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The Zn Finger protein Iguana impacts Hedgehog signaling by promoting ciliogenesis

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Abstract

Hedgehog signaling is critical for metazoan development and requires cilia for pathway activity. The gene *iguana* was discovered in zebrafish as required for Hedgehog signaling, and encodes a novel Zn finger protein. Planarians are flatworms with robust regenerative capacities and that utilize epidermal cilia for locomotion. RNA interference of *Smed-iguana* in the planarian *S. mediterranea* caused cilia loss and failure to regenerate new cilia, but did not cause defects similar to those observed in *hedgehog(RNAi)* animals. *Smed-iguana* gene expression was also similar in pattern to the expression of multiple other ciliogenesis genes, but was not required for expression of these ciliogenesis genes. *iguana*-defective zebrafish had too few motile cilia in pronephric ducts and in Kupffer's vesicle. Kupffer's vesicle promotes left-right asymmetry and *iguana* mutant embryos had left-right asymmetry defects. Finally, human Iguana proteins (dZIP1 and dZIP1L) localize to the basal bodies of primary cilia and, together, are required for primary cilia formation. Our results indicate that a critical and broadly conserved function for Iguana is in ciliogenesis and that this function has come to be required for Hedgehog signaling in vertebrates.

Introduction

The Hedgehog (Hh) signaling pathway is of broad importance for development and disease (Varjosalo and Taipale, 2008). In vertebrates, Hh signaling can regulate limb and digit patterning (Tabin and McMahon, 2008), patterning of neurons in the neural tube (Dessaud et al., 2008), and has been implicated in diseases including cancer (Jiang and Hui, 2008). In recent years, primary cilia have been demonstrated to be required for Hh signaling in vertebrates (Eggenchwiler and Anderson, 2007).

Cilia are cellular projections that grow from a basal body and that involve nine doublet microtubules arranged in a ring with two singlet central pair microtubules (a 9+2 microtubule arrangement) in the case of motile cilia or no central microtubules (a 9+0

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microtubule arrangement) in the case of primary cilia (Eggenschwiler and Anderson, 2007; Gerdes et al., 2009; Huangfu et al., 2003). Cilia dysfunction in humans can result in chronic bronchitis, *situs inversus*, immotile sperm, polydactyly, mental retardation, kidney cysts, and other disorders (Badano et al., 2006). Mutations in genes required for ciliogenesis in vertebrates can cause defects in Hh signaling (Huangfu et al., 2003; Liu et al., 2005). Several Hh signaling components can localize to cilia in vertebrates, including the Smoothed transmembrane protein (Corbit et al., 2005) and Gli transcription factors (Haycraft et al., 2005). The requirement of cilia for Hh signaling, however, may be an innovation that occurred during the evolution of the deuterostomes because no evidence exists to link cilia and Hh signaling in *Drosophila*. Given the significance of both Hh signaling and cilia for development and disease, understanding the identity and roles of proteins that impact Hh signaling and cilia is of great importance.

Iguana is a novel, C2H2-type, zinc finger protein (Sekimizu et al., 2004; Wolff et al., 2004), also referred to as DZIP1 (Moore et al., 2004). The zebrafish *iguana* gene is required for normal Hedgehog signaling (Odenthal et al., 2000; Sekimizu et al., 2004; Wolff et al., 2004), but the reason it is required for Hedgehog signaling is unknown. The planarian *S. mediterranea* is an emerging model system for studies of metazoan gene function in regeneration and tissue turnover (Reddien and Sánchez Alvarado, 2004). The essentially complete planarian genome sequence combined with the ability to perform RNAi screens has opened the door to molecular genetic studies of the robust biological attributes these animals display (Reddien et al., 2005). Because there are numerous ciliated cell types in planarians, including the ventral epidermis for accomplishing animal locomotion (Hyman, 1951), ciliogenesis genes can readily be studied. We found that an *iguana*-like gene is required for ciliogenesis in the planarian *S. mediterranea*. This observation suggested the possibility that it is a role in ciliogenesis that explains the requirement for Iguana in Hedgehog signaling. We therefore also studied the role of Iguana/DZIP1 proteins in zebrafish and in human cells and found that these proteins are required for vertebrate ciliogenesis and are components of the basal bodies of cilia. We conclude that Iguana proteins are novel, and broadly conserved, components of basal bodies with a critical role in ciliogenesis.

Materials and methods

planarian assays

Time to move across a defined distance in a light gradient was measured to determine speed. Head rim cilia length and density at the lateral animal edge were determined using differential interference contrast (DIC) microscopy and still images (Zeiss Axiovision).

planarian *in situ* hybridizations and antibody labeling

Fixations and hybridization methods were largely based upon Pearson et. al (Pearson et al., 2009). For antibody labeling, planarians were first placed in 5% N-acetyl cysteine in PBS for 5 min, followed by fixation with Carnoy's fixative, and labeling as previously described (Reddien et al., 2005) using an anti-acetylated tubulin antibody (Sigma).

RNAi

RNAi involved feeding a mixture of dsRNA-containing bacteria and liver, as previously described (Reddien et al., 2005). 10mL of pelleted culture was resuspended in either 100uL or 33uL of a 66% blended liver solution in planarian water, with similar results.

molecular biology

Smed-iguana sequence was obtained using 5' RACE (RLM-RACE, Ambion) and from the NB.10.6h cDNA. The NBE.7.10a RNAi construct was used for RNAi (Reddien et al., 2005). Riboprobes to *BBS2* and *dnah1* were obtained using RT-PCR and other riboprobes were generated from cDNAs (*BBS1*, saah-aac61a11.g1; *BBS9*, saah-aab84c07; *IFT88*, saah-aaa85e05). The *rootletin* riboprobe was generated from the H.2.8b cDNA. Zebrafish *cmlc2* (AF425743) riboprobe template was obtained using RT-PCR.

zebrafish lines and morpholinos

Zebrafish lines used included wild type AB, *igu^{tm79a}* (Brand et al., 1996; Karlstrom et al., 1996) and *smo^{hi229Tg}* (Chen et al., 2001). Control embryos for experiments involving *igu^{tm79a}* were phenotypically wild type and from a cross of two *igu^{tm79a/+}* animals. Wild-type embryos were injected with 1nL of 1-2 mM *igu* splice targeted morpholinos (MOs) (Gene Tools; Splice MO1, 5'-GTACAGACCTTGTGGTAATTGGCAC-3'; Splice MO2, 5'-CAGATTGAACTCACTCATGTCGAAT-3'). 5 base-pair mismatched MOs were used as controls. 50 picograms of mRNA encoding CAAX-eGFP (membrane GFP; kindly provided by J.B. Green, Dana-Farber Cancer Institute Boston, MA) served as an injection control.

zebrafish immunohistochemistry

Embryos were fixed in 2% trichloroacetic acid (TCA) for 3 hours, blocked (phosphate buffer with 0.5% Triton-X100, 10% goat serum, 0.1% BSA), and labeled with anti-acetylated tubulin (1:1000) overnight at 4°C. *in situ* hybridizations utilized standard procedures.

