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Primary cilia as signaling organelles in development and disease

by

William E. Dowdle

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Developmental Biology

in the

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by

William E Dowdle

ACKNOWLEDGEMENTS

The ambitious goal of the biomedical sciences graduate education framework is to train young scientists to think critically about science and to design experiments that challenge the boundaries of our current understanding of the stuff of life. Implicit in this enterprise is the necessity for support. The NIH is the bedrock of financial support, and without it no graduate training program would exist. I am grateful to live in a society that values the search for knowledge and encourages the free dissemination of these truths.

Shortly before beginning graduate school, I attended a seminar given by James Watson the co-discoverer of the structure of DNA. During his talk he provided a word of advice to budding scientists that I will never forget. He said that his key to success was to “never to be the smartest person in the room”. As I have never felt this way about any room that I have ever been in... including ones in which I am the sole inhabitant, I thought this was advice worth following. Given UCSF’s reputation, I knew it would be the proper venue to keep this agenda alive, and it has not let me down. I cannot thank the UCSF community enough for providing the perfect challenging and supportive environment for my graduate training. I want to thank all of our instructors for relentlessly challenging us to question everything and for giving us the tools answer these questions. My classmates were the most unique, insightful, intelligent, and quirky group of people I could ever hope to meet. It was never hard to adhere to Watson’s advice in that crowd.

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Primary cilia as signaling organelles in development and disease
William E. Dowdle

ABSTRACT

In this dissertation, I describe the regulation of signal transduction by primary cilia, microtubule-based organelles present on nearly every vertebrate cell, during embryonic development and in the pathogenesis of disease. Using a combination of molecular biology, biochemistry, cell biology, and mouse genetics, I have investigated the role of primary cilia in canonical Wnt signaling and the role of B9-domain containing proteins in the formation and function of cilia during mouse development and in the etiology of Meckel-Gruber syndrome.

I show that the primary cilium restricts the activity of the canonical Wnt pathway in mouse embryos, primary fibroblasts, and embryonic stem cells. Non-ciliated cells activate transcription in response to Wnt stimulation, but do so more robustly than ciliated cells. Loss of *Kif3a*, but not *Ift88* and *Odf1* genes, causes constitutive phosphorylation of Dishevelled (Dvl) in a casein kinase I (CKI) activity dependent manner. These results suggest that Kif3a restrains canonical Wnt signaling both by restricting the CKI-dependent phosphorylation of Dvl and through a separate cilia dependent mechanism. More generally, these findings reveal that, in contrast to its role in promoting Hedgehog (Hh) signaling, the cilium restrains canonical Wnt signaling.

In addition, I describe the role of B9-proteins, found in nearly every ciliated organism, in the formation and function of cilia. Mice lacking B9d1 displayed phenotypes identical to those caused by Meckel syndrome (MKS), a severe inherited human disorder, and neural tube patterning defects concomitant with compromised ciliogenesis, ciliary protein localization and Hedgehog (Hh) signal transduction. With

coimmunoprecipitation and mass spectrometric analysis, we found that all three B9- proteins encoded in the genome interact physically with one another and with additional proteins implicated in MKS. In collaboration, we screened MKS patients for mutations in *B9D1* and *B9D2* and identified a homozygous *B9D2* mutation that segregates with MKS, affects an evolutionarily conserved residue, and is absent from controls. I also observed that B9d1 is not required for the formation of all cilia in tissues, but is required for the localization of ciliary membrane proteins to the cilia that remain in its absence. Cumulatively, these data suggest that primary cilia are important in the regulation of multiple signaling pathways including Wnt and Hh and in the development and homeostasis of multiple organ systems in vertebrates.

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Chapter 1: Introduction

In the early stages of vertebrate development, the single cell that will produce the entire animal undergoes a series of rapid divisions to create a field of genetically identical and impressionable progeny. These cell divisions drive an increase in complexity that leads to the division of the embryo along several planes of symmetry. Sheets of tissue differentiate into the three primary germ layers, the dorsal and ventral surfaces separate, the body axis elongates, and the head becomes distinct from the tail. At embryonic day 7.5, half way between the anterior and posterior of the organism, monociliated cells rotate their cilia in a fluid filled indentation called the node and propel fluid towards the left of the embryo, which participates in establishing the left/right asymmetry of the body plan. These same ciliated cells move forward out of the node to form the notochord, a long cord-like structure that defines higher organisms known as chordates. Cells of the notochord secrete a molecule (sonic hedgehog, SHH) that patterns the overlying neural tube, a structure that becomes the spinal cord and brain of the animal. Secretion of SHH from the notochord creates a gradient of signal from high to low concentration from the ventral surface of the neural tube (near the notochord) to the dorsal surface of the neural tube (bordering the external environment). The un-patterned cells of the neural tube interpret this gradient of SHH and differentiate into neural subtypes dependent on the concentration of SHH they are exposed to. The transduction of SHH signal from the surface of neural tube cells to the nucleus is dependent on the presence of non-motile primary cilia (microtubule based membrane projections) on the surface of these cells.

These events are representative of the role of the nearly omnipresent primary cilia in the development and homeostasis of vertebrate organisms.

Left-Right determination

The consistent establishment of the asymmetric vertebrate body plan is essential for the efficient functioning of critical organs like the heart. The complex series of events required to establish this asymmetry about the left-right axis is likely to be different in divergent species, yet recent empirical evidence from mouse studies and clues from inherited human disorders have suggested a conserved mechanism in vertebrates that is dependent on cilia [1]. One of the first suggestions that cilia might be involved in the establishment of asymmetry in the vertebrate body plan came from the observations of the Swedish scientist Björn Afzelius in 1976 [2]. Afzelius brilliantly postulated that the motility of cilia might link the symptoms of chronic bronchitis, male infertility and situs inversus observed in patients diagnosed with Kartagener syndrome. He observed the absence of dynein arms in the cilia of patients with this disorder and thus hypothesized that the gene mutated in Kartagener syndrome might encode the dynein protein itself, or another protein that links dynein to the microtubules of the cilium. He also suggested that the movement of cilia on the embryonic epithelium might be responsible for left-right determination and put forth the idea that chance alone might establish the situs of the viscera in the absence of dynein [2].

Two decades later, the discovery of a spontaneous mouse mutant with Kartagener-like features and the advent of modern mouse genetic manipulation lead to the confirmation that both dynein and kinesin motors are involved in visceral situs

determination [3-7]. Positional cloning identified *left-right dynein (lrd)* as the gene mutated in the *iv* mutant, which displays situs inversus. Despite strong expression of *lrd* in the ciliated node of early mouse embryos, it was concluded that mechanisms other than ciliary movement must regulate LR patterning in the early embryo because of previous information that the cilia of the node were immotile and lacked dynein arms [4,8]. Shortly after, evidence emerged that supported all of Afzelius' hypotheses and revealed that leftward fluid flow is generated in the embryonic node by motile dynein-containing monocilia [5-7]. Importantly, direct experimental evidence also demonstrated a role for fluid flow in breaking the bilateral symmetry of the embryo. Artificial fluid flow, generated by a mechanical apparatus, was able to reverse situs in WT embryos, and was able to rescue LR determination in *iv* mutants [9]. Furthermore, these studies established that nodal flow precedes the asymmetrical expression patterns of genes like *Nodal*, *Lefty1/2*, and *Pitx2* required for LR determination [4,6,7,9-17].

Despite these conclusive experiments, many unanswered questions regarding the role of nodal flow in establishment vs. maintenance of LR asymmetry, the nature of the signal (*i.e.*, soluble factor vs. mechanical) dependent on nodal flow, and the mechanism by which information is transmitted from the node to the lateral plate mesoderm several cell distances away, and to distant sites of organogenesis remain open [18]. In chapter 3, I describe the randomization of LR patterning in a mouse model of the human ciliopathy Meckel syndrome and describe the role of a novel protein in forming the cilia of the node [19].

Primary cilia as signaling organelles

Evidence from nearly a century of historical texts implicates the cilium as a signaling organelle. In “To the knowledge of the histogenesis and growth of the retina”, Carl Magnus Fürst, using nothing more than a light microscope, observed the following: “The emergence of rods and cones begins from protoplasmic extensions, which are first initiated from the large cone cells and then from the cells between them and then migrate over the external limiting membrane. These extensions display diplosomes on their surface or tips. The outgrowths then close to form a cuticle-like structure, display some irregularly arranged characteristics and are connected to the appendages of the pigment cells. In general, a fine thread extends from the outer centriole of the diplosome.” (translated from original German)[20]. This description of threads extending from the mother centriole in a photoreceptor might very well be the first description of a primary cilium in a mammalian cell.

When the electron microscope came into wide-use, there was a revolution in our understanding of the substructure of the cell. Upon observation of mouse and rabbit retinas, Eduardo De Robertis made the first definitive suggestion that the connecting region between the inner and outer segment of the rod photoreceptor appeared similar in structure to a cilium, and thus named this region the “connecting cilium” [21,22]. Furthermore, De Robertis observed the retina at different stages of development, and observed that in an 8-day old white mouse, a “primitive cilium” exists before the fully formed membranous outer stacks are present, thus suggesting that ciliogenesis is an essential component of retina development [23]. In a similar study, this view of cilia driven photoreceptor development was supported, and an accurate model of a cilium was

illustrated [24]. It thus appears that what Fürst described as fine threads protruding from the centrioles of rods and cones were what we now call “ciliary fibers” (microtubules), which are continuous with the connecting cilia of photoreceptors [20].

In parallel with developments in microscopy, advances in biochemistry allowed researchers to break open cells and to separate components from one another, which allowed detailed characterization of crude cell extracts. One such extract from the retina, known as visual purple (the G-protein coupled receptor [GPCR] rhodopsin), was observed to change from a deep purple to light yellow in the presence of bright sunlight, and to recover its violet hue in darkness. In 1931, Katharine Tansley made detailed measurements of the bleaching and recovery of this visual pigment from the retinas of rats. By describing the biophysical properties of this extract, we gained insight into signal transduction and intimate details regarding the physiology of the eye.

A decade later, new techniques allowed for the direct visualization of rhodopsin molecules in the photoreceptors, and it was observed that densely packed rhodopsin was concentrated in the inner membrane of the inner-segment, within the connecting cilium, and in the membranous stacks of the outer segment [25]. These observations are consistent with the hypothesis that rhodopsin produced in the inner segment rapidly traffics through the connecting cilium and into the membranous stacks of the outer segments. If one were to consider the entire outer segment of the photoreceptor as merely a dramatic extension of the cilium itself, then it is not hard to view the cilium as a signaling organelle. In support of the idea that cilia are signaling organelles, GPCRs were identified in pure cilia preparations from olfactory sensory neurons, and proposed to be the molecules necessary for olfaction [26-29]. We use cilia to see and smell.

The identification of GPCRs as important signaling proteins and the advent of modern molecular biology led to an explosion in the broad characterization of GPCRs of unknown function, and some surprising findings. In 1999 and 2000 two different GPCRs were found to be highly enriched specifically in the primary cilia of adult neurons, thus expanding the role of cilia outside of sensory organs, and implicating these nearly ubiquitous organelles as signaling hubs [30,31]. The identification of yet another GPCR in the primary cilia of the kidney epithelium made it clear that this phenomenon was not limited to neural tissue [32].

Both forward and reverse genetic studies pushed these finding beyond correlation and established a likely requirement for cilia in the function of signaling molecules and in the pathogenesis of human disease [32-34].

Primary cilia dependent Hedgehog (Hh) signaling and development

Forward genetics, the random mutagenesis of the genome and analysis of interesting resultant phenotypes, in model organisms has been a favorite approach of developmental biologists as it can reveal new physiology and tie it to molecular function often in new and unexpected ways. One such unexpected discovery linked the function of the Hh signaling pathway, which was well studied in *Drosophila*, to primary cilia. A forward genetic screen in mice to discover genes involved in early development revealed that mutation of *Ift88* and *Ift172*, essential components of the intraflagellar transport (IFT) machinery required to build primary cilia, resulted in striking developmental defects reminiscent of Hh pathway mutants [35]. The essential components of the Hh signaling pathway were discovered in similar forward genetic screens in *Drosophila*, but

strikingly, primary cilia are largely absent from non-sensory nervous cells in the fly, and are not required for Hh pathway activity.

In *Drosophila*, when the Hh pathway is in the off state, the 12-pass transmembrane protein Patched (Ptc) represses the activity of the GPCR-like 7-transmembrane protein Smoothened (Smo). The transcriptional effector of the pathway, Cubitus interruptus (Ci) is processed by the proteasome into a repressor molecule (CiR) to keep transcription off. This processing is promoted by the interaction of a portion of Smo with the the kinesin-like protein Costal-2 (Cos2), which acts as a scaffold to induce the phosphorylation of Ci via protein kinase A (PKA) casein kinase I (CKI), glycogen synthase kinase-3 β (GSK3 β) and Fused (Fu). CiR retains DNA binding activity, but lacks the co-activator domain, thus inhibiting transcription of target genes. A portion of Ci is associated with Suppressor of Fused (Sufu), which inhibits its activity by sequestering it in the cytoplasm. Upon binding of the Hh ligand Sonic Hedgehog (Shh) to Ptc, the suppression of Smo is relieved, and the processing of Ci into a repressor form is blocked, leaving full length Ci to translocate into the nucleus and activate the transcription of target genes with its co-activator CREB-binding protein (CBP). For a thorough review, see [36].

The organization of the Hh signaling pathway in vertebrates is similar to that of *Drosophila*, but with some key differences. There are multiple Hh ligands that are expressed in specific tissues and function at distinct times during vertebrate development. There are also multiple Ci orthologues, called Gli1, Gli2 and Gli3 in vertebrates. The Gli proteins are distinct from Ci in that they are not all processed into repressor forms. Gli1 is an activator only, Gli3 is processed into a repressor and likely has no role as an activator,

and Gli2 is similar to Ci in that it likely acts both as an activator and repressor. Cos2 has a distant vertebrate orthologue (Kif7), but it does not function exactly as Cos2 [37]. Instead, the primary cilium is required for the proper processing of Gli proteins and likely is a functional orthologue of Cos2. Genetically and biochemically, it has been shown that mutation of genes required for the formation of primary cilia disrupt both the activator and repressor function of Gli proteins and their processing [38-40].

