Centrosomal Protein DZIP1 Regulates Hedgehog Signaling by Promoting Cytoplasmic Retention of Transcription Factor GLI3 and Affecting Ciliogenesis*

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Background: All known cilia-related proteins regulate Hedgehog signaling through their role in ciliogenesis. The centrosomal protein DZIP1 interacts with and sequesters GLI3 transcription factor in the cytoplasm and also regulates ciliogenesis.

Results: The centrosomal protein DZIP1 interacts with and sequesters GLI3 transcription factor in the cytoplasm and also regulates ciliogenesis.

Conclusion: DZIP1 is the first known cilia-related protein that regulates Hedgehog signaling through a dual mechanism.

Significance: Understanding how DZIP1 regulates Hedgehog signaling provides new insights into the molecular mechanism of Hedgehog signal transduction.

The primary cilium is required for Hedgehog signaling. So far, all known ciliogenic proteins regulate Hedgehog signaling through their role in ciliogenesis. Here we show that the mouse DZIP1 regulates Hedgehog signaling through two mechanisms. First, DZIP1 interacts with GLI3, a transcriptional regulator for Hedgehog signaling, and prevents GLI3 from entering the nucleus. Second, DZIP1 is required for ciliogenesis. We show that DZIP1 colocalizes and interacts with CEP164, a protein localizing at appendages of the mother centrioles, and IFT88, a component of the intraflagellar transport (IFT) machinery. Functionally, both CEP164 and Ninein appendage proteins fail to localize to ciliary appendages in Dzip1 mutant cells; IFT components are not recruited to the basal body of cilia. Importantly, the accumulation of GLI3 in the nucleus is independent of loss of primary cilia in Dzip1 mutant cells. Therefore, DZIP1 is the first known ciliogenic protein that regulates Hedgehog signaling through a dual mechanism and that biochemically links IFT machinery with Hedgehog pathway components.

The family of secreted Hedgehog (Hh) molecules plays an important role in the development of almost all embryonic tissues in vertebrates. An inappropriate activation of the Hh pathway is also associated with several types of human cancer (1). Thus, a thorough understanding of the molecular mechanism of Hh signal transduction is essential not only for the elucidation of the molecular mechanism of the embryonic developmental process but also for the prevention and treatment of Hh-related cancers. The molecular mechanism of Hh signal transduction has been extensively studied. In vertebrates, Hh ligands bind to the cell surface receptor, Patched (PTCH) (2, 3). This binding prevents PTCH from inhibiting another transmembrane protein known as Smoothened (SMO), which then transduces signals to activate the GLI transcription factors downstream. Of the three GLI family members, GLI2 and GLI3 act as transcriptional repressors (GLI2Rep and GLI3Rep) in the absence of Hh signaling, because the C-terminal activation domains of the full-length proteins (GLI2FL and GLI3FL) are proteolytically processed (4, 5). Hh signaling inhibits this proteolytic processing and converts the latent full-length proteins into activators, which subsequently activate the downstream transcriptional targets. One of these targets is Gli1, which further enforces the Hh pathway activation, whereas Gli2 is also a target to establish a negative feedback regulation of the pathway.

One of the recent advances in Hh signal transduction is the discovery that vertebrate Hh signaling occurs in primary cilia (6). Consistent with this, the Hh pathway core components, such as PTCH, SMO, GLI2 and GLI3 proteins, SUFU, and KIF7, localize to the cilia (7–11). Primary cilia, also called non-motile cilia, are solitary microtubule-based organelles that protrude from the cell surface and serve as a site for transducing extracellular signals. Defects in cilia structure are associated with a diverse array of developmental abnormalities, collectively termed “ciliopathies,” including polycystic kidney disease, respiratory and visual disorders, hydrocephalus, obesity, and mental retardation (12).

Primary cilia assemble during the G1 phase from the mother centriole. The mother centriole is characterized by unique distal and subdistal appendages made of specific proteins, including Odf2, Ninein, and CEP164 (13–16). The mother centriole serves as a basal body and a docking station for ciliary microtubules and intraflagellar transport (IFT) machinery during primary cilia elongation. IFT machinery is also essential for the maintenance of primary cilia. There are two IFT protein complexes, IFT-A and IFT-B. IFT-B, together with a kinesin motor, is responsible for anterograde trafficking of protein complexes

