Convergent extension movements in growth plate chondrocytes require gpi-anchored cell surface proteins

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Proteins that are localized to the cell surface via glycosylphosphatidylinositol (gpi) anchors have been proposed to regulate cell signaling and cell adhesion events involved in tissue patterning. Conditional deletion of Piga, which encodes the catalytic subunit of an essential enzyme in the gpi-biosynthetic pathway, in the lateral plate mesoderm results in normally patterned limbs that display chondrodysplasia. Analysis of mutant and mosaic Piga cartilage revealed two independent cell autonomous defects. First, loss of Piga function interferes with signal reception by chondrocytes as evidenced by delayed maturation. Second, the proliferative chondrocytes, although present, fail to flatten and arrange into columns. We present evidence that the abnormal organization of mutant proliferative chondrocyte results from errors in cell intercalation. Collectively, our data suggest that the distinct morphological features of the proliferative chondrocytes result from a convergent extension-like process that is regulated independently of chondrocyte maturation.

KEY WORDS: Glycosylphosphatidylinositol (gpi), Chondrocyte, Polarity, Morphogenesis, Convergent extension

INTRODUCTION

Growth of endochondral bones derives from regulation of the rate of chondrocyte maturation in the growth plate cartilage (reviewed by Kronenberg, 2003). In the growth plate, resting chondrocytes are a pool of progenitor cells that reside at the ends of embryonic long bones (Abad et al., 2002). Resting chondrocytes mature into proliferative chondrocytes that are characterized by the upregulation of cell proliferation and a change in cell morphology, such that round resting cells give rise to proliferative progeny that are discoid and arranged in columns resembling a stack of coins (Dodds, 1930). As maturation continues, proliferative chondrocytes undergo cell cycle arrest and enlarge, forming prehypertrophic cells. Subsequently, these cells undergo extensive growth to form hypertrophic chondrocytes, which deposit a specialized cartilage matrix that serves as a scaffold for blood vessels and osteoblasts to invade and lay down a true bony matrix (Howlett, 1979; Howlett, 1980; Noonan et al., 1998; Hunziker et al., 1999).

Establishment and maintenance of this cellular architecture is essential for proper function of the growth plate. Previous studies have revealed a complex network of interacting signaling pathways that generate a robust system to regulate the growth plate cartilage. At the heart of this system lie the secreted signaling proteins Indian hedgehog (Ihh) and parathyroid hormone-related peptide (Pthrp; also known as Pthlh) (reviewed by Kronenberg, 2003). Ihh produced by the proliferative chondrocytes acts to induce proliferative chondrocyte formation while simultaneously inducing Pthrp expression in resting chondrocytes (Vorkamp et al., 1996). In turn, Pthrp secreted by resting chondrocytes antagonizes both the action of Ihh and the maturation of proliferative chondrocytes into prehypertrophic chondrocytes. Overlaid on this central interaction are the antagonistic actions of the Wingless/Int-1 factors Wnt5a and Wnt5b that coordinately control the transitions from resting to proliferative and proliferative to prehypertrophic chondrocyte (Yang et al., 2003). Bone morphogenetic proteins (Bmp) and fibroblast growth factors (Fgf) provide additional regulation of chondrocyte maturation (Kronenberg, 2003). Together, these positive and negative interactions help to establish distinct zones of resting, proliferative, and hypertrophic chondrocytes.

Previously, we demonstrated that columns of proliferative chondrocytes form by a process of convergent extension (CE) involving cell intercalation (Li and Dudley, 2009). Thus, cell division displaces daughter cells lateral to the column before cell intercalation movements promote re-integration. Orientation of the division plane and the stacking of chondrocytes depend on non-canonical frizzled (Fzd) signaling and Rho GTPase activity, two components of pathways that additionally regulate CE movements in the neural tube and planar cell polarity (PCP) in epithelial tissues (Klein and Mlodzik, 2005). Proteins displayed on the cell surface via glycosylphosphatidylinositol (gpi) linkages are also important components of the CE pathway (Topoczewski et al., 2001; Shao et al., 2009), and reduction in gpi-anchored proteins affects CE in the axolotl and zebrafish (Drawbridge and Steinberg, 2000; Shao et al., 2009). Therefore, we sought to determine whether gpi-anchored proteins are also key regulators of CE and PCP in other tissues.