Consistent with the hypothesis that cilia are required for the function of the Hh pathway, most components of the pathway including Ptc, Smo, Gli, and Sufu are enriched in primary cilia, [39,41,42]. In fact, the Hh transduction canon can be revised as follows: in the off-state Ptc is in the membrane of primary cilia and represses the function of Smo, which is present on cytoplasmic vesicles. Gli proteins are processed via the proteasome into GliR in a cilium-dependent manner to keep the transcription of Hh regulated genes off. Upon binding of Shh to Ptc, Ptc vacates the cilium and, within minutes, Smo becomes highly enriched there. The Hh ligand also promotes the enrichment of Gli proteins within the cilium and blocks their processing into repressor forms [43].

There are multiple differentially expressed Gli proteins in vertebrates, and tissues have distinct requirements for either repression or activation of Hh targets via one or more of the Gli proteins. Given this complexity, genetic disruption of primary cilia, and thus processing of the Gli proteins, can result in many Hh dependent phenotypes that are seemingly in opposition to one another. For example, mutation of a gene required for ciliogenesis results in disruption of the patterning of the neural tube in a way that mimics genetic ablation of Shh or Gli2 (a transcriptional activator), thus resembling a loss of Hh

signaling. In the limb bud however, disruption of cilia leads to extra digits (polydactyly), which mimics disruption of Ptc or Gli3R (pathway repressors), and resembles a de-repression, or partial activation of the Hh pathway [35]. In a similar way, cancers driven by mutations of Hh pathway components might be either exacerbated or inhibited by the perturbation of cilia [44,45].

It is also interesting to note that in multiple organisms where orthologues of the *Drosophila* Hh pathway proteins, like Fused, do not seem to be required for Hh signaling *per se*, these proteins are often involved in ciliary function [46,47]. The evolutionary relationship between Hh signaling and cilia thus appears to be ancient.

Primary cilia dependent Wnt signaling and development

Similar to the discovery that mutation of intraflagellar transport genes disrupts Hh signaling, mutation of cilia genes has linked cilia function to both non-canonical and canonical Wnt signaling [48-56]. The first suggestion that a ciliary gene might modulate Wnt signaling came from the observation that *Inversin (INV)*, a gene linked to the ciliopathy nephronophthisis (NPHP), regulates canonical and non-canonical Wnt signal transduction [56].

The Wnt signaling pathway consists of secreted lipoproteins called Wnts that bind to members of the Frizzled family of transmembrane receptors and transduce signals to the LEF/TCF family of transcription factors. Genes controlled by LEF/TCF transcription factors regulate developmental programs of cell fate and morphogenesis. The main node of regulation in the canonical Wnt signaling pathway is the control of β -catenin protein levels. In the off-state, β -catenin degradation is promoted by its interaction with axin,

adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3b), and casein kinase 1 (CK1). Degradation of β -catenin is promoted by GSK3b and CK1 mediated phosphorylation, which target it for ubiquitination and then degradation by the proteasome. During signaling, Wnt ligands interact with Frizzled receptors to promote the assembly of a complex involving Dishevelled (Dvl), axin and GSK3b, thus preventing the phosphorylation of β -catenin. Stabilized β -catenin enters the nucleus and interacts with TCF/LEF transcription factors to promote activation. Non-canonical Wnt signaling is a β -catenin independent mode of Wnt signaling that stimulates the planar cell polarity pathway by activating the small GTPases Rho and Rac [57]. Planar cell polarity refers to polarity within the plane of a cell sheet and is thought to regulate the convergence and extension movements (cellular intercalation) that narrow and elongate vertebrate body plans [58].

Core PCP genes were first identified in arthropods and *Drosophila melanogaster* where genetic mutations result in planar cell polarity defects in arrangement of hair bristles and photoreceptors in the eye [59]. Mutation of the core PCP orthologues Vangl2 (Van Gogh), Celsr1 (Flamingo), Scrib1 (Scribbled), and Ptk7 in mice result in a dramatic convergent extension defect called craniorachischisis (neural tube closure defect) and disrupted stereocilia bundle organization in the cochlea hair cells [60-64].

Mutations in *Apc* and *β -catenin* that cause activation of the canonical Wnt pathway lead to formation of renal cysts [65,66]. Similarly, morpholino (MO) knockdown of *inv* in zebrafish results in renal cyst formation [56]. Inversin interacts biochemically with Dvl and when overexpressed results in decreased levels of β -catenin and Dvl [56]. Furthermore, MO knockdown of *inv* in *Xenopus laevis* embryos results in

convergence extension defects indicative of altered non-canonical Wnt signaling [56]. In addition to inversin, mutations in genes that cause Bardet-Biedl syndrome (BBS, a severe ciliopathy) can interact genetically with core PCP genes and cause PCP defects in the mouse including exencephaly (neural tube closure defect) and disrupted stereocilia bundle organization in cochlear sensory hair cells [55].

We sought to identify the molecular mechanism governing the connection between cilia and the regulation of Wnt signaling. In chapter 2, we investigate the requirement of cilia in canonical Wnt signaling and describe the localization of canonical Wnt signaling components to primary cilia. In collaboration with Kevin Corbit, Amy Shyer and other members of the Reiter Lab, we describe the hyperactivation of canonical Wnt signaling in cells devoid of the ciliogenesis factors Kif3a, Ift88, and Odf1. Specifically in *Kif3a* null cells, Dvl2 and 3 are constitutively phosphorylated, β -catenin is stabilized and the pathway is hyperactivated in response to ligand in a CK1 dependent way. We found that components of the canonical Wnt signaling pathway localize to centrioles or cilia, which supports the idea that cilia play a role in canonical Wnt signaling [53]. As we published this work in Nature Cell Biology in 2007, the laboratory of Nicholas Katsanis published a report supporting these findings [54].

Subsequent to these publications, several studies have contradicted our core argument that cilia regulate canonical Wnt signaling and several other studies have bolstered this argument by uncovering the nuances of the regulation of Wnt signaling by cilia [49-51,67-70]. It is clear from further developmental studies that the genetic elimination of cilia does not result in dramatic phenotypic anomalies in tissues where canonical Wnt signaling is required, yet the role for cilia in non-canonical signaling and

PCP is evident [71]. We hypothesized that loss of cilia might result in enhanced Wnt signaling in tissues where canonical Wnt signaling is important for development or homeostasis. To this end, we analyzed the role of cilia in the intestinal epithelium, as canonical Wnt signaling is often activated in colon cancers, and found that conditional deletion of *Kif3a* or *Ift88* with Villin::Cre does not result in the development of intestinal neoplasias (data not shown). Upon close inspection, it is clear that the intestinal epithelium is one of the few cell types that does not possess primary cilia (data not shown).

In specific tissues and developmental contexts, however, cilia proteins have proven indispensable for the regulation of Wnt signaling. Mutation of the ciliopathy protein Ahi1 (Joubertin) alters Wnt signaling and causes a cerebellar midline closure defect during development [49]. A prevailing molecular model posits that Ahi1 translocates to the nucleus to promote Wnt signaling, and in its absence, tissue specific Wnt defects can occur [50]. Recently, other groups have discovered novel mutations in the potential cilia genes *Stk11* and *Tmem237* that also result in hyperactivation of Wnt signaling, and a constitutive phosphorylation of Dvl [51,67]. We found that Dvl is constitutively phosphorylated when *Kif3a* is mutated. Thus it is plausible that a complex composed of Stk11, Tmem237, and Kif3a regulates the phosphorylation status of Dvl [51,53,67].

Similar to Hh signaling, several orthologues of *Drosophila* PCP pathway components have been shown to be required for ciliogenesis, and thus the link between Wnt signaling and cilia also appears to be ancient [72-77].

Ciliopathies

Inherited human mutations that result in the broad disruption of cilia cause severe developmental and adult phenotypes. Cilia are found on nearly every cell of the human body and are diverse in form and function. There are cilia with 9+0 and 9+2 arrangements of microtubules and examples of either which are motile and non-motile. Cilia function to clear mucus, move fluid, to sense fluid flow and other mechanical forces, to intercept extracellular signaling molecules and to concentrate and activate intracellular signaling molecules. Cilia participate in developmental processes from breaking the left-right symmetry of the embryo to tooth formation, and in adult processes ranging from kidney tubule homeostasis to vision, olfaction, and hearing [78]. Mutations could affect a gene that disrupts an isolated cilia function and thus cause a mild disease like retinal degeneration, or they could affect a gene essential for all cilia functions and thus cause severe disease in all tissues where cilia are found and result in embryonic lethality. Here I discuss the functions of cilia as they relate to human disease (ciliopathies) in an attempt to illuminate how a group of seemingly unrelated, yet overlapping conditions are linked by cilia dysfunction. I will discuss ciliopathies in order of increasing severity and end with a discussion of ciliopathy protein and disease interaction networks, patterns that have emerged from these networks, and what we have learned from these patterns. The history, evolution and general functions of cilia have been reviewed exhaustively and are outside of the scope of this text [79-90].

Motile cilia play an important role during the development of, and in the adulthood of, humans. As discussed above, the motile cilia of the node play a role in patterning the left-right axis of vertebrates, and thus mutations that affect cilia in this

structure can result in situs inversus. Motile cilia with a 9+2 arrangement of microtubules are found in the ependymal cells of the brain, epithelial cells of the trachea and bronchioles, in sperm tails and throughout the female reproductive tract. As discussed above, primary cilia dyskinesia (CILD), also known as Kartagener's syndrome, affects the motility of all cilia while leaving other functions intact. Without direct evidence, Afzelius predicted that the gene mutated in his Kartagener patients might encode the protein comprising the axonemal dynein arms that allow for the motility of cilia [2]. Incredibly, nearly every gene variant linked to Kartagener's syndrome since then has been found to encode an axonemal dynein (See "CILD" Table 1.1).

Of the 80 genes now linked to ciliary diseases, nearly three-quarters of them are linked to cilia functions other than motility, thus highlighting the complexity of these fascinating organelles (Table 1.1). As discussed above, connecting cilia of retinal photoreceptors are essential for vision. Rhodopsin, as well other proteins, are produced in the inner segment of the photoreceptor and moved into the outer segment via intraflagellar transport along the microtubules of the connecting cilium. In the absence of this transport, photoreceptors die via apoptosis and the retina degrades, resulting in progressive loss of vision. Retinitis Pigmentosa and Leber Congenital Amaurosis are two types of retinal degeneration that have been linked to mutations in gene products associated with the cilium. RPGR localizes to the connecting cilium of retina and mutations in the gene encoding it account for 20% of all retinitis pigmentosa cases [91]. The role of this protein is likely intimately tied to the function of the connecting cilium in the retina as mutations in *RPGR* have not been discovered in more severe ciliopathies. Interestingly, however, RPGR localizes to the transition zone of motile cilia in the

trachea, and mutations in *RPGR* have been identified in CILD (A mild ciliopathy, Table 1.1) [91].

Autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), and nephronophthisis (NPHP) are progressive cystic diseases of the kidney. Positional cloning of the gene mutated in the Oak Ridge Polycystic Kidney disease mouse (*Tg737^{orpk}*), a mouse model of ARPKD, uncovered *Ift88* as the genetic culprit and implicated primary cilia in ARPKD etiology. The products of other genes mutated in ADPKD, ARPKD, and NPHP localize to the primary cilia lining the tubules of the kidney, where many cysts originate. This supports the hypothesis that cilia are involved in these diseases. Two such gene products polycystin-1, and polycystin-2, encoded by *Pkd1* and *Pkd2* respectively, are hypothesized to form a mechanosensory complex that can sense the bending of primary cilia in response to the flow of fluid in kidney tubules. A persistent intracellular Ca^{2+} increase can be observed in response to flow-induced ciliary bending, and is dependent on polycystin-1 and polycystin-2 [92]. It is likely that the sensation of flow in the kidney tubule is required both for the proper formation of tubules during development, and for the maintenance of these tubules in adulthood. Mutation of *Pkd1* or *Pkd2* likely abolishes flow sensation and causes a compensatory proliferation or rearrangement of tubular cells [78]. Mutation of *Pkd1* or *Pkd2* causes renal cystic disease in isolation, so while they may be reused as flow sensors in other tissues, it is unlikely that they are broadly required for the function of cilia. Interestingly, a few genes, when mutated, can cause simultaneous cystic kidney disease and retinal degeneration, a condition known as Senior Løken syndrome. It is likely that these gene products are required for a highly specialized function of kidney

and retinal cilia (Table 1.1), or that these tissues are more sensitive to the partial loss of ciliary function. Mutation of genes that severely affect IFT and cilia formation are unlikely to cause cystic kidney disease or retinal degeneration in isolation unless they are hypomorphic mutations as in the case of the *Tg737^{orp}* allele of *Ift88* mentioned above. A more severe allele of *Ift88*, likely to be a true null, results in death in a mouse at embryonic day 9.5, before kidney development begins [93]. Early embryonic lethality likely explains why no mutations in IFT genes have been found in human ciliopathies (table 1.1).

Bardet-Biedl syndrome (BBS), Joubert syndrome (JS) and Meckel syndrome (MKS) are all multisystem, complex, recessively inherited human diseases, which display distinct but partially overlapping phenotypes. Here I focus on the relation of these diseases to one another genetically and phenotypically, attempt to highlight important similarities and differences between them, and discuss what this suggests about the function of cilia and their components. Common to these three disorders are ocular/retinal abnormalities, polydactyly, renal cystic disease, CNS malformations or impairments, and situs inversus (although the latter is not common in BBS) [94,95]. Unique among these disorders is cognitive impairments and obesity in BBS, cerebellar vermis hypoplasia in JS and encephalocele and perinatal lethality in MKS. Genetically, there are numerous commonalities between JS and MKS, yet very little overlap between BBS and any other ciliopathy (table 1.1).

If we accept genetic mutation of core cilia components (IFT particles) in the mouse as a baseline for the most severe cilia related disease possible, then one can posit that genes causing equally severe disease in humans are likely to represent components

essential to all cilia functions, while mutations in genes that cause less severe disease may be involved in functional sub-specialties of cilia. Of course the “strength” of the two inherited alleles in a recessive disorder can determine the severity of the disease phenotype. For example, two strong mutations in *CEP290* cause MKS, which is embryonically lethal, while the presence of one “weak” mutation in *CEP290* causes NPHP, which only affects the kidney [94].

The relative uniqueness of BBS phenotypes suggests a relationship between genes whose variants cause this disease and suggests they support a specialized cilia function. Indeed, it has been shown that most BBS proteins interact biochemically and assemble a protein coat required to traffic membrane proteins and GPCRs to cilia [96-98]. This functional characterization of the BBS complex is intriguing given the occurrence of obesity in BBS patients, and the involvement of GPCRs in human obesity [99]. It appears clear that the BBS protein complex is important for some, but not all cilia functions.

