Disruption of PCP signaling causes limb morphogenesis and skeletal defects and may underlie Robinow syndrome and brachydactyly type B

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Brachydactyly type B (BDB1) and Robinow syndrome (RRS) are two skeletal disorders caused by mutations in ROR2, a co-receptor of Wnt5a. Wnt5a/Ror2 can activate multiple branches of non-canonical Wnt signaling, but it is unclear which branch(es) mediates Wnt5a/Ror2 function in limb skeletal development. Here, we provide evidence implicating the planar cell polarity (PCP) pathway as the downstream component of Wnt5a in the limb. We show that a mutation in the mouse PCP gene Vangl2 causes digit defects resembling the clinical phenotypes in BDB1, including loss of phalanges. Halving the dosage of Wnt5a in Vangl2 mutants enhances the severity and penetrance of the digit defects and causes long bone defects reminiscent of RRS, suggesting that Wnt5a and Vangl2 function in the same pathway and disruption of PCP signaling may underlie both BDB1 and RRS. Consistent with a role for PCP signaling in tissue morphogenesis, mutation of Vangl2 alters the shape and dimensions of early limb buds: the width and thickness are increased, whereas the length is decreased. The digit pre-chondrogenic condensates also become wider, thicker and shorter. Interestingly, altered limb bud dimensions in Vangl2 mutants also affect limb growth by perturbing the signaling network that regulates the balance between Fgf and Bmp signaling. Halving the dosage of Bmp4 partially suppresses the loss of phalanges in Vangl2 mutants, supporting the hypothesis that an aberrant increase in Bmp signaling is the cause of the brachydactyly defect. These findings provide novel insight into the signaling mechanisms of Wnt5a/Ror2 and the pathogenesis in BDB1 and RRS.

INTRODUCTION

Autosomal dominant brachydactyly type B (BDB1, OMIM no. 113000) and autosomal recessive Robinow syndrome (RRS, OMIM no. 268310) are two congenital disorders caused by mutations in ROR2. ROR2 encodes a transmembrane protein with an extracellular Frizzled (Fz) like cysteine-rich domain (CRD) and a cytoplasmic tyrosine kinase (TK) domain. BDB1 is the most severe form of brachydactyly and is characterized by loss of nails and varying number of phalanges (1,2). In contrast, RRS patients display more broad skeletal dysplasia including mesomelic limb shortening and dwarfism, and may or may not display brachydactyly (3–6). BDB1 is associated with heterozygous mutations that truncate the cytoplasmic portion of ROR2, either before or after the TK domain (1–3). RRS, in contrast, is caused by different homozygous missense, nonsense and frameshift mutations in either the extracellular or cytoplasmic region of ROR2 (3–5). Because most of the ROR2 mutations in RRS are predicted to be loss-of-function mutations and heterozygous carriers of these mutations do not display brachydactyly phenotypes, it has been proposed that ROR2 mutations associated with BDB1 cause gain-of-function or dominant-negative effects (1,3–5,7).

In vitro biochemical studies indicate that Ror2 binds to Wnt5a directly through its Fz-like CRD and may serve as a co-receptor in Wnt5a-induced signaling (8–10). Interestingly, in mice carrying null mutations in the Wnt5a gene, all the...
phalanges fail to form and the metacarpals are extremely short. The remaining limb skeletal elements are significantly shortened and the severity of the phenotype follows a gradient, with distal bones more affected than proximal ones (11,12), reminiscent of mesomelic limb shortening in RRS patients. More recently, two missense mutations in \textit{WNT5A} have been found in an autosomal dominant form of RRS (OMIM no. 180700) (13,14). Collectively, these studies strongly implicate that disruption of Wnt5a signaling may underlie both RRS and BDB1 (9,15).

What remains unclear, however, is the intracellular signaling pathway(s) through which Wnt5a/Ror2 regulates limb skeletal formation. Wnt5a is unique among Wnt family of signaling ligands, in that it can signal through different Fz receptors and co-receptors to activate either the canonical or the non-canonical Wnt pathway (8,16). Activation of the canonical Wnt pathway requires the co-receptors Lrp5 and Lrp6, which signal through cytoplasmic protein Dishevelled (Dvl) to stabilize $\beta$-catenin. Subsequently, $\beta$-catenin accumulates and translocates to the nucleus to activate gene transcription (17,18). Although the canonical Wnt pathway is known to be involved in early limb patterning as well as endochondral bone formation (19–23), the distinct phenotypes between \textit{Wnt5a} and $\beta$-catenin as well as Lrp5/Lrp6 mutants (24) indicate that Wnt5a does not signal through the canonical Wnt pathway during limb development (15).

In contrast to the canonical Wnt pathway, the non-canonical Wnt pathway includes multiple branches that do not involve stabilization of $\beta$-catenin. In one of the better characterized branches of the non-canonical Wnt pathway known as the planar cell polarity (PCP) pathway, Fz and Dvl are also involved, but they interact with a distinct set of core PCP proteins including Van gogh (Vang) and Prickle (PK) (25).

Initially identified in \textit{Drosophila}, the PCP pathway is involved in establishing cellular polarity in the plane of the epithelium, perpendicular to the apical–basal (a–b) polarity of the cell (25). Studies in vertebrate model systems, including \textit{Xenopus} and zebrafish, indicate that the PCP pathway also regulates the convergent extension (CE) morphogenetic process. CE was first demonstrated in gastrulating \textit{Xenopus} embryos by in which mesodermal cells underwent mediolaterally oriented intercalation, leading to concomitant tissue lengthening and narrowing (26). Imaging experiments in zebrafish indicate that, in addition to polarized cell intercalation, the PCP pathway also regulates directional cell migration and oriented cell division during CE (27–30). Both \textit{Wnt5a} and another non-canonical \textit{Wnt}, \textit{Wnt11}, are linked to CE in frogs and zebrafish (28,31,32). Studies in \textit{Xenopus} have further demonstrated that Ror2 interacts with Wnt11 and Fz7 to regulate CE, suggesting that Ror2 may also be part of the PCP pathway (10). In the mouse, we and others have demonstrated that the mammalian PCP pathway regulates both cellular polarity and polarized tissue morphogenesis reminiscent of CE in \textit{Xenopus} and zebrafish (33–45).

In addition to the PCP pathway, Wnt5a has been shown to activate at least two other branches of the non-canonical Wnt pathway. The first is known as the Wnt–Ca$^{2+}$ pathway, in which Wnt5a stimulation induces Ca$^{2+}$ release and subsequent activation of the Ca$^{2+}$-sensitive kinases protein kinase C and Ca$^{2+}$/calmodulin-dependent kinase (46–49). Over-expression of the core PCP proteins, Dvl and Pk, can also activate the Wnt–Ca$^{2+}$ cascade in zebrafish and \textit{Xenopus}, suggesting that the Wnt–Ca$^{2+}$ and PCP pathways either overlap substantially or are components of the same signaling network (50–52). Secondly, in mammalian cell culture, Wnt5a has been found to antagonize the canonical Wnt pathway by either promoting GSK3$\beta$-independent $\beta$-catenin degradation (53) or down-regulating $\beta$-catenin-induced transcription (8). It is unclear whether core PCP proteins such as Dvl and Vang are required for Wnt5a-induced suppression of canonical Wnt signaling.

