, in conjunction with the LIM-HD transcription factors expressed in the limb bud, acts in limb mesenchymal cells to control the production of some output signals in response to Shh, such as Grem1.

Lhx2, Lhx9 and their co-factor Ldb1 are required for PD limb outgrowth by regulating Fgf10 expression

Lhx2 and Lhx9 expression in the distal limb bud resembles that of Fgf10 (Ohuchi et al., 1997), which is required for PD limb outgrowth (Min et al., 1998; Sekine et al., 1999). Fgf10 and other Fgfs expressed in the AER, mainly Fgf8, regulate each other’s expression, and such reciprocal regulation primarily controls PD limb outgrowth. At 9.5 dpc when the hindlimb bud is initiated, expression of Fgf10 was normal in the Ldb1c/–;T-Cre mutant compared with the wild-type control (Fig. 5A). However, Fgf10 expression was rapidly reduced during successive stages of limb development. Shortly after limb initiation, Fgf10 expression began to disappear in patches in the dorsal forelimb at 9.5 dpc (Fig. 5A). At 10.25 dpc and 10.5 dpc, Fgf10 expression was much reduced (Fig. 5A). Surprisingly, the distal expression of sprouty 4 (Spry4), a transcription target of Fgf signaling (Minowada et al., 1999), was almost normal in the Ldb1c/–;T-Cre hindlimb bud at 10.5 dpc and 11.5 dpc (Fig. 5B and data not shown), suggesting that in the to Ldb1c/–;T-Cre hindlimb, Fgf8 signaling is transduced at normal levels, but unable to maintain Fgf10 expression. In the Lhx2–/–;Lhx9–/– mutant limb bud, we also observed reduction of Fgf10 expression, although not as severe as that in the Ldb1c/–;T-Cre limb bud (Fig. 5A).

Fgf8 expression was detected in the AER in the Ldb1c/–;T-Cre limb bud at 10.25 dpc, but its expression domain was thinner and truncated posteriorly (Fig. 5C). In the Lhx2–/–;Lhx9–/– mutant, Fgf8 expression was also lost in the posterior AER, but this happens at a slightly later developmental stage (Fig. 5D). At 10.5 dpc, in the Ldb1c/–;T-Cre mutant limb, Fgf8 expression in the AER became very spotty and the AER was completely flattened by 11.5 dpc (Fig. 5C). These results indicate that Lhx2, Lhx9 and Ldb1 are not required for the initial expression of Fgf10 and Fgf8, but they are likely to mediate the positive-feedback loop between Fgf10 and Fgf8 by regulating Fgf10 expression in the limb mesenchyme in response to Fgf8 signaling.

The distal limb bud of the Ldb1c/–;T-Cre embryo at 11.5 dpc still expressed Lhx9, which marks the distal domain (Fig. 5E), indicating that loss of Fgf10 expression later in limb development did not result in loss of distal limb bud cells. However, when the zeugopod and autopod skeletal primordia were detected by Sox9 in situ hybridization at 12.5 dpc, both were much smaller, and distal chondrogenesis was much reduced in the Ldb1c/–;T-Cre mutant limb.
Lhx2–/– implanted into the hindlimb buds at 10.5 dpc and Grem1 expression was examined. (B) Grem1 expression was reduced in the 10.5 dpc Lhx2–/–;Lhx9–/– hindlimb bud, most notably in the posterior part. Ectopic Grem1 expression in the anterior limb bud was induced by Shh beads in the control Lhx2–/–;Lhx9–/– limb, but not in the Lhx2–/–;Lhx9–/– limb (arrows). Control beads were inserted to the contralateral limb buds. D, dorsal; V, ventral. (C) Shh-coated beads were implanted into 10.25 dpc forelimb buds and Fgf8 expression was not induced in the Lhx2–/–;Lhx9–/– limb (arrow). (D) Shh-coated beads were implanted into the hindlimb buds at 11.0 dpc. Pch1 expression was upregulated in the Ldb1f/–;T-Cre and wild-type limb buds.

(Fig. 5F). As premature AER loss due to AER specific removal of fibroblast growth factor receptor 2 (Fgfr2) delayed generation of above threshold number of progenitors required to form normal autopod skeletons (Lu et al., 2008; Yu and Ornitz, 2008), reduced autopod skeleton formation in the Ldb1c/–;T-Cre mutant limb bud may also be a result of reduced limb progenitor cells owing to reduced Fgf and Shh signaling.