The differences between JS and MKS are less well defined than those between BBS and other ciliopathies. Many differences might be accounted for by the strength of inherited alleles, as some genes like *CEP290* can cause both JS and MKS (Table 1.1). MKS, the most severe ciliopathy is likely to be caused by mutation of genes encoding proteins that are essential for the function of all cilia, while mutated genes that cause JS and never MKS may encode another sub-special function of the cilium. JS is characterized by cerebellar-vermis hypoplasia, which is diagnosed radiographically by the presence of the molar tooth sign (MTS) on magnetic resonance imaging scan of the brain. The role of *AHI1*, a gene mutated in JS, in the development of the cerebellar vermis has been carefully analyzed and a mechanism by which AHI1 plays a role in

canonical Wnt signaling in the development of this structure has been proposed [49,50,52,100]. This unique phenotype may further support the link between primary cilia and canonical Wnt signaling as discussed above. Thus a specific requirement for primary cilia, AHI1, or other proteins linked to JS, in the development of the cerebellar vermis may explain the enigmatic MTS.

Similar to BBS proteins, extensive biochemical interactions between ciliopathy-associated proteins have been uncovered by several groups [19,101-103]. Interestingly, proteins linked to a given disease often interact biochemically (Figure 1.1) [102]. Furthermore, clusters of genes linked to a given disease often cooperate in a specialized ciliary function, as is the case with BBS proteins in GPCR transport. Proteins linked to NPHP occupy a distinct space within the network and may function together. The CEP290 protein is perhaps the most interconnected protein within this network, with mutations linking its function to proteins that cluster around MKS, JS, BBS, SLSN, and LCA.

Working from this hypothesis that cilia disorders may link to defects in cellular mechanisms, a group recently identified the proteins that interact with nine NPHP-JS-MKS associated proteins. Based on biochemical interactions and localization studies, the authors defined three complexes: NPHP 1-4-8 (apical surface function), NPHP 5-6 (centrosomal function) and MKS (Hh signaling) [101].

In chapter 3 of this dissertation, we provide data that links two B9 domain-containing proteins, B9D1 and B9D2, to the MKS protein module and describe their localization to the transition zone at the base of the cilium, which is required for the localization of numerous proteins to the cilium. We also discuss the predictive power of

these interaction networks and their usefulness in identifying novel mutations in ciliary diseases.

Overview

The work herein highlights the role of primary cilia as signaling centers in vertebrate development and disease. In chapter 2, I show that proteins important for the formation and function of primary cilia also have a role in restraining canonical Wnt signaling. Kif3a, Ift88 and Odf1, proteins required for ciliogenesis, restrain the transcriptional response of the canonical Wnt signaling pathway in the presence of the ligand Wnt3a. Additionally, Kif3a functions outside of the cilium to prevent phosphorylation of Dvl2 and Dvl3, Wnt pathway components, by Casein kinase 1.

In chapter 3, I describe the role of B9d1, an evolutionarily conserved protein found in nearly every ciliated organism, in the function of cilia. Mice lacking B9d1 displayed polydactyly, kidney cysts, ductal plate malformation, and abnormal patterning of the neural tube, concomitant with compromised ciliogenesis, ciliary protein localization and Hh signal transduction. In collaboration, we screened MKS patients for mutations in *B9D1* and *B9D2*, and identified a mutation in *B9D2* that segregated with MKS, affected an evolutionarily conserved residue, and was absent from controls. With coimmunoprecipitation and mass spectrometric analyses, I found that all three B9-proteins encoded in the genome, Mks1, B9d1 and B9d2 interact physically with each other, and with other proteins mutated in Meckel syndrome. I also found that genetic disruption of this protein complex perturbs ciliary protein localization in tissues where ciliogenesis is not compromised.

In chapter 4, I identify unanswered questions suggested by these works and propose follow up studies to address these.

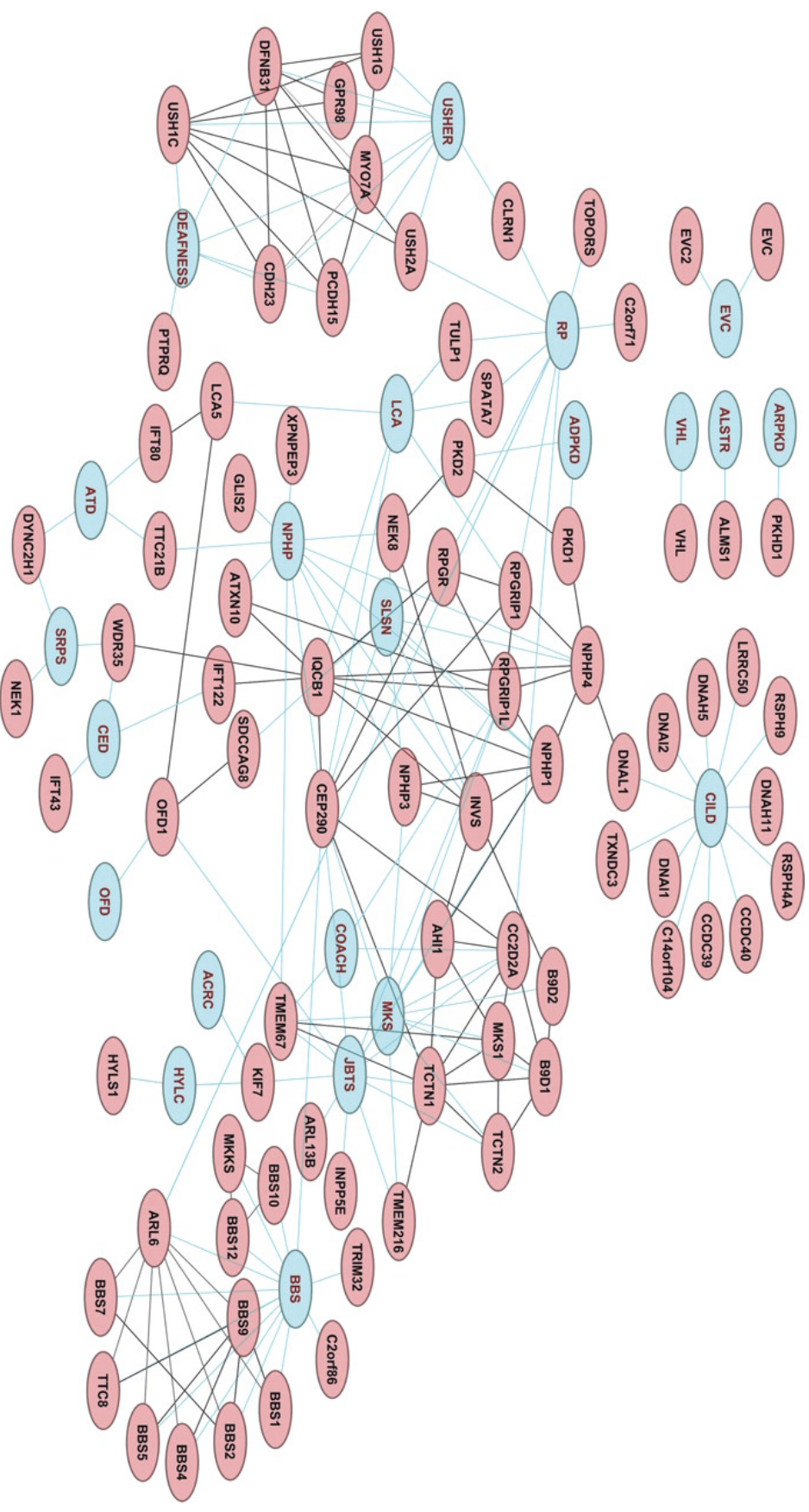


Figure 1.1 (Adapted from Reeuwijk et al. 2011) *Biochemical and disease ciliopathy interaction network* [102]
This network illustrates biochemical interactions (dark lines) between proteins (red spheres) known to be associated with human ciliopathies (blue spheres). Disease and biochemical modules likely define distinct ciliary or cellular functions.

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Chapter 2: Kif3a constrains β -catenin-dependent Wnt signaling through dual ciliary and nonciliary mechanisms

Kevin C. Corbit, Amy E. Shyer, William E. Dowdle, Julie Gaulden, Veena Singla and Jeremy F. Reiter. (2007). Kif3a constrains beta-catenin-dependent Wnt signaling through dual ciliary and non-ciliary mechanisms. *Nature Cell Biology*, Dec 16(10): 70-76. 2007.

ABSTRACT

Primary cilia are microtubule-based organelles involved in signal transduction that project from the surface of most vertebrate cells[1]. Proteins that can localize to the cilium such as Inversin and Bardet-Biedl syndrome (BBS) proteins are implicated in both β -catenin-dependent and independent Wnt signaling[2-4]. Given that Inversin and BBS proteins are found both at the cilium and elsewhere in the cell, it has not been clear what role the cilium itself plays in Wnt signaling. Using three separate mutations that disrupt ciliogenesis (affecting *Kif3a*, *Ift88* and *Odf1*)[5-7], we show here that the primary cilium restricts the activity of the canonical Wnt pathway in mouse embryos, primary fibroblasts, and embryonic stem cells. Interestingly, unciliated cells activate the transcriptional response only in response to Wnt stimulation, but do so much more robustly than ciliated cells. Loss of *Kif3a*, but not other ciliogenic genes, causes constitutive phosphorylation of Dishevelled (Dvl). Blocking the activity of Casein kinase I (CKI), reverses this constitutive Dvl phosphorylation and abrogates pathway hyper-responsiveness. These results suggest that Kif3a restrains canonical Wnt signaling both by restricting the CKI-

dependent phosphorylation of Dvl and through a separate ciliary mechanism. More generally, these findings reveal that, in contrast to its role in promoting Hedgehog (Hh) signaling, the cilium restrains canonical Wnt signaling.

INTRODUCTION

Wnts are secreted lipoproteins involved in diverse developmental and pathological events that bind to Frizzled receptors to activate several downstream signaling pathways[8]. Both the canonical and noncanonical pathways make use of members of the Dvl family, enigmatic proteins that act at the branch point between the two pathways[9]. Dvl regulates activation of the canonical pathway by binding to and inhibiting the protein complex that phosphorylates β -catenin to target it for proteasome-mediated destruction. This stabilization allows β -catenin to accumulate in the nucleus where it interacts with transcription factors of the TCF/Lef family to turn on the Wnt transcriptional program.

Primary cilia are sophisticated microtubular projections found on the surface of most mammalian cells[1]. Generation of primary cilia depends on intraflagellar transport (IFT) to build the ciliary axoneme on the basal body, which uses the heterotrimeric, microtubular anterograde motor Kinesin-2 (ref. [10]). Interference with IFT or basal body function disrupts ciliogenesis[5-7]. To examine the role primary cilia play in canonical Wnt signaling, we investigated the effects of disrupting ciliogenesis by using three different genetic models: disruption of the Kinesin-2 motor, inhibition of IFT, and deletion of a basal body protein required for ciliogenesis.

RESULTS/DISCUSSION

In the absence of Kif3a, an essential component of Kinesin-2, cells cannot generate cilia[6]. We generated mouse *Kif3a*^{-/-} embryos that express a β -galactosidase reporter under the control of canonical Wnt signals (BATgal)[11]. As compared to somite-matched *Kif3a*^{+/-} littermates, embryonic day (E) 9.0 *Kif3a*^{-/-} embryos display markedly higher reporter activity (Fig. 1a). Interestingly, the BATgal reporter is not activated ectopically but, rather, increased activity occurs in regions in which the reporter is normally expressed, including the midbrain, somites and tailbud. The cells in these regions are ciliated at this stage, whereas younger embryos only have cilia in the cells of the notochord and node[1,12]. At earlier stages (e.g., late headfold stage), BATgal activity in *Kif3a*^{-/-} embryos is normal (see Supplementary Information, Fig. S1a), consistent with the involvement of Kif3a in Wnt signal transduction specifically in ciliated cells.

To determine the mechanism by which Wnt signaling is modulated by primary cilia, we generated mouse embryonic fibroblasts (MEFs) from *Kif3a*^{lox/lox} BATgal embryos. Stimulation of the *Kif3a*^{lox/lox} BATgal MEFs with 100 ng/ml Wnt3a for 18 hours strongly activates the BATgal reporter, whereas stimulation with Wnt5a, a Wnt protein that does not activate the canonical pathway in many contexts, fails to activate the reporter (see Supplementary Information, Fig. S1b). Infection of these cells with Cre-expressing adenovirus deletes *Kif3a* and consequently prevents ciliogenesis (Fig. 1b,c). Infection of *Kif3a*^{lox/+} cells with Cre-expressing adenovirus does not affect BATgal reporter activity (data not included). However, upon Wnt3a stimulation, unciliated *Kif3a*^{-/-}

^{-/-} cells show a dramatically increased response relative to the normally ciliated *Kif3a*^{lox/lox} parent line (Fig. 1d). An alternative reporter of Wnt activity, SuperTOPFlash[13], shows the same increased responsiveness in immortalized *Kif3a*^{-/-} MEFs (Fig. 1e). Of note, reporter basal activity is unchanged in *Kif3a*^{-/-} cells. It was recently found that, in contrast, shRNA-mediated depletion of *Kif3a* in HEK293 cells increases β -catenin levels in the absence of exogenous Wnts[4]. This may reflect inherent differences between HEK293 cells and MEFs, or may simply reflect the endogenous production of Wnt3a by HEK293 cells but not by MEFs (ref. [14] and data not included). Regardless, both *Kif3a*^{-/-} embryos and MEFs display hyper-responsive activation of the canonical pathway specifically upon Wnt stimulation.

The increased Wnt responsiveness in embryos and cells lacking cilia could reflect either the direct involvement of cilia in β -catenin-dependent signaling or the indirect consequence of dysregulation of an interacting pathway. Hh signaling is dependent on IFT and the primary cilium[15]. In addition, the Hh pathway has been shown to both positively and negatively regulate Wnt signaling[16,17]. To test whether cilia-dependent modulation of the Wnt pathway is due to altered Hh pathway activity, we used *Smoothed* (*Smo*) mutant MEFs devoid of Hh pathway activity[18]. *Smo*^{-/-} MEF response to Wnt3a is indistinguishable to that of wild-type MEFs (see Supplementary Information, Fig. S1c), suggesting that ciliary restriction of Wnt signaling is not due to changes in Hh pathway activity.