The divergent signaling cascades triggered by Wnt5a and Ror2 in different experimental systems have made it difficult to understand how Wnt5a/Ror2 regulates limb skeletal formation. We approached this problem by analyzing limb skeletal formation in mice carrying mutations in the PCP gene \textit{Vangl2}, one of the two \textit{Vang} orthologs in mammals (38,54).

Our results support the hypothesis that Wnt5a signals through the PCP pathway to regulate limb skeletal development and suggest that PCP signaling regulates limb morphogenesis to control limb shape and dimensions. Furthermore, our embryological and genetic analyses indicate that altered limb bud shape and dimensions in \textit{Vangl2} mutants also perturb the signaling cross-talk critical for limb growth, leading to loss of phalanges. Collectively, these findings provide novel insight into the signaling mechanism of Wnt5a/Ror2 during limb development and the pathogenesis of BDB1 and RRS.

**RESULTS**

**Mutation in the mouse PCP gene \textit{Vangl2} causes digit skeletal defects**

To assess the potential role of the mammalian PCP pathway in limb development, we examined \textit{Looptail} (\textit{Vangl2}Lp) mice that harbored a loss-of-function point mutation in the core PCP gene \textit{Vangl2} (38,54), one of the two \textit{Vang} homologs in mammals. Interestingly, in embryonic day (E) 18.5 \textit{Vangl2}Lp/Lp homozygous mutants, the primary claw field located at the digit tip appeared rudimentary in digits 3, 4 and 5 in the forelimb (black arrowheads, Fig. 1B). The primary claw field is a layer of thickened epithelium that gives rise to the claw, a structure homologous to the nail in humans. This digit appendage defect in \textit{Vangl2}Lp/Lp mutant mice is reminiscent of the clinical symptom in some human BDB1 patients (2,3).

When we stained the skeleton of E18.5 embryos with Alcian blue and Alizarin red, we found that the middle phalanges were missing in all the digits in the forelimb of \textit{Vangl2}Lp/Lp mutants (compare Fig. 1D and E). In wild-type or \textit{Vangl2}Lp/+ mice, digits 2–5 consisted of the metacarpal (mc) and three phalanges (the proximal p1, the middle p2 and the distal p3, Fig. 1D). In \textit{Vangl2}Lp/Lp forelimbs, however, only the metacarpal, one proximal phalanx and one distal phalanx resembling the wild-type terminal phalanx p3 formed (Fig. 1E). We refer to the proximal phalanx as p1 and the distal one as p2/3 for reasons that we will explain in the Discussion. Also, wild-type digit 1 consisted of two phalanges, whereas only one phalanx formed in the forelimb digit 1 in \textit{Vangl2}Lp/Lp mutants (data not shown).
similar digit defects are consistent with the hypothesis that Wnt5a/Ror2 signals through the PCP pathway, they may also arise through independent mechanisms. To test whether Wnt5a and Vangl2 function in the same pathway during limb skeletal formation, we studied their genetic interaction. We crossed a Wnt5a null allele (Wnt5a<sup>−/−</sup>) (11) into Vangl2<sup>−/−</sup> mutants. As reported previously, Wnt5a<sup>−/−</sup> heterozygous mutants do not display any limb defects (11), and we observed no limb defects in Wnt5a<sup>−/+</sup>; Vangl2<sup>−/−</sup> double heterozygous mutants either (data not shown). Interestingly, however, we found that reducing the dosage of Wnt5a by 50% in Vangl2<sup>−/−</sup> mutants significantly enhanced both the severity and the penetrance of digit skeletal defects. In Wnt5a<sup>−/−</sup>; Vangl2<sup>−/−</sup> mutants, digits 4 and 5 in the forelimb displayed more severe defects, in that both the proximal and medial (p1 and p2) phalanges were lost and only a single phalanx resembling p3 formed (Fig. 1F, red arrows). In the hindlimb, the digit skeletal defects became more penetrant as all digits were affected and displayed a loss of the middle phalanges (Fig. 1I).

More significantly, long bones in the limb became significantly shortened and misshapen in Wnt5a<sup>−/−</sup>; Vangl2<sup>−/−</sup> mutants (Fig. 1L and O, red arrowheads). Similar to Wnt5a and Ror2 null mice and human RRS patients, the distal bones were more affected than the proximal ones. For instance, in the forelimb of Wnt5a<sup>−/−</sup>; Vangl2<sup>−/−</sup> mutants, although the scapula appeared normal (95% the length of control littermates), the humerus, radius and ulna were only 77, 72 and 69% the length of controls (n = 6, P < 0.03), respectively. The hindlimb of Wnt5a<sup>−/−</sup>; Vangl2<sup>−/−</sup> mutants was even more severely affected, where the femur, fibula and tibia were reduced to 80,}

Vangl2 genetically interacts with Wnt5a during limb skeletal development

The hypoplastic claws and the loss of phalanges in Vangl2<sup>−/−</sup> mutants are reminiscent of the clinical symptoms in BDB1 (1,2). A similar loss of phalanges in the digits has also been reported in two mouse mutants, in which Ror2 was truncated either immediately after the transmembrane domain or after the TK domain (2,7,55). Although the similar digit defects are consistent with the hypothesis that Wnt5a/Ror2 signals through the PCP pathway, they may also arise through independent mechanisms. To test whether Wnt5a and Vangl2 function in the same pathway during limb skeletal formation, we studied their genetic interaction. We crossed a Wnt5a null allele (Wnt5a<sup>−/−</sup>) (11) into Vangl2<sup>−/−</sup> mutants. As reported previously, Wnt5a<sup>−/−</sup> heterozygous mutants do not display any limb defects (11), and we observed no limb defects in Wnt5a<sup>−/+</sup>; Vangl2<sup>−/−</sup> double heterozygous mutants either (data not shown). Interestingly, however, we found that reducing the dosage of Wnt5a by 50% in Vangl2<sup>−/−</sup> mutants significantly enhanced both the severity and the penetrance of digit skeletal defects. In Wnt5a<sup>−/−</sup>; Vangl2<sup>−/−</sup> mutants, digits 4 and 5 in the forelimb displayed more severe defects, in that both the proximal and medial (p1 and p2) phalanges were lost and only a single phalanx resembling p3 formed (Fig. 1F, red arrows). In the hindlimb, the digit skeletal defects became more penetrant as all digits were affected and displayed a loss of the middle phalanges (Fig. 1I).