Cell culture and siRNA transfection

cDNAs for the human Iguana (dZIP1) and Iguana-like (dZIP1L) protein were obtained as IMAGE clones (5271595 and 4940443, respectively). GFP-tagged proteins were transiently transfected using effectene (Qiagen) into HeLa or hTERT-RPE1 cells and processed 30 to 72 hours later. For experiments in hTERT-RPE1 cells, primary cilia formation was induced by serum starvation for 48 hours. RNAi experiments were conducted as described previously (Kline et al., 2006). All cells were transfected with Oligofectamine (Invitrogen). 6 hours post-transfection, OPTI-MEM media was replaced with DMEM and cells processed 48-72 hours later. siRNAs were obtained from Dharmacon as a pool of 4 sequences; DZIP1 (CCGCAGGAGUCGCCGUUAA, GGAGUGAGAUCGUAUU, ACAACAACUUUGCGAUGUA, and GUGACUGAUUGGAGCGACA) and dZIP1L (AGAGGGAAAUAGAAGCUAA, CAAAGGACCACACGUGUAA, CUGACGAGCUCAAGGGUGU, and GGAACAAGCUAGGAUCAUU). Control RNAi was performed with siCONTROL Non-targeting siRNA Pool #1 (Dharmacon).

Immunofluorescence and microscopy with human cells

Cells were fixed with methanol for 5 minutes in -20°C. Cells were stained with anti-rabbit centrin, and acetylated-tubulin (6-11B-1, Sigma-Aldrich). Transiently transfected HeLa and hTERT-RPE1 cells were also stained with anti-goat GFP. Cy2, Cy3, and Cy5-conjugated secondary antibodies (Jackson Laboratories) were used at 1:100. DNA was visualized using 10 µg/ml Hoechst. Images were acquired using a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 CCD camera. 40 Z-sections were acquired at 0.2-micron steps using a 100×, 1.3 NA Olympus U-PlanApo objective with 1×1 binning. Images were deconvolved using the DeltaVision software. Equivalent exposure conditions and scaling was used between controls and RNAi-depleted cells.

Results

***Smed-iguana* is required for ciliogenesis in planarians**

The *Smed-iguana* gene (abbreviated as “*iguana*”) encodes an Iguana-like Zn finger protein (Fig. S1) and is required for normal planarian locomotion (Reddien and Sánchez Alvarado, 2004). Inhibition of *iguana* with RNAi in adult planarians results in loss of the ability of animals to glide (Reddien et al., 2005); resultant animals display an inching behavior with slower than normal locomotion (Fig. 1A) accomplished by muscular contractions (Supplementary movies 1, 2). Regenerating planarians produce an epidermis-covered mesenchymal bud at the wound site called a blastema, within which some missing tissues are produced (Reddien and Sánchez Alvarado, 2004). *iguana(RNAi)* animals were capable of normal blastema formation and regeneration (Fig. 1B), but these regenerating animals also showed inching behavior (n=274/289 inching with 15 not moving versus 286/288 gliding and 2 not moving for the control). Regenerating *iguana(RNAi)* animals ultimately bloat with fluid and blister (Reddien et al., 2005), indicating a candidate defect in water homeostasis (Fig. 1B). Planarian locomotion is accomplished with the use of ventral motile cilia and water balance is maintained by a ciliated excretory/osmoregulatory system referred to as protonephridia (Hyman, 1951). Together, the locomotory and osmoregulatory defects of *iguana(RNAi)* animals suggested a possible defect in cilia function.

We examined planarian cilia using differential interference contrast (DIC) microscopy at the head rims of *iguana(RNAi)* and control animals. Cilia number was grossly abnormal in *iguana(RNAi)* animals (Fig. 2A, B). Cilia began to disappear from the animals between five and seven days following RNAi, and reached a low point following approximately 14 days of RNAi (Fig. 2B). During the period of cilia loss, remaining cilia maintained normal length (Fig. 2B) and were capable of beating (Supplementary movies 3, 4). These data indicate a requirement for *iguana* in maintenance of the ciliated state of cells at the head rim.

To test for a general requirement for *iguana* in maintenance of cilia and in the genesis of new cilia, we utilized an anti-acetylated tubulin antibody that can label planarian cilia. Prominent ciliated features labeled with this antibody include the entire ventral surface and a dorsal, midline stripe of cilia (Reddien et al., 2007; Robb and Sánchez Alvarado, 2002). *iguana(RNAi)* animals displayed a near complete loss of ventral and dorsal cilia (Fig. 3A). We also failed to observe cilia on *iguana(RNAi)* regeneration blastemas (Fig. 3B, Fig. S2). Because no cilia were observed on *iguana(RNAi)* blastemas during timepoints of regeneration at which cilia normally are forming (Fig. S2), *iguana* is likely required for ciliogenesis as opposed to the maintenance of cilia on blastemas following a normal phase of formation. The ciliated cells (flame cells) of the planarian excretory/osmoregulatory system can also be visualized with the anti-acetylated tubulin antibody. The ‘flame bulb’ of the flame cells in this system is a cluster of cilia that creates fluid flow. Bloating and blistering in planarians can be caused by disruption of microtubule function (Reddien et al., 2005), consistent with a possible defect in motile cilia explaining the bloating and blistering observed in *iguana(RNAi)* animals. We found that the regeneration blastemas of *iguana(RNAi)* animals had fewer flame bulbs than did the blastemas of control animals (Fig. 3C). Therefore, *iguana* is required for the regeneration of ciliated protonephridia. Together these data indicate that *iguana* is required for maintenance of a ciliated epidermis and for ciliogenesis in newly regenerated tissues.

***Smed-iguana* is expressed with other ciliogenesis genes in ciliated planarian cell types**

If *iguana* primarily functions in planarian ciliogenesis, it should be expressed predominantly in ciliated cell types. *iguana* expression was detected using whole-mount *in situ* hybridizations and was observed within the ciliated epidermis, including along a stripe on

the dorsal epidermis (Fig. 4A). Expression was also observed within the pharynx, the highly ciliated feeding organ of planarians (Fig. S3). An additional site of *iguana* expression was within the region peripheral of the brain in the anterior, where sensory neurons containing sensory cilia reside (Fig. 4A). We compared the *iguana* expression pattern to that of numerous planarian genes predicted to encode proteins associated with cilia formation or function (Gerdes et al., 2009). Specifically, we identified and cloned the planarian genes *BBS1* (*Smed-BBS1*; BBS, Bardet-Biedl Syndrome), *BBS2* (*Smed-BBS2*), *BBS9* (*Smed-BBS9*), *IFT88* (*Smed-IFT88*; IFT, intraflagellar transport), and dynein *Dnah1* (*Smed-dnah1*, *Dnah1* is required for ciliary motility). All of these genes were expressed in a pattern similar to that of the *Smediguana* gene (Fig. 4A). We also found that the planarian *rootletin* gene (*Smed-rootletin*) was expressed in the ciliated epidermis (Fig. 4A); Rootletin is a component of ciliary rootlets (Yang et al., 2002). The expression pattern of *iguana* in planarians, and the similarity of this pattern to that of genes involved in cilia function, is consistent with a primary role for planarian *iguana* being in ciliogenesis and maintenance of a ciliated state.