In addition to its functions in ciliogenesis, Kinesin-2 is implicated in the trafficking of melanosomes and N-cadherin, and mitotic spindle formation[19-21]. To test whether the observed Wnt hyper-responsiveness is due to a ciliary or nonciliary

function of Kif3a, we assessed Wnt signal transduction in two other genetic models of cilia dysfunction; *Ift88^{Orpk/Orpk}* and *Odf1*⁻. *Orpk* is a hypomorphic mutation in the gene encoding Ift88, a participant in intraflagellar transport[5,10]. MEFs derived from *Ift88^{Orpk/Orpk}* homozygous embryos have short, dysmorphic cilia (Fig. 1f and ref. [22]). Similar to *Kif3a*^{-/-} cells, *Ift88^{Orpk/Orpk}* *BATgal* MEFs display increased reporter activity in response to Wnt3a stimulation (Fig. 1h). These data indicate that IFT is required for cells to properly modulate canonical Wnt pathway tone.

Odf1 is an X-linked gene encoding a basal body protein required for ciliogenesis[7]. We have developed mouse embryonic stem (ES) cells lacking *Odf1*. In contrast to wild-type ES cells, cilia are entirely absent in *Odf1*⁻ ES cells (Fig. 1g). Like *Kif3a*^{-/-} and *Ift88^{Orpk/Orpk}* MEFs, ES cells lacking cilia are hyper-responsive to Wnt3a (Fig. 1i). These results demonstrate that disruption of ciliogenesis through loss of a basal body protein also increases canonical Wnt responsiveness.

As a final test to correlate the presence of primary cilia and Wnt responsiveness, we assessed Wnt activity in *Kif3a*^{+/+} and *Kif3a*^{-/-} MEFs under conditions under which wild-type cells are not ciliated. MEFs cultured at subconfluency (5•10⁴ cells per cm²) do not possess primary cilia (see Supplementary Information, Fig. S2a). Consistent with the hypothesis that the ciliary functions of Kif3a are important for Wnt signaling, subconfluent *Kif3a*^{+/+} and *Kif3a*^{-/-} cells show equal responses to Wnt3a (see Supplementary Information, Fig. S2b). However, confluent (10⁵ cells per cm²) *Kif3a*^{+/+} MEFs are ciliated and display less Wnt responsiveness than do unciliated *Kif3a*^{-/-} MEFs (see Supplementary Information, Fig. S2). The finding that disrupting ciliogenesis through culture conditions or mutation of the anterograde motor (*Kif3a*), intraflagellar

transport (*Ift88*), or a basal body component (*Odf1*) increases Wnt responsiveness indicates that the primary cilium modulates Wnt signal transduction.

To assess whether ciliary effects on Wnt signaling are due to effects on β -catenin, we measured β -catenin levels in *Kif3a*^{+/+} and *Kif3a*^{-/-} MEFs both in the presence and absence of Wnt3a. As expected, stimulation with Wnt3a increases cytoplasmic β -catenin levels in control *Kif3a*^{+/+} MEFs after 3 hours and maximal β -catenin levels are reached by 6 hours (Fig. 2a,b). In contrast, cytoplasmic β -catenin levels are stabilized even in the absence of Wnt3a in MEFs lacking cilia (Fig. 2a,b). Consistent with these findings, E9.5 *Kif3a*^{-/-} embryos also show increased β -catenin levels (Fig. 3c). Furthermore, whereas nuclear β -catenin, a hallmark of Wnt pathway activation[8], is evident following Wnt3a stimulation, immunostaining reveals that unciliated *Kif3a*^{-/-} MEFs accumulate markedly more nuclear β -catenin (Fig. 2c,d). Although it is not able to induce nuclear localization of β -catenin in either ciliated or unciliated MEFs (Fig. 2c,d), Wnt5a does promote rearrangement of the actin cytoskeleton (see Supplementary Information, Fig. S3a), as previously reported[23].

Wnt activation of Frizzleds induces the phosphorylation of Dvl, a key regulator of both canonical and noncanonical Wnt signaling[24-26]. Stimulation of *Kif3a*^{+/+} MEFs with 100 ng/ml of either Wnt3a or Wnt5a induces time-dependent mobility shifts of both Dvl2 and Dvl3 (Fig. 3a,b). Remarkably, in unciliated *Kif3a*^{-/-} MEFs, Dvl-2 and -3 are phosphorylated even in the absence of Wnts. Similarly, E9.5 *Kif3a*^{-/-} embryos show constitutive phosphorylation of Dvl3 (Fig. 3c). In contrast, neither *Ift88*^{Orpk/Orpk} nor *Odf1*^{-/-} cells display constitutive Dvl phosphorylation (see Supplementary Information, Fig. S3b,c).

Several kinases are implicated in Dvl phosphorylation, including PAR-1, Casein kinase 2 and Casein kinase I ϵ [8]. Treatment of ciliated *Kif3a*^{+/-} MEFs with the CKI inhibitor D4476 (ref. [27]) inhibits Wnt3a- and Wnt5a-induced phosphorylation of both Dvl-2 and -3 (Fig. 3d). Importantly, D4476 also inhibits the constitutive phosphorylation of Dvl in unciliated *Kif3a*^{-/-} MEFs, suggesting that CKI is responsible for the constitutive activation of Dvl in cilia-deficient cells (Fig. 3d). In agreement with this hypothesis, treatment with D4476 also abrogates the hyper-responsiveness to Wnt3a exhibited by unciliated *Kif3a*^{-/-} MEFs (Fig. 3e). These findings could reflect either a global increase in CKI activity, or increased activity of CKI specifically towards Dvls. Total cellular CKI ϵ activity is unchanged in *Kif3a*^{-/-} MEFs (see Supplementary Information, Fig. S3d), suggesting that the latter possibility is true. Taken together, these results indicate that Kif3a constrains the ability of CKI to phosphorylate Dvls, and that loss of Kif3a results in uncontrolled phosphorylation of Dvl by CKI leading to hyper-responsiveness of the downstream pathway.

What accounts for the hyper-responsive Wnt response of *Ift88*^{Orpk/Orpk} and *Odf1*⁻ cells? The fundamental structural element of the primary cilium is an array of stabilized microtubules on which Kinesin-2 and dynein motors move. Both APC, a negative regulator of canonical Wnt signaling, and β -catenin have been shown to associate with and be transported along microtubules by Kinesin-2 (ref. [28]). Indeed, a portion of the cellular pool of APC localizes to the primary cilia (Fig. 4a and Supplementary Information, Fig. S3e). In addition, a small fraction of the total cellular β -catenin is apparent in cilia (Fig. 4b,c). In contrast, phosphorylated β -catenin that has been targeted for subsequent proteasome-mediated degradation is present at one end of the cilium (Fig.

4d). Consistent with this interpretation, LiCl-mediated inhibition of GSK3 β reduces the amount of phospho- β -catenin near the cilium (Fig. 4e). Phospho- β -catenin colocalizes with γ -tubulin, a marker of the basal body, indicating that inactive β -catenin accumulates at the base of the cilium (Fig. 4f). In *Kif3a*^{-/-} cells, phospho- β -catenin localizes to the centrosome, a structure analogous to the basal body of ciliated cells (Fig. 4g). Thus, several Wnt signal transduction components are present either at the cilium or the associated basal body, raising the possibility that the cilium directly restrains some of the Wnt signaling machinery (Fig. 5). One possibility is that the cilium promotes turnover of β -catenin by pericentrosomal proteasomes, as recently suggested for BBS4 (ref. [4]).

It is interesting that loss of *Kif3a* is not sufficient to activate TCF/Lef dependent transcription in the absence of Wnt stimulation, despite constitutive CKI-dependent phosphorylation of Dvl and stabilization of cytoplasmic β -catenin. It appears that an additional regulatory step prevents ligand-independent pathway activation in unciliated cells, as increased transcriptional activation only occurs in the presence of Wnts. These findings reveal that a critical role for the cilium in the Wnt pathway is to restrict the magnitude of the canonical response. This role for the primary cilium is thus distinct from its role in Hh and PDGF signaling in that it acts, not to promote, but to restrain signaling. Therefore, cilia have diverse roles in intercellular communication and, depending on the pathway, may either stimulate or restrict pathway activity.

It remains unresolved how ciliary defects lead to the wide spectrum of human diseases in which they are implicated. The most common ciliary pathology is polycystic kidney disease (PKD), a disease in which the dysregulated growth of kidney epithelial cells produces cysts that impinge on surrounding nephrons. Canonical Wnt signaling has

previously been implicated in promoting renal epithelial cell proliferation, and planar cell polarity defects are implicated in renal cyst formation[29,30]. Our finding that the primary cilium restrains Wnt pathway tone supports the hypothesis that ciliary defects cause PKD cyst formation through increased Wnt signaling.

METHODS SUMMARY

Mice

Ift88^{Orpk} (ref. [31]), *BATgal* (ref. [11]), and *Kif3a^{lox}* (ref. [32]) mice have been described. The null allele of *Kif3a* was generated by crossing *Kif3a^{lox/lox}* males to *Tie2Cre* transgenic mice, which delete in the female germline (a gift from Rong Wang, UCSF). To assess *BATgal* activity, embryos were stained for whole-mount β -galactosidase activity as previously described [11].

Cell culture

MEFs were derived from E12.5 embryos, except for *Smo^{-/-}* MEFs (a gift from J. Taipale). Conditioned media from parental as well as Wnt3a- and Wnt5a-stably transfected L cells was carried out according to ATCC. MEFs derived from E12.5 embryos by trypsinization and cultured in Dulbecco's Minimal Essential Media (DMEM) with 10% fetal bovine serum and antibiotics. To delete *Kif3a* in primary cells, *Kif3a^{lox/lox}BATgal* MEFs were infected with adenovirus expressing either eGFP or Cre-eGFP (Vector Development Laboratory, Baylor College) at a multiplicity of infection of 10, cultured for 24 hours, and then double sorted to purity using a FACS Aria with propidium iodide exclusion and a 100 μ m filter. MEFs were cultured for a further 3-4 days prior to analysis. The large T

antigen-immortalized *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs were a gift from Pao-tien Chuang (UCSF) and were derived from E9.5 *Kif3a*^{+/-} and *Kif3a*^{-/-} embryos. E14 embryonic stem cells were grown in GMEM media (Sigma) supplemented with 10% fetal bovine serum, glutamine, pyruvate, non-essential amino acids, β -mercaptoethanol and LIF on gelatin-coated plates. *Ofd1*⁻ (BayGenomics line RRF427) are derived from male E14 ES cells. Hemizygous *Ofd1*⁻ ES cells (BayGenomics line RRF427) produce no full-length *Ofd1* (V. Singla, J. Reiter, unpublished). Inhibition of GSK3 β was accomplished by treating cells with 30 mM LiCl for 18 hours.

Activity assays

BATgal activity was detected using the Galacto-Light Plus System (Applied Biosystems). Canonical Wnt activity was determined in immortalized MEFs treated with 100 ng/ml recombinant Wnt (R&D Systems) by transient transfection of the SuperTOPFlash reporter plasmid (a gift from Randall Moon, University of Washington) and pRL-TK expressing renilla luciferase using Fugene 6 (Roche) or jetPEI (Polyplus transfection) as described [13]. SuperTOPFlash reporter values were normalized to renilla luciferase activity to account for differences in transfection efficiency using the Dual-Luciferase Reporter Assay (Promega). Firefly luciferase values were normalized to Renilla luciferase. Variability in assay activity is partially attributable to inherent differences in MEF line responsivity. Therefore, multiple independent MEF lines were derived and tested, and mutant lines were always compared to control lines derived from littermate embryos. All data was subjected Student's t-test.

For the CK1 ϵ activity assays, confluent 10 cm plates of wild type or mutant cells were starved overnight in DMEM with 1% delipidated serum, and lysed in 1ml RIPA buffer supplemented with protease and phosphatase inhibitors. CK1 ϵ was immunoprecipitated with 2 μ g of CK1 ϵ antibody (Santa Cruz) for 1.5 hours and captured with protein G conjugated agarose beads (Roche) for 1 hour. CK1 ϵ autophosphorylation was carried out in a buffer consisting of 20mM Tris-Cl pH7.5, 5mM MgCl₂, 0.5mM DTT, 150mM KCl, 5 μ M ATP and 5 μ Ci γ -³²P-ATP at 30°C for 20 minutes. The reaction was resolved via SDS-PAGE, visualized with a phosphor-imager, quantified with image-quant software, and normalized to immunoprecipitated CK1 ϵ amount as determined by Li-Cor Odyssey fluorescent western blotting with anti-CK1 ϵ antibody (BD transduction labs). BATgal activity in embryos was detected as previously described[11]. BATgal activity in cells was determined using the Galacto-Light Plus System (Applied Biosystems). Wnt activity was measured by transfection of the SuperTOPFlash reporter plasmid (Randall Moon, University of Washington) and pRL-TK expressing renilla luciferase[13]. All assays were conducted in triplicate in at least three independent experiments.

Western blotting

MEFs were starved overnight in DMEM with 0.5% delipidated serum (Cocalico), pre-treated with D4476 (Calbiochem) as described and subsequently stimulated with 100 ng/ml recombinant Wnt (R&D) for the times indicated. Lysates were prepared in RIPA buffer, separated by 8% SDS-PAGE, and analyzed by Western blotting. Embryo lysates were prepared in RIPA buffer (Upstate Biotechnology), separated by 8% SDS-PAGE, and analyzed by Western blotting. Antibodies used were anti- β -catenin (1:5000, BD

Transduction Labs 610153), anti-Dvl2 (1:1000, Cell Signaling 3224), anti-Dvl3 (1:1000, Cell Signaling 3218), anti- β -actin (1:3000, Abcam 8227), anti- α -tubulin (1:3000, Santa Cruz 12462), and anti-Kif3a (1:5000, Sigma K3513).