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54 and 48% of the length of those in control littermates ($n = 6$, $P < 0.01$), respectively. Theibia and fibula in $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants also appeared grossly misshapen (Fig. 1O, red arrowheads).

Collectively, these data strongly support the hypothesis that $Wnt5a$ may signal through the PCP pathway to regulate limb skeletal development and suggest that disruption of PCP signaling may underlie both BDB1 and RRS in humans.

**Limb skeletal defects in PCP mutants arise at the early stage of endochondral bone formation**

The skeletal elements in the limb form through endochondral bone formation, a complicated, multiple step process. In the mouse, this process starts at E11.5 and continues through the remaining gestational period. To investigate how the endochondral bone formation process was affected, we first examined Alcian blue-stained $Vangl2^{Lp/Lp}$, $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ and control embryos at different developmental stages. Interestingly, we found obvious defects starting at E12.5, the earliest time point when the chondrogenic primordia of the limb skeleton can be detected by Alcian blue staining. At this early stage, the mesenchymal cells in the limb buds undergo condense packing to form the pre-chondrogenic condensate that serves as a template of the future bone. In each digit, the condensate is a single unsegmented column known as the digit ray (Fig. 2A).

By E13.5, the digit condensate extends further distally and some cells de-differentiate to form the metacarpal–phalangeal joint (m/p, red arrow in Fig. 2I) and inter-phalangeal joint (p1/p2, blue arrow in Fig. 2I).

In both $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants, the digit condensates appeared shorter and wider at E12.5. When we measured the condensate of digit 4 in the forelimb, we found that in the two mutants, the length was reduced by 41–46%, whereas the width was increased by 21–44% (compare the red brackets in Fig. 2A–C and Fig. 2M and N). The digit condensates in $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ double heterozygous embryos were not significantly changed from wild-type controls (data not shown), consistent with normal digit formation in these mice.

Furthermore, when we micro-dissected digit 4 from the forelimb and visualized it transversely from the side (Fig. 2E–G, dorsal to the right and ventral to the left), we found that the thickness of the digit condensates along the dorsal–ventral axis was also significantly increased in the mutants (black brackets in Fig. 2E–G). In E12.5 control embryos, the thickness of digit 4 condensate was 135 μm. In $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants, however, the thickness was increased by as much as 37–42% to 185–192 μm (Fig. 2O).

The third abnormality we found in both $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutant limbs was that the digit condensate did not extend into the distal limb mesenchyme as far as those in the wild-type (compare the blue brackets in Fig. 2E with those in Fig. 2F and G). In digit 4, the distance from the distal tip of the digit condensate to the distal edge of the limb was increased by 46 and 53% in $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants, respectively (Fig. 2P). We noticed that digit condensates in the hindlimb also appeared shorter and wider and failed to extend into the distal limb mesenchyme in $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants at E12.5 (data not shown). However, Alcian blue staining was overall weaker and less distinct in the hindlimb at this stage, making it difficult to carry out morphometric analysis.

The abnormal morphology of the digit condensates persisted during subsequent development (Fig. 2I–K). By E13.5, digit condensates in wild-type limbs extended further distally and m/p and p1/p2 joints were visible (Fig. 2I, red and blue arrows). In $Vangl2^{Lp/Lp}$ mutants, however, only m/p joints appeared to form (Fig. 2J, red arrow). In $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants, even the m/p joints were only visible in digits 2 and 3 (Fig. 2K, red arrow). Furthermore, in both $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants, only very small condensates formed beyond the m/p joints. The distance between the distal tip of the condensates and the distal edge of the limb remained significantly greater in both mutants. Therefore, the lack of condensation in the distal limb mesenchyme is most probably the cause of loss of phalanges at later stages (Fig. 1E and F).

We also performed Alcian blue staining on different stage $Wnt5a^{−/−}$ mutants (Fig. 2D, H and L). Similar to an earlier analysis using a noggin-LacZ knock-in line to mark pre-chondrogenic condensates (11), our analysis revealed that the digit pre-chondrogenic condensates were shortened and failed to extend into the distal zone of the limb. Overall, the defects in $Wnt5a^{−/−}$ mutants appeared similar to, but more severe than, those in $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants. Collectively, these data are consistent with the idea that $Wnt5a$ signals through the PCP pathway to regulate digit morphogenesis.

We also found that the condensates of limb long bones (humerus, radius and ulna in the forelimb and femur, fibula and tibia in the hindlimb) were shortened and widened to varying degrees in $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants at E12.5 and 13.5 (Fig. 2B, C, J and K). Similar to the digits, the long bones in $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants appeared to be more affected than those in $Vangl2^{Lp/Lp}$ mutants, especially by E13.5 (compare Fig. 2J and K). As $Vangl2^{Lp/Lp}$ mutants possessed relatively normal long bones at E18.5 (Fig. 1K and N), whereas those in $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants were either shorter (Fig. 1L) or missshapen (Fig. 1O), this difference in length at early stages may suggest that a threshold size of pre-chondrogenic condensates has to be reached to ensure normal endochondral bone formation subsequently. Alternatively, $Wnt5a$ and/or the PCP pathway may have additional functions during later steps of endochondral bone formation (12,56), which contribute to the different long bone phenotypes in $Vangl2^{Lp/Lp}$ and $Wnt5a^{+/+}$; $Vangl2^{Lp/Lp}$ mutants at E18.5. These two possibilities need to be investigated further in the future. In the remaining part of the current study, we focussed on determining how the digit defects arose in $Vangl2$ mutants.

**The early limb bud shape and dimensions are altered in mouse PCP mutants**

Given that the PCP pathway is known to regulate tissue morphogenesis, we tested whether an earlier change in limb bud shape and dimensions might underlie the aberrant morphology of the digit pre-chondrogenic condensates in the mutants. In E11 (approximately 40–42 somites) $Wnt5a^{+/−}$; $Vangl2^{Lp/Lp}$
Figures 2 and 3. Early limb bud shape and dimensions are altered in mouse PCP mutants. Forelimb buds from 40 somites (approximately E11) wild-type (A and C) and Wnt5a+/−; Vangl2−/− mutants were imaged from the dorsal side (A and B) or from the posterior side (C and D). In (A) and (B), two perpendicular double arrow lines (black and red) were drawn to measure the maximum length and width of the limb buds. The white brackets in (C) and (D) represent the presumptive progress zone region, defined as 100 μm from the AER. A blue line was drawn perpendicular to the dorsal–ventral midline to determine the thickness of the progress zone.

The lack of chondrogenic condensation in the distal limb of Vangl2 mutants is due to reduced cell number and density

The altered early limb bud shape and dimensions in Vangl2−/− and Wnt5a+/−; Vangl2−/− mutants may explain the thickening and widening of the digit pre-chondrogenic condensates, but do not explain why the condensates fail to form in the distal limb mesenchyme (Fig. 2E and F). To identify the defects that inhibit condensate formation in the distal limb mesenchyme in the mutants, we performed detailed histological analyses.