We sought to determine whether *iguana* acts by promoting the expression of a ciliogenesis program or by promoting the process of ciliogenesis. If *iguana* acts to specify a ciliated cell fate, a gross general defect in expression of genes involved in cilia biology would be anticipated in *iguana*(RNAi) planarians. However, we failed to detect any defect in expression of cilia genes in intact and regenerating *iguana*(RNAi) planarians that lacked cilia (Fig. 4B, C). Although it is possible that the IGUANA protein has a role in the expression of one or more critical cilia proteins, these data are most consistent with a role for IGUANA in the physical process of ciliogenesis as opposed to a role in the specification of a cilia program as part of a cell fate.

***Smed-iguana* is not required for planarian Hedgehog signaling**

There is no evidence that cilia are required for Hedgehog signaling in protostomes, nor is there evidence that Hedgehog signaling is required for ciliogenesis. Because the defects associated with RNAi of *iguana* in planarians are in ciliogenesis, the possibility therefore exists that *iguana* is not required for Hedgehog signaling in protostomes. We identified a single *hedgehog* gene in the *S. mediterranea* genome (*Smed-hedgehog*), and inhibited the gene with RNAi. *hedgehog*(RNAi) animals did not have detectable defects in ciliogenesis, but did display defects in tail regeneration (Fig. 5A, B). Similar results were obtained following inhibition of a gene encoding a Gli2-like protein, *Smed-gli2-1* (Fig. 5A, B). The similar phenotypes caused by RNAi of *hedgehog* and *gli2-1* indicates that tail formation is promoted by a Hedgehog signaling pathway similar to that of other organisms. By contrast, *iguana*(RNAi) animals did not display tail regeneration defects (Fig. 5A, B). These data suggest that Iguana is not a component of planarian Hedgehog signaling.

***iguana* is required for ciliogenesis and left/right asymmetry in zebrafish**

Because cilia have recently been demonstrated to be required for Hedgehog signaling in vertebrates the implication of *iguana* in cilia biology in planarians raised the intriguing possibility that it is a cilia role for Iguana that explains its requirement for Hedgehog signaling in zebrafish. Zebrafish Smoothed protein can localize to primary cilia (Aanstad et al., 2009), and genetic manipulations that disrupt cilia can cause Hedgehog signaling defects (Aanstad et al., 2009; Beales et al., 2007; Huang and Schier, 2009). These observations suggest the role for cilia in Hedgehog signaling is conserved in zebrafish.

The Hh pathway works, in brief, as follows. The Hh receptor, Patched, can inhibit translocation of the Smoothed protein to cilia (Varjosalo and Taipale, 2008). Hh signals by binding to and inhibiting Patched. Within cilia, Smoothed acts with other factors to regulate the activity state of Gli transcription factors. In the presence of Hh, formation of

repressive Gli proteins is inhibited, and formation of activating Gli proteins is promoted (Varjosalo and Taipale, 2008). The genetic attributes of the zebrafish *iguana* gene (*igu*), with respect to action of the Hh pathway, share similarities to the attributes of ciliogenesis genes in mice. Aspects of the *igu* mutant phenotype are similar to mutants for the *Sonic hedgehog*-like *sonic-you* (*syu*) gene, a *smoothened* (*smo* or *smu*, *smooth muscle omitted*) gene, the *Gli1*-like *detour* (*dtr*) gene, and the *Gli2*-like *you-too* (*yot*) gene (Brand et al., 1996; Chen et al., 2001; Karlstrom et al., 1999; Karlstrom et al., 2003; Schauerte et al., 1998; Varga et al., 2001). However, the *igu* phenotype is consistent with defects in both activating and repressive roles of Gli proteins. *igu* mutants show defects in Hh-dependent marker expression in the ventral neural tube (Sekimizu et al., 2004), a decrease in somite cell populations promoted by low and high Hh levels, and an increase in somite cell populations promoted by intermediate Hh levels (Wolff et al., 2004). Multiple experiments indicate that *igu* acts at a step in the Hh pathway downstream of *smoothened* and that *igu* may affect the relative activities of repressive and activating Gli proteins (Sekimizu et al., 2004; Wolff et al., 2004). Loss of function of cilia genes in mice can also affect the Hh pathway downstream of *smoothened* and have impacts on the activity states of Gli proteins (Eggenschwiler and Anderson, 2007; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005). The similarities between genetic data for *igu* in zebrafish and for cilia genes in mouse, with respect to Hh signaling, are consistent with a hypothesis of a cilia-related role for Iguana in vertebrates.

We sought to determine whether the zebrafish *iguana* gene is broadly required for zebrafish ciliogenesis by examining the cilia of multiple tissues in *igu*-defective zebrafish embryos. We utilized the *tm79a* allele (Brand et al., 1996; Karlstrom et al., 1996) of *igu*, which is a nonsense mutation (Sekimizu et al., 2004), and also used splice-site morpholinos to create *igu* morphants (morpholino antisense oligonucleotide-injected embryos). *igu* mutants were recently reported to contain fewer cilia in the zebrafish neural tube and floor plate (Huang and Schier, 2009). We also detected fewer motile cilia in the pronephric ducts of *igu* mutant embryos than were observed in control embryos (Fig. 6A). The similar phenotypes observed in *igu* mutants and morphants indicate that the observed reduction in cilia number was the result of loss of *igu* function. If Iguana has a primary function in ciliogenesis it should be required for the functioning of ciliated tissues. The cilia of Kupffer's vesicle generate a counter-clockwise flow of fluid that is required for the production of left-right asymmetry in zebrafish (Essner et al., 2005; Kramer-Zucker et al., 2005). *iguana* mutant zebrafish were previously described to affect cardiac asymmetry (Chen et al., 1997), consistent with a hypothesized general role in the functioning of ciliated tissues. We examined left-right asymmetric gene expression in the lateral plate mesoderm of control and *igu* morphant zebrafish embryos. The heart is formed on the left side of zebrafish embryos, and the developing heart can be visualized with *in situ* hybridizations using a riboprobe corresponding to the *cardiac myosin light chain 2* (*cmlc2*) gene. *igu*-defective embryos displayed a highly penetrant left-right asymmetry abnormality, with the heart forming either on the left, centrally, or on the right side of embryos (Fig. 6B). By contrast, embryos mutant for the Hh pathway gene *smoothened* (*smo*) displayed normal left-right *cmlc2*-expression asymmetry (Wilson et al., 2009) and data not shown). *igu* morphants had a marked reduction in the number of cilia in Kupffer's vesicle (Fig. 6C). By contrast, Hedgehog signaling does not appear to be required for left-right asymmetry in zebrafish (Chen et al., 2001) and we failed to observe cilia number abnormalities in Kupffer's vesicle in *smoothened* (*smo*) morphants (Fig. 6C). The fact that many ciliated tissues displayed a reduction in cilia number in *igu* mutants and morphants supports the hypothesized primary role for *iguana* in ciliogenesis. Together these data demonstrate a Hedgehog-independent role for Iguana in vertebrate ciliogenesis.