Reference:
2 The abbreviations used are: IFT, intraflagellar transport; ES, embryonic stem; En, embryonic day n; MEF, mouse embryo fibroblast; LMB, leptomycin B; aa, amino acids.
and vesicles in the cilia, whereas IFT-A, together with a dynein motor, is responsible for retrograde trafficking (17, 18). Increasingly, more ciliary proteins are found to be involved in Hh signaling. These proteins include motor proteins, components of the IFT-A and IFT-B complexes, centrosomal proteins, small GTPases (Rab and Arf or Arl), etc. (6). Among them, only KIF7, a kinesin motor and core component of the Hh cytoplasmic signaling complex, and MIM, a protein enriched in the basal body, have been shown to associate with GLI proteins physically (7, 19–21), although the in vivo role of MIM in Hh signaling and ciliogenesis needs to be further determined (22). However, none of these proteins have thus far been biochemically shown to bridge Hh pathway components and IFT machinery. The effect of these proteins on Hh signaling also appears to be only through their regulation of ciliogenesis or protein transport in the cilia.

In this study, we show that a mutation in the Dzip1 gene, the mouse homolog of the zebrafish Iguana (Igu) (23, 24), results in phenotypes consistent with loss of Hh signaling. DZIP1 regulates Hh signaling through two mechanisms. First, DZIP1 binds to GLI3 but not to GLI2 or GLI1, thus preventing the GLI3 protein from entering the nucleus. Consistent with this, the DZIP1 mutation leads to accumulation of GLI3 in the nucleus. Second, DZIP1 plays an essential role in ciliogenesis. We show that DZIP1 specifically accumulates at the distal appendage of the mother centrioles. In DZIP1 mutant cells, primary cilia are not formed; Ninein and Cep164, two ciliary appendage proteins, fail to localize to the appendages of the mother centrioles. In addition, IFT machinery is not recruited to the basal body. More interestingly, DZIP1 can interact with both CEP164 and IFT88. Thus, DZIP1 is necessary not only for recruiting IFT machinery to the distal appendages of the mother centrioles but also for bridging IFT88 to the cargo that contains GLI3. The sequestration of GLI3 in the cytoplasm and the role of ciliogenesis by DZIP1 are two independent functions. Therefore, DZIP1 is the first known protein that regulates Hh signaling through this dual mechanism and biochemically connects Hh signaling components with IFT machinery.

**EXPERIMENTAL PROCEDURES**

**Mouse Strains and the Generation of the Dzip1 Mutant Allele—** A PAC clone containing mouse Dzip1 genomic DNA sequences (Geneservices, Inc.) was used to create a Dzip1 targeting construct. The construct was engineered by replacing the first two exons of the Dzip1 gene with a neomycin cassette flanked by loxp sites in reverse orientation relative to the Dzip1 gene (Fig. 1A). The linearized construct was electroporated into W4 ES cells, and targeted ES cell clones were identified by digestion of genomic DNA with EcoRI, followed by a Southern blot analysis of ES cell DNA with 5'- and 3'-probes (Fig. 1B). Two Dzip1-targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeric founders, which were then bred with C57BL/6 to establish F1 heterozygotes. The Dzip1 heterozygotes were maintained in a 129/SVE and C57BL/6 mix background. The neomycin cassette was removed by crossing the Dzip1 mutant mice with actin-cre mice. PCR analysis was used for routine genotyping with the following primers: forward primer P1 (5’-ATC GAC GTG GAC AAG GTT GC-3’) and reverse primer P2 (5’-GCC AGC CTG CTT GTG GAG CAG C-3’) for the wild type allele, which produced a 282-bp fragment and forward primer P3 (5’-CAG TGT TAT CTT TAC TGT GGA GT-3’) and reverse primer P4 (5’-AGG TCC CTC GAC CTG CAG CCC AAG-3’) for the targeted Dzip1 allele, which produced a 260-bp fragment.

**Cell Lines and Cell Culture—** Wild type and mutant Dzip1 mouse embryonic fibroblasts (MEFs) were generated from E9.5 mouse embryos. IFT88 mutant MEFs were derived from E10.5 mouse embryos (25). The MEFs were cultured in DMEM supplemented with heat-inactivated 10% FBS, penicillin, and streptomycin until cells were immortalized. HEK293 cells and human RPE-1 cells were cultured in the same medium. C3H10T1/2 cells were also grown in the same medium supplemented with ZnSO4 (0.1 μg/ml) and β-mercaptoethanol (0.1 μM).

cDNA and shRNA Constructs and Cloning—Mouse and human full-length Dzip1 cDNAs were purchased from Open Biosystems and cloned into the pCMV-3xFLAG vector (Sigma) by restriction enzyme digestion and/or PCR. pLNCX-FLAGHA-Dzip1 was created by cloning FLAGHA-Dzip1 into the pLNCX retroviral vector (Clontech). The Dzip1 mutant constructs were created by the same method. Human GLI3NT (aa 1–478), GLI3ΔZF (aa 478–645 deleted), GLI3CT (aa 645–1596), and GLI3CTΔPDD (aa 860–1596) were cloned into the pRK expression vector by the same method. The scramble and hDZIP1 shRNA set were purchased from Open Biosystems (RHS4533). Two of the five constructs (TRCN0000146638 and TRCN0000151587) from the hDZIP1 shRNA set were able to knock down endogenous hDZIP1 in RPE-1 cells to undetectable levels (data not shown). Data presented in this study were obtained using one of the two shRNA constructs.