We performed serial sagittal sections of forelimbs from E12.5 control, Vangl2−/− and Wnt5a+/−; Vangl2−/− embryos and stained every third section with Alcian blue to identify chondrogenic condensates (Fig. 4A, F and K). We then stained one of the adjacent sections with hematoxylin and eosin (H&E) to examine the histology of the limb (Fig. 4B, G and L). When we super-imposed the images of Alcian blue and H&E staining (Fig. 4C, H and M), we found that the regions located immediately distal to the condensates had dramatically decreased cell density in Vangl2−/− and Wnt5a+/−; Vangl2−/− mutants (black boxes in Fig. 4C, H and M and enlarged views in Fig. 4D, I and N, respectively). To examine the cell morphology in greater detail, we stained another adjacent section with the
Table 1. Shape and dimensions of early limb buds are altered in E11 Wnt5a+/-; Vangl2Lp/Lp mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Progress zone thickness (along D–V axis, 100 µm from the AER)</th>
<th>Length/width ratio</th>
<th>Limb bud length (along P–D axis)</th>
<th>Limb bud width (along A–P axis)</th>
<th>Length/width ratio</th>
<th>% change</th>
<th>P-value</th>
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<tr>
<td>Wild-type (n = 4)</td>
<td>284 ± 13</td>
<td>134 ± 12</td>
<td>162 ± 10</td>
<td>92 ± 10</td>
<td>1.73 ± 0.08</td>
<td>0.02</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Vangl2Lp/Lp (n = 3)</td>
<td>323 ± 13</td>
<td>126 ± 10</td>
<td>217 ± 15</td>
<td>101 ± 8</td>
<td>1.77 ± 0.08</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Wnt5a+/-; Vangl2Lp/Lp (n = 6)</td>
<td>139 ± 12</td>
<td>19.3 ± 3.1</td>
<td>261 ± 27</td>
<td>142 ± 6</td>
<td>1.85 ± 0.04</td>
<td>0.04</td>
<td>0.01</td>
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 fluorescent dye calcein, which stained cell bodies uniformly on paraffin sections, and performed confocal microscopy (Fig. 4E, J and O). In wild-type forelimbs, we found that the cells located in the most distal 100 µm region, in the presumptive progress zone, are densely packed and have varied shapes (Fig. 4E). The cells in the 100–200 µm range (red scale bar in Fig. 4E), however, appeared slightly less densely packed but elongated along the anterior–posterior axis, suggesting that they were in the early stages of undergoing pre-chondrogenic condensation (57).

In Vangl2Lp/Lp and Wnt5a+/-; Vangl2Lp/Lp mutants, the densely packed, distal progress-like region was reduced to only 50 µm (Fig. 4J and O, white scale bars). The cells located in the 50–200 µm range (Fig. 4J and O, red scale bars) had completely different morphologies: they appeared loosely packed and isolated from each other, extended multiple long projections and were not flattened along the A–P axis. Overall, they resemble stellate cells in the interdigital region at this stage or undifferentiated mesenchyme in E10–11 limb buds (57).

Because a threshold number and density of cells are required for mesenchymal condensation to occur (58–61), we reason that the significant reduction in cell number and density is the direct cause of the failure in forming distal digit pre-chondrogenic condensates in Vangl2Lp/Lp and Wnt5a+/-; Vangl2Lp/Lp mutants at E12.5 and the loss of phalanges at later stages.

The reduction in cell number and density in Wnt5a and Vangl2 mutants is associated with increased cell death and reduced cell proliferation

We reasoned that the reduction in progress zone size as well as cell number and density in the distal limb mesenchyme could be due to either increased cell death or reduced cell proliferation. To examine cell death, we sectioned E11.5 control and mutant forelimbs transversely and stained the sections for cleaved caspase 3 (62). Although apoptotic cells were not found in wild-type distal limb mesenchyme within 200 µm from the AER (white circle in Fig. 5A), they could be detected in this region in Vangl2Lp/Lp and Wnt5a+/-; Vangl2Lp/Lp mutant forelimbs (white arrowheads in Fig. 5B and data not shown).

To examine cell proliferation, we performed immunostaining with anti-phospho-histone H3 (pHH3) antibody to label mitotic cells in the M phase. On transverse sections of E11.5 control and Vangl2Lp/Lp forelimbs, we counted the total number of cells in a 200 µm diameter circle adjacent to the AER (white circles in Fig. 5C and D) and measured the proliferation rate in this region by calculating the percentage of pHH3-positive cells. We found that although the total number of cells in the 200 µm circle was not changed in the Vangl2Lp/Lp mutant forelimb at E11.5 (465 ± 38 in controls versus 479 ± 22 in Vangl2Lp/Lp), the proliferation rate was reduced by 30.3% (6.5 ± 1.7% in controls versus 4.5 ± 1.2% in Vangl2Lp/Lp). This reduction is statistically significant (P = 0.015). In E11.5 Wnt5a+/-; Vangl2Lp/Lp mutant forelimb, we found that the total cell number within the distal 200 µm circle also was not changed (470 ± 16), but the cell proliferation rate was reduced by 46.6% compared with the...
wild-type (3.54 ± 1.5%). This further reduction in cell proliferation rate was statistically significant when compared with the Vangl2Lp/Lp single mutant (P = 0.014) and might explain the more severe digit defects in the Wnt5a+/−; Vangl2Lp/Lp mutant.

Collectively, these data indicate that the reduction in cell number and density in the distal limb mesenchyme in Vangl2 mutants occurred after E11.5, following a significant decrease in cell proliferation and increase in apoptosis.

**Vangl2 and Wnt5a mutation alters limb bud dimensions and disrupts the Fgf–Shh–Grem1 signaling loop during limb development**

We were intrigued by the alteration in cell death and proliferation in Vangl2 mutant limb buds. The PCP pathway is known to coordinate cell polarity and directional cell behavior during tissue morphogenesis, but has not been associated directly with regulating cell proliferation or cell survival. When we isolated distal limb mesenchymal cells from E11 Vangl2Lp/Lp limb buds and maintained them for 3 days in cell culture, we found neither increased cell death nor decreased cell proliferation (data not shown). This suggested that the cell death and proliferation defects observed in Vangl2 mutant limbs might be due to the in vivo environment, rather than a disruption of PCP signaling.

