Disp1 regulates growth of mammalian long bones through the control of Ihh distribution

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Dispatched1 (Disp1) is required for the release of cholesterol modified hedgehog (Hh) proteins from producing cells. We investigated the role of Disp1 in Indian hedgehog (Ihh) signaling in the developing bone by bypassing the lethality of the Disp1 C829F allele at early somite stages through the supply of non-cholesterol modified Sonic hedgehog (N-Shh). The long bones that develop in the absence of wild-type Disp1, while clearly shorter, have a juxtaposition of proliferating and non-proliferating hypertrophic chondrocytes that is markedly more normal in organization than those of Ihh null mutants. Direct analysis of Ihh trafficking in the target field demonstrates that Ihh is distributed well beyond Ihh expressing cells though the range of movement and signaling action is more restricted than in wild-type long bones. Consequently, a PTHrP–Ihh feedback loop is established, but over a shorter distance, reflecting the reduced range of Ihh movement. These analyses of the Disp1 C829F mutation demonstrate that Disp1 is not absolutely required for the paracrine signaling role of Ihh in the skeleton. However, Disp1 is critical for the full extent of signaling within the chondrocyte target field and consequently the establishment of a normal skeletal growth plate.

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Introduction

The bones of vertebrates form through two different mechanisms. Intramembranous ossification involves the direct differentiation of mesenchymal progenitors into osteoblasts (e.g. cranium and facial bones). In contrast, endochondral ossification refers to the differentiation of mesenchymal progenitors into chondrocytes and the eventual replacement of some regions by bone matrix secreting osteoblasts (e.g. long bones, axial skeleton) (Olsen et al., 2000). For the long bones of mice, this process begins with the condensation of mesenchymal cells originating from the lateral plate mesoderm in the limb at embryonic day 9.5 (E9.5). These differentiate into a chondrocyte-based skeletal anlage surrounded by a flattened layer of perichondrial cells. Following a skeletal element specific program, chondrocytes in the middle of the element exit the cell cycle, then undergo a period of hypertrophic cell growth before undergoing apoptosis. Osteoblasts initially arise in the perichondrial/perioseal region adjacent to the prehypertrophic chondrocyte where they give rise to cortical bone. The bone of the primary spongiosa forms in the center of the long bone on vascular invasion following hypertrophic apoptosis. In this way, growth of the long bone is regulated by the actions at both ends of a growth plate (Olsen et al., 2000; Karsenty and Wagner, 2002).

In the growth zone, chondrocytes can be classified by morphology, gene expression and behavior into four distinct regional subpopulations. Closer to the epiphyseal surface, resting chondrocytes show low levels of proliferation (postnatal stages to puberty), proliferating chondrocytes, prehypertrophic chondrocytes (mitotically inactive, non-hypertrophic and Ihh expressing), and the enlarged quiescent hypertrophic chondrocytes that express a number of distinct molecular markers, including a distinct collagen form, collagen X (Kronenberg, 2003).

Ihh is a major regulator of the ordered proliferation and differentiation of chondrocytes (for review see Kronenberg, 2003). Ihh induces chondrocytes to proliferate (St-Jacques et al., 1999) in a cell autonomous manner (Long et al., 2001). Ihh also induces expression of parathyroid hormone related peptide (PTHrP) in periarticular chondrocytes (Vortkamp et al., 1996; Lanske et al., 1996; St-Jacques et al., 1999). PTHrP acts to maintain PTHrP receptor expressing chondrocytes within the range of the signal in a mitotic state, blocking the activation of a hypertrophic differentiation program (Karaplis et al., 1994; Vortkamp et al., 1996; Karp et al., 2000). Thus, the Ihh–PTHrP interaction establishes and maintains an appropriate growth zone throughout skeletal development.

It is unclear how Ihh regulates PTHrP expression in periarticular cells. In one model, Ihh acts directly on PTHrP producing cells to regulate PTHrP production (Koziel et al., 2004; Hilton et al., 2007). Alternatively, a secondary signaling relay has been invoked (Vortkamp et al., 1996; Alvarez et al., 2002; Dentice et al., 2005). BMPs have been postulated to play a secondary relay role as they do in...
Fig. 1. Long bones lacking functional Disp1 are reduced in length but are more similar to wild-type than Ihh mutants. This is evident in skeletal preparations of Disp1^C829F/−:Shh^+/−Sox2Cre (A), Disp1^C829F/C829F;Shh^+/−Sox2Cre (B) and Ihh^−/−;Sox2Cre (C) E18.5 embryos, and close-up of limb skeletons from control (D), mutant (E) and Ihh null (F) embryos with the forelimb on the top and hindlimb on the bottom. Control embryos have an extra digit 1 in the forelimb (*) as a result of N-Shh expression. Alizarin red stains mineralized matrix and alcian blue stains cartilage. Scale bar, 0.5 cm.