**Tissue Immunohistochemistry and in Situ Hybridization—** For immunohistochemistry, mouse embryos at 9.5 days postcoitus (E9.5) were dissected, fixed in 4% paraformaldehyde plus PBS for 20–30 min at 4 °C, equilibrated in 30% sucrose plus PBS overnight at 4 °C, and embedded in OCT. The frozen embryos were transversely cryosectioned at the forelimb areas (10 μm). Tissue sections were immunostained using antibodies against Foxa2 (concentrated), Nkx2.2, Hb9, Isl1/2, Nkx6.1, and Pax6 (Developmental Study Hybridoma Bank) as described (26). The secondary antibodies (Jackson ImmunoResearch) were Cy3-conjugated donkey anti-mouse IgG. In situ hybridization of embryonic sections with Ptch1 or GlI1 digoxigenin-labeled probes was performed as described (26).

**Cell Immunofluorescence and Microscopy—** For GLI3 protein nuclear translocation, cells were incubated with a culture medium containing 50 nM leptomycin B (LMB) for 6 h prior to immunofluorescence. For cell ciliation studies, cells were plated on coverslips coated with poly-d-lysine overnight and then incubated with DMEM supplemented with 0.1% FBS and penicillin/streptomycin for 1 day to arrest the cells. For centrosome staining, cells were fixed 2–3 min in −20 °C 100% methanol. For the cytoplasmic and cilia staining, cells were fixed for 15 min in 4% paraformaldehyde plus PBS. After washing with PBS, the cells were incubated in PBS with 0.2% Triton X-100 for 15 min, followed by a wash with PBS. The cells were then incubated with primary antibodies in PBS, 1% BSA, 4% donkey...
serum for 1 h at room temperature. The cells were washed with PBS and then incubated with secondary antibodies in the same solution for 1 h at room temperature. After three washes with PBS, the coverslips were mounted to glass slides with Vectashield mounting fluid with DAPI (Vector Labs). The staining was visualized using a Zeiss Axiovert fluorescent microscope.

Antibodies—Antibodies to DZIP1, GLI2, IFT88, and GFP were generated by Covance, Inc. Rabbits were immunized with an insoluble His-tagged mouse DZIP1 fragment (aa 1–552), a GLI2 fragment (aa 605–1460), a soluble GST-IFT88 (aa 693–824), or an insoluble GST-GFP purified from bacteria. The DZIP1 and GFP antibodies were used at a 1:1000 dilution for Western blotting. The IFT88 antibody was used at a 1:1000 dilution for cell staining. Other antibodies used for cell staining included FLAG M2 mAb (1:1000), acetylated tubulin (1:2000), γ-tubulin (1:2000) (Sigma), CP110 (1:200) (Bethyl Laboratories, A301-343A), GLI3 crude serum (1:1000) (5), ODF2 (Abcam, ab43840, 1:200), Ninein (1:5000) (27), CEP97 (1:5000) (28), CEP164 (1:200) (Sigma), SAB3500022, IFT57 (1:1000), and IFT140 (1:1000) (29). Secondary antibodies Dylight 488-conjugated donkey anti-rabbit IgG and Cy3-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch, Inc.

Immunoprecipitations and Immunoblots—E9.5 mouse embryos or cells grown in dishes were washed once in PBS and lysed in radioimmunoprecipitation buffer buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitors) for 10 min on ice. After being cleared by centrifugation, the lysates were mixed with protein loading buffer, resolved in SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane, followed by Western blotting as described (5). For immunoprecipitation, the lysates were incubated with the indicated antibodies for 2 h on ice and subsequently with 15 μL of protein G-conjugated agarose beads with rotation at 4 °C for 1 h. The beads were washed with radioimmunoprecipitation buffer buffer, and proteins were eluted with protein loading buffer and subjected to Western blotting.