Cell death and proliferation in developing limbs is known to be regulated by a delicate balance between Fgf, Shh and Bmp signaling sustained by the Fgf–Shh–Grem1 signaling loop. Fgf ligand secreted from the AER (AER-FGfs), an ectodermal structure forming the rim of the distal tip of the limb, is essential for cell proliferation and survival in the
underlying limb mesenchyme (60,62,63). AER-FGFs also stimulate cells in the posterior distal limb mesenchyme, in a region known as the zone of polarizing activity (ZPA), to produce Shh. Shh in turn maintains the expression of Msx2, a target gene of Bmp, was increased by 34.2% at 45ss (approximately E11.5), whereas Msx2 was increased by 2.1-fold. The levels of Fgf4 and Spry4 were also reduced by 42.9 and 35.3%, respectively, but the expression of Fgf8 was not altered. ***P \leq 0.005 and *P \leq 0.05. Transverse sections of E11.5 control (F) and Vangl2Lp/Lp mutant (H) forelimb buds were stained with an anti-phospho-Smad1/5/8 antibody to examine Bmp signaling activity. In wild-type embryos, pSmad1/5/8 staining was only detected in the proximal region of the forelimb (green signals in (F)). In Vangl2Lp/Lp mutants, however, pSmad1/5/8 staining could be detected in both the proximal and distal limb mesenchyme (H). (G) and (I) are higher magnification views of the distal region in (F) and (H), showing that pSmad1/5/8 staining could be detected in the AER in Vangl2Lp/Lp mutants (I), but not in controls (G). Whole-mount in situ hybridization indicated that by E13.5, although Fgf8 remained to be expressed in the distal region of each digit primordial in the wild-type (J), its expression was completely abolished in Vangl2Lp/Lp mutant forelimbs (red arrows in (J)). (K) and (L) are higher magnification views of the boxed areas in (J).

To test this hypothesis, we first performed in situ hybridization to examine the expression of Grem1, a critical node of the Fgf–Shh–Grem1 signaling loop. To more quantitatively assess the change in the Fgf–Shh–Grem1 signaling loop, we performed quantitative real-time (RT) PCR in Vangl2Lp/Lp mutants. We found that although Grem1 expression was normal at 39ss in Vangl2Lp/Lp forelimb buds, it was reduced by 34.2% at 45ss (E11.5) (Fig. 5E). Simultaneously, the expression of Msx2, a target gene of Bmp, was increased by over 2-fold. Moreover, the level of Fgf4, an AER-Fgf whose expression was known to rely on Grem1 inhibition of Bmp (66,69), was reduced by 42.9% in Vangl2Lp/Lp mutants. Spry4, a target gene of AER-Fgf, was also decreased by 35.3%, indicating that Fgf signaling in the limb mesenchyme was compromised in Vangl2Lp/Lp mutants.

To confirm the RT-PCR result and to assess Bmp signaling activity more directly in Vangl2Lp/Lp mutants, we performed immunostaining for phospho-Smad1/5/8 (Fig. 5F–I), a direct readout for Bmp signaling activation (70,71). In E11.5 wild-type embryos, pSmad1/5/8 staining was only detected in the proximal region of the forelimb (Fig. 5F), most probably in cells that were in the early stages of mesenchymal condensation. In Vangl2Lp/Lp mutants, we detected pSmad1/5/8 staining in both the proximal and the distal limb mesenchyme (Fig. 5H). Furthermore, pSmad staining was also significantly increased in the AER in Vangl2Lp/Lp mutants (compare Fig. 5G–I).
Although Bmp signaling was increased in the AER of Vangl2<sup>Lp/Lp</sup> mutants at E11.5, we found that the expression of Fgf8, the most important AER-FGF (60,72–74), was not altered at this stage (Fig. 5E). This could be due to the fact that at this stage, Bmp signaling in the AER does not directly regulate Fgf8 expression, but only impacts on Fgf8 expression through interfering with AER maintenance (66). Indeed, our in situ analysis indicated that by E13.5, although Fgf8 remained to be expressed in the distal region of each digit primordial in the wild-type, its expression was completely abolished in the distal region in Vangl2<sup>Lp/Lp</sup> mutant forelimbs (Fig. 5J, red arrows; also compare higher magnification views in Fig. 5K and L). This suggests that AER maintenance may be compromised in Vangl2<sup>Lp/Lp</sup> mutants or that a prolonged increase in Bmp signaling may lead to a down-regulation of Fgf8 expression.

The loss of phalanges in Vangl2<sup>Lp/Lp</sup> mutants can be partially rescued by reducing the dosage of Bmp4

Our findings so far support the hypothesis that altered limb bud shape and dimensions in Vangl2 mutants perturb the Fgf–Shh–Grem1 signaling loop, leading to an imbalance between Fgf and Bmp signaling in the distal limb mesenchyme and the AER. To determine whether a perturbed Fgf–Shh–Grem1 signaling loop might be responsible for the loss of phalanges in Vangl2<sup>Lp/Lp</sup> mutants, we tested whether reducing the dosage of Bmp might rescue this defect. We reasoned that by decreasing the dosage of Bmp in Vangl2<sup>Lp/Lp</sup> mutant limbs, we might be able to partially compensate for the reduced suppression of Bmp by Grem1, thereby attenuating Bmp’s negative regulation on Fgf signal transduction (64) and AER maintenance (65,66). Consequently, the balance between Fgf and Bmp signaling might be maintained longer to allow additional phalanges to form in Vangl2<sup>Lp/Lp</sup> mutants, although each digit skeletal element might still display an aberrant shape due to defects in limb morphogenesis.

Of the three Bmps expressed in the limb (Bmp2, 4 and 7), Bmp4 appears to be the primary target antagonized by Grem1, because reducing the dosage of Bmp4 by 50% using a Bmp4 null allele, Bmp4<sup>LacZ</sup> (75,76), is sufficient to rescue some of the digit skeletal defects in Grem1 null mutants (65). We therefore used the Bmp4<sup>LacZ</sup> allele to reduce the dosage of Bmp4 by 50% in Vangl2<sup>Lp/Lp</sup> mutants. In Vangl2<sup>Lp/Lp</sup> embryos, reducing the Bmp4 dosage by 50% did not alter limb skeletal formation (Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup>, Fig. 6A). In contrast, in Vangl2<sup>Lp/Lp</sup> mutants, 50% reduction of Bmp4 allowed the normal number of phalanges to form in digits 2 and 4 in the forelimb (Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup>, Fig. 6C, red arrowheads). When we compared digits 2 and 4 from Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup> mutants with those from Vangl2<sup>Lp/Lp</sup> littermates, we further found that the rescue was due to altered segmentation of the digit pre-chondrogenic condensates, because the length of the metacarpal and p1 in Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup> mutants remained the same as those in Vangl2<sup>Lp/Lp</sup> mutants (compare Fig. 6D with E and F with G). In contrast, the distal phalanges in Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup> mutants were significantly longer than those in Vangl2<sup>Lp/Lp</sup> mutants (compare Fig. 6D with E and F with G). For instance, the length of the fused p2 and p3 (p2/3, green arrows in Fig. 6B and F) in digit 4 of Vangl2<sup>Lp/Lp</sup> was 250 ± 22 μm, whereas the total length of p2 and p3 in Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup> mutants was 540 ± 32 μm, an increase of 116% (n = 4; P < 0.02). Similarly, the total length of p2 and p3 in digit 2 of the Bmp4<sup>LacZ/Lp</sup>; Vangl2<sup>Lp/Lp</sup> mutant was increased by 36% when compared...
with the Vangl2^Lp/Lp mutant (526 ± 18 versus 388 ± 17 μm, n = 4; P < 0.03; Fig. 6D and E). These morphological analyses suggest that the additional phalanges in Bmp4^LacZ/+; Vangl2^Lp/Lp mutants arose from longer pre-chondrogenic condensate formation in the distal limb mesenchyme. The partial rescue of digit skeletal defects in Bmp4^LacZ/+; Vangl2^Lp/Lp mutants provided strong support for our hypothesis that the loss of phalanges in mouse PCP mutants is due to an aberrant increase in Bmp signaling.