The human Iguana-like proteins dZIP1 and dZIP1L localize to the basal bodies of primary cilia and are required for primary ciliogenesis

Because Iguana proteins are required for ciliogenesis, we sought to determine if Iguana proteins localize to cilia. Two Iguana-like genes are found in humans, *dZIP1* and a second dZIP1-like gene, *dZIP1L* (Moore et al., 2004). The localization of GFP-tagged versions of the human Iguana-like proteins was examined in RPE1-hTERT cells, which allow induction and visualization of primary cilia (Vorobjev and Chentsov Yu, 1982). dZIP1 and dZIP1L localized to two discrete puncta. These puncta localized in close proximity to centrin, a centriolar component (Fig. 7A and S4). Furthermore, dZIP1, dZIP1L, and centrin localized near to the basal body of primary cilia in hTERT-RPE1 cells. In cells lacking cilia (HeLa cells), dZIP1 localized to the centrioles (Fig. S4). Potential centriolar and basal body localization of Iguana proteins was unexamined in prior localization studies (Sekimizu et al., 2004; Wolff et al., 2004). RNAi was utilized to determine if human Iguana proteins are required for human primary cilia formation. RNAi of *dZIP1* and *dZIP1L* together resulted in a reduction in the percentage of hTERT-RPE1 cells that were capable of primary cilia formation (Fig. 7B). Together, our observations indicate that dZIP1/Iguana proteins are previously unrecognized centriole and basal body-associated proteins required for normal ciliogenesis.

Discussion

Data presented here demonstrate a broad requirement for Iguana-like proteins in ciliogenesis in planarians, zebrafish, and in human cells. A requirement was found for both motile and primary cilia formation and Iguana/dZIP1 proteins localized to the basal bodies of cilia in human cells. These observations explain two seemingly disparate pieces of initial information about Iguana proteins: requirements for planarian locomotion and for zebrafish Hedgehog signaling. The highly abundant and prominent usage of motile cilia in planarians for locomotion (Hyman, 1951), coupled with the efficient use of RNAi (Sánchez Alvarado and Newmark, 1999), can allow for ease of discovery of novel ciliogenesis genes by study of planarians. In this case, the identification of a ciliogenesis role for the planarian *iguana* gene generated several predictions. First, a ciliogenesis role could explain the similarity of the *iguana* loss-of-function phenotype to that caused by loss of function of other Hedgehog signaling genes in zebrafish because cilia can be required for vertebrate Hedgehog signaling. We found that *iguana* mutant zebrafish had fewer cilia in pronephric ducts and in Kupffer's vesicle. Furthermore, these embryos displayed left-right asymmetry defects that can be explained by a lack of cilia in Kupffer's vesicle. Second, *iguana*-like genes should be required for primary cilia formation, because primary cilia can be the site of events of Hedgehog signaling. We found that primary cilia induction in human cells was disrupted by RNAi of the human *iguana*-like *dZIP1* and *dZIP1L* genes. Finally, Iguana proteins may localize to cilia if they are directly involved in the process of ciliogenesis. We found that both human Iguana-like proteins localized to the basal bodies of cilia and in cells lacking cilia, to the centrioles.

Cilia are not apparently required for Hedgehog signaling in *Drosophila*. However, it is possible that *Drosophila* have lost the use of cilia in many cell types in the course of evolution, leaving only sensory neurons and sperm as ciliated cells (Jiang and Hui, 2008). We observed a clear defect in tail formation following inhibition of the planarian *hedgehog* gene, but did not observe any defect in tail regeneration in *iguana(RNAi)* animals. Because we failed to see a Hedgehog-defective phenotype in *iguana(RNAi)* planarians, our data further support the idea that the use of cilia in Hedgehog signaling is an evolutionary innovation of the deuterostomes.

The linkage between ciliogenesis and Hh signaling in the course of evolution is poorly understood. For example, the gene *fused (fu)* in *Drosophila* is required for Hh signaling together with an atypical kinesin Costal2 (Robbins et al., 1997; Sisson et al., 1997). However, a *Fused* gene is required for normal ciliogenesis but not apparently for Hh signaling in the mouse (Chen et al., 2005; Merchant et al., 2005; Wilson et al., 2009). In zebrafish, *fused* is required for both Hedgehog signaling and ciliogenesis (Wilson et al., 2009). There may exist multiple additional genes with unidentified roles in ciliogenesis that have impacts on Hh signaling.

Ciliogenesis involves the modification of one of the centrioles of the cell to produce a basal body (Sorokin, 1962). The basal body becomes modified to display features such as a basal foot and ciliary rootlets, localizes near the cell surface, and is associated with growth of both primary and motile cilia (Hoyer-Fender, 2009). Because the human Iguana-like DZIP1 proteins co-localized with a marker of centrioles and basal bodies, Iguana proteins are newly identified basal body components. We conclude that Iguana is a novel regulator of ciliogenesis broadly in the Metazoa and that this activity in cilia biology has evolved to be critical for normal Hedgehog signaling in vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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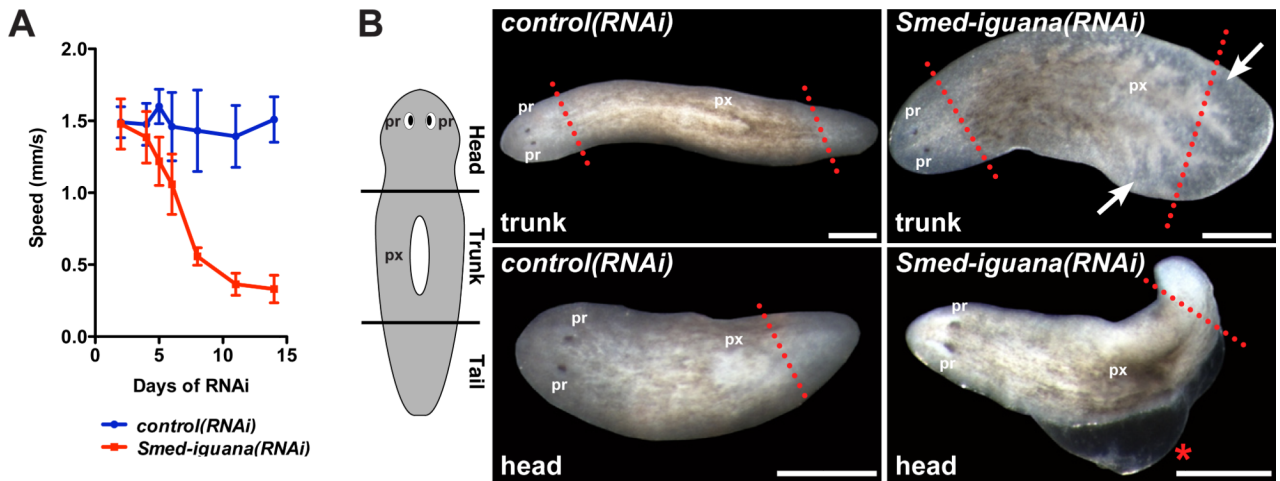