RESULTS

A Dzip1 Mutation Impairs Hh Signaling in Embryos—Genetic evidence shows that the zebrafish Igu gene product acts downstream of Smo to modulate both Gli activator and repressor function (23, 24). To determine whether DZIP1, the mouse homolog of Igu, is involved in Hh signaling, we mutated the Dzip1 gene by deleting the first two exons to create the Dzip1Δ1,2 allele (Fig. 1, A and B). Only about one-third of the homozygous Dzip1 mutant mice (7 of 22) died around E9.5, whereas the rest did not show noticeable phenotypes and survived to adulthood. The mutant embryos that showed abnormal phenotypes were smaller than their wild type littersmates. In particular, the neural tube and somites were underdeveloped (Fig. 1D). The incomplete penetration of the mutant phenotype could have resulted from the possibly partial inactivation of the Dzip1 gene, because a truncated DZIP1 protein was still expressed in the mutant (Fig. 1C).

To determine the possible initiation codon for the mutant DZIP1 protein, we inspected the DZIP1 amino acid sequence and found that any of the four methionine residues at positions 193, 229, 258, and 285 could serve as an initiation codon. To determine which residue is most likely to be the initiation codon, four expression constructs, each starting from one of these methionines, were created and transfected into HEK293 cells. Western blot analysis indicated that the size of the mutant DZIP1 protein was similar to that of the DZIP1 protein initiated at residue 258 (data not shown). Therefore, the DZIP1 mutant protein most likely lacks the N-terminal 257 residues (DZIP1Δ257), which includes the zinc finger domain (aa 185–230).

To determine whether the Dzip1 mutant phenotypes are caused by dysfunctional Hh signaling, we examined the neural tube patterning of the affected embryos. Several transcription factors that are normally expressed in the developing ventral neural tube and whose expression is induced by Sonic Hedgehog (Shh) (30) were missing in the Dzip1 mutant. These included Foxa2, a floor plate marker; Hb9, a motor neuron marker; Nkx2.2, a marker for V3 progenitors; and Nkx6.1, a protein normally expressed in the V1–V3 domains. In contrast, Pax6 expression, normally restricted by Shh signaling, expanded throughout the entire ventral neural tube (Fig. 1E).

To determine if the Dzip1 mutation directly affects Hh signaling, we examined the expression of Gli1 and Ptc1 RNA, transcriptional targets of Hh signaling. In situ hybridization showed that the expression of both Gli1 and Ptc1 was significantly reduced in the mutant neural tube (Fig. 1E), indicating that Hh signaling was impaired in the Dzip1 mutant.

To investigate whether the mutant phenotypes are related to GLI2 and GLI3 function, we examined GLI2 and GLI3 protein levels in the mutant embryos by immunoblotting with GLI2 and GLI3 antibodies. The results showed that levels of both of the full-length GLI2 and GLI3 proteins, GLI2FL and GLI3FL, were increased, whereas GLI3 repressor (GLI3Rep) levels were significantly reduced (Fig. 1F). Taken together, these results indicate that the Dzip1 mutation impairs both GLI2 activator function and GLI3 processing.

DZIP1 Interacts with and Sequesters GLI3 in the Cytoplasm—To explore the potential mechanism by which DZIP1 regulates GLI protein activity, we first determined whether DZIP1 interacts with the three GLI proteins. A FLAG-tagged DZIP1 was coexpressed with each of GLI1, GLI2, and GLI3 in HEK293 cells. GLI proteins were precipitated with Sepharose beads conjugated with a GLI-binding oligonucleotide, and the precipitates were then immunoblotted with FLAG antibody (4). The results showed that DZIP1 interacted with GLI3 but not GLI1 or GLI2 (Fig. 2A). The interaction between endogenous DZIP1 and GLI3 was also detected by coimmunoprecipitation using wild type MEFs. Interestingly, the interaction between endogenous GLI3 and the mutant DZIP1 was significantly reduced, indicating that the DZIP1 N-terminal region is necessary for high affinity binding to GLI3 (Fig. 2B). In addition, human DZIP1 was able to interact with hGLI3 (Fig. 2C), indicating that DZIP1 and GLI3 interaction is conserved in mice and humans.
To map the regions in DZIP1 that interact with GLI3, GLI3 was coexpressed with various Dzip1 mutant constructs in HEK293 cells. The interaction between GLI3 and the DZIP1 mutant proteins was examined as described above. The results revealed that two C-terminally truncated DZIP1 proteins, DZIP1(1–350) and DZIP1(1–437), which mimic two Igu mutations (23, 24) and lack the coiled coil domain, were still able to bind to GLI3, whereas DZIP1 C-terminal fragments containing the coiled coil domain were not (Fig. 2, D and G). Thus, the coiled coil domain is not required for GLI3 binding. Conversely, coimmunoprecipitation using protein lysates made from cells co-expressing FLAG-DZIP1 and various GLI3 mutants showed that both the N-terminal and C-terminal regions of GLI3 bind to DZIP1. The binding site in the C terminus is located in the processing determinant domain (PDD) region, which determines the extent of GLI3 processing (31), because the GLI3 C terminus lacking the PDD failed to bind DZIP1 (Fig. 2, E (lane 8) and H). The binding of GLI3PDD to DZIP1 was specific, because glutathione S-transferase (GST) fused with GLI2 PDD did not bind to DZIP1 (Fig. 2, F). Taken together, the results indicate that the N-terminal region of DZIP1 and the GLI3 PDD domain are essential for the two proteins’ interaction. This is consistent with the fact that binding of the DZIP1 mutant protein to GLI3 in Dzip1 mutant embryos is significantly reduced (Fig. 2B).
Dual Regulation of Hedgehog Signaling by DZIP1