We did not observe consistent rescue in digits 1 and 5, and the length of the distal p2/3 of digit 3 was only increased slightly (14%) in Bmp4^LacZ/+; Vangl2^Lp/Lp mutants. In addition to Bmp4, Bmp2 and Bmp7 are also expressed in the limb. We speculate that the variation in the rescue of individual digits may relate to the regional difference in the levels of the three Bmps.

**DISCUSSION**

In this study, we examined the limb skeletal defects in mice carrying loss-of-function mutations in the PCP gene Vangl2. Our combined genetic, morphological and embryological analyses provide strong evidence that PCP signaling functions downstream of Wnt5a to promote limb bud distal extension while restricting its expansion in width and thickness. Altered limb bud shape and dimensions in Vangl2 mutants affect not only the dimensions of the chondrogenic primordia of the digits, but also the signaling network important for limb growth (Fig. 6H). These findings provide novel insight into the signaling mechanisms of Wnt5a/Ror2 during limb development and the pathogenesis of BDB1 and RRS.

The PCP pathway mediates Wnt5a/Ror2 function during limb skeletal formation and may underlie both BDB1 and RRS in humans

BDB1 and RRS are two distinct skeletal dysplasias caused by mutations in ROR2. Extensive *in vitro* biochemical and cell biological studies have demonstrated that Ror2 functions as a co-receptor of Wnt5a, but the signaling events downstream of Ror2 have remained controversial. The proposed downstream events of Wnt5a/Ror2 signaling include activation of JNK signaling (9), inhibition of canonical Wnt signaling (8), promoting filopodia formation (77) and membrane recruitment, and activation of Src (78). How these *in vitro* findings relate to the *in vivo* function of Ror2 during limb development, however, is not clear.

Our analyses of the limb defects in mouse Vangl2^Lp mutants provide the first *in vivo* evidence that the PCP pathway mediates Wnt5a/Ror2 function during limb development and that disruption of PCP signaling may underlie BDB1. First, the rudimentary primary claw field at the tip of some digits in the Vangl2 mutant forelimb is reminiscent of the nail defects in some human BDB1 patients (2,3). Secondly, Vangl2 mutants display loss of the middle phalanges in the digits. Identical defects have also been reported in mice carrying the Ror2^W749X mutation that truncates Ror2 after the TK domain and causes the most severe form of BDB1 in humans (55). Thirdly, Wnt5a and Vangl2 genetically interact^2, reducing the Wnt5a dosage by 50% significantly enhanced both the severity and the penetrance of the loss of phalanges in Vangl2^Lp/Lp mutants. Our results also support the previous study in *Xenopus*, in which Ror2 has been suggested to function as a co-receptor for the PCP pathway during CE (10).

Although the digit defects in Wnt5a^+/−; Vangl2^Lp/Lp mutants are more severe than those in Vangl2^Lp/Lp mutants, they are still less than those in Wnt5a^−/− mutants, in which all phalanges fail to form (11). We speculate that Vangl1, the other Vang homolog also expressed in the limb mesenchyme (79), may partially compensate for the loss of Vang function in Vangl2^Lp/Lp and Wnt5a^+/−; Vangl2^Lp/Lp mutants, leading to incomplete disruption of PCP signaling.

The genetic interaction between Wnt5a and Vangl2 also implies that disruption of PCP signaling may underlie RRS as well. Although Vangl2^Lp/Lp mutants do not display any detectable limb long bone defects, Wnt5a^+/−; Vangl2^Lp/Lp mutants display a shortened radius and ulna in the forelimb and a misshapen tibia and fibula in the hindlimb. These defects resemble the mesomelic limb shortening in RRS patients and are very similar to the long bone phenotype observed in mice carrying the Ror2^Tmiac2 mutation that truncates the entire cytoplasmic domain of Ror2 (7,55). Our results imply that different degrees of PCP signaling disruption may cause different spectra of limb defects observed in BDB1 and RRS.

How disruption of PCP signaling affects limb long bone development needs to be investigated further in the future. Our skeletal staining indicates that the chondrogenic condensates of the radius and ulna in Wnt5a^−/−; Vangl2^Lp/Lp mutants are already shortened significantly by E13.5 (Fig. 2I), suggesting that an early abnormality in condensate formation may be a contributing factor. In contrast, a recent study indicates that at later stages of endochondral ossification in chick, PCP signaling plays an essential role in regulating cell polarity and cell arrangement during chondrocyte proliferation and differentiation (56). A conditional knock-out approach will be needed in the future to investigate the contribution of PCP signaling at different stages of endochondral ossification.

PCP signaling regulates limb bud morphogenesis and the shape and dimensions of pre-chondrogenic condensates of limb skeleton

PCP signaling is known to regulate cell polarity and directional cell behavior during polarized tissue morphogenesis. Our morphometric analyses indeed indicate that limb buds in Wnt5a^+/−; Vangl2^Lp/Lp and Vangl2^Lp/Lp mutants are thicker, wider and shorter, suggesting that PCP signaling is required for limb distal extension while restricting its excessive expansion in width and thickness. The limb bud thickening and shortening defects in Wnt5a^+/−; Vangl2^Lp/Lp are more severe than those in Vangl2^Lp/Lp mutants (Table 1), suggesting that Wnt5a signals through Vangl1 and Vangl2 to regulate limb morphogenesis. Consistent with this notion, we have found that limb buds in the Wnt5a^+/− mutant display even more severe thickening and shortening defects than those in Wnt5a^+/−; Vangl2^Lp/Lp mutants (Wang et al., unpublished data).
A recent study revealed that Wnt ligands secreted from the surface ectoderm and Fgf derived from the AER synergistically stimulated Nmyc expression to promote rapid cell proliferation in the subjacent distal limb mesenchyme (80). This distally localized mitotic zone provides a basis for the distal outgrowth of the limb. However, computational simulation indicates that the presence of a distal zone with a higher proliferation rate is not sufficient to account for the morphological changes occurring during limb bud outgrowth (81,82). The computation models suggest that directional cell behavior is also required (81,82). This has been demonstrated experimentally with Dil labeling in chick limbs, in which cells in the progress zone are found to migrate directionally towards the AER (83,84). Because the AER rims the distal tip of the limb bud, the highly oriented cell migration may simultaneously promote limb bud elongation along the P–D axis and restrict its expansion along the D–V axis following rapid cell proliferation. Furthermore, a recent study also reveals that the mitotic orientation in the limb mesenchyme also displays some distal bias (81). Whether these directional cell behaviors occur in a PCP-dependent manner in the mouse needs to be examined in the future. We note that Wnt5a is expressed in a graded fashion in the distal limb mesenchyme, with highest expression directly underlying the AER (11). Therefore, it is conceivable that Wnt5a may activate PCP signaling specifically in the distal limb to guide directional cell migration/division (85) or enable cells to respond to other chemotactic cues (83,86,87).