For Li-Cor quantification of cytoplasmic β -catenin levels, T-antigen-immortalized *Kif3a* MEFs were seeded onto 6-well plates and allowed to grow to confluency for 24 hours. MEFs were starved in high glucose DMEM supplemented with 1% delipidated serum for 12 hours. MEFs were then stimulated for the times indicated with 250 ng/ml recombinant Wnt3a. Cells were fractionated with the NE-PER cytoplasmic and nuclear extraction kit (Pierce Biotechnology), using the conditions specified by the manufacturer for a 10 μ l cell pellet. 2 μ g of protein was loaded on a 7% Tris-glycine SDS-PAGE gel and resolved. The gel was transferred to nitrocellulose and blotted according to the recommended protocol for the Li-Cor Odyssey infrared scanner, using a 1:1 dilution of Odyssey blocking solution to TBS (pH 7.5). Primary antibodies used were mouse anti- β -catenin (1:10,000 BD Transduction Labs 610153), and rabbit anti- β -actin (1:5000, Abcam 8227) as a loading control. Secondary antibodies were Alexa 680-conjugated goat anti-mouse and goat anti-rabbit, both used at 1:10,000. The blots were scanned using the Li-Cor Odyssey infrared scanner. The average integrated pixel intensity of identical sized boxes drawn around the major β -catenin band was normalized to the intensities of the β -actin loading control.

Immunofluorescence

MEFs were grown on gelatin-coated cover slips or 8-well chamber slides (BD Falcon) were fixed with 4% paraformaldehyde at room temperature for 20 minutes or 100% methanol at -20°C for 3 minutes. 10^5 E14 or *Ofdl*⁻ embryonic stem (ES) cells were plated on 12 mm coverslips coated with poly-D-lysine and Matrigel (BD Biosciences). ES cells were treated with 0.5 mM mimosine (Sigma) for 6-8 hrs to enhance percent ciliated cells and then fixed with 4% paraformaldehyde for 5 minutes. Following extensive washing, cells were blocked with 2% bovine serum albumin for 45 minutes at room temperature. Cells were then incubated with primary antibodies overnight at 4°C in block buffer. Primary antibodies were detected with Alexa-conjugated fluorophores (1:400, Molecular Probes) following a secondary block with 2% bovine serum albumin and 10% serum. Antibodies used were anti- β -catenin (1:500, BD Transduction Labs 610153 and 1:100 Cell Signaling 9582), anti-acetylated Tubulin (1:1000, Sigma T6793), anti-glutamylated Tubulin (1:750, Chemicon AB3201), anti-APC (1:100, Santa Cruz Ali 12-28 and 1:50, Cell Signaling 2504), anti-phospho- β -catenin (S33/37/T41) (1:100, Cell Signaling 9561), and anti- γ -tubulin (Santa Cruz 7396). Microscopy was performed on a Leica SP2 confocal microscope, and images are maximum projections of complete confocal stacks except for Fig. 3c which uses only z-sections that include cilia.

Figure 1. Loss of primary cilia increases responsiveness of the canonical Wnt

pathway. (A) Increased Wnt activity in mouse embryos lacking *Kif3a* is revealed by

whole-mount β -galactosidase staining of 12 somite *Kif3a*^{+/-}*BATgal* and *Kif3a*^{-/-}*BATgal*

littermates. **(B)** Deletion of *Kif3a* by Cre-mediated excision. *Kif3a*^{lox/lox}*BATgal* primary

MEFs were infected with control (Ad-GFP) or Cre-expressing (Ad-Cre-GFP) virus and

sorted for GFP expression. The Western blot reveals a complete absence of Kif3a in Cre-

infected MEFs. β -actin controls for loading. **(C)** Loss of Kif3a disrupts ciliogenesis.

Kif3a^{lox/lox}*BATgal* MEFs were infected with Ad-GFP (*Kif3a*^{lox/lox}) or Ad-Cre-GFP (*Kif3a*^{-/-})

virus. Staining for acetylated Tubulin (green) reveals primary cilia (arrows) in

Kif3a^{lox/lox} but not *Kif3a*^{-/-} cells. DAPI stains nuclei (blue). Scale bars in all micrographs

depict 10 μ m. **(D)** Deletion of *Kif3a* increases transcriptional activation in response to

Wnt3a. *Kif3a*^{lox/lox}*BATgal* MEFs infected with Ad-GFP (*Kif3a*^{lox/lox}) or Ad-Cre-GFP

(*Kif3a*^{-/-}) virus were untreated (control) or stimulated with Wnt3a or Wnt5a. *Kif3a*^{lox/lox}

cells show a 2.5-fold increase in Wnt3a-induced *BATgal* activity, whereas *Kif3a*^{-/-} cells

show a 10-fold increase in activity ($P < 0.005$). Recombinant Wnt5a has no effect. The

experiment was conducted three times, and averages and standard deviations are shown.

(E) Wnt-dependent transcriptional activation measured in *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs

transfected with SuperTOPFlash reporter plasmid and pRL-TK expressing renilla

luciferase. Wnt3a induces reporter activity 7-fold above control in *Kif3a*^{+/-} MEFs,

whereas Wnt3a induces reporter activity over 15-fold in *Kif3a*^{-/-} MEFs ($P < 0.005$). The

experiment was conducted three times, each with three samples, and averages and

standard deviations are shown. **(F)** Immunofluorescence of *Ift88*^{+/+} and *Ift88*^{Orpk/Orpk}

MEFs stained for cilia (arrows, acetylated Tubulin, green) and basal bodies (Rootletin,

red). **(G)** Immunofluorescence of *Odf1*⁺ and *Odf1*⁻ embryonic stem cells stained for cilia (arrows, acetylated Tubulin, green). **(H)** *Ift88*^{+/+} *BATgal* MEFs stimulated with Wnt3a show a 14-fold increase in reporter activity above control, whereas *Ift88*^{Orpk/Orpk} *BATgal* MEFs show a 23-fold increase ($P<0.05$). The experiment was conducted three times, each with three samples, and averages and standard deviations are shown. **(I)** *Odf1*⁺ embryonic stem cells stimulated with Wnt3a show a 4.4-fold increase in reporter SuperTOPFlash activity over non-stimulated cells, whereas *Odf1*⁻ cells show a 22.7-fold increase ($P<0.01$). The experiment was conducted three times, each with three samples, and averages and standard deviations are shown.

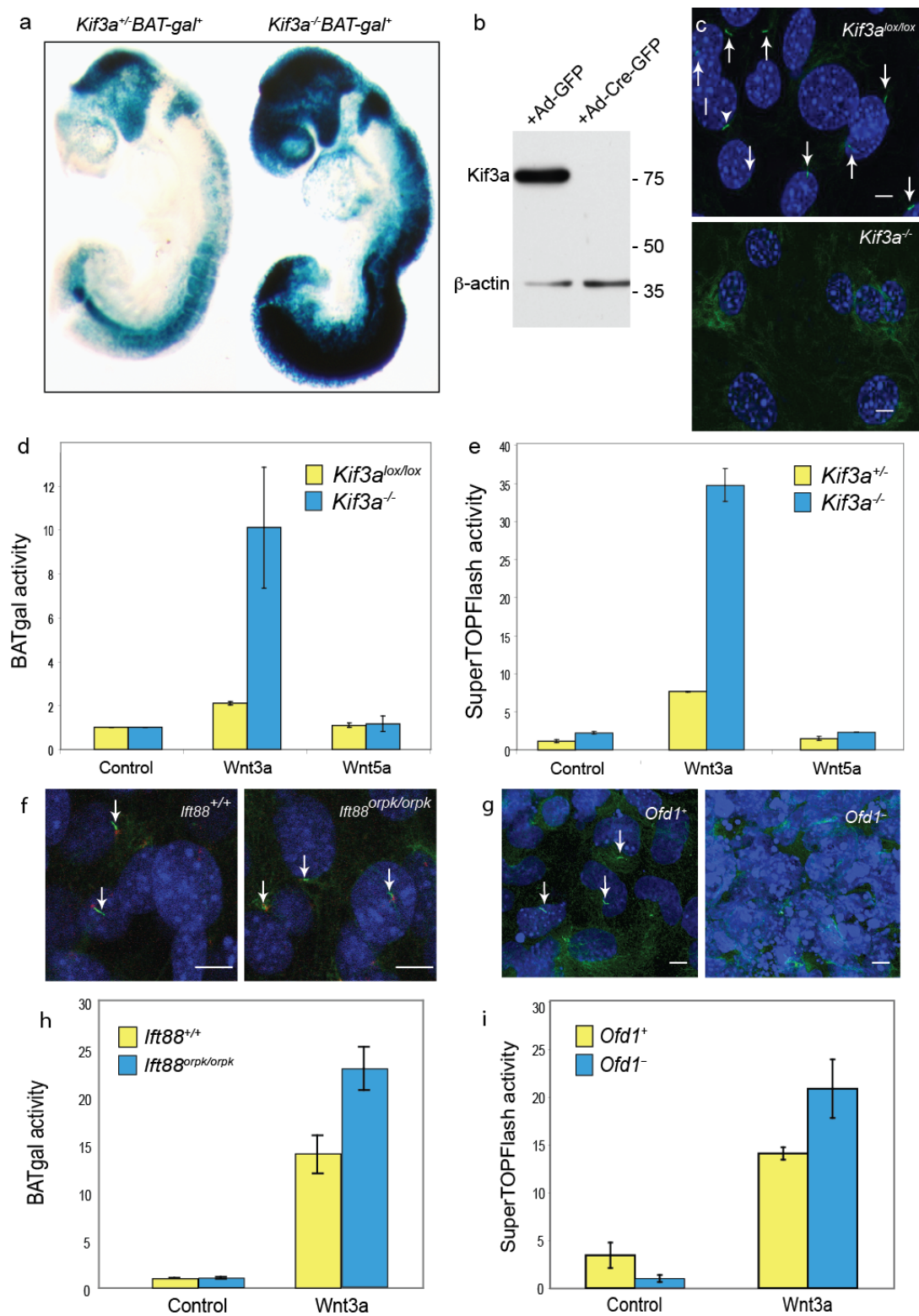


Figure 2. Mutation of *Kif3a* stabilizes β -catenin. (A) Western blot showing a time-dependent increase in Wnt3a-induced levels of cytoplasmic β -catenin in *Kif3a*^{+/-} cells, whereas β -catenin is constitutively stabilized in *Kif3a*^{-/-} cells. α -tubulin controls for loading. (B) Western blot quantitation demonstrates that Wnt3a stimulation of wild-type MEFs stabilizes cytoplasmic β -catenin. *Kif3a*^{-/-} MEFs display high levels of cytoplasmic β -catenin even in the absence of Wnt3a. (C) Immunofluorescence of *Kif3a*^{+/-} MEFs detects nuclear β -catenin (red, arrow) in Wnt3a-treated cells, but not in untreated or Wnt5a-treated cells. Cilia are apparent by glutamylated Tubulin staining (green, white arrowheads). (D) Immunofluorescence of Wnt3a-stimulated *Kif3a*^{-/-} MEFs reveals higher levels of nuclear β -catenin than in normally ciliated control cells. Nuclear β -catenin is not observed in untreated or Wnt5a-treated cells.