Because DZIP1 interacts with GLI3, we next investigated whether DZIP1 regulates GLI3 subcellular localization. When FLAG-DZIP1 was expressed in C3H10T1/2 Hh-responsive cells, the DZIP1 protein localized exclusively to the cytoplasm (Fig. 3, B panels). DZIP1 does not shuttle between the nucleus and cytoplasm, because treatment of cells with LMB, an inhibitor for nuclear protein export, did not affect DZIP1 cytoplasmic localization (Fig. 3F). On the other hand, although GLI3 was predominantly found in the cytoplasm of the C3H10T1/2 cells, LMB treatment caused the GLI3 protein to translocate to the nucleus (Fig. 3, A panels and E panels). This observation indicates that GLI3 shuttles between the nucleus and cytoplasm. However, when coexpressed with FLAG-DZIP1, GLI3 was retained in the cytoplasm even upon LMB treatment (Fig. 3, F panels). However, upon coexpression of DZIP1(1–350) and DZIP1(1–437), which localize to the cytoplasm and both the nucleus (Fig. 3, C panels and D panels), respectively, GLI3 protein was not retained in the cytoplasm following LMB treatment (Fig. 3, G panels and H panels). Because both the DZIP1(1–350) and DZIP1(1–437) mutant proteins can

FIGURE 2. DZIP1 interacts with GLI3 in the cell. A, DZIP1 interacts with GLI3, but not GLI1 and GLI2. FLAG-DZIP1 was coexpressed with GLI1, GLI2, or GLI3 in HEK293 cells, as indicated. The protein lysates made from the cells were subjected to coimmunoprecipitation analysis, as indicated. IP, immunoprecipitation; PI, preimmune serum. B, coimmunoprecipitation analysis shows that the human DZIP1 also interacted with hGLI3 protein when the two proteins were coexpressed in HEK293 cells. The protein lysates from the cells were subjected to coimmunoprecipitation with either FLAG or GLI3 antibodies (middle or bottom panels) or pull-down with Gli-binding oligonucleotides followed by immunoblotting with FLAG antibody (top panels). The specific DZIP1 proteins are indicated by asterisks. E and F, GLI3 CT domain is necessary for the interaction of GLI3 with DZIP1. The proteins shown at the top were coexpressed in HEK293 cells. The protein lysates from the cells were subjected to coimmunoprecipitation (C, top panels), GST pull-down (D, top), or immunoblotting with the indicated antibodies (middle and bottom panels). G and H, diagrams summarize the results from A–E. ZF, zinc finger; CC, coiled coil.
bind GLI3, these results indicate that it is the DZIP1 C terminus that prevents GLI3 from entering the nucleus by tethering GLI3 in the cytoplasm.

If DZIP1 is required for GLI3 to be retained in the cytoplasm, one would predict that overexpressed GLI3 in Dzip1 mutant MEFs would localize to the nucleus. To test this prediction, both wild type and Dzip1 mutant MEFs were transfected with a GLI3 expression construct, and the GLI3 subcellular localization was determined by immunofluorescence with a GLI3 antibody. As shown in Fig. 4, A, A’, and E, GLI3 localized to the cytoplasm in most transfected wild type MEFs (>80%). In contrast, GLI3 was found in the nucleus in most transfected Dzip1 mutant MEFs (nearly 70%) (Fig. 4, B, B’, and E). This nuclear localization could be partially reversed by coexpression of exogenous DZIP1 in the mutant cells (reduced to about 40%, Fig. 4, D, D’, and E). The partial rescue of Gli3 cytoplasmic localization is probably due to the variation in the levels of DZIP1 expression.