Gpi-anchored proteins include heparan sulfate proteoglycans (HSPGs) of the glypican family, ephrin A ligands (for Eph receptors), putative adhesion/signaling molecules of the Ly6 family, and enzymes such as alkaline phosphatase (Bernfield et al., 1999; Paulick and Bertozzi, 2008). In addition to regulating CE movement, gpi-anchored proteins are thought to play important roles in patterning tissue by regulating cell signaling and cell adhesion. For example, loss of the glypican Daily interferes with Wingless (Wg) signaling and results in defects in patterning of the ventral denticles and wing of Drosophila (Lin and Perrimon, 1999; Tsuda et al., 1999). In vertebrates, manipulation of gpi-anchored ephrin A (Ide et al., 1994; Wada and Ide, 1994; Stadler et al., 2001), a ligand for Eph receptors, or of the ortholog of the gpi-anchored Ly6 family protein CD59 (da Silva et al., 2002; Kumar et al., 2007) in the limb mesenchyme results in defects in cell sorting and skeletal patterning. Therefore, gpi-anchored proteins might mediate the cellular interactions that impart unique identities to subgroups of cells within a field to promote tissue patterning.

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Simple genetic tests of these models are confounded by the presence of diverse families of gpi-anchored molecules. As a first approach, we knocked out display of all gpi-anchored proteins on the cell surface using a conditional allele of Piga, which encodes the catalytic subunit of the enzyme complex that generates gpi moieties (Kawagoe et al., 1994; Keller et al., 1999; Keller et al., 2001). Conditional deletion of Piga in the lateral plate mesoderm (LPM) – progenitors of the limb skeleton – surprisingly resulted in a normally patterned limb skeleton. Here, we show that Piga null chondrocytes have normal maturation signatures, but abrogation of gpi-anchors causes cell autonomous defects in maturation rate, in cell morphology, and in chondrocyte organization. Although chondrocytes progress through each stage of the maturation process, mutant growth plates fail to form columns of discoid chondrocytes. Failure of column formation results from a defect in cell intercalation, a coordinated cell movement that depends on cell polarity. In addition, Piga expression is required to properly orient hair cells in the inner ear, a process regulated by the PCP pathway. Collectively, our data firmly establish that gpi-anchored proteins are common components of polarity pathways, including one that regulates CE-like movements in proliferative chondrocytes that in part define the structure and the growth properties of developing long bones.

MATERIALS AND METHODS

Mouse strains and animal care

The mouse strains used are Hoxb6: cre (Lowe et al., 2000), lox-Piga-lacZ (Tremml et al., 1999), Prox1: cre (Logan et al., 2002), Ctnnb1tm2Kem (Braught et al., 2001), Foxg1tm1(cre)Skm (Hebert and McConnell, 2000), and TOPGAL [Jackson Laboratories (DasGupta and Fuchs, 1999)]. Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of Northwestern University. The morning after mating was designated as 0.5 days post coitum (dpc).

Histology

Skeletons were prepared as described (McLeod, 1980). Briefly, dissected hindlimbs were dehydrated in 95% ethanol followed by acetone, stained with Alcian Blue/Alizarin Red (Sigma), washed in 95% ethanol, cleared with 1% KOH, followed by a glycerol/decreasing KOH series, and stored in 80% ethanol.

Antibody staining

To analyze cell morphology, limbs were dissected, fixed in Bouin’s Fixative (Ricca Chemical Company, Texas, USA) at 4°C overnight, dehydrated through an ethanol series followed by xylenes, and embedded in paraffin (Richard-Allan Scientific, MI, USA). Sections were stained with Masson’s Trichrome using a standard protocol.

β-galactosidase staining on P0 hindlimbs from TOPGAL mice was performed as described (Kawaguchi et al., 2002). Briefly, limbs were fixed in 1% paraformaldehyde (PFA)/0.5% glutaraldehyde in PBS for 1.5 hours at room temperature, and then stained in X-gal staining solution at 37°C overnight.

Antibody staining

To analyze cell proliferation, cells in S-phase were labeled with bromodeoxyuridine (BrDU) for 2 hours prior to tissue harvest. Tissue was fixed in 4% PFA in PBS at 4°C overnight and prepared for paraffin sectioning as described. Sections of BrDU-labeled tissue were digested in trypsin and depurinated in hydrochloric acid. Incorporated BrDU was detected using a mouse anti-BrDU monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa, USA) and Cry2-labeled donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, Pennsylvania, USA). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The BrDU labeling index was calculated as the percentage of total nuclei that were BrDU positive in each designated box.

P0 cryosections were used for staining with the following antibodies: cleaved caspase-3 (1:500, Cell Signaling); phospho-Smad 1Ser463/465 and Ser426/428 (1:500, Cell Signaling); phospho-p44/42 (1:100, Cell Signaling); β-galactosidase (1:2000, a gift from R. Holmgren, Northwestern University, IL, USA); acetylated tubulin (1:1000, Sigma). Samples were washed in PBS plus 0.1% Triton X-100 (PBSTx), blocked for 1 hour in 10% FBS/PBStx at room temperature, then incubated overnight at 4°C with the primary antibody in 2% FBS/PBStx. Samples were washed and incubated with donkey anti-rabbit Alexa 488 (1:500, Invitrogen) for 4 hours at room temperature, and counterstained with DAPI.