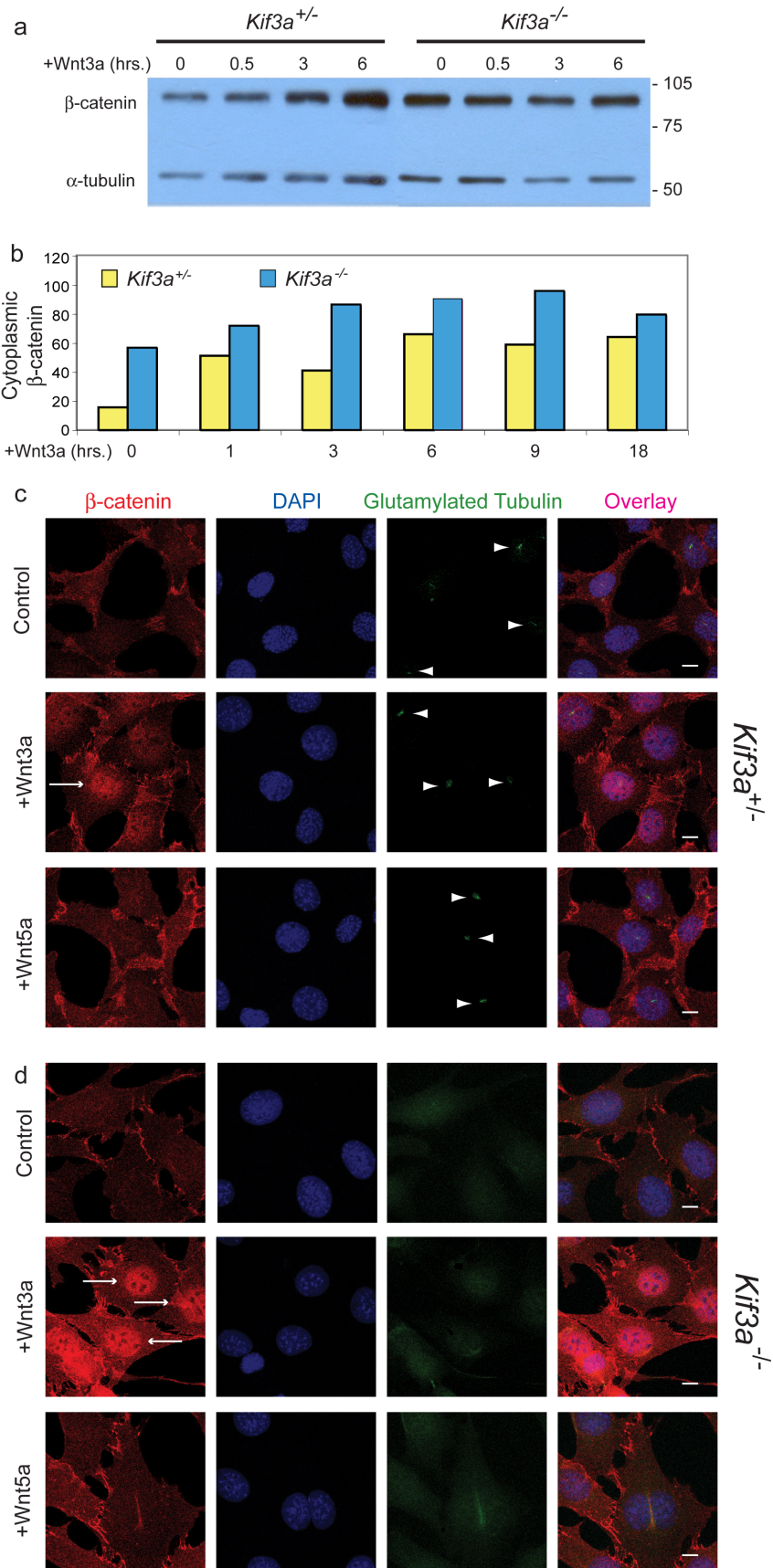


Figure 3. In the absence of Kif3a, Dvl is constitutively phosphorylated by CKI. (A) Dvl2 from *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs stimulated with Wnt3a or Wnt5a for 90 minutes. Dvl2 resolves as a doublet in untreated *Kif3a*^{+/-} MEFs, whereas *Kif3a*^{-/-} MEFs contain only the phosphorylated (**) form. Stimulation of *Kif3a*^{+/-} MEFs with either Wnt3a or Wnt5a shifts Dvl to the phosphorylated state. Anti-Kif3a identifies mutant samples. **(B)** Western blot analysis of Dvl3 from *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs stimulated with Wnt3a or Wnt5a for 90 minutes. As with Dvl2, stimulation of *Kif3a*^{+/-} MEFs with either Wnt3a or Wnt5a shifts Dvl3 to the phosphorylated state, but *Kif3a*^{-/-} MEFs contain only the phosphorylated form even in the absence of Wnts. **(C)** Western blotting of lysates from E9.5 *Kif3a*^{+/-} and *Kif3a*^{-/-} littermates. Dvl3 resolves as a doublet in *Kif3a*^{+/-} embryos, whereas animals lacking cilia contain only the phosphorylated form. **(D)** Western blot analysis of Dvl2 (top) and Dvl3 (bottom) from *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs untreated (CTRL), or starved and pre-treated with 100 μM D4476 (+) or vehicle (-) for one hour, and then stimulated with 100 ng/ml recombinant Wnt3a or Wnt5a for 2 hours. Inhibition of CKI with D4476 blocks Dvl2 phosphorylation in both *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs. The same effect is seen with Dvl3, although the inhibition of phosphorylation is not as complete. **e**, Inhibiting CKI activity abrogates the hyper-responsiveness of unciliated *Kif3a*^{-/-} MEFs to Wnt3a. Wnt-dependent transcriptional activation measured in *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs transfected with SuperTOPFlash reporter plasmid. MEFs were starved and left untreated (control), or pre-treated with 100 μM D4476 for one hour, and stimulated with 100 ng/ml recombinant Wnt3a for 18 hours. Wnt3a induces reporter activity 9-fold above control in *Kif3a*^{+/-} MEFs, whereas Wnt3a induces reporter activity over 20-fold in *Kif3a*^{-/-} MEFs (*P*<0.01). The experiment was conducted three times, each

with three samples, and averages and standard deviations are shown. Both *Kif3a*^{+/-} and *Kif3a*^{-/-} MEFs treated with D4476 have a reduced transcriptional response to Wnt3a.

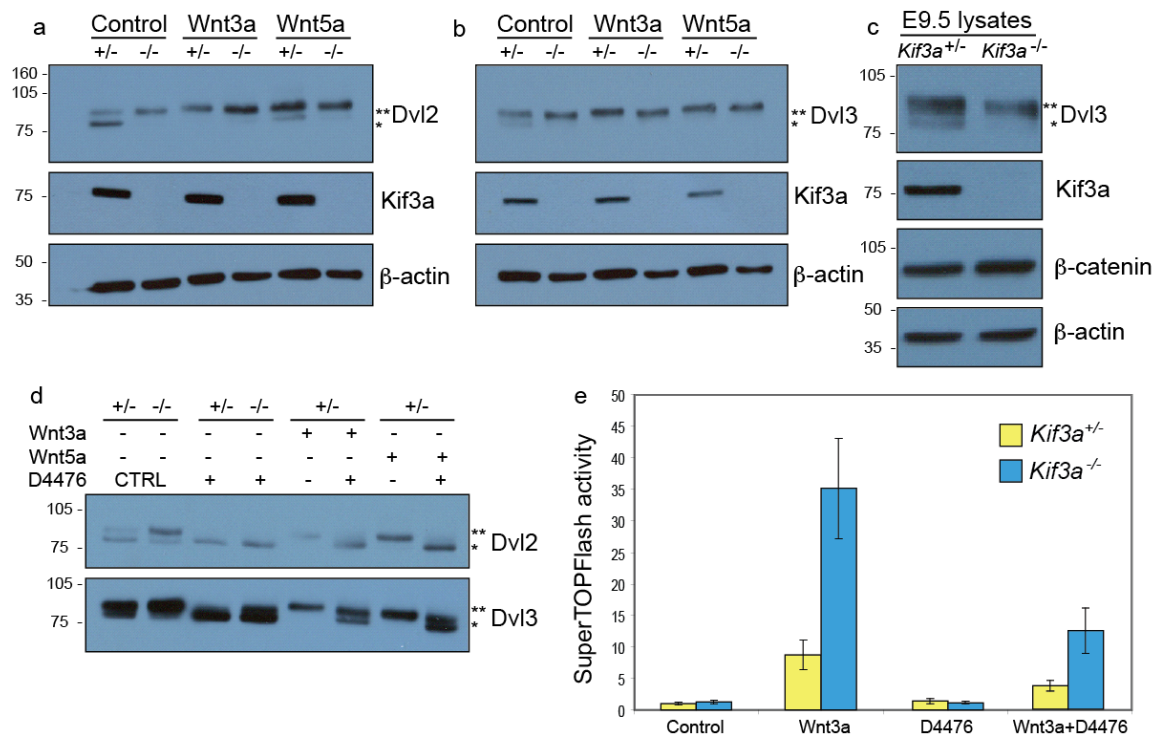


Figure 4. Wnt pathway components are present at cilia. (A) A portion of APC (red, arrows) is present in primary cilia (green, acetylated Tubulin) of *Kif3a*^{+/-} MEFs. (B) Only a small proportion of total β -catenin (red, arrows) is present in primary cilia (green, glutamylated Tubulin). (C) High magnification image of cilia revealing ciliary β -catenin. (D) Phospho-(S33/37/T41)- β -catenin (red) is present at one end of primary cilia (green, acetylated Tubulin). (E) Inhibition of GSK3 β with LiCl reduces the amount of phospho- β -catenin (red) proximal to cilia (green, acetylated Tubulin). (F) β -catenin targeted for degradation is present at the basal body. Phospho- β -catenin (green) localizes to the basal bodies (red, γ -tubulin) of primary cilia (blue, acetylated Tubulin) in *Kif3a*^{+/-} MEFs. (G) In *Kif3a*^{-/-} MEFs, phospho- β -catenin (green) is present at centrosomes (red, γ -tubulin). Acetylated Tubulin (blue) staining demonstrates the lack of primary cilia.

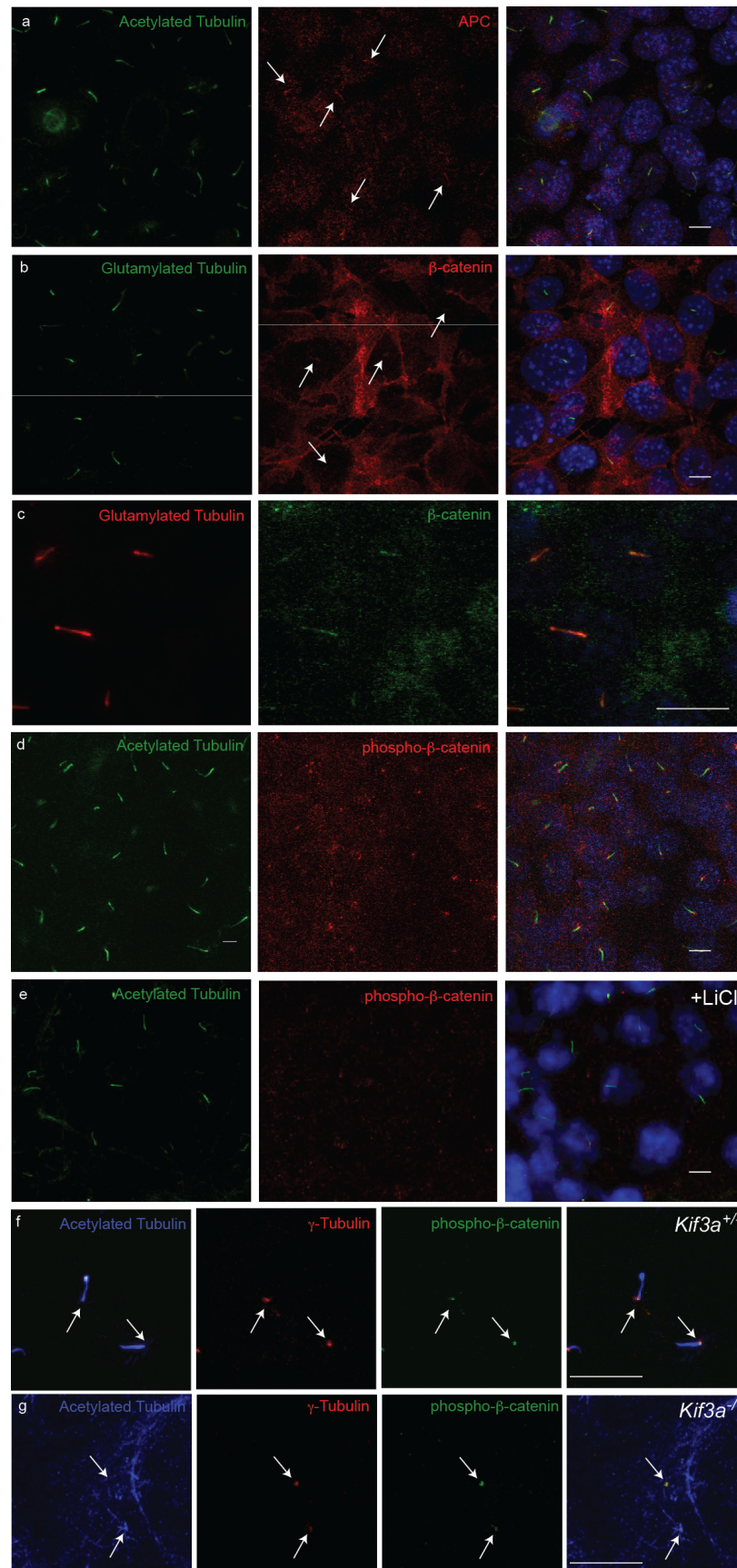


Figure 5. Ciliated and unciliated cells respond differently to Wnt stimulation. (A)

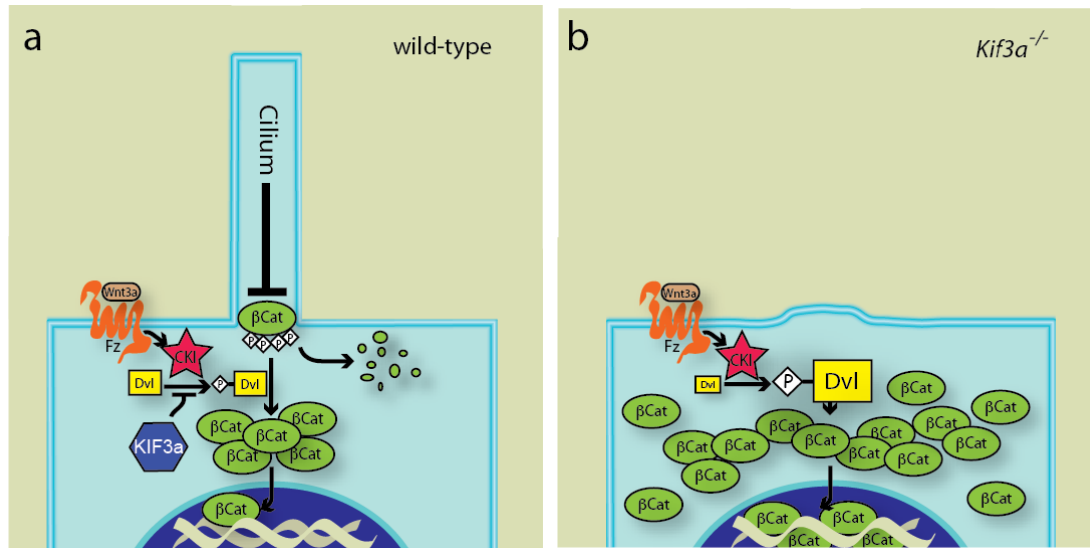
Kif3a inhibits CKI phosphorylation of Dvl, providing a brake to the Wnt3a-induced

stabilization of β -catenin. **(B)** In the absence of Kif3a, this brake is lost and CKI shifts

Dvl to the phosphorylated form. An additional ciliary brake on Wnt signaling is also lost.

Consequently, β -catenin is stabilized and, in the presence of Wnt3a, accumulates in the

nucleus where it overactivates the Wnt transcriptional program.



Acknowledgements

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Figure S1. Loss of Kif3a does not affect Wnt signaling in unciliated cells in vivo, BATgal activity is stimulated by Wnt3a but not Wnt5a, and Wnt-induced transcriptional activation is normal in *Smo*^{-/-} cells. (A) *Kif3a*^{-/-} embryos display no increased Wnt pathway activity prior to the point at which most cells become ciliated. Whole-mount β -galactosidase staining of late headfold stage *Kif3a*^{+/-} *BATgal* and *Kif3a*^{-/-} *BATgal* littermates. (B) Wnt3a, but not Wnt5a, activates the *BATgal* reporter. MEFs derived from *Kif3a*^{lox/lox} *BATgal* embryos were stimulated with 100 ng/ml recombinant Wnt3a or Wnt5a for 18 hours. Whereas Wnt5a does not stimulate BATgal activity over control conditions ($P=0.42$), Wnt3a increases BATgal activity 25-fold ($P<0.0001$). The experiment was conducted three times, each with three samples, and averages and standard deviations are shown. (C) Wnt-dependent transcriptional activation measured in *wild-type* (*WT*) and *Smo*^{-/-} MEFs transfected with SuperTOPFlash reporter plasmid and stimulated with 100 ng/ml recombinant Wnt3a for 18 hours. Wnt3a stimulates transcriptional activity in wild-type and *Smo*^{-/-} cells 43.2- and 47.8-fold ($P=0.99$), respectively, over control conditions. The experiment was conducted one time with three samples, and averages and standard deviations are shown.