Because Dzip1 mutant cells lack primary cilia (see below), we wanted to know whether the nuclear localization of GLI3 resulted from this or from a loss of the ability of DZIP1 to sequester GLI3 in the cytoplasm. To address this question, GLI3 subcellular localization was also examined in transfected MEFs with various DZIP1 constructs.
To determine whether DZIP1 localizes to the cilia. To this end, A formation.

Thus, we asked whether primary cilia are present in is reminiscent of mouse embryos exhibiting cilia defects (6).

This indicates that the ability of DZIP1 to sequester GLI3 in the cytoplasm is function, not the lack of cilia. Together, these results indicate that the accumulation of DZIP1 at the basal body of cilia and is required for cilia formation. A, Dzip1 mutant cells lack cilia. WT and Dzip1 mutant MEFs were stained with acetylated α-tubulin antibody (red) for cilia and DAPI for the nucleus (blue). B, endogenous DZIP1, but not the Dzip1 mutant protein, accumulates at the mother centrioles. WT MEFs were co-stained with antibodies, as indicated. Note that the strong DZIP1 staining overlapped with that of Ninein. C, human DZIP1 is also required for ciliogenesis. Human RPE-1 cells were transduced with lentivirus carrying control shRNA or hDZIP1 shRNA expression constructs and subsequently subjected to immunostaining antibodies, as indicated. The percentage of cells with cilia is shown by a graph (more than 100 cells were counted, p < 0.0001). D, the N terminus is required for the localization of DZIP1 at the basal body. WT MEFs were transfected with the indicated FLAG-DZIP1 constructs. The transfected cells were co-stained for FLAG-DZIP1 proteins (red), IFT88, which localizes at the basal body and axoneme of cilia, and the nucleus (DAPI) (blue). E, diagrams showing the constructs used in D. Arrows, basal bodies. ZF, zinc finger; CC, coiled coil; BB loc, basal body localized.

Ift88 mutant MEFs, which lack cilia (11). The results showed that, as that in wild type cells, overexpressed GLI3 in Ift88 mutant cells was predominantly found in the cytoplasm (about 80%) (Fig. 4, C, C’, and E). Therefore, the nuclear localization of GLI3 in the Dzip1 mutant cells is due to the loss of DZIP1 function, not the lack of cilia. Together, these results indicate that the ability of DZIP1 to sequester GLI3 in the cytoplasm is independent of its function in ciliogenesis (see below).

DZIP1 Localizes to the Basal Body and Is Required for Ciliogenesis—The fact that Hh signaling and GLI3 processing are impaired in Dzip1 mutants suggests that DZIP1 is required for both GLI2 and GLI3 activator and repressor functions. This is reminiscent of mouse embryos exhibiting cilia defects (6). Thus, we asked whether primary cilia are present in Dzip1 mutant MEFs. Immunofluorescent staining for acetylated tubulin, which marks the axoneme of primary cilia, showed that primary cilia were present in most wild type MEFs but not in the mutant MEFs, except for a small dotted staining (Fig. 5A). Thus, DZIP1 is required for cilia formation.

The involvement of DZIP1 in ciliogenesis prompted us to determine whether DZIP1 localizes to the cilia. To this end, wild type MEFs were co-immunostained for DZIP1 and γ-tubulin, a marker for centrioles. The results showed that DZIP1 localized to two centrioles asymmetrically. The weaker DZIP1-immunoreactive signal completely overlapped with one centriole, whereas the stronger signal only partially overlapped with the other centriole (Fig. 5B, top panels). Interestingly, only the weaker DZIP1-immunoreactive signals were detected in the centrioles of Dzip1E1–2/ΔE1–2 MEFs (Fig. 5B, middle panels), indicating that the Dzip1 mutation abolishes DZIP1 accumulation to one of the centrioles. To determine whether the DZIP1 accumulation occurred at the mother or daughter centrioles, wild type MEFs were co-immunostained for DZIP1, Ninein (a subdistal appendage marker for the mother centrioles) (13), and γ-tubulin. The results showed that the stronger DZIP1 staining overlapped with that of Ninein (Fig. 5B, bottom panels), indicating that DZIP1 accumulates at the basal body and may be a part of the ciliary appendage structure. The role of DZIP1 in ciliogenesis is also conserved in human cells, because knockdown of hDZIP1 expression by shRNA significantly reduced cilia number in RPE-1 cells (Fig. 5C).