Figure 1. *Smed-iguana*(RNAi) planarians display defects in locomotion and osmoregulation (A) *Smed-iguana*(RNAi) animals lost the ability to locomote away from a source of light. Data are mean velocities from animals at different times following initial RNAi \pm standard deviations. *unc-22* dsRNA was used as the RNAi control. At least five animals per condition were analyzed at each timepoint. (B) *Smed-iguana*(RNAi) animals were capable of regenerating normally, but displayed bloating and blistering defects. Animals were fed dsRNA-containing food on days 0 and 4, cut on day 5, and cut again on day 14. Animals were photographed 28 or 29 days following initiation of RNAi. Anterior, left. Bar, 0.5 mm. Top, trunk fragments (see cartoon, oriented with anterior up, for amputation sites). Bottom, head fragments. 3/14 head fragments were blistered (not shown, 8/20 tail fragments blistered). 3/21 trunks displayed bloating (not shown, 19/20 tail fragments bloated). Red dotted lines indicate approximate blastema boundary. pr, photoreceptors. px, pharynx. white arrows, bloating. red asterisk, blistering.

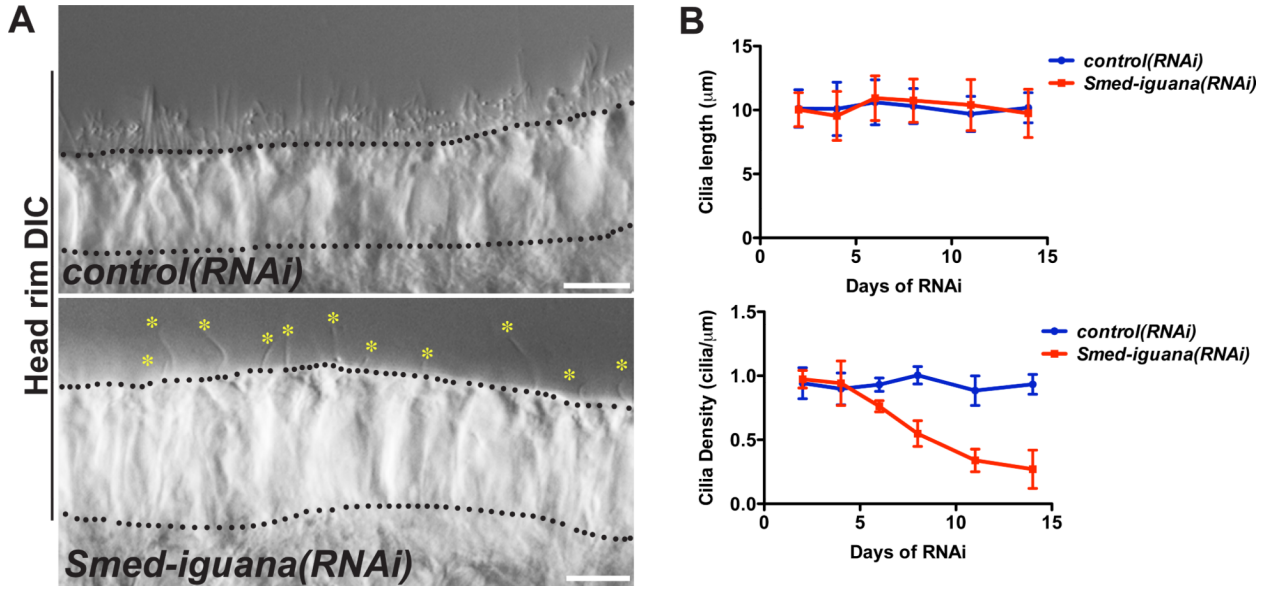


Figure 2. *Smed-iguana(RNAi)* planarians lack head rim cilia
 (A) Differential interference contrast (DIC) images of the head rims of control and *Smed-iguana(RNAi)* animals. Black dotted lines demarcate the single-cell thick epidermis at the edge of the animal. Cilia protrude from this epidermis. Yellow asterisks label the few remaining cilia of a *Smed-iguana(RNAi)* animal. Animals were examined 14 days after initiation of RNAi and are representatives from the data shown in panel B. (B) The length and number of cilia were determined from *unc-22* and *Smed-iguana* RNAi animals. Cilia lengths were determined for at least seven animals and cilia density for at least five animals per timepoint (the day six *iguana(RNAi)* timepoint involved four animals). Data are means from animals at different times following initial RNAi \pm standard deviations.