Gene expression analysis

For in situ hybridization (ISH) analysis, RNA probes were produced using T7, T3 or SP6 polymerase in the presence of non-radioactive digoxigenin-labeled rNTPs (Roche) or S35 (PerkinElmer), as described (Murtaugh et al., 1999). For quantification, the length of the expression domain was measured along the longitudinal axis of the cartilage and calculated as a ratio over the total length of the growth plate (articular surface to the mineralized region) using non-radioactive samples.

Chondrocyte isolation and culture

Growth plate cartilage was dissected free of the perichondrium/periesteum, minced with a razor blade, and incubated in 0.5% collagenase in DMEM+10% heat inactivated FBS (Invitrogen) at 37°C for 3 hours. After gentle trituration the resulting cell suspension was passed through a cell strainer (70 μm pore size, Becton-Dickinson), pelleted, and washed twice with serum-free DMEM. For transfection experiments, 2.5×105 chondrocytes were seeded into 12-well tissue culture clusters (Corning) in DMEM+10% heat-inactivated serum with penicillin/streptomycin. Eighteen to 24 hours later chondrocytes were transfected with GFP-gpi or GFP-myr (gifts from A.-K. Hadjantonakis, Sloan-Kettering Institute, NY, USA) using Superfect transfection reagent (Qiagen), according to the manufacturer’s instructions. Thirty-six hours later, cultures were washed twice with PBS and imaged by epifluorescence.

Flow cytometry

All incubations were performed on ice in PBS containing 0.1% sodium azide unless otherwise noted. Isolated chondrocytes were washed three times in PBS/azide, blocked in 5% heat-inactivated normal donkey serum (Jackson ImmunoResearch), and incubated for 4 hours in 5% serum containing 1 μg/105 chondrocytes of sheep IgG (Jackson ImmunoResearch) or anti-Gpc3 (R&D Systems). After washing three times in PBS/azide, cells were incubated in Alexa-Fluor 488-anti-sheep antibody (Invitrogen) for 1 hour. Cells were washed three times in PBS/azide then fixed in 2% PFA and analyzed using a FACScalibur and Cell Quest Pro software (Becton-Dickinson). For experiments involving removal of gpi-anchored proteins, cells were initially incubated in αMEM (Invitrogen) containing 0.1 units of gpi-specific phospholipase C (Sigma Aldrich) for 1 hour at 37°C.

Alkaline phosphatase activity

P3 limbs were fixed in 4% PFA for 3 hours at room temperature and then prepared for cryosectioning. Alkaline phosphatase staining was performed as described in the development step of the non-radioactive ISH protocol.

Image analysis

Orientation of cell division was determined as described (Li and Dudley, 2009). For analysis of stereocilia, P0 lox-Piga-lacZ/Foxg1tm1(cre)Skm pup inner ears were dissected in PBS/2 mM CaCl2/0.5 mM MgCl2 and fixed in 4% PFA/2 mM CaCl2/0.5 mM MgCl2 at 4°C overnight. The following day, the cochlea were dissected from the temporal bone, and the tissue was permeabilized in PBS plus 1% Triton X-100 (PBSTx) for 1-2 hours, and then blocked overnight in 20% heat-inactivated sheep serum/PBStx. Cochlea were stained with anti-acetylated tubulin (1:200, Sigma) to visualize the kinocilium or anti-Vangl2 (1:400, gift from M. Montcouquiol, INSERM, Bordeaux, France). All specimens were additionally incubated with Alexa-Fluor 568 phalloidin (Invitrogen) to label the stereocilia and actin cytoskeleton. Images were acquired using a Zeiss apotome deconvolution microscope or Leica Confocal Laser Scanning System and analyzed as described (Qian et al., 2007).

Statistical methods

A two-way ANOVA was used to determine whether the distributions of gene expression domain length were distinct between wild-type and mutant mice. The model contained the main effects of genotype (wild type and mutant) and
zone (an interaction effect between the genotype and zone). Different contrasts were constructed to test whether the gene expression of a particular zone was the same between wild-type and mutant mice; if two zones had the same gene expression within the same genotype; and if the gene expression difference between two zones of interest was the same for wild type and mutants.