To determine whether overexpressed DZIP1 was able to recapitulate the basal body localization of endogenous DZIP1, wild type MEFs were transfected with FLAG-DZIP1. The cells were then immunostained for FLAG-DZIP1 and IFT88, an IFT component that localizes to the basal bodies and accumulates in the tips of the primary cilia in quiescent cells (32). As expected, the DZIP1 full-length protein, DZIP1FL, was enriched in the base of the cilia and colocalized with IFT88 at the basal body in addition to its diffuse localization in the cytoplasm (Fig. 5D, top panels). To define the region responsible for DZIP1 basal body localization, the same experiments were performed using several DZIP1 mutant constructs. DZIP1(1–437) and DZIP1(1–350) remained localized to the basal body (Fig. 5D, middle panels). However, DZIP1–350–E, which contains the coiled coil domain, failed to localize to the basal body (Fig. 5D, bottom panels). These results are consistent with the observation that the DZIP1ΔE1–2 mutant protein, which presumably lacks the N-terminal 257 residues, fails to localize to the basal body (Fig. 5, B (middle panels) and E). Taken together, these observations indicate that the accumulation of DZIP1 at the basal body is dependent on the DZIP1 N-terminal region but not the DZIP1 coiled coil domain.

DZIP1 Is Required for the Localization of CEP164 and Ninein to the Appendages of the Mother Centrioles—To understand the molecular mechanism underlying the absence of primary cilia in Dzip1 mutant cells, we examined the localization of several centrosomal and ciliary proteins in the wild type and mutant MEFs. Immunostaining of cells with antibodies against γ-tubulin and CP110 or CEP97, two proteins localizing to the distal end of the centrioles (28, 33), showed that the Dzip1 mutation did not affect the localization of either, suggesting that the distal centriole structure of centrioles still forms in Dzip1 mutant cells. CEP164, however, was not detected in the mutant cells at the distal appendage of the mother centrioles (Fig. 6A), where it normally localizes (16). This absence of CEP164 in Dzip1 mutant cells could not have been due to a change in expression, because the levels of CEP164 expression in the mutant cells were comparable with those in wild type cells (data not shown).
It also cannot simply be a consequence of the loss of cilia in the Dzip1 mutant cells, because CEP164 still localized to the distal appendage of the mother centrioles in the Ift88 mutant MEFs (Fig. 6B).

We also examined whether the Dzip1 mutation affects the localization of Ninein. In the quiescent wild type cells, Ninein staining usually showed three dots in the mother centrioles, one in the proximal end and two in the subdistal appendages, and one dot in the daughter centrioles (Fig. 6A) (13, 34). However, in Dzip1 mutant cells, only a single dotted signal in the mother centrioles was detected and presumably localized to the proximal end of the mother centrioles (Fig. 6A). The absence of Ninein at the mother centriole appendages is not simply due to the lack of primary cilia in Dzip1 mutant cells, because Ninein was still found at the appendages in Ift88 mutant cells (Fig. 6B). Unlike that of Ninein, the localization of ODF2 to the appendages of the mother centrioles (34) in the mutant cells was unaffected (Fig. 6A). Taken together, these data indicate that DZIP1 is required for CEP164 and Ninein to localize to the distal and subdistal appendages of the mother centrioles, respectively.

To gain insight into the mechanism by which CEP164 and Ninein are absent in the distal and subdistal appendages, we asked whether DZIP1 co-localizes with these two proteins. Wild type MEFs were transfected with a FLAG-DZIP1 expression construct, and the transfected cells were subjected to co-immunostaining with antibodies against FLAG and CEP164 or Ninein. The results showed that DZIP1 interacts with CEP164. The colocalization of DZIP1 with CEP164 prompted us to determine whether DZIP1 interacts with CEP164. FLAG-DZIP1 or FLAG-DZIP1Δ257 and GFP-tagged CEP164 (GFP-Cep164) were coexpressed in HEK293 cells, and the cell lysates were subjected to coimmunoprecipitation. The results showed that FLAG-DZIP1, but not FLAG-DZIP1Δ257, could coin-
Because IFT88 and IFT57 are components of the IFT-B complex in the wild type cells but not in the mutant cells (Fig. 7A), both IFT57 and IFT140 were recruited to the mother centrioles detected in the mother centrioles of the mutant cells. Similarly, DZIP1 body and the tips of the cilia in wild type cells but was not with the indicated antibodies (Fig. 6A). This interaction was confirmed in HEK293 cells that stably express modest levels of FLAGHA-DZIP1 (Fig. 7B), suggesting that the DZIP1 and IFT88 interaction is unlikely to be due to the overexpression. Therefore, DZIP1 recruits IFT components to the base of the cilia through its interaction with IFT88.