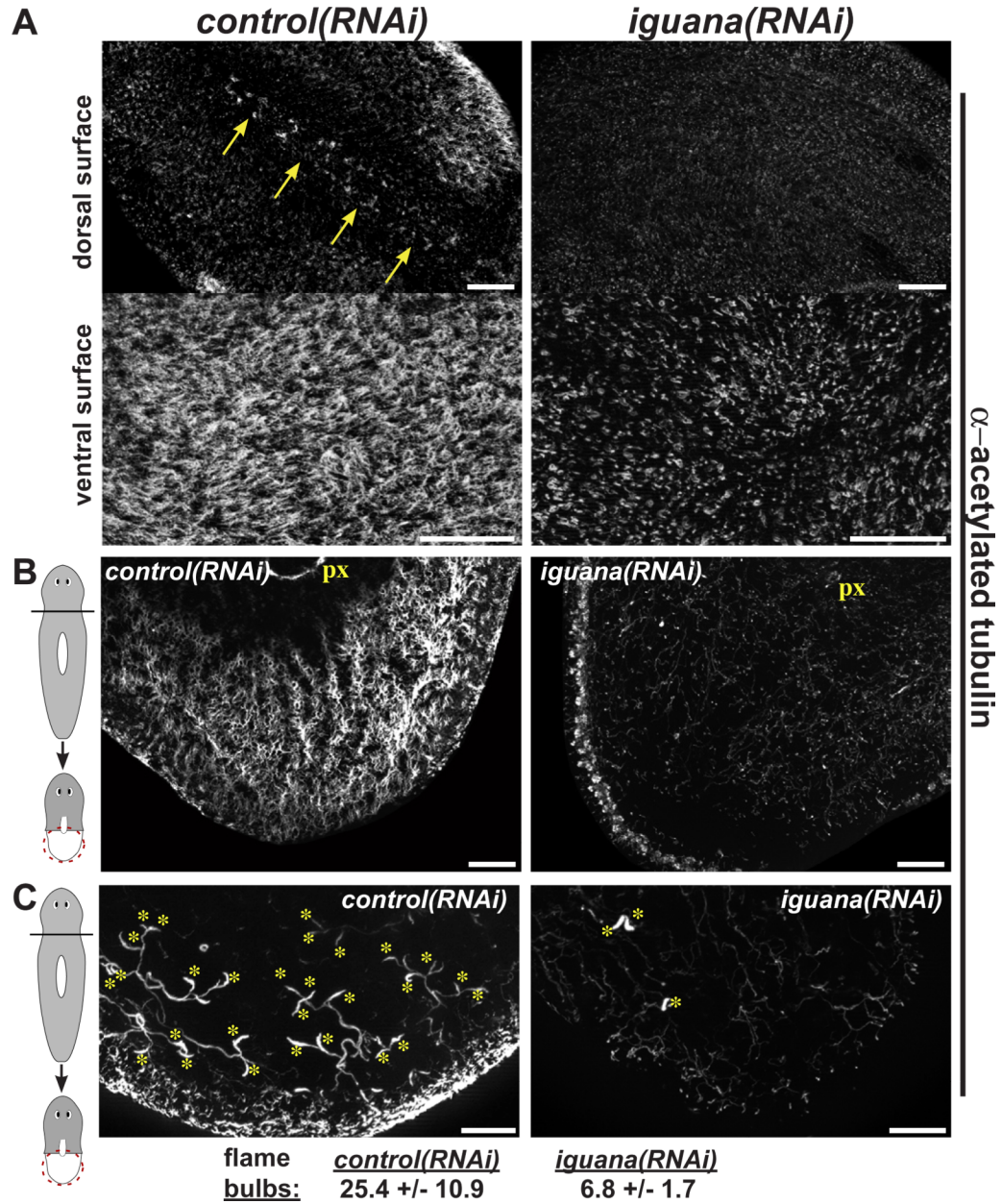


Figure 3. *Smed-iguana(RNAi)* planarians cannot maintain or regenerate cilia

(A) The cilia of intact, fixed planarians were labeled with an anti-acetylated tubulin antibody. *unc-22* dsRNA was used as the RNAi control. Top, views of the dorsal animal surface. A stripe of dorsal cilia is visible in the *control(RNAi)* animal (yellow arrows), but was absent from a *Smed-iguana(RNAi)* animal (*iguana(RNAi)*). Bottom, the ventral surface of a *control(RNAi)* animal is covered with cilia, but an *iguana(RNAi)* animal failed to maintain cilia. The anti-acetylated tubulin antibody also labels numerous sub-epidermal tubules that remained visible as small fluorescent dots in the dorsal and ventral images of *iguana(RNAi)* animals. 15/15 animals showed a similar phenotype to the representatives shown. Animals were fixed 14 days after initial RNAi feeding. Bar, 100 microns. (B, C) Animals were fed dsRNA on days 0 and 4, decapitated on day 5, and resultant head fragments that were regenerating tails (cartoon) were fixed 7 days later. The tail blastemas of head fragments (circled in red in the cartoon) are depicted. Anterior, up. Bars, 50

microns. (B) Ventral surfaces of tail blastemas on head fragments, labeled with an anti-acetylated tubulin antibody are shown. Control animals displayed coverage of the ventral blastema epidermis with motile cilia whereas an *iguana(RNAi)* animal did not. 15 control and 15 *iguana(RNAi)* animals displayed results similar to the representative shown. px, pharynx opening. (C) Ciliated flame bulbs of the planarian excretory system in the tail blastemas of control and *iguana(RNAi)* head fragments were labeled with an anti-acetylated tubulin antibody and visualized with optical sectioning. Z-stacks of optical sections taken internal to the labeled epidermis are shown; not all flame bulbs can be visualized in these images. Flame bulbs are indicated with a yellow asterisk. The numbers of flame bulbs in the tail blastemas of 13 animal head fragments were determined. The region posterior to the regenerated pharynx was used for counting. Below, mean numbers of flame bulbs per blastema \pm standard deviations are shown. Data were determined to be significantly different, $P < 0.0001$, unpaired t-test.