**Lhx2, Lhx9, Lmx1b and their co-factor Ldb1 integrate limb patterning and growth along all three axes**

The growth and patterning defects along the AP, DV and PD axes in the Ldb1f/–;T-Cre mutant limb suggest that Lhx2, Lhx9, Lmx1b and their co-factor Ldb1 coordinate limb patterning and growth along all three axes. To test this hypothesis, we first inserted Fgf8-coated beads into the hindlimb mesenchyme. Fgf8 beads upregulated Fgf10 expression in the control limb bud, but failed to maintain or activate Fgf10 expression in the Ldb1f/–;T-Cre hindlimb bud (Fig. 6A). By contrast, Fgf10 beads rescued Fgf8 expression in the AER of the Ldb1f/–;T-Cre and Lhx2–/–;Lhx9–/– limb buds (Fig. 6B,C), indicating that one of the primary defects in the Ldb1f/–;T-Cre and Lhx2–/–;Lhx9–/– limb buds was reduced Fgf10 expression. Furthermore, Fgf8 beads induced Spry4 expression around the beads in the 10.5 dpc Ldb1f/–;T-Cre limb bud (Fig. 6D). This finding is consistent with the normal Spry4 expression in the Ldb1f/–;T-Cre hindlimb bud (Fig. 5B). When cell death was examined in the cultured limb bud by whole-mount TUNEL assay (see Fig. S4 in the supplementary material), no difference was found between cultured wild-type and Ldb1f/–;T-Cre hindlimb buds. Thus, weakened or loss of gene expression in the mutant cultured limb bud is not a result of excessive cell death. Our results confirm that Fgf8 signal itself is transduced at normal levels in the Ldb1f/–;T-Cre limb bud and that the LIM-HD factors are required in the positive-feedback loop to regulate Fgf10 expression.

Next, we tested whether the LIM-HD factors are also required in the positive-feedback loop between Fgf signaling from the AER and Shh expression in the ZPA. In the Shh–/– mutant limb, PD limb outgrowth was reduced with prematurely degenerated AER and reduced Fgf8 expression (Chiang et al., 2001). The zygopod phenotype of Shh–/– mutant (Chiang et al., 2001) is similar to that of the Ldb1f/–;T-Cre limb. To test whether Ldb1 also acts to maintain Shh expression in response to Fgf signaling from the AER, we inserted Fgf8-coated beads into the posterior hindlimb mesenchyme of 10.5 dpc embryos. Fgf8 beads expanded Shh expression domain in the control hindlimb (Fig. 6E), but failed to do so in the Ldb1f/–;T-Cre mutant (Fig. 6E). We did notice that small patches of distal limb bud cells under the AER and around the Fgf8 bead expressed Shh in the Ldb1f/–;T-Cre mutant. These are probably the Ldb1f/– cells that have escaped recombination by the T-Cre and can therefore respond to the recombinant Fgf8. As Grem1 also plays an essential role in limb mesenchymal-epithelial signaling interactions, and both A-P and P-D limb defects were observed in the Grem1–/– limb bud (Khokha et al., 2003; Michos et al., 2004), another mechanism used by Lhx2, Lhx9, Lmx1b and Ldb1 to coordinate limb development along the AP and PD axes is to regulate Grem1 expression in response to Shh signaling (Fig. 3C).

**Ldb1 is required to maintain cell proliferation in the limb bud**

The smaller limb sizes in the Lhx2–/–;Lhx9–/– and Ldb1f/–;T-Cre embryos suggest that Ldb1 in conjunction with the transcriptional regulators that depend on this co-factor, regulates cell proliferation and/or cell survival. To test this, we examined cell proliferation and cell death in the hindlimb bud of the Ldb1f/–;T-Cre embryos. At early stages (i.e. 10.5 dpc) of development, cell proliferation was similar throughout the entire limb bud. We observed a significant reduction of cell proliferation in the Ldb1f/–;T-Cre embryos (Fig. 7A,B). By 11.5 dpc, cell proliferation progressively reduces in the distal to proximal direction in the wild-type limb bud. In the mutant limb bud, there was a significant reduction in cell proliferation in all regions of the limb (Fig. 7A,B). We did not detect significant difference of cell death in most regions of the limb between the mutant and wild type (Fig. 7C). However, consistent with reduced Fgf signaling from the AER, we found that cell death was slightly increased in the proximal limb bud of the Ldb1f/–;T-Cre mutant (Fig. 7C).

**DISCUSSION**

Our report is focused on transcriptional regulation in response to cell-cell signaling that controls limb patterning and outgrowth. We show that the LIM-HD transcription factors Lhx2 and Lhx9 play redundant roles to integrate limb outgrowth and patterning along the
PD and AP axes by regulating the expression of the crucial signaling molecules Shh, Grem1 and Fgf10 in the posterior distal limb mesenchyme (Fig. 7D). In addition, by removing Lhx2 and Lhx9, we uncovered a previously undescribed role of the dorsal determinant Lmx1b. In our mutants, it can partially substitute for the function of Lhx2 and Lhx9 in regulating AP and PD limb patterning and outgrowth through Grem1 (Fig. 7D). Furthermore, conditional inactivation of Ldb1, an essential co-factor of LIM-HD genes, causes additional phenotypes that affect limb development not only along the PD and AP, but also the DV axis. Curtailing Ldb1 function in the limb bud leads to signaling defects that can be ascribed to the malfunction of several LIM-HD factors, including Lhx2, Lhx9 and Lmx1b. Our data revealed that Ldb1 and the associated LIM-HD transcription factors act in concert to integrate the signal exchange between limb mesenchyme and ectoderm that regulates limb growth and patterning along all three axes.