**DISCUSSION**

In the present study, we investigate the role and mechanism of DZIP1 in Hh signaling in mice. The deletion of the first two exons of Dzip1 compromises Hh signaling during embryogenesis. DZIP1 regulates Hh signaling through two mechanisms. First, DZIP1 directly binds the GLI3 protein and prevents GLI3 from entering the nucleus. Second, DZIP1 is required for activation of GLI proteins through a cilia-dependent mechanism. Therefore, DZIP1 is the first known ciliogenic protein to regulate Hh signaling through this dual mechanism.

Dzip1 mutant embryos that show severe developmental defects have dorsIALIZED neural tubes and underdeveloped somites, indicating that GLI activator function is impaired. Consistent with this, the expression of *Ptc1* and *Gli1* is dramatically reduced. However, levels of both GLI2FL and GLI3FL are increased, whereas the GLI3 repressor level is reduced (Fig. 1, D–F). Thus, like its zebrafish homolog Igu (23, 24) and many other known cilia-related proteins (6), DZIP1 is required for the functions of both GLI2 and GLI3 activators and repressors. However, unlike many known ciliary proteins, DZIP1 has also been implicated in the regulation of embryonic stem cells and germ cells (35). Thus, DZIP1 may have a broader function than the other known ciliary proteins.

The Dzip1 mutant phenotypes reported here are only partially penetrant. The embryos with the most severe phenotypes, about a third of the mutant embryos, died around E9.5, whereas the rest of the animals were born and grew to maturity without any noticeable phenotypes. The phenotypes appear to correlate with the presence of the primary cilia, because those without noticeable phenotypes had normal cilia (data not shown), whereas those with severe phenotypes lacked cilia (Fig. 5A). The partial penetrance of the Dzip1 mutant phenotypes could be related to the possibility that the mutant allele reported in this study might be hypomorphic, because a truncated protein is expressed in the mutant (Fig. 1C). It could also be attributed to potentially functional redundancy of different members of the DZIP1 family, such as DZIP1L. Further studies are necessary to determine the cause of the partial penetrance of the DZIP1 mutant phenotypes.

Most GLI3FL proteins localize to the cytoplasm, and the protein shuttles between the nucleus and cytoplasm (31, 36, 37). One protein that has been reported to regulate the GLI3 local-
izations of the mother centrioles. Second, the components of both ages of the mother centrioles. First, DZIP1 accumulates at the mother centrioles because of the lack of primary cilia in mutant cells (Fig. 7A). This is probably due to a failed interaction between the Dzip1 mutant protein and IFT88 (Fig. 7, B and C). These results suggest that the recruitment of IFT machinery to the basal body, which is essential for cilia elongation, is through the interaction of IFT88 with DZIP1. These data may explain why cilia are not formed in Dzip1 mutant cells.

Hh signaling is generally thought to occur in primary cilia. Consistent with this, several of the Hh pathway components are found in cilia. Hh signaling leads to the accumulation of Smo and GLI2/GLI3 proteins in the cilia (10, 44–47). The transport of Hh signaling components in cilia is presumably mediated by IFT machinery. However, the biochemical evidence that links the Hh signaling components with IFT machinery remains missing. The finding that DZIP1 colocalizes with IFT88 in the basal body and interacts with both IFT88 and GLI3 is the first biochemical evidence that GLI3 is transported by IFT machinery in cilia (Fig. 7B).

Thus far, all known ciliary proteins to regulate Hh signaling do so because they are essential for ciliogenesis or protein and vesicle transport in the cilia (6). Compared with these proteins, DZIP1 is unusual in that it regulates Hh signaling through two different mechanisms. One is by sequestering GLI3 in the cytoplasm, similar to the function of SUFU, and the other is by its regulation of ciliogenesis through the recruitment of cilia appendage and IFT components to the cilia basal body. It is worth noting that the localization of GLI3 to the nucleus in Dzip1 mutant cells is not the result of the loss of primary cilia, because GLI3 in Ift88 mutant cells, which also lack primary cilia, is predominantly in the cytoplasm (Fig. 4). Therefore, the dual functions (the sequestration of GLI3 in the cytoplasm and the regulation of ciliogenesis through the recruitment of cilia appendage and IFT components to the cilia basal body) appear to be independent of each other and unique to DZIP1. As discussed above, the retention of the GLI3 weak activator in the cytoplasm by DZIP1 ultimately results in a net increase in the output of overall Gli activating function. The formation and maintenance of functional cilia by ciliary proteins, such as DZIP1, are essential for the activation of GLI2/GLI3 proteins. Therefore, it is through this dual mechanism that DZIP1 positively regulates Hh signaling. To our knowledge, DZIP1 is the first ciliary protein that has been identified so far to regulate Hh signaling in this fashion.

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