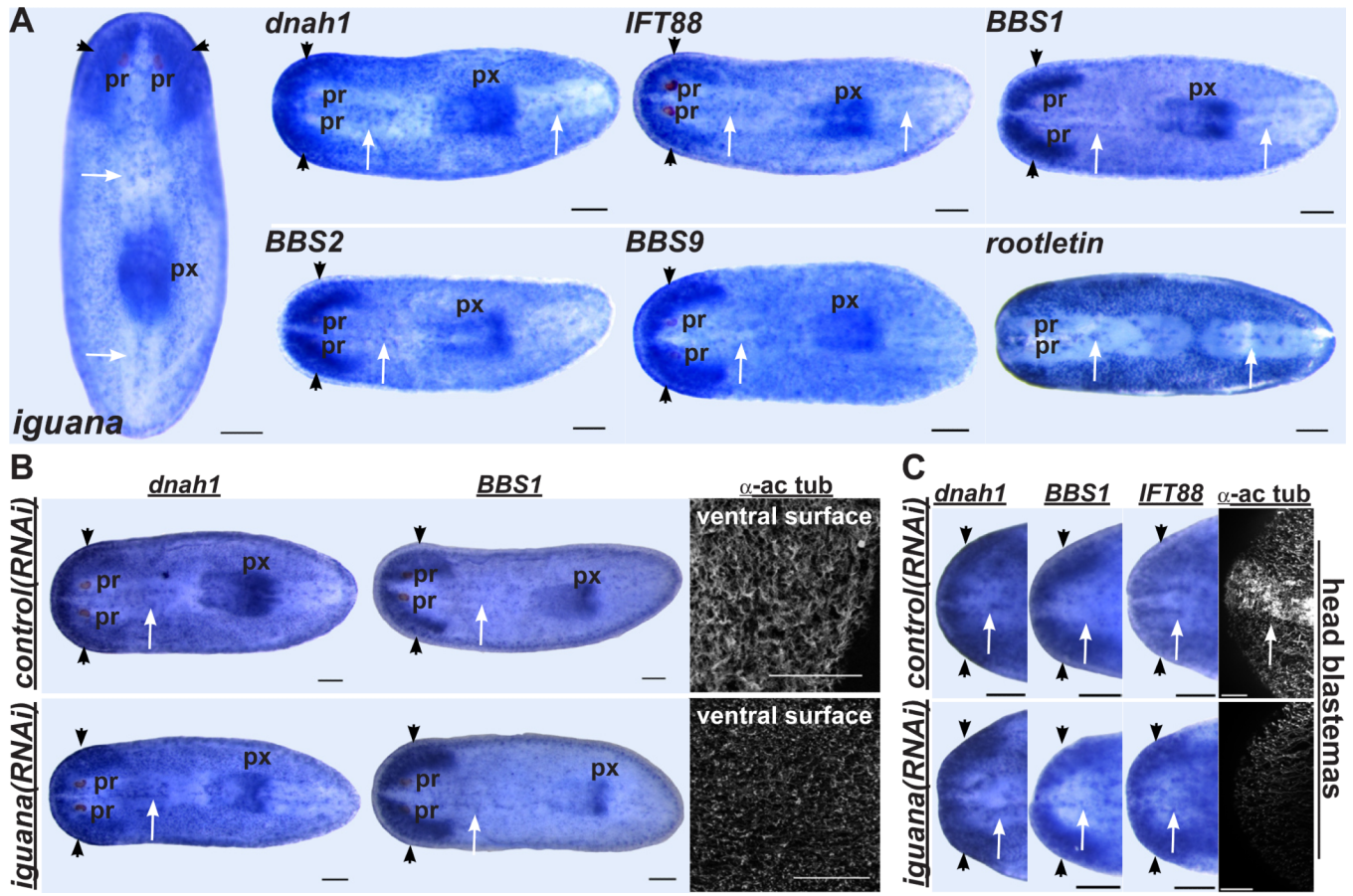


Figure 4. *Smed-iguana* is expressed in ciliated cell types

(A) Wild-type animals were labeled with *Smed-iguana*, *dnah1*, *ift88*, *BBS1*, *BBS2*, *BBS9*, and *rootletin* riboprobes in a whole-mount *in situ* hybridization and similar expression patterns were observed. The animal labeled with the *iguana* riboprobe is oriented with anterior up and all other animals are oriented with anterior left. (A-C) White arrows, dorsal stripe cilia. Black arrows, peripheral head signal. pr, photoreceptors. px, pharynx. Bar, 100 microns. (B) Intact *iguana* and control RNAi animals were labeled with riboprobes from cilia genes. There was no detected difference in expression of cilia genes, despite the absence of detectable cilia on *iguana*(RNAi) animals as determined with anti-acetylated tubulin antibody (α -ac tub) labeling, right. Animals were fixed 14 days after initiation of RNAi. (C) The head regeneration blastemas of trunk fragments are shown (see Figure 1b for depiction of trunk fragment). Animals were fixed 16 days after initiation of RNAi, and following 7 days of regeneration. *iguana*(RNAi) animals were able to regenerate cells expressing cilia markers, despite absence of ability to regenerate ciliated cells (right, dorsal cilia are labeled with an anti-acetylated tubulin antibody. Bar, 50 microns. Arrow, dorsal cilia stripe).

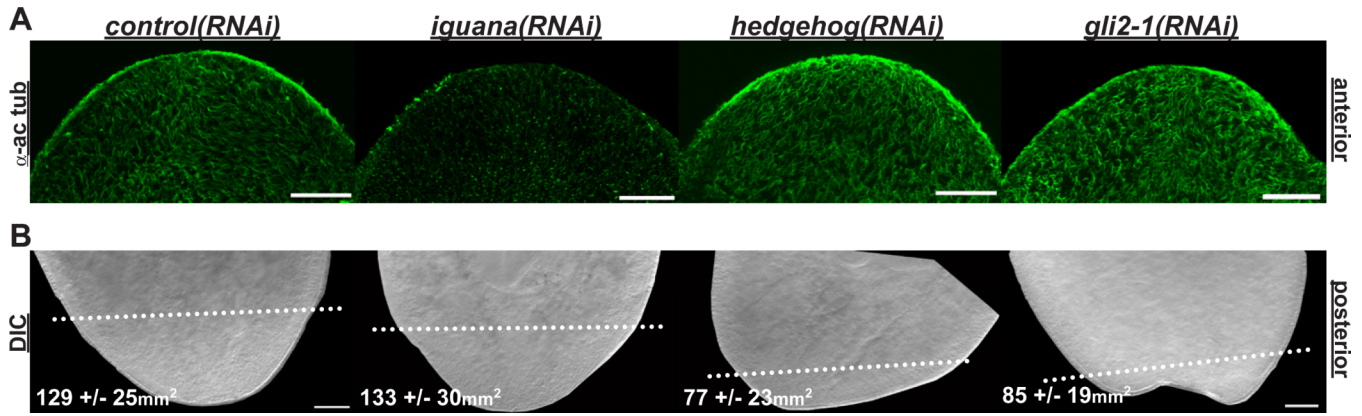
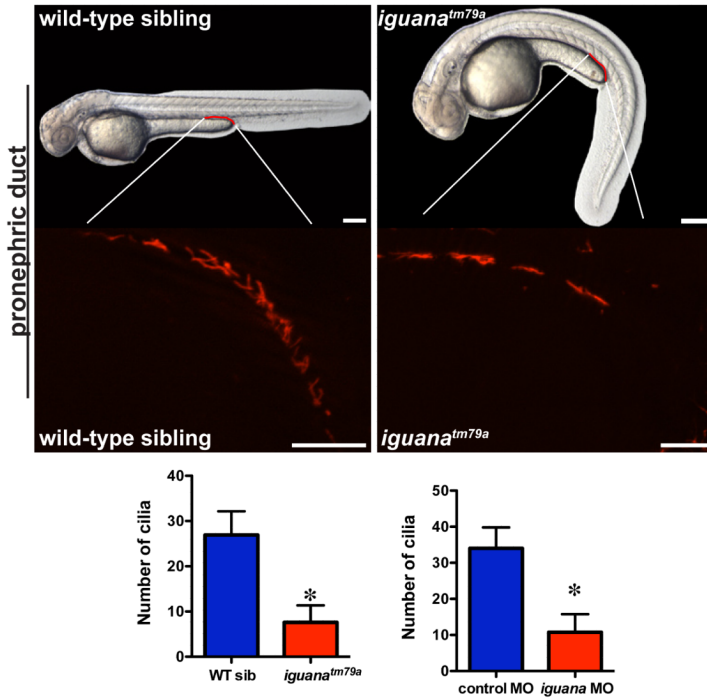
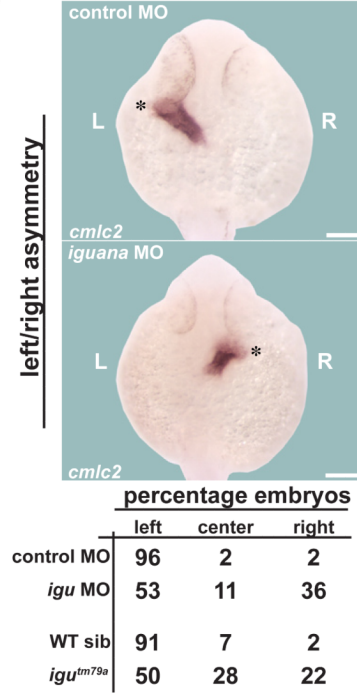