**LIM-HD transcription factors act as a central integrator for patterning and growth along the three axes of the limb**

Crosstalk among the signaling centers that control limb development along the PD, AP and DV axes have been previously identified as a signal relay in which a cell receives a signal and emits its own signal(s) to elicit responses in neighboring cells. It was unknown prior to our study how such signal relays were transcriptionally controlled. We show that a transcriptional complex whose essential elements include Ldb1 and LIM-HD protein is instrumental in controlling signaling interactions and integrations in the early limb bud. Shh and Fgf10 expression in response to Fgf8 signaling and Grem1 expression in response to Shh signaling are controlled by the same family of transcription factors directly or indirectly (Fig. 7D). Therefore, Ldb1, together with a select group of LIM-HD transcription factors, acts as a central intracellular integrator to

**Fig. 5. Reduced Fgf10 and Fgf8 expression in the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> and Ldb<sup>−/−</sup>;T-Cre limbs.** Whole-mount in situ hybridization was performed. (A) Fgf10 expression was rapidly lost in the mutant limb bud. Loss of Fgf10 expression was first observed in the distal-most limb bud (arrow). Loss of Fgf10 expression in the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> hindlimb bud was less significant (arrow). (B) Spry4 was expressed almost normally in the Ldb<sup>−/−</sup>;T-Cre mutant hindlimb bud at 10.5 dpc. (C) Fgf8 expression was progressively reduced in the AER of the Ldb<sup>−/−</sup>;T-Cre hindlimb bud (black arrows). The AER was completely flattened (yellow arrows) at 11.5 dpc, as shown by the scanning EM. (D) Fgf8 expression was lost in the posterior AER of the Lhx2<sup>−/−</sup>;Lhx9<sup>−/−</sup> forelimb bud at 10.5 dpc (arrow and broken line). (E) Lhx9 expression in the 11.5 dpc hindlimb. (F) Sox9 expression in the 12.5 dpc hindlimb bud. Small Sox9-expressing domains the Ldb<sup>−/−</sup>;T-Cre mutant are indicated by arrows. Z, zeugopod; A, autopod.
coordinate limb development along all three axes by mediating at least two distinct signaling feedback loops: Shh-AER and Fgf10-Fgf8 (Fig. 7D). This is quite intriguing because Shh and Fgfs signal through completely different intracellular pathways that can be distinguished by their well-established signaling read-outs. The Ldb1/LIM-HD transcription complex appears to control some specific responses to Shh and Fgf8 signaling, rather than regulating these signaling activities per se in the limb bud mesenchyme. Thus, one system the limb bud has employed is a remarkably efficient signaling integration strategy, whereby LIM-HD/Ldb1 factors act as a central node in the distal limb mesenchymal cells. This central node first receives signaling inputs from any one of the three axes and subsequently controls the production of signals that can regulate patterning and growth along other axes. One possibility is that LIM-HD/Ldb1 factors regulate expression of some downstream genes of both Fgf and Shh signaling. However, one cannot rule out that some or even all of these regulations may be indirect. As the expression of Shh, Grem1 and Fgf10 in the mesenchyme gradually decreases as the limb mesenchymal cells differentiate, it is possible that the LIM-HD transcription factors and co-factors may also maintain limb progenitor cell states by regulating responses to AER-Fgfs and Shh. Recent studies have shown that both Fgf and Shh signaling act to sustain progenitor pools required for proper limb patterning and growth (Lu et al., 2008; Mariani et al., 2008; Towers et al., 2008; Yu and Ornitz, 2008; Zhu et al., 2008). Entirely compatible with this idea is our observation that Lhx2 and Lhx9 expression was lost in the older distal limb bud when chondrogenesis started (Fig. 1). Furthermore, Lhx2 has been shown to control an early progenitor cell state during hair follicle formation or in the hematopoietic system (Rhee et al., 2006).