Fig. 2. Growth plates retain functionality in the absence of wild-type copy of Disp1. E18.5 tibiae sections (6 μm) stained with hematoxylin/eosin (H&E), from control (A), mutant (B) and Ihh null embryos (C) show the presence of the same cell types in mutant and control tibiae (green: hypertrophic chondrocytes (H.C.), red: proliferating chondrocytes (Prol. C.), orange: round proliferating chondrocytes (R. Prol. C.), brown: columnar flat proliferating chondrocytes (C. F. Prol. C.)) in contrast to Ihh null tibiae where all chondrocytes are hypertrophic. BrdU labeling in control (D), mutant (E) and Ihh null (F) growth plates indicates the presence of proliferating chondrocytes in the first two while in the absence of Ihh chondrocyte proliferation is almost completely lost. Von Kossa staining in control (G), mutant (H) and Ihh null (I) growth plates demonstrates the ability of the mutant growth plate to induce ossification of perichondrium in contrast to the situation of Ihh absence, when the only mineralized matrix is deposited by the hypertrophic chondrocytes. Arrow (_ve) indicates the area of bone collar formation. Scale bar, 100 μm.
imaginal disc patterning in response to *Drosophila* Hh (Torroja et al., 2005). However, BMPs appear to be unable to substitute for Ihh in *in vitro* systems (Minina et al., 2001; Koziel et al., 2004; TGFβ2 appears to be required for Sonic hedgehog (Shh), a surrogate for Ihh-mediated effects *in vitro*, but the growth of bones is not significantly affected in TGFβ2 mutants (Sanford et al., 1997). Direct visualization of expression of Hh-targets (e.g. Ptc1) in PTHrP expressing cells has not given a clear picture at different stages of long bone growth (Koziel et al., 2004). In addition to the aforementioned roles, Ihh plays a third vital role in the development of osteoblasts in the perichondrium adjacent to the prehypertrophic chondrocytes (St-Jacques et al., 1999; Long et al., 2004).

Hh ligands undergo two lipid modifications; addition of a C-terminal cholesterol moiety in conjunction with cleavage of the precursor protein to its active signaling form, and a covalent attachment of an N-terminal palmitate (Porter et al., 1996; Pepinsky et al., 1998). Each ligand signals through a single membrane receptor protein, patched1 (Ptc1), Hh-Ptc1 binding leads to the derepression of the seven-pass membrane protein smoothened (Smo), and pathway activation in the responsive cell (reviewed in Ingham and McMahon, 2001; Hooper and Scott, 2005). Interestingly, paracrine signaling by cholesterol modified Hh ligands requires the action of a Ptc1 related membrane protein, Disp1, within Hh producing cells both in the fruit fly (Burke et al., 1999) and in early stage mouse embryos (Ma et al., 2002; Caspary et al., 2002; Kawakami et al., 2002; Tian et al., 2005).

Here we investigated the specific function of long range Ihh signaling in mouse skeletal development by examining skeletal development in mice homozygous for a mutant Disp1 allele (C829F) that removes Disp1 activity (Caspary et al., 2002). As a result of the critical role of Disp1, Disp1C829F/C829F mutants die at E9.5 due to the loss of paracrine Ihh and Shh signaling resembling Shh−/−;Ihh−/− compound mutants (Zhang et al., 2001). The similar phenotype of Disp1C829F/C829F mutants and those deleting most of Disp1 suggest that this allele is likely a null allele, precluding analysis of later action during Ihh regulated skeletal development (Caspary et al., 2002; Ma et al., 2002; Kawakami et al., 2002; Tian et al., 2005). One solution to this problem (Tian et al., 2005) uses the conditional activation of N-Shh, a non-lipid containing but bioactive ligand (Lewis et al., 2001) to rescue the early lethality. Disp1 is not essential for N-Shh release and activity (Tian et al., 2005). In a Disp1C829F/C829F;Shh−/−;Sox2Cre embryo (referred to as mutant hereafter), early Sox2Cre activity in the epiblast (Hayashi et al., 2002) activates production of N-Shh, from the modified Shh locus, in a Disp1C829F/C829F background. N-Shh is sufficient for early embryonic patterning. This enables later arising skeletal elements to develop in the Disp1C829F/C829F background to then determine the role of Disp1 in Ihh export into the skeletal target field.

Surprisingly, although we observe a marked reduction in skeletal growth, a growth plate forms. Proliferating chondrocytes are present and we observe a stratification of cell types in the growth zone that represents a significantly less severe phenotype than that observed in Ihh mutants. Although the physical area over which Ihh is distributed in the target field is reduced, we observe Ihh ligand directly, and Ihh signaling, beyond the prehypertrophic chondrocyte cells that synthesize the ligand. Our data indicate that Disp1 is required for a normal range of action of Ihh in bone growth regulation. They also raise interesting questions about possible organ specific differences in Disp1 function.