Bonferroni’s procedure was used to control the family-wise error rate at significance level 0.05 as multiple tests were performed simultaneously. That is, a test was considered to be statistically significant if the corresponding $P$-values were below 0.05 divided by the total number of tests. Analysis was based on a minimum of three independent samples per genotype.

**RESULTS**

**Chondrodysplasia in Piga mutant limbs**

To determine the role of gpi-anchored proteins in development, we analyzed loss of Piga expression in developing limbs. Piga encodes PIG-A, the catalytic subunit of an essential enzyme for the initial step of gpi-anchor biosynthesis (Fig. 1A) (Miyata et al., 1993; Watanabe et al., 1998; Kostova et al., 2000). Since null Piga alleles are early embryonic lethal (Kawagoe et al., 1994; Rosti et al., 1997), a conditional allele was employed to selectively delete Piga using...
Cre recombinase (Keller et al., 1999; Tremml et al., 1999). We took advantage of the fact that Piga resides on the X chromosome and mated females homozygous for the conditional allele (X<sup>Piga</sup>/X<sup>Piga</sup>) to males heterozygous for the autosomal Hoxb6::cre transgene (cre/+), which expresses Cre recombinase in the posterior LPM (Lowe et al., 2000), which gives rise to the mesenchyme from which the hindlimb skeleton forms. Analysis using reporter alleles shows that Hoxb6::cre function is initiated at 8.5 days post coitum (dpc) and promotes near complete recombination in the hindlimb mesenchyme by 9.5 dpc prior to limb bud outgrowth (Lowe et al., 2000). Our breeding scheme yields equal numbers of genotypically wild-type (X<sup>Piga</sup>Y; +/+ ) and mutant (X<sup>Piga</sup>Y; cre/+ ) males (data not shown). By contrast, all females are heterozygous for the conditional allele (X<sup>Piga</sup>/X). However, due to random X-inactivation, females carrying the Hoxb6::cre transgene (X<sup>Piga</sup>Y; cre/+) are mosaics composed of both wild-type and null cells. The following studies are based on the analysis of both null mutant (X<sup>Piga</sup>Y; cre+) and mosaic (X<sup>Piga</sup>Y; X<sup>Piga</sup>/X; cre+) limbs.

Given the proposed roles for gpi-anchored proteins in embryogenesis, we were surprised to find normal patterning of the limb skeleton in both mutant and mosaic animals (Fig. 1B). The slight delay in formation of distal cartilage elements that was observed at 12.5 dpc did not translate into patterning defects at later stages. However, Piga mutant hindlimb long bones were significantly shorter (P<0.0001) and wider (P<0.0001) at birth, although heterozygous females presented an intermediate phenotype (Fig. 1C).

A substantial delay in ossification was observed at 14.5 dpc but was absent twelve days after birth (P12). By 15.5 dpc, wild-type cartilage displayed domains of collagen type X alpha 1 (Col10a1)-positive hypertrophic chondrocytes between the collagen type II alpha 1 (Col2a1)-expressing immature chondrocytes and the primary ossification center (Fig. 1D). By contrast, only a single small domain of Col10a1 expression occupies the central diaphysis in Piga mutants. Furthermore, a reduction in mineralization was observed in the periosteum and the primary ossification center at birth, and Piga mutants did not form an extensive trabecular network as observed in wild-type cartilage (see Fig. S1 in the supplementary material). Because matrix mineralization is one function of osteoblasts, we investigated whether gpi-anchored proteins are required for osteoblast formation. Analysis of collagen type I alpha 1 (Col1a1), osteocalcin (OC; also known as Bglap2) and osteopontin (Opn; also known as Spp1) suggested normal or slightly decreased numbers of mature and progenitor osteoblasts (Kratochvil et al.,...
Thus, gpi-anchored proteins are not required for skeletal patterning or osteoblast formation, but might have additional roles in osteoblast cells.

Gpi-anchored proteins are absent from Piga mutant chondrocytes

The absence of major developmental defects could result from a perdurance of gpi-anchored proteins produced prior to recombination or from partial compensation of Piga function by other cellular enzymes. To test these possibilities, we examined whether gpi-anchored proteins were lost in Piga mutant chondrocytes. Alkaline phosphatase (Alp) is a gpi-anchored enzyme displayed on the surface of hypertrophic chondrocytes and osteoblasts. Alp activity was not detectable in Piga null cartilage, but in situ hybridization (ISH) analysis confirmed expression of Akp2 (also known as Alpl), the gene encoding bone/liver/kidney alkaline phosphatase (Fig. 2A,B) (Pizauro et al., 1994; Magnusson et al., 1999). Interestingly, in mosaic females, we observed clusters of Alp-positive cells generated from morphologically wild-type columnar chondrocytes beside Alp-negative cells generated from round mutant chondrocytes (Fig. 2A). The phenotypes appeared to remain spatially segregated, as ‘salt-and-pepper’ patterns were never observed with regard to cell shape or Alp activity. We additionally showed by flow cytometry that the gpi-anchored protein glypican 3 (Gpc3) is not displayed on Piga mutant chondrocytes (see also Figs S2, S3 in the supplementary material). Moreover, mutant chondrocytes transfected with gpi-anchored green fluorescent protein (gfp) lacked fluorescence in contrast to wild-type chondrocytes (Fig. 2D). By contrast, transfection with a myristilated gfp construct resulted in equivalent fluorescence on intracellular membranes of wild-type and mutant chondrocytes (Fig. 2D).