An altered limb bud shape and dimensions may directly impact on the mesenchymal condensation process in Vangl2 mutants. For instance, the expression of canonical Wnts in the limb ectoderm can inhibit the condensation process (80). Consequently, mesenchymal condensation only occurs in the center of the limb, where the cells are out of the range of Wnt signals. Thus, an increase in limb bud thickness in Vangl2 mutants is likely to lead to a corresponding increase in the thickness of digit pre-chondrogenic condensates.

**PCP-mediated limb morphogenesis is necessary to sustain the signaling network important for limb bud growth**

Our studies also reveal the unexpected result that altered limb bud shape and dimensions in mouse PCP mutants affect the signaling network critical for limb growth. Limb skeletal development is critically dependent on rapid expansion of limb bud mesenchyme to produce the required number of chondrogenic progenitors (58–60,63). Cell death and proliferation in the limb ectoderm is regulated in part by a delicate balance between the Fgf and Shh signaling that promotes cell survival and proliferation (60,62,63,88) and the counteracting Bmp signaling (64,65,67,89,90). The Bmp antagonist Greml1 plays a key role in the integration of the Fgf, Shh and Bmp pathways into an interlinked, self-regulatory signaling network (Fig. 6H). At the center of this signaling network is the Fgf–Shh–Greml1 signaling loop, in which AER-Fgf promotes Shh expression to maintain Greml1 transcription. Greml1 in turn suppresses Bmp to sustain AER-Fgf expression, AER maintenance and AER-Fgf signaling activity (64–67). In addition, experimental manipulation in chick limb has demonstrated that Greml1 transcription is promoted in a dose-dependent manner when the Bmp level is moderate, but is inhibited when the Bmp level is high (91,92). This concentration-dependent regulation of Greml1 by Bmp is likely to be another critical feedback mechanism that maintains a consistent low level of Bmp signaling during early limb development. Furthermore, although Bmp negatively regulates the feedback loop between AER-Fgf and Shh, its expression is positively regulated by Fgf signaling (64,69). Therefore, these interconnected feedback loops allow the establishment of a robust, self-regulatory signaling network that can buffer certain levels of variation in the dosage of Fgf, Shh and Bmp pathway components (65).

An interesting aspect of this signaling network is that Fgf, Shh and Greml1 are secreted from spatially distinct signaling centers (the distal AER, posterior ZPA and dorsal–ventral margin of the limb, respectively, Fig. 6H) and have limited signaling range. Therefore, its duration and robustness are likely to be affected by changes in limb dimensions along the D–V, A–P and P–D axes. We speculate that in Vangl2 mutants, the expansion in limb bud thickness may render Greml1 secreted from the D/V margin to be insufficient to constrain Bmp signaling within its normal threshold. An aberrant increase in Bmp signaling may in turn interfere with Fgf signaling, AER-Fgf expression and AER maintenance. The expansion in limb bud width, in contrast, increases the distance between the Shh-producing ZPA and the limb mesenchyme at the D/V margin capable of expressing Greml1 (92,93), leading to a down-regulation of Greml1 that further enhances ectopic Bmp signaling. A combination of the two events in Vangl2 mutants may perturb the fine balance between Fgf, Shh and Bmp signaling and trigger a premature breakdown of the signaling network, leading to limb growth defects and loss of phalanges.

Our genetic compensation experiment provides the strongest evidence for the above hypothesis. Reducing the dosage of Bmp4 by 50% allows the normal number of phalanges to form in digits 2 and 4 in Vangl2 mutants, and our morphometric analyses suggest that the rescue is due to formation of longer distal digit condensates, but not alteration of digit ray segmentation. Presumably, the reduction in Bmp4 dosage is able to partially compensate for the insufficient antagonist effect of Greml1 in Vangl2 mutant limb buds, allowing the balance between Fgf and Bmp signaling to be maintained for longer. Bmp4 is expressed in both the anterior and posterior limb mesenchyme, but not in the central region (94). This expression pattern probably explains why digits 2 and 4 are rescued in Bmp4+/-; Vangl2+/Lp mutants, whereas digit 3 is not. Furthermore, Bmp2 is expressed most prominently in the posterior limb mesenchyme, whereas Bmp7 is expressed ubiquitously in the limb mesenchyme (94). The expression of Bmp2 and 7 may prevent digits 1 and 5 from being rescued significantly in Bmp4+/-; Vangl2+/Lp mutants.

Although the results collectively support our hypothesis that the limb growth defects and loss of phalanges in Vangl2 mutants originate from abnormal limb bud morphogenesis that perturbs the signaling cross-talk between Fgf, Shh and Bmp, we note that other PCP-related functions may also be responsible. For instance, Vangl2 mutation may lead to altered cell adhesion properties or a loss in cell polarity that...
results in the observed reduction in cell proliferation or survival.

It is also interesting to note that in the Vangl2Lp/Lp mutant, p2 is lost but a terminal phalanx resembling p3 continues to form. We think that this defect may be an imbalance between Bmp and Fgf signaling that reduces cell survival and proliferation. It is thought that the pre-chondrogenic condensate formation of each limb skeletal element occurs on a fixed schedule and progresses in a proximal-to-distal fashion (59). Therefore, it is likely that in the Vangl2Lp/Lp mutant, reduced proliferation results in a situation in which an insufficient number of cells is present when mesenchymal condensation of p2 is scheduled to happen. In contrast, by the time the p3 condensate is scheduled to form, the cells in the mutant progress zone, although reduced in number and proliferating at a slower rate, are able to generate sufficient pool of cells to allow mesenchymal condensation to occur. Thus, we refer to the terminal phalanx in Vangl2 mutants as p2/3 because it is likely to be derived from progenitors that normally give rise to both p2 and p3 in wild-type limbs. We note that the same phenomenon, in which middle phalanges are lost but the terminal phalanx remains, is also observed in mouse mutants in which Fgf signaling is attenuated but not completely disrupted, such as Fgf8 single mutants or those in which Fgfr3 is disrupted in the limb mesenchyme (73,95,96).