### Materials and methods

Mutant embryos were generated by crossing Disp1C829F;Sox2Cre female mice to Disp1C829F;ShhCre;Ctnmb1 null males. Embryos were collected at E16.5 and E18.5. The left forelimbs and hindlimbs were removed for sectioning while the remaining embryo was subjected to alcian blue/alizarin red staining as previously described to visualize the general organization of cartilage and bone, respectively (Long et al., 2001). Samples for histological and *in situ* hybridization analysis were fixed in 4% paraformaldehyde overnight, washed in PBS and stored in 70% ethanol prior to paraffin-wax embedding and sectioning. *In situ* hybridization using 32P-labeled probes was carried out as described previously (Long et al., 2001; Wilkinson, 1993). Quantitation of the Ptc1 mRNA distribution was done using imageJ software. Analysis of BrdU incorporation was performed as previously described (Long et al., 2001). Hematoxylin/eosin and von Kossa staining were carried out according to standard histological methods. Samples that were destined for immunohistochemistry were fixed with St Marie’s fixative (95% ethanol, 1% acetic acid) overnight, and then washed with 95% ethanol before embedding in paraffin-wax. Antibody Ab80 (Bumcrot et al., 1995), originally raised against Shh but cross-reactive with Ihh (Griffit-Linde et al., 2001), was used in the current study for immunohistochemical analysis of Ihh distribution according to a described protocol (Griffit-Linde et al., 2001).

### Results and discussion

The mutant embryos collected at E18.5 have severe problems with many aspects of skeletal development, when compared to mice that

**Fig. 3.** The number of hypertrophic and proliferating chondrocytes is proportionately reduced in the Disp1C829F/C829F mutant growth plates. (A) When the number of cell diameters in the middle of the element along the axis of bone elongation is quantified, all the histologically identified cell types are represented but each domain is reduced in the mutants. In all cases, the *p*-value calculated was <0.005 (*). Six control and 8 mutant elements were examined. (B) Expressing the cell numbers as a percentage of the total cells counted indicates that the difference between control and mutant is not significant. (C) The percentage of BrdU-labeled cells is the same in control and mutant growth plates despite the smaller size of the second. Three control and 3 mutant elements were examined.
have one wild-type copy of Disp1 allele (Fig. 1, Supplementary Fig. 1). Shh is required for intramembranous ossification and the interpretation of the results for certain aspects of skeletal development becomes complicated, when examining the N-Shh mediated rescue. However, only Ihh is expressed in the developing long bones, and thus it is fair to view the developing long bones as a simple Disp1(C829F/C829F) homozygous mutant rescue. As N-Shh is expressed from the endogenous Shh locus, and because Shh is not expressed in the growth plate, the phenotype is due exclusively to the absence of wild-type Disp1 activity.

Long bones are significantly shorter in the mutant mice, but unlike Ihh mutants there is a visible growth zone consistent with a partial but not complete loss of Ihh actions (Fig. 1). A closer look at the reduced growth zone by histological analysis identifies a normal stratification of cells types in the mutant limbs in contrast to that of Ihh mutants where hypertrophic chondrocytes predominate (Fig. 2). At E16.5, growth plates display these same characteristics (Supplementary Fig. 2). A quantitative analysis of the relative numbers of hypertrophic, columnar flat proliferating and round proliferating chondrocytes revealed that each population was proportionately reduced, suggesting that normal Ihh signaling occurs, but over a reduced target field (Fig. 3). The reduction of hypertrophic chondrocytes is most likely not a direct effect of altered Ihh signaling. The hypertrophic pool increases by the entry of postmitotic cells exiting the proliferating pool and decreases by the loss of hypertrophic chondrocytes through apoptosis. In the mutant, the decrease in hypertrophic chondrocyte number results from a decreased rate of chondrocytes through apoptosis. Remarkably, each distant site of paracrine Ihh is expressed from the endogenous Shh locus, and because Shh is not expressed in the growth plate, the zone of high proliferation activity is shorter following the general shortening of the growth plate. von Kossa staining indicates that Ihh-dependent ossification of the bone collar, a paracrine Ihh activity, is observed in Disp1(C829F/C829F) mutants again suggestive of some level of Ihh paracrine signaling function (Fig. 2).

Expression of marker genes reveals that each population of cells maintains their special order and appropriate identity in the mutant, despite the shortening of their specific domains. Thus, col2a1 and colX, markers of undifferentiated and hypertrophic chondrocytes, respectively, are appropriately positioned, an Ihh prehypertrophic chondrocyte zone is established and expression of the PTHrP receptor (PPR) partially overlaps Ihh in the growth zone (Fig. 4).