Together, these data demonstrate that gpi-anchored proteins are absent from the cell surface of Piga mutant chondrocytes.

Piga mutant chondrocytes proceed through normal stages of maturation

The observation of morphologically abnormal chondrocytes suggested the presence of defects in chondrocyte maturation in mutants. To determine whether loss of Piga expression affects early steps in chondrocyte maturation, we first examined the cellular organization in Piga mutant limbs. (A) The growth plate contains distinct zones, including resting (RZ), proliferative (PZ), prehypertrophic, and hypertrophic chondrocyte (HZ) zones. (B) Masson's trichrome-stained sections of P0 cartilage reveals that the transition from round disorganized cells to discoid columns is perturbed in Piga mutant chondrocytes. BrdU staining shows that the proliferation rate in Piga mutant RZ is increased compared with in wild type (9.8±0.2% versus 5.7±0.7%); the proliferation rate is increased in the RZ of Piga mutants compared with wild type (14.7±2.2% versus 12.3±1.6%). Cleaved caspase 3 antibody staining shows that apoptosis is absent in the growth plate (box) and normal in mineralized regions (arrow). (C) Trichrome staining of mosaic growth plates demonstrates that stacked discoid wild-type chondrocytes (black arrow) are found adjacent to round disorganized mutant chondrocytes (red arrow). Scale bars in B: 50μm for trichrome staining; 100μm for immunostaining.
structure of developing tibiae. In Piga null hindlimbs, resting chondrocytes appeared normal but proliferative chondrocytes failed to flatten or form columns (Fig. 3A,B). Heterozygous females are mosaics with round mutant chondrocytes adjacent to discoid wild-type chondrocytes (Fig. 3C). Bromodeoxyuridine incorporation (Fig. 3B) demonstrated upregulation of the cell cycle in the growth plate cartilage of wild-type (5.7±0.7% versus 12.3±1.6%, resting versus proliferative chondrocytes) and mutant (9.8±0.2% versus 14.7±2.2%, resting versus proliferative chondrocytes) samples. Curiously, we observed a small but significant increase in cell proliferation in mutants compared with littermate controls (e.g. 9.8±0.2% versus 5.7±0.7%, in the resting chondrocytes). In addition, the absence of cleaved caspase-3-positive apoptotic chondrocytes demonstrates that cell viability does not depend on gpi-anchored proteins (Fig. 3B) (Nicholson et al., 1995). Upregulation of the cell cycle suggests that Piga growth plates contain proliferative chondrocytes. To test this possibility, we used ISH analysis to show that mutant growth plate chondrocytes expressed markers appropriate to immature (Col10a1) chondrocytes of the resting (Fgfr1) and proliferative (Bapx1, Fgfr3, Prelp) zones, prehypertrophic chondrocytes (Ihh), and hypertrophic chondrocytes (Col10a1; see Fig. S4 in the supplementary material; see also Fig. 4A). Thus, Piga null growth plates contain a bona fide proliferative zone.

Although each is present, the length of some maturation zones is altered in mutants indicating imbalances in chondrocyte maturation. To quantify this effect, we measured the length of gene expression domains along the epiphyseal-metaphyseal axis and determined the ratio of this measurement to total length of the growth plate (articular surface to the mineralized region). These measurements revealed that total growth plate length is similar at birth (P0) for wild-type (1.05±0.16 mm), mosaic (1.03±0.10 mm) and mutant (1.04±0.16 mm) femurs. The domain of least mature chondrocytes was not significantly shorter in mutants compared with wild type (Fig. 4B; Fgfr1, P=0.033) unlike, the proliferative (Prelp, P<0.0001) and prehypertrophic zones (Ihh; P<0.005; Fig. 4B). By contrast, the region between the Fgfr1 and the Prelp expression domains was expanded (P<0.0001), consistent with the observed upregulation of cell proliferation (Fig. 4B). Collectively, our results demonstrate that morphologically abnormal Piga mutant chondrocytes undergo normal phases of maturation and that the delay in maturation begins at the transition between resting and proliferative chondrocytes.