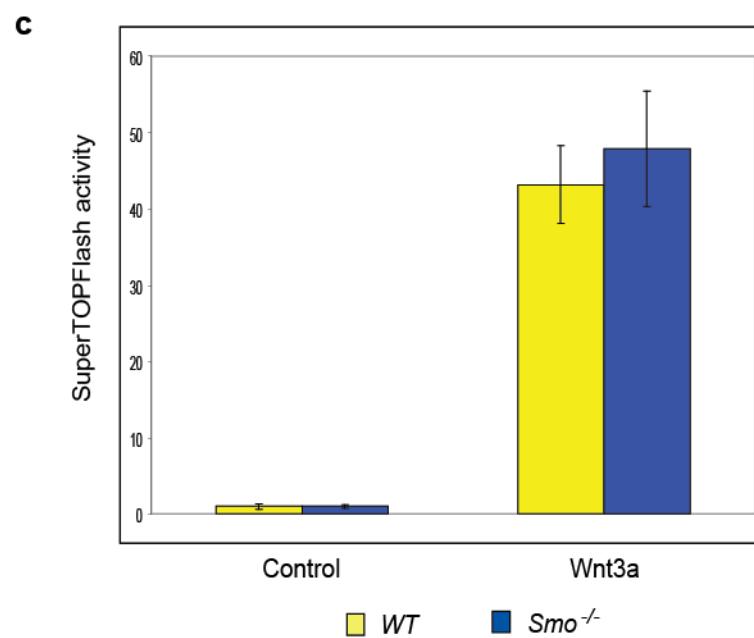
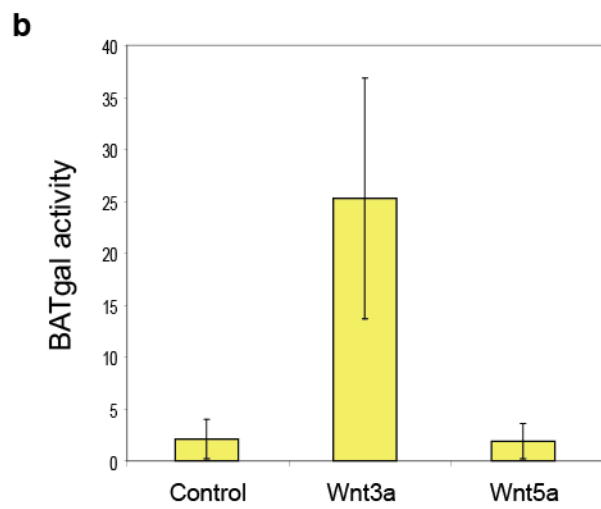
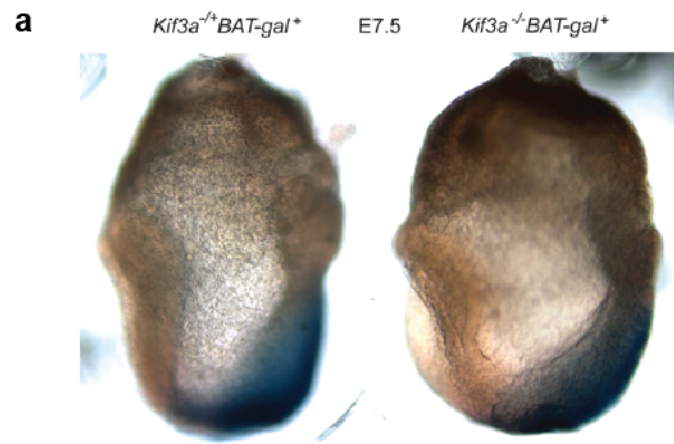
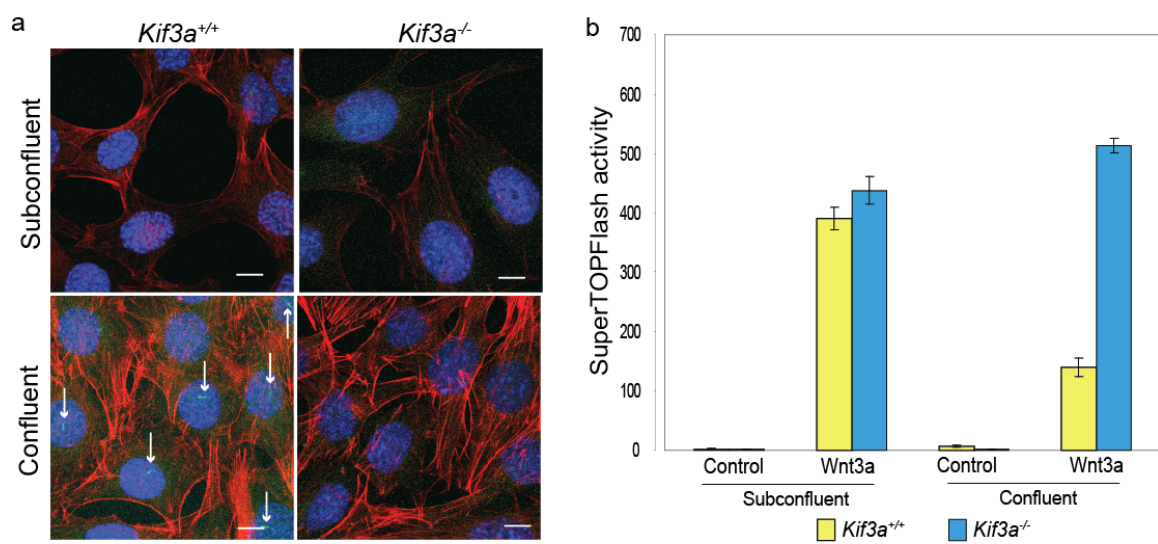
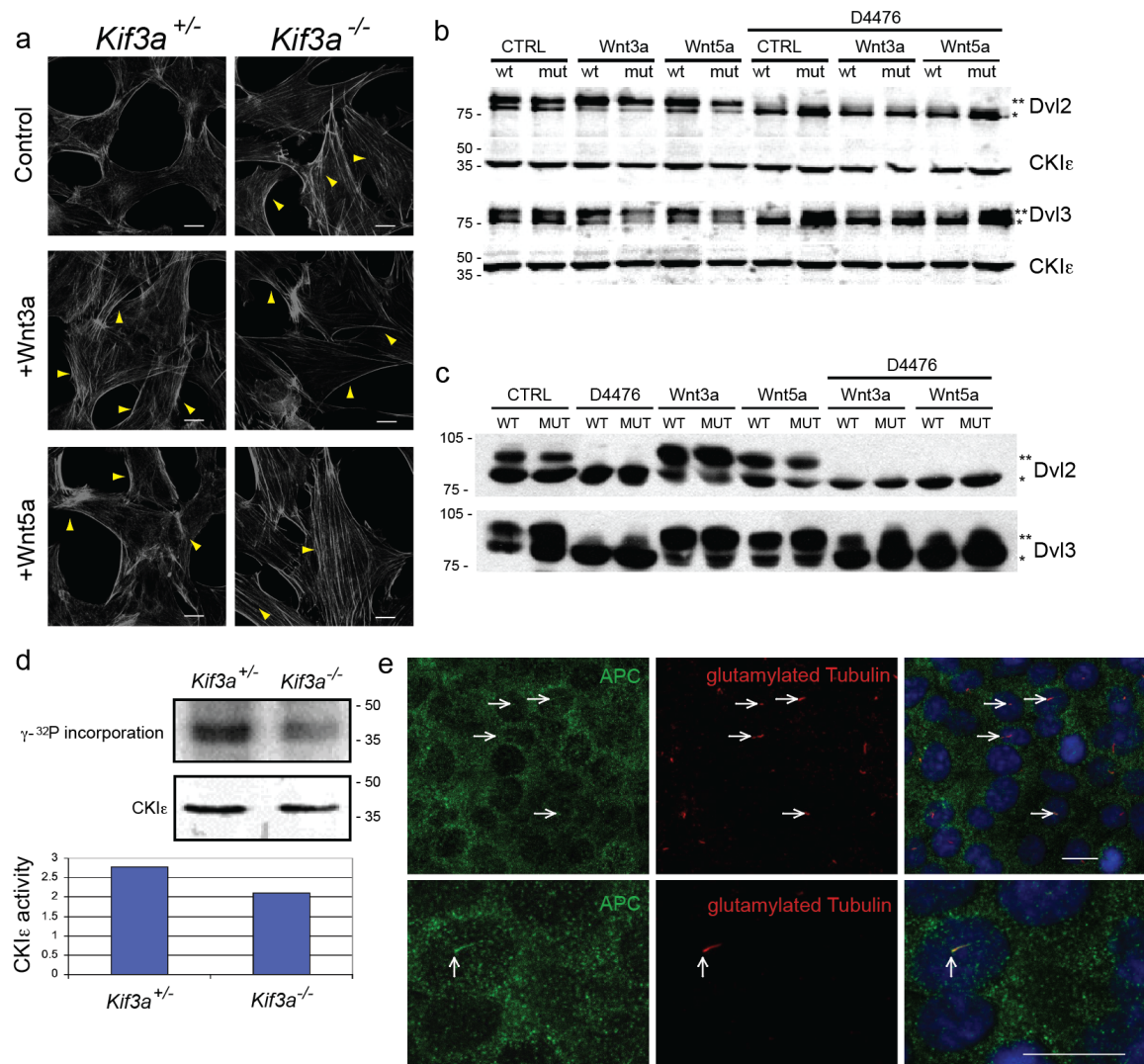


Figure S2. Subconfluent MEFs are unciliated and show increased Wnt

responsiveness. **(A)** Immunofluorescence of *Kif3a*^{+/+} and *Kif3a*^{-/-} MEFs plated at 5•10⁴ or 10⁵ cells per cm² stained for cilia (arrows, acetylated Tubulin, green). Nuclei are blue, and actin (phalloidin, red) demonstrates the degree of confluency. **(B)** Wnt-dependent transcriptional activation measured in *Kif3a*^{+/+} and *Kif3a*^{-/-} MEFs transfected with SuperTOPFlash reporter plasmid and pRL-TK expressing renilla luciferase. Wnt3a induces reporter activity to similar levels in subconfluent *Kif3a*^{+/+} and *Kif3a*^{-/-} MEFs plated at 5•10⁴ cells per cm². At confluency (10⁵ cells per cm²), Wnt3a-induced pathway activation is maintained at high levels in unciliated *Kif3a*^{-/-} MEFs, whereas it is dampened in ciliated *Kif3a*^{+/+} MEFs.



Figures S3. *Kif3a*^{-/-} cells display increased cortical actin, and neither *Odf1* nor *Ift88* inhibit Dvl phosphorylation. (A) Phalloidin staining (white) reveals an increase in cortical actin bundles following both Wnt3a and Wnt5a stimulation (yellow arrowheads). Interestingly, the actin cytoskeleton of *Kif3a*^{-/-} MEFs contains stress fibers even in the absence of Wnt stimulation. (B) Dvl2, Dvl3, and CKIε from *Ift88*^{Orpk/Orpk} and wild-type MEFs untreated (CTRL), or starved and pre-treated with 100 μM D4476 or vehicle for one hour, and then stimulated with 100 ng/ml recombinant Wnt3a or Wnt5a for 2 hours. Inhibition of CKI with D4476 blocks Dvl2 and Dvl3 phosphorylation. (C) Dvl2 and Dvl3 from *Odf1*⁻ and wild-type embryonic stem cells untreated (CTRL), or starved and pre-treated with 100 μM D4476 or vehicle for one hour, and then stimulated with 100 ng/ml recombinant Wnt3a or Wnt5a for 2 hours. As with MEFs, inhibition of CKI with D4476 blocks Dvl2 and Dvl3 phosphorylation. (D) CKIε activity is not globally increased in the absence of *Kif3a*. Activity (graph) is measured by in vitro autophosphorylation of immunoprecipitated CKIε (top) normalized to CKIε amount (middle). (E) A portion of APC (green, arrows) is present in primary cilia (red, glutamylated Tubulin) of *Kif3a*^{+/-} MEFs.



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Chapter 3: Disruption of a ciliary B9 protein complex causes Meckel Syndrome

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ABSTRACT

Nearly every ciliated organism expresses three B9 domain containing proteins, MKS1, B9D1, and B9D2. Mutations in human *MKS1* cause Meckel Syndrome (MKS), a severe ciliopathy characterized by occipital encephalocele, liver ductal plate malformations, polydactyly and kidney cysts. Mouse mutations in either *Mks1* or *B9d2* compromise ciliogenesis and result in phenotypes similar to those of MKS. Given the importance of these two B9 proteins to ciliogenesis, we examined the role of the third B9 protein, B9d1. Mice lacking B9d1 displayed polydactyly, kidney cysts, ductal plate malformations, and abnormal patterning of the neural tube, concomitant with compromised ciliogenesis, ciliary protein localization and Hedgehog (Hh) signal transduction. These data prompted us to screen MKS patients for mutations in *B9D1* and *B9D2*. We identified a homozygous c.301A>C (p.Ser101Arg) *B9D2* mutation that segregates with MKS, affects an evolutionarily conserved residue, and is absent from

controls. Unlike wild type *B9D2* mRNA, the p.Ser101Arg mutation failed to rescue zebrafish phenotypes induced by the suppression of *b9d2*. Using coimmunoprecipitation and mass spectrometric analyses, we found that Mks1, B9d1, and B9d2 interact physically, but that the p.Ser101Arg mutation abrogates the ability of B9d2 to interact with Mks1, further suggesting that the mutation compromises B9d2 function. Our data indicate that B9d1 is required for normal Hh signaling, ciliogenesis, ciliary protein localization and that B9d1 and B9d2 are essential components of a B9 protein complex, disruption of which causes MKS.

INTRODUCTION

Meckel syndrome (MKS [MIM 249000]) is a rare autosomal recessive disorder resulting in perinatal lethality and characterized by renal cysts, hepatic ductal plate malformation, and central nervous system defects, such as occipital encephalocele [1]. Mutations in six genes are known to cause MKS, *MKS1* (MIM 609883), *TMEM216* (*MKS2* [MIM 613277]), *TMEM67* (*MKS3* [MIM 609884]), *CEP290* (*MKS4* [MIM 610142]), *RPGRIP1L* (*MKS5* [MIM 610937]), and *CC2D2A* (*MKS6* [MIM 612013]) [2-7]. In addition, mutations in *NPHP3* (*MKS7* [MIM 608002]) and *TCTN2* (*MKS8* [HUGO]) cause an MKS-like phenotype [8,9]. The products of these genes localize to cilia and/or basal body, supporting the hypothesis that ciliary dysfunction underlies MKS.