Next, we examined directly the distribution of Ihh ligand within the skeletal target field and the target field response to that ligand as measured by the transcriptional upregulation of Ptc1, a conserved Hh specific feedback response in all Hh target fields. Long bones of genetic controls, similar to wild-type embryos, exhibit extensive Ptc1 upregulation toward the articular direction beyond the source of Ihh ligand (Fig. 5). In addition, Ptc1 is upregulated in periosteal precursors adjacent to the hypertrophic Ihh producing chondrocytes and in a third distant population of cells at the hypertrophic/bone marrow interface. Remarkably, each distant site of paracrine Ihh is observed by Ptc1 upregulation in Disp1(C829F/C829F) mutants. Furthermore, immunostaining demonstrated an extended distribution of the ligand many cell diameters from the ligand source, a distribution that closely correlates with Ptc1 regulation (Fig. 5). Thus, although the range of Ihh signaling and the level of signaling were significantly reduced (Fig. 5), the result clearly demonstrates that distant trafficking of Ihh ligand occurs within the skeleton, a surprising result given the loss of paracrine Shh and Ihh signaling in Disp1(C829F/C829F) mutants at earlier developmental stages (Caspar et al., 2002).

To compare the Ihh gradient in the field of proliferative chondrocytes of control and mutant growth plates, we assumed
that the concentration of Ihh in steady state along the long axis of
the bone is \( C_{\text{Ihh}} = C_{\text{Ihh source}} e^{-x/(K/D)} \). In this equation, \( C_{\text{Ihh source}} \) is the concentration of Ihh protein at the source, \( C_{\text{Ihh}} \) is the concentration of Ihh protein at distance \( x \) from the source while \( K \) and \( D \) are the
rate of absorption and diffusion of Ihh protein. We calculated the
value of the proportionality factor in the exponent \( 1/(K/D) \) using data
based on the Ptch1 gradient. Thus, the exponential decay of Ihh
signaling strength is being measured rather than Ihh protein itself,
but the first will follow the second in the same way for both mutant
and control and this makes it useful to compare the qualitative
behavior of the decay. The \( 1/(K/D) \) is 0.0027 for the control and
0.0026 for the mutant indicating that the effect of Disp1 mutation is
the result of a decrease in the output of Ihh protein from the
expressing cells rather than the trafficking of Ihh protein in the
target field.

These results raise an interesting possibility, that while Disp1
clearly plays a role in Ihh action in the skeleton, other Disp1
independent processes may also regulate long-range Ihh signaling
specifically within this tissue. A second Disp1 related gene has been
reported, Disp2 (Ma et al., 2002). However, Disp2 is unlikely to
substitute for Disp1 in the skeleton, as it is not expressed in the
developing growth plate (Supplementary Fig. 3). The possibility that
Ihh and Shh have different requirement for Disp1 cannot be excluded
although it seems unlikely because the Disp1 mutants seem more
similar to Shh\(^{-/-}\);Ihh\(^{-/-}\) and Smo\(^{-/-}\) mutants than to single Shh\(^{-/-}\)
mutants (Casparry et al., 2002; Ma et al., 2002; Kawakami et al., 2002;
Tian et al., 2005). An alternative possibility is that while Disp1\(^{C829F}\) has
lost much of its activity, relatively little activity is required for its
action in Ihh export in the skeleton compared to earlier stages of
mouse development.

Fig. 5. Ihh protein is found at some distance from the source in growth plates lacking wild-type Disp1 function. Ptch1 expression domain is similar in control (A) and mutant (C) growth plates. In both cases, Ptch1 expression expands away from the Ihh expression zone (arrows). Similarly, detection of Ihh protein with immunohistochemistry reveals a gradient in control (B) and mutant growth plates (D). Quantitation of the Ptch1 gradient, using the imageJ software, illustrates a reduction in intensity and breadth of the Ptch1 response (E).
The fact that Ihh proteins move into the target field precludes a simple analysis of whether Ihh directly or indirectly maintains articular PTHrP expression. Nonetheless, the proportional reduction in the lengths of each chondrocyte cell population is consistent with Ihh acting directly. If a secondary signal was required, the range of this signal could reasonably be expected to show a wild-type like distribution. Hence, the expectation in this circumstance would be the expression of PTHrP at the appropriate distance from the Ihh source rather than a shortening of the growth plate to accommodate a direct Ihh–PTHrP feedback loop model.

Overall, the data indicate that Disp1 is essential for establishing a normally organized and functional growth zone in the prenatal long-bone skeleton. In the absence of Disp1 activity, Ihh does not extend as far over its target field. This is reflected by a reduction in its signaling domain. The reduction enables a normal stratification of chondrocyte populations to still occur, but leads to a proportional reduction in these populations as the growth zone accommodates to the new realities of the Ihh–PTHrP loop that is established under the condition of Disp1 inactivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.039.

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