Additionally, we compared the length of gene expression domains for wild-type and null cells in mosaic cartilage. The Prelp expression domain was similar in size for wild-type chondrocytes in both mosaic and wild-type growth plates (P=0.062; Fig. 5A,B). Similarly, the expression domain of Ihh was the same for wild-type cells in mosaic and wild-type samples (P=0.364; Fig. 5A,B). Likewise, gene expression domains were similar for mutant chondrocytes expressing Prelp (P=0.944) and Ihh (P=0.610; Fig. 5A,B), whether the Piga null cells were in a mutant or a wild-type environment. The delayed maturation of Piga mutant chondrocytes in mosaic cartilage suggests a defect in signal reception. However, we did not detect a decrease in Ihh, Bmp, Fgf, or canonical Wnt signaling in mutant chondrocytes (Fig. 4A,B, Ptc1; see Fig. S5 in the supplementary material; see also Fig. 6C). Moreover, to confirm that canonical Wnt signaling is unaffected, we demonstrated that conditional inactivation of β-catenin (Cnmnb<sup>tm2Kem</sup>) (Brau et al., 2001) in the cartilage does not alter morphology or organization of proliferative chondrocytes (Fig. S6 in the supplementary material).

Piga mutant chondrocytes fail to intercalate

One intriguing observation is that although mutant chondrocytes ultimately progress through all stages of maturation, columns of discoid cells never form, suggesting that cell morphology and maturation are regulated by distinct mechanisms. Column formation in chondrocytes occurs in two steps with cell division...
progeny into adjacent sections.

Piga function regulates cell polarity

**A**

Prep Mosaic  Ihh Mosaic  Ptc1 Mosaic

Failure of column formation could also result from defects in cytokinesis, as shown for integrin β1 mutant chondrocytes (Aszodi et al., 2003). However, in Piga mutants, chondrocytes showed normal cell surface display of integrin β1, and multinucleate or apoptotic cells normally associated with cytokinesis defects were not observed (Fig. 3B; see also Fig. S7 in the supplementary material; data not shown). Moreover, we found in both wild-type and mutant cartilage that dividing cells in the resting and proliferative zones showed a normal flattened interface that included a well-formed midbody (Fig. 6E), a microtubule-based structure assembled during anaphase that is important for completion of cytokinesis (Raich et al., 1998). These observations suggest that cell division occurs normally in Piga mutants and thus the requirement for gpi-anchored proteins is in the downstream cell intercalation process.

**Gpi-anchored proteins regulate cell polarity**

The requirement for gpi-anchored proteins for polarized cell movements in cartilage and in axis formation in the axolotl and zebrafish suggests that gpi-anchored proteins are general components of polarity pathways (Drawbridge and Steinberg, 2000; Topczewski et al., 2001; Shao et al., 2009). We tested this hypothesis using the sensory epithelium of the vertebrate inner ear. The sensory epithelium contains four rows of hair cells each displaying a ‘V’-shaped bundle of actin-rich stereocilia with a single microtubule-based kinocilium at the apex (reviewed by Kelly and Chen, 2007). Alignment of stereocilia and kinocilium across all hair cells in wild-type cochlea is regulated by the PCP pathway (reviewed by Klein and Mlodzik, 2005). To test whether gpi-anchored proteins regulate cell polarity, we crossed X<sup>Foxg1<sup>tm1(cre)Skm</sup></sup> females to Foxg1<sup>tm1(cre)Skm</sup> males, which express Cre recombinase in the otic vessel (Hebert and McConnell, 2000). Hair cells in Piga mutant ears display disrupted architecture at the base of the hair cells and poorly aligned stereocilia and kinocilium of the outer hair cell layers (Fig. 7A–C), as has been shown for PCP mutants such as Vangl2 and scribble (Montcouquiol et al., 2003). Additionally, Vangl2 is mislocalized in Piga mutant males, which express Cre recombinase in the otic vessel (Hebert and McConnell, 2000). Hair cells in Piga mutant ears display disrupted architecture at the base of the hair cells and poorly aligned stereocilia and kinocilium of the outer hair cell layers (Fig. 7A–C), as has been shown for PCP mutants such as Vangl2 and scribble (Montcouquiol et al., 2003). Additionally, Vangl2 is mislocalized in Piga mutant males.
outer hair cells (Fig. 7C). Collectively, our data establish a link between gpi-anchored protein activity and tissue polarity pathways that pattern the sensory epithelium of the inner ear.