In two mouse mutants in which Ror2 is truncated immediately after the transmembrane domain or after the TK domain, a similar brachyactly defect is observed where p2 is lost but p3 remains. Skeletal staining at E14.5 also revealed a lack of condensate formation in the distal limb mesenchyme (55). It will be important to test in the future whether the loss of phalanges in Ror2 mutants arises similarly to that in Vangl2Lp/Lp mutants and whether it can also be suppressed by a reduced dosage of Bmp. This will confirm whether an aberrant increase in Bmp signaling may also be the pathogenic mechanism for the loss of phalanges in BDB1 patients.

Our work also contrasts with two published studies on the digit skeletal defects in Wnt5a−/− mutants (11,53). In the first study (11), Wnt5a was proposed to be a mitogen, on the basis of a decrease in the proliferation rate in the distal zone in Wnt5a−/− limbs at E12.5. Our unpublished results also confirm that reduction in cell proliferation can be detected in Wnt5a−/− distal limbs by E11.5, similar to the Vangl2Lp/Lp mutant. However, because the PCP pathway has never been known to directly regulate cell proliferation, we reasoned that the reduced cell proliferation in Wnt5a−/− mutants may also be due secondarily to an aberrant increase in Bmp signaling, similar to the Vangl2Lp/Lp mutant.

In the second study, Wnt5a was proposed to promote mesenchymal condensation by regulating GSK-3β-independent β-catenin degradation to antagonize canonical Wnt signaling in the distal limb mesenchyme (53). It is intriguing to note that several publications reported that in wild-type embryos, canonical Wnt signaling was in fact high in the distal limb mesenchyme (80,97), where Wnt5a was specifically expressed. Secondly, as canonical Wnt signaling keeps distal limb mesenchymal cells in a highly proliferative state (80), if Wnt5a−/− distal limbs indeed possessed higher levels of canonical Wnt signaling activity, we would expect an increased cell proliferation rate in the distal zone. However, both the previous publication (11) and our unpublished result on Wnt5a−/− mutants revealed the opposite. Nevertheless, it remains possible that Wnt5a activates a pathway parallel to the PCP pathway to modulate canonical Wnt signaling to promote mesenchymal condensation. This hypothesis can be tested by determining whether reducing the dosage of β-catenin can rescue the digit defects in Wnt5a−/− mutants.

In conclusion, our study provides the first evidence that Wnt5 functions, at least in part, through the PCP pathway to regulate limb bud morphogenesis and control its shape and dimensions. Proper control of limb bud shape and dimensions is important not only for forming pre-chondrogenic condensates of proper dimensions, but also for maintaining a signaling network that sustains limb growth and patterning (Fig. 6H). These results will shed new light on the pathogenic mechanisms in BDB1 and RRS and provide new insight into the biology of limb development.

MATERIALS AND METHODS

Mice

Wnt5a and Vangl2Lp mutant mice were obtained from the Jackson Laboratory and genotyped as described (11,54). Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Alabama at Birmingham.

Skeletal staining

Skeletal staining of E12.5–18.5 embryos was performed as described (98). For E12.5 and 13.5 embryos, Alizarin red was omitted from the staining solution, and clearing time in 1% KOH was limited to 1 h. Cleared limb skeletons were imaged with a Leica MZ16FA stereoscope equipped with a Leica DFC 490 CCD camera. Morphometric measurement and analyses were carried out using the LAS Interactive Measurement Module.

Histology, in situ hybridization and immunostaining

Embryos were collected between E11.5 and 13.5 and fixed in 4% paraformaldehyde at 4°C overnight. For sectioning, limb buds were removed and embedded in Histogel (Thermo/Fisher, Pittsburgh, PA) in either sagittal or transverse orientation, dehydrated and infiltrated with paraffin. Samples were sectioned at 7 μm, mounted on slides and stained with H&E to examine histology or with skeletal staining solution (4 vol of 0.1 M HCl and 1 vol of 0.5% Alcian blue stock solution) to detect chondrogenic condensates. To examine cell morphology in greater detail, sections were rehydrated and stained in 0.2% Calcein (Sigma, St Louis, MO) in deionized water. After extensive washing in water, sections were imaged with an Olympus FV1000 confocal scanning microscope.

For immunostaining, sections were boiled for 15 min in citrate buffer, blocked with 10% goat serum for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies used were anti-Cleaved
Caspase-3 (Asp175) (Cell Signaling, Danvers, MA), anti-pHH3 H3 (Ser10) (Millipore, Billerica, MA) and anti-phospho-Smad1/5/8 (Cell Signaling). Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) was used as secondary antibody. A tyramide signal amplification kit (PerkinElmer, Covina, CA, #NEL741E001KT) was used for pSmad detection (70, 71). Slides were mounted with ProLong Gold antifade with DAPI (Invitrogen) and analyzed using confocal microscopy. Whole-mount in situ hybridization was carried out with a standard protocol (99).

Quantitative real-time PCR

Forelimb buds from 45 somite (E11.5) Vangl2 mutants and control littermates were removed at the base and lysed with Trizol reagent (Invitrogen) to collect total RNA. Quantification of total RNA was performed using a Bio-Rad spectrophotometer, and samples with an optical density 260:280 of 1.7–1.9 were used for subsequent reverse transcription with a High-Capacity cDNA reverse transcription Kit from Applied Biosystems, Foster City, CA. Real-time Q-PCR was performed using LightCycler480 SYBR Green I Master according to the manufacturer’s instructions (Roche, Indianapolis, IN, #04707516001). GAPDH was run in parallel with test genes to normalize gene expression. cDNA samples from three mutants and three controls were tested, and each cDNA sample was tested in duplicate within an assay. Real-time PCRs were carried out using the LightCycler rapid thermalycler system (Roche), according to the manufacturer’s instructions with the following conditions: 95°C for 10 min, 45 repeated cycles including denaturation (95°C) for 30 s, annealing (55°C) for 30 s and extension (72°C) for 60 s with fluorescence detection at the end of each 72°C step and then melting with continuous fluorescence detection to 95°C. The data were analyzed with the LightCycler 480 sw1.5 software. Primers used were:

GAPDH [TGAAGGTGGATGTCACCGATTTGGGT; AAATGAGCCCCAGCTTTCCATG (100)]; Grem1 [CCCACGGAATGACAGATGA; AAGCAACGC TCCCACAGTGTA (65)]; Msx2 [ATACAGGACCGCAGATCACT; TCCGTTGG TCTTGTGTTC (65)]; Fgf4 [TCTAATGCAACGTTCGATCT; CTTCAATGTAAG CGACACT (101)]; Fgf8 [GTGCGAACCAGTCTTGG; GCCCAAGTCTC TGGCTGCC (102)]; Spry4 [TAGAAGGCCTGACCCTTGGTA; TGGAGCCATGT GATCTAGGA (103)].

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Conflict of Interest statement. None declared.

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