Growth and patterning signals are integrated in the distal limb mesenchyme

Lhx2 and Lhx9 are expressed throughout the early limb bud. Later in limb development, their expression is restricted to the distal limb mesenchyme (Fig. 1). Lmx1b expression overlaps with that of Lhx2 and Lhx9 in the dorsal limb bud. The distal limb mesenchyme is the location where signaling inputs from the AER (Fgf8), ZPA (Shh) and the dorsal ectoderm (Wnt7a) are integrated. The LIM-HD transcription factors Lhx2, Lhx9 and Lmx1b, and possibly additional members of this family, regulate the production of output signals, including Fgf10, Shh and Grem1 in the limb mesenchyme (Fig. 1). A fate-mapping study in the early chick limb bud indicates that regionalization along the PD axis progresses in a proximal-to-distal direction. Cells located more distally are less restricted in terms of their final regional identities and thus able to contribute to limb structures that span a larger domain (Sato et al., 2007). Therefore, it appears that more distal limb cells are more expandable and have more plasticity in this regard. This further underscores the likelihood that the LIM-HD factors Lhx2, Lhx9 and Lmx1b may regulate a transcription profile required for maintaining a progenitor state and that this profile may also allow the distal limb cells to express downstream genes regulated by Fgf and Shh signals. Our model predicts that, in the absence of Lhx2 and Lhx9 function, mesenchymal cells of the limb bud will assume a more determined and regionalized state such that their ability to expand is severely compromised. Indeed, the limb buds were smaller, narrower and limb skeletal segments were either much shorter or missing in the Lhx2−/−;Lhx9−/− and the Ldb1−/−;T-Cre mutant, although zeugopods and autopods still formed. Some elements (i.e. the tarsal bones) were fused together. These
phenotypes are very similar to those observed in the Fgf and Shh signaling-deficient limbs, in which missing skeletal elements are thought to be caused by reduced progenitor cell numbers (Sun et al., 2002; Zhu et al., 2008). However, Fgfl8/4 signaling does not control Lhx2/9 expression (see Fig. S5 in the supplementary material). In addition, in contrast to the Shh, Grem1 and all Fgf mutants, reduced cell proliferation, rather than cell death, is the major contributing factor to the small limb phenotypes in the Ldb1 mutant limb. This may be due to incomplete loss of Shh, Fgf and Grem1 signaling in the Ldb1 mutant.

It appears that Lhx2 and Lhx9 are the major LIM-HD transcription factors acting in conjunction with Ldb1 to coordinate early limb patterning and outgrowth. This by no means excludes the possibility that additional factors participate in the transcriptional regulation of this process. Ldb2, a close family member of Ldb1, is also expressed in the developing limb bud (see Table S1 and http://www.informatics.jax.org/searches/image. cgi?11751). Ldb2−/− mice do not have limb defects (H.W., unpublished). However, these two genes may play redundant roles in limb development. Besides mediating the function of the three LIM-HD factors analyzed in this report, they are likely to affect the function of additional Ldb-associated regulators in the developing limb. For example, our RT-PCR analysis (Table S1 in the supplementary material) revealed the presence of appreciable amounts of Islet1 transcripts at transient stages of early limb bud formation. Also, GATA and Sdop factors are regularly observed in the context of Ldb/LIM-HD transcriptional activity (van Meyel et al., 2003; Vyas et al., 1999). Furthermore, in the Twi1−/− limb bud, the Fgf10-Fgf8 feedback loop is also disrupted (Zuniga et al., 2002), raising the possibility that the LIM-HD/Ldb1 factors in the limb may also interact with Twi1.

**Different molecular mechanisms underlie the function of Lhx2 and Lhx9 during evolution in limb development**

Despite the functional similarity between *ap* in *Drosophila* and Lhx2, Lhx9 and Lmx1b in the mouse, the underlying molecular mechanisms by which these transcription factors regulate limb development may not be conserved. In the *Drosophila* wing primordium, *ap* induces the expression of the Notch ligand Serrate in dorsal cells and restricts the expression of Delta, another Notch ligand, to the ventral cells (Diaz-Benjumea and Cohen, 1995; Milan and Cohen, 2000). Serrate and Delta activates Notch signaling. This induces Wg expression in cells along the DV boundary, which is required for PD outgrowth (Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). Limb development in vertebrates is more complicated, as it involves two distinct germ layers: ectoderm and the mesoderm. The ectoderm emits signals (i.e. Wnt7a and Fgf8), whereas the mesoderm responds to these signals by expressing Lmx1b, Shh and Fgf10, which, in turn, control DV, AP and PD limb patterning and outgrowth, respectively. Lhx2, Lhx9 and Lmx1b are expressed only in the limb mesenchyme, whereas the action of Notch ligand jagged 2 (a homologue of *Drosophila* Serrate) appears to be mainly confined to the AER, which is an ectodermal structure (Sidow et al., 1997; Valsecchi et al., 1997). Therefore, Lhx2, Lhx9 and Lmx1b may not act through controlling the activation of Notch signaling by directly regulating the expression of jagged 2, its major ligand in the developing limb. Rather, a new mechanism may have been evolved in higher vertebrates whereby Lhx2, Lhx9 and Lmx1b coordinate three-dimensional limb patterning and outgrowth.
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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/8/1375/DC1

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