MKS1, a variant of which was the first gene linked to MKS, encodes a protein containing a B9 domain, structurally similar to the lipid binding domains of the C2 family [10]. Although none of the other previously recognized MKS proteins contain B9

domains, bilaterians including mammals, *Drosophila* and *C. elegans* possess two other B9 domain-containing proteins, B9d1 and B9d2 (also known as Stumpy [MIM 611951]). B9 domain-containing proteins are also present in diverse ciliated protists, including Trypanosomes, Naegleria, and Batrachochytrium, but are absent from non-ciliated eukaryotes, including most yeast and higher plants (Figure S1). Consistent with a role in cilia function, all three B9 domain proteins localize to the transition zone of *C. elegans* cilia, or the basal body and axoneme of mammalian cilia [11-14]. Mouse embryos homozygous for a loss of function mutation in *Mks1* display hallmarks of MKS, such as renal cysts, ductal plate malformation, and brain malformations similar to occipital meningoencephalocele. In addition, these mutants displayed cleft palate and polydactyly, phenotypes associated commonly with MKS1 (MIM 249000) [13,15-18]. Conditional inactivation of *B9d2* in the brain and kidney cause hydrocephalus and renal cysts, accompanied by defects in ependymal and kidney cilia [14].

Primary cilia are required for mammalian development, in part because of their roles in mediating Hh signal transduction [19]. Hh signals participate in the patterning of a large number of tissues, including the neural tube and limb buds. Components of the Hh pathway, including Patched1 (*Ptc1*), Smoothened (*Smo*) and the Gli transcriptional effectors, localize to primary cilia, suggesting that critical Hh signal transduction events occur within cilia [20-22]. Consistent with this hypothesis, vertebrate mutations that disrupt ciliogenesis cause defects in Hh signaling and concomitant developmental phenotypes [23].

To investigate further the role of B9 proteins in ciliogenesis and in the pathogenesis of MKS, we analyzed mice mutant for the third member of the B9 genes,

B9d1. Strikingly, loss of B9d1 resulted in MKS-like phenotypes in mouse embryos which, taken together with the mouse mutant data for *B9d2*, suggested that these two loci are candidates for causing severe ciliopathies. Therefore, we screened MKS patients for mutations in *B9D1* and *B9D2* and identified a homozygous mutation in *B9D2*. We also found that all three B9 proteins interact physically with each other, but that the MKS-associated disease allele of *B9d2* exhibited decreased affinity for Mks1. These findings reveal that a complex comprised of the three B9 proteins support mammalian ciliogenesis, disruption of which results in Hh signaling defects and MKS.

MATERIALS AND METHODS

B9 protein conservation

We identified MKS1, B9D1, and B9D2 orthologs using reciprocal best match by BLAST. MKS1 orthologs include *Tetrahymena* TTHERM_00630490, *Batrachochytrium* BDEG_00264, *Trichoplax* JGI 61316, and *Naegleria* JGI 29577. B9D1 orthologs include *Batrachochytrium* BDEG_03036, *Chlamydomonas* Au9.Cre01.g066000, *Paramecia* GSPATP00029931001, *Tetrahymena* TTHERM_00219110, *Leishmania major* LmjF32.0280, *Trypanosoma brucei* Tb10.61.2290, *Trichoplax* JGI 52971, *Monosiga* JGI 24999, *Naegleria* JGI 52666, *Thalassiosira* JGI 264691, and *Phytophthora* JGI 71279. B9D2 orthologs include *Batrachochytrium* BDEG_03725, *Chlamydomonas* Au9.Cre03.g196050, *Naegleria* JGI 30379, *Trypanosoma brucei* Tb11.03.0750, *Leishmania major* LmjF25.0320, *Tetrahymena* TTHERM_00633350, *Paramecium* ICIS-1, *Trichoplax* JGI 52819, *Monosiga* JGI 17074, and *Thalassiosira* JGI 38153. Protein

sequences were aligned using the ID weight matrix of ClustalW, and the alignment used to create an unrooted nearest-joining tree.

Mouse Strains

All mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco. *B9dI*^{+/-} mice (*B9dI*^{tm1a(EUCOMM)Wtsi}) were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM). The targeted mutation was made in C57BL/6N ES cells, which were injected into B6(Cg)-Tyr^{c-2J}/J blastocysts. The mice were maintained by crossing to C57BL/6J mice (Jackson Laboratory). The mixed background strain was created by outcrossing *B9dI*^{+/-} mice with CD1 mice (Charles River Laboratory) for two generations. The *B9dI* exon 3 deletion allele was created by crossing *B9dI*^{tm1a(EUCOMM)Wtsi} mice to mice expressing FLPe recombinase (*Tg(ACTFLPe)9205Dym/J*) to remove the En2 splice acceptor and βGeo cassette (*B9dI*^{flox/flox}). Subsequently, *B9dI*^{flox/flox} mice were crossed to mice expressing Cre recombinase (*Tg(ACTB-cre)2Mrt/J*) to delete exon 3. We considered noon on the day a copulation plug was detected to be 0.5 days post coitus.

Immunohistochemistry, histology and in situ hybridization

For whole-mount staining of nodes, E8.0 embryos were dissected in PBS + 10% FBS, rinsed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min on ice, blocked for 30 min in 2% BSA + 5% donkey serum in PBS + 0.1% TritonX-100 (PBT), and incubated with primary antibodies overnight at 4°C in 2% BSA + 1% donkey serum in PBT. For neural tube analysis, E9.5 embryos were harvested in PBS + 10% FBS,

rinsed in PBS and fixed for 1 h in 4% PFA in PBS on ice. Embryos were equilibrated in 30% sucrose overnight and mounted in O.C.T. mounting media (Fisher). 12 μ m sections were stained as previously described [24]. For liver and kidney analysis, organs were fixed in 10% formalin overnight at 4°C, sectioned, and stained with hematoxylin and eosin according to standard protocols. Immunofluorescent staining of liver and kidney sections was performed as described [25]. Biotinylated lectin staining of kidney sections was performed as described [26], with the exception that avidin/biotin blocking solution (Vector Labs) was applied according to the manufacturer's instructions after sections were dewaxed.

The antibodies employed were mouse α -acetylated tubulin 6-11B-1 (1:1,000, Sigma), rabbit α -N99 (1:20,000, gift of James Silbourn), mouse α -E-cadherin (1:100, BD), mouse α -Sox9 (1:100), goat α - γ tubulin (1:100, Santa Cruz Biotechnology), mouse α -Cytokeratin-19 (1:130, Novacastra), rabbit α -Pax6 (1:100, Covance), mouse α -Nkx2.2 (1:20), mouse α -FoxA2 (1:20) and mouse α -Islet1/2 (1:20, Developmental Studies Hybridoma Bank). These last three antibodies were developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242, USA. Biotinylated Lectins employed were *Dolichos biflorus* agglutinin (10 μ g/ml, Vector Labs B-1035), and *Lotus tetragonolobus* lectin (10 μ g/ml, Vector Lab B-1325). Avidin conjugated Alexa 488 (1:300, Invitrogen). Nodal and neural tube cilia were observed with a Nikon C1 spectral confocal microscope. Neural tube pattern, as well as cilia staining of liver and kidney sections was observed with a Leica SP5 confocal microscope. Kidney and liver H&E, as well as Sox9 and E-cadherin staining of liver sections were observed with a Zeiss Observer D1 inverted epifluorescent

microscope. Post-acquisition image processing was performed with Metamorph imaging software (Molecular Devices) or Image J (NIH).

To visualize cartilage, E13.5 embryos were harvested in PBS + 10%FBS, frozen at -20°, and stained with alcian blue as described [27]. *In situ* hybridization was performed as described [27,28]. Cartilage preparations and whole-mount embryos were observed with a Zeiss Discovery V12 steREO microscope.

Electron microscopy

For SEM analysis, E8.0 (2-3 somite) embryos were fixed for 1h in 2% sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in sodium cacodylate buffer, and dehydrated through a graded ethanol series. Samples were mounted on aluminum stubs with carbon dots, critical point dried in a Tousimis AutoSamdri 815, and sputter coated with gold in a Tousimis chamber. Samples were visualized on a Hitachi S-5000 scanning electron microscope. For TEM analysis, E9.5 embryos were fixed for 24h at RT in 0.1M phosphate buffer (pH 7.4, PB) containing 2% paraformaldehyde and 2.5% glutaraldehyde. Embryos were rinsed in PB and postfixed for 2h with 2% osmium tetroxide. After a graded ethanol dehydration series and propylene oxide infiltration, embryos were embedded in Araldite resin (Durcupan, Fluka). To select the area of interest, we cut semithin sections (1.5 µm) with a diamond knife and stained with 1% toluidine blue. Subsequently, we cut ultrathin sections (60-90nm) from the area of interest with a diamond knife, stained them with lead citrate, and examined them using a Fei Tecnai Spirit electron microscope.

Plasmid construction

pGLAP5-Rer1, pGLAP5-Mks1, pGLAP5-B9d1, pGLAP5-B9d2, pEF/V5/Frt-Rer1, pEF/V5/Frt-Mks1, pEF/V5/Frt-B9d1, pEF/V5/Frt-B9d2 were created by polymerase chain reaction (PCR) amplification of the coding sequences from verified mouse cDNA clones (Open Biosystems). PCR products were TOPO cloned into the pCR8/GW/TOPO Gateway entry vector (Invitrogen), and recombined subsequently into either pGLAP5 (Addgene plasmid 19706) [29] or pEF/V5/Frt (Invitrogen) destination vectors with Clonase II (Invitrogen). The B9d2 p.Ser101Arg mutation was introduced into pCR8/GW/-B9d2 via site directed mutagenesis using Quikchange II XL (Agilent). pCR8/GW-B9d2 S101R was used to create pGLAP5-B9d2 S101R and pEF/V5/Frt-B9d2 S101R via recombination, as described above. The p.Ser101Arg mutation was introduced into human *B9D2* cDNA clone IOH4997 of the Invitrogen ORF collection using QuickChange site-directed mutagenesis (Agilent). Wild type and p.Ser101Arg *B9D2* cDNAs were cloned into pEGFP-N1 (Clontech) to generate pGFP-wtB9D2 or pGFP-B9D2-S101R. B9D2 and B9D2-S101R were subcloned into the pCS2+ Gateway destination vector (Invitrogen) from pENTR221 (Invitrogen) using Clonase II (Invitrogen) to generate pCS2-wtB9D2 or pCS2-B9D2-S101R.

Cell culture, immunofluorescence, and microscopy

hTERT-RPE1 cells were grown to 70% confluency in DMEM/F12 media supplemented with 10% FBS and transfected with pGFP-wtB9D2 or pGFP-B9D2-S101R using FUGENE6 (Invitrogen). 18h post-transfection, cells were switched to low serum (0.5% FBS) medium; 48h after serum starvation, the cells were washed with PBS and

fixed in 4% PFA, permeabilized and blocked with PBS-T (0.1% Triton-X 100) containing 10% goat serum, stained with primary antibodies as indicated, and observed with a Zeiss LSM 510 META confocal microscope.

B9d1 wild type and mutant MEFs were derived from E12.5 mouse embryos, as described, and grown in DMEM + 15% FBS [27]. For Smo localization analysis, MEFs were grown to confluency, starved in Opti-MEM (Invitrogen) for 24h and treated for 6h with 1 μ M of the small molecule Smoothed AGonist (SAG) (Enzo Life Sciences) in dimethyl sulfoxide (DMSO, Sigma). MEFs were then fixed in 4% PFA for 5' at 37°C, and -20°C methanol for 3'. For analysis of Hh pathway activation, MEFs were treated for 20 hours with 1 μ M SAG.

Antibodies employed were rabbit α -Smo (1:100), rabbit α -Ift88 (1:100, Proteintech), rabbit α -Arl13b (1: 1,5000, gift of Tamara Caspary), rabbit α -ACIII (1:100, Santa Cruz Biotechnology), mouse α -acetylated tubulin 6-11B-1 (1:1,000, Sigma), and goat α - γ tubulin (1:100, Santa Cruz Biotechnology). Anti-Smo antibodies were generated by injection of a Smo-MBP fusion protein comprising the C-terminal tail of mouse Smo into rabbits (Pacific Immunology) and affinity purified using recombinant GST-Smo C-terminus.

qRT-PCR

For analysis of Hh pathway activation, RNA was extracted from MEFs with an RNAeasy kit (Qiagen), and cDNA synthesis was performed with a Maxima first-strand cDNA synthesis kit (Fermentas). qRT-PCR was performed with Maxima SYBR Green qPCR 2x master mix with ROX (Fermentas) and primers homologous to *Ptch1*, *Gli1*, and

β-actin (Table S2) on an ABI 7900HT real-time PCR machine. Levels of *Ptch1* and *Gli* were normalized to levels of *β-actin*. The qRT-PCR of each RNA sample was performed in quadruplicate and the experiment was replicated. The fold increase is the average of the fold increase of the *Ptch1* or *Gli1* mRNA levels (normalized to *β-actin*) in the experimental treatments compared to the average of the *Ptch1* or *Gli1* mRNA levels (normalized to *β-actin*) in the vehicle only control. Error bars represent the standard deviation. Statistical significance was assessed with the Student's t-test.

Human genetics

The study was approved by the review board and ethics committee of the RWTH Aachen University and the participants gave informed consent.

Linkage analysis

DNA samples from the index family were used for linkage analysis of genes, mutant versions of which cause MKS, and related disease loci. A minimum of two informative flanking markers were typed [5]. Primers for PCR amplification were as published by the Genome Data Base. Microsatellite markers were analyzed on an ABI PRISM 3130 genetic analyzer (Applied Biosystems) with a universal fluoresceinated primer ComF [30]. Each marker was amplified in a three-primer PCR reaction in which the forward primer of the two specific primers was tailed with the sequence corresponding to ComF (specFcom). A 10:1 molar ratio of primers ComF to specFcom ensured that specFcom was exhausted during the early amplification cycles.

Mutation analysis

We sequenced the exons encoding **B9D2** (MIM 611951, Table S2) on chromosome 19q13 (GenBank: NM_030578.3, NP_085055; mutation numbering +1 corresponds to the A of the initiation codon in exon 2). Genomic DNA from a parent and an affected individual was amplified by PCR with oligonucleotide primers complementary to flanking intronic sequences (available upon request). PCR products were purified with the DNA Clean & ConcentratorTM-5 kit (Zymo Research) and sequenced with ABI BigDye chemistry (