**DISCUSSION**

**Gpi-anchored proteins function cell autonomously**

Conditional inactivation of *Piga* results in the loss of gpi-anchored cell surface proteins, as determined by the absence of Alp activity on mutant chondrocytes and osteoblasts, the lack of Gpc3 on mutant chondrocytes, and the failure of transfected mutant chondrocytes to display gpi-anchored Gfp, findings that are consistent with previous studies in the hematopoietic lineage (Tremml et al., 1999). Interestingly, Alp activity is not observed in the matrix of mutant cartilage, demonstrating that secreted forms are not generated by the absence of anchor addition. This finding is consistent with previous studies demonstrating that failure of anchor addition leads to retention in the endoplasmic reticulum (Field et al., 1994; Doering and Schekman, 1996; Doering and Schekman, 1997) and protein degradation (Ali et al., 2000). In this context, the presence of secreted epitope-tagged gpi-anchored Knypek in zebrafish embryos injected with morpholinos directed against *pigp* (Shao et al., 2009) suggests an incomplete knockdown of transamidase activity. However, secreted forms, if they exist, probably do not act dominantly because small patches of wild-type or mutant cells surrounded by cells of the opposite genotype are readily observed in mosaic females. Moreover, in mosaics, transitions in maturation state for cells of one genotype are unaffected by the presence of large numbers of cells of the opposite genotype. Together, these data indicate that the observed phenotype is the result of cell autonomous *Piga* expression and does not reflect global defects in signaling pathways that regulate growth plate function.
Gpi-anchored proteins are not required for tissue patterning

Numerous studies have demonstrated important roles for gpi-anchored cell surface proteins in controlling cell signaling and cell adhesion during embryogenesis. For example, dally and dally-like encode two gpi-anchored HSPGs of the glypican family that contribute to patterning of the Drosophila wing through the regulation of signaling by secreted factors of the Wg/Wnt and Hh families (Lin and Perrimon, 1999; Tsuda et al., 1999; Lum et al., 2003; Kirkpatrick et al., 2004). In the vertebrate limb, gpi-anchored
Gpi-anchored proteins regulate chondrocyte maturation

Loss of gpi-anchored proteins leads to a reduced rate of chondrocyte maturation that is first observed at the transition from resting to proliferative chondrocytes, as demonstrated by the decreased size of the proliferative zone despite an increase in resting chondrocyte proliferation. The size of and transitions between maturation zones are controlled by the action of a complex network of synergistic and antagonistic pathways regulated by secreted signaling proteins (Karsenty and Wagner, 2002; Kronenberg, 2003; Yang et al., 2003). HSPGs, including gpi-anchored glypicans, are important regulators of these signaling pathways (reviewed by Häcker et al., 2005).

In cartilage, HSPGs might determine the extracellular distribution of secreted ligands (Koziel et al., 2004). However, in mosaics, in which wild-type cells display normal zones of maturation, of wild-type neighbors cells suggests that Piga null cells either do not correctly detect or fail to respond appropriately to growth plate signals.

Defects in signaling are consistent with a role for gpi-anchored proteins as co-receptors for signals that promote chondrocyte maturation, or as antagonists of factors that repress maturation.

Likely candidates for these functions are gpi-anchored HSPGs of the glypican family. Consistent with this interpretation, many similarities exist between mutants in Ext1 (Koziel et al., 2004), an enzyme involved in the production of HSPGs, and Piga (see Table S1 in the supplementary material). Interestingly, a hypomorphic allele of Gpc3 displays defects in chondrocyte maturation similar to those of Piga mutants, suggesting that Gpc3 is the primary gpi-anchored regulator of chondrocyte maturation in the growth plate (Viviano et al., 2005). If so, it will be important to determine the functions of the other glypicans expressed in the growth plate (see Fig. S3 in the supplementary material).

Gpi-anchored proteins regulate tissue polarity

The fact that Piga null cells eventually pass through all phases of chondrocyte maturation while defects in cell shape and cell arrangement in proliferative chondrocytes are absolute suggests that distinct mechanisms regulate chondrocyte maturation and proliferative chondrocyte behavior. The arrangement of proliferative chondrocytes into columns of stacked cells is a multi-step process that includes two planar processes – aligned division planes and cell intercalation – and that is regulated by non-canonical Fzd signaling (Dodds, 1930; Li and Dudley, 2009).