Figure 5. *hedgehog(RNAi)* and *iguana(RNAi)* planarians have different phenotypes
 RNAi animals were amputated to remove heads and tails, and were analyzed following eight days of regeneration. (A) Ventral view of the head blastemas of animals labeled with an anti-acetylated tubulin antibody (α -ac tub). *iguana(RNAi)* animals had greatly reduced or complete absence of cilia (n=27). By contrast, *hedgehog(RNAi)* and *gli2-1(RNAi)* animals displayed normal ciliogenesis (n=29). Anterior, up. Bar, 100 microns. (B) Differential interference contrast (DIC) images of tail blastemas in fixed animals. The approximate boundary between the blastema and the preexisting tissue was determined by fluorescence differences between these regions. Values listed are average areas of blastemas determined from multiple images as shown. *hedgehog* and *gli2-1* RNAi caused animals to regenerate grossly aberrant tails that were small (n=17; $P < 0.0001$, unpaired t-test). By contrast, *iguana(RNAi)* animals regenerated tails of normal size (n=18). Anterior, up. Bar, 100 microns.

A



B



C

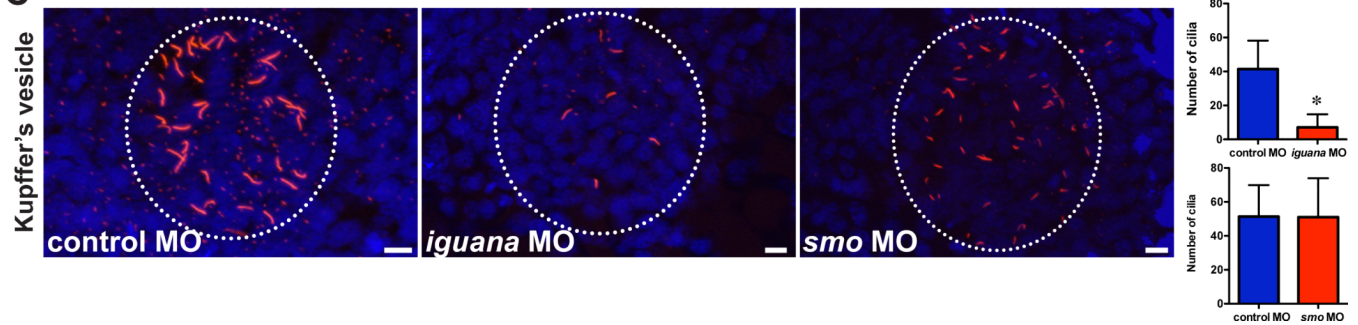


Figure 6. *iguana* mutant zebrafish have defects in ciliogenesis and left-right asymmetry
 (A) Top, brightfield images of an *igu* mutant embryo and a wild-type sibling embryo (labeled at right as ‘WT sib’, see methods), 48 hours post fertilization (hpf). The red line indicates the region of the pronephric duct that was analyzed for cilia. Anterior, left. Dorsal, up. Bar, 200 microns. Bottom, animals were labeled at 48 hpf with an anti-acetylated tubulin antibody. Bar, 20 microns. Right, the numbers of cilia within the posterior-most 100 microns of the pronephric duct were determined. The numbers of cilia in *igu* morphants and control morpholino-injected embryos were also determined. Data are means ± standard deviations. Asterisk, data were determined to be significantly different, $P < 0.0001$, unpaired t-test. (B) Animals were fixed at 28 hpf and labeled with a riboprobe from the *cardiac myosin light chain 2* gene (*cmlc2*). Anterior, up; dorsal view. Bar, 100 microns. Asterisks indicate the labeled heart. A control morpholino-injected embryo displayed heart development on the left side of the embryo and an *iguana* morpholino-injected embryo displayed heart development on the right side. Right, percentages of embryos displaying left, medial, or right heart development are shown. 44 control morphants (control MO), 55 *igu* morphants (*igu* MO), 49 wild-type siblings of *igu^{tm79a}/tm79a* embryos (WT sib, see methods), and 9 *igu* mutant embryos were analyzed. (C) Cilia in Kupffer's vesicle from control morpholino, *iguana* morpholino, and *smoothened* (*smo*) morpholino-injected embryos were labeled with

an anti-acetylated tubulin antibody (red). Nuclei are labeled with DAPI (blue). White dotted circle, approximate boundary Kupffer's vesicle. Embryos were fixed at the six somite stage. Bar, 10 microns. Right, mean numbers of cilia in Kupffer's vesicle \pm standard deviations. Asterisk, data were determined to be significantly different, $P < 0.0001$, unpaired t-test.

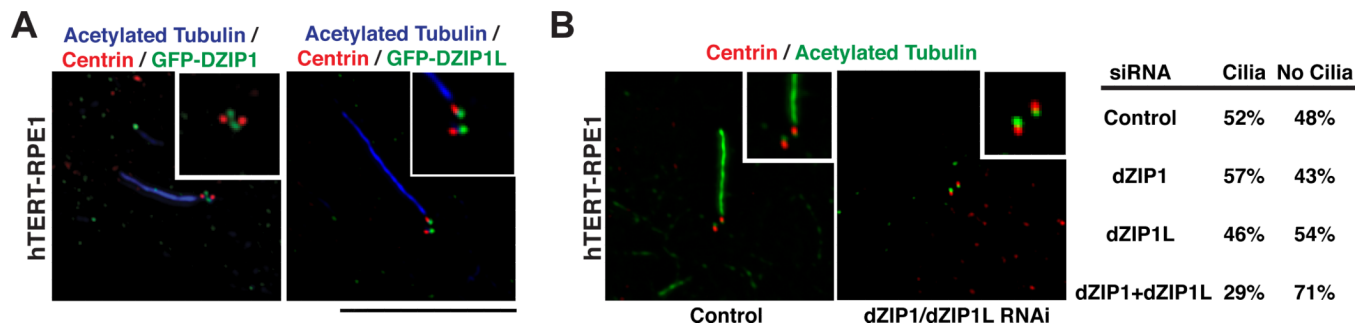


Figure 7. The human Iguana-like DZIP1 and DZIP1L proteins localize to basal bodies and are required for primary ciliogenesis
 (A) GFP-tagged human Iguana-like proteins dZIP1 and dZIP1L localized to the basal bodies of cilia. An anti-centrin antibody labeled the basal bodies and an anti-acetylated tubulin antibody labeled primary cilia. hTERT-RPE1 cells were used to visualize primary cilia. (E) RNAi of *dZIP1* and *dZIP1L* resulted in a defect in formation of primary cilia. 295 control, 282 double RNAi, 84 *dZIP1*, and 99 *dZIP1L* RNAi cells were analyzed. Only cells for which there was a clear presence or absence of a cilia were counted. Differences between the double RNAi and control cells were significant ($P < 0.0001$)