In the absence of gpi-anchored proteins, the spindle orienting mechanism is functional, but imprecise, resulting in a percentage of chondrocytes that divide out of plane. A similar percentage of misaligned division planes are observed in kidney tubules lacking the antipodal cadherin Fat4, a component of the PCP pathway (Saburi et al., 2008). However, the Fat4 mutant phenotype is more severe than that of Piga mutants in that a substantial number of Fat4 mutant cells align the mitotic spindle orthogonal to wild type. In this respect, the Fat4 phenotype approximates, but is less severe than, the uniform distribution of division planes in chick chondrocytes deficient in non-canonical Fzd signaling (Li and Dudley, 2009). Although gpi-anchored glypicans are components of the non-canonical Fzd pathway (Caneparo et al., 2007; Shao et al., 2009), the weak division plane phenotype of Piga mutants suggests that gpi-anchored proteins either are largely expendable because of redundancy or are not core components of the Fzd pathway that regulates the division plane. In this latter model, one possibility is that gpi-anchored proteins regulate a parallel process that influences the division plane. For example, division of most cells occurs according to Hertwig’s rule, which predicts alignment of the mitotic spindles parallel to the long axis of the cell (Wilson, 1900; Wilson, 1925), and might involve interaction between cell-matrix adhesion and the actin cytoskeleton (Toyoshima and Nishida, 2007). Even though tissue polarity pathway function can override Hertwig’s rule (Gong et al., 2004), perhaps the system is more robust when components of polarity pathways are aligned with a prominent axis defined by cell shape/cell adhesion that might be lacking in round Piga chondrocytes.

Interestingly, although the plane of cell division is largely normal, mutant cells fail to intercalate and, as a result, clones of Piga null cells expand laterally across the proliferative zone instead of forming columns aligned to the longitudinal axis. It remains to be determined if the small number of stacked chondrocytes in a mutant clone results from occasional, normal cell intercalation events or from aberrant division planes in a non-intercalating clone. Nonetheless, these results refine our previous model (Li and Dudley, 2009) by demonstrating that establishment of the division plane and the process of cell intercalation are differentially regulated, despite both events being sensitive to non-canonical Fzd signaling. Moreover, lateral expansion of the proliferative chondrocytes...
correlates with the increased width and decreased longitudinal growth of mutant cartilage, providing further evidence that cell intercalation events orient both local and tissue-wide vectors of growth in cartilage through a process of CE.

Towards a mechanism of convergent extension in cartilage

How cell polarity pathways influence cell shape and promote cell intercalation is currently under debate. One possibility is that gpi-anchored proteins regulate non-canonical Fzd signaling. In this regard, two non-exclusive models have been proposed for the role of the gpi-anchored proteins in CE. First, the glypic an Knypek has been shown to act in conjunction with the secreted canonical pathway inhibitor Dickkopf (Dkk) to alter the specificity of Fzd signaling from activating canonical to activating non-canonical pathways (Caneparo et al., 2007). Second, gpi-anchored proteins might stabilize Fzd receptors on the cell surface potentially promoting membrane localization of Dishevelled, an event associated with non-canonical PCP signaling (Shao et al., 2009). In this context, the observation of reduced VanG1 localization in hair cells might reflect defects in Fzd signaling rather than a direct interaction between VanG1 and gpi-anchored proteins.

The effectors of cell polarity pathways are most probably regulators of cytoskeletal dynamics and cell-matrix interactions. In particular, matrix structure is an important determinant of cell shape and cell intercalation (Davidson et al., 2006). Consistent with this, null mutants in cartilage-specific Col9a1 (Blumbach et al.; Dreier et al., 2008) and the cell-matrix adhesion receptor integrin β1 (Aszodi et al., 2003) result in round, disorganized proliferative chondrocytes that are grossly similar to those of Piga mutants. However, loss of either integrin β1 or Col9a1 function results in a more severe phenotype than displayed by Piga null cartilage, and expression of Col9a1 and cell surface localization of integrin β1 are unaffected in Piga mutant cartilage (see Figs S4, S6 in the supplementary material). Together, these data suggest that gpi-anchored proteins do not regulate the production of Col9a1 or integrin β1. Thus, as with Fzd receptors, gpi-anchored proteins might be required for the appropriate trafficking or stability of cell adhesion molecules, or to properly organize the extracellular matrix to promote column formation.

Conclusions

CE and PCP are two processes that define the cellular architecture of many tissues. Although once thought to be highly specific to certain tissues, these processes are being discovered in diverse cell types, including chondrocytes. The involvement of non-canonical Fzd signaling, Rho GTPase activity, and now gpi-anchored proteins in both CE and PCP suggests that common mechanisms regulate the architecture of all tissues.

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and surface area of their cytoplasmic organelles. Staining with GlcNAc binding lectin 5 (GALC5) revealed that the planar cell polarity genes were expressed in a manner consistent with the formation of a complex membrane-anchoring structure for proteins. Biochemistry 47, 6991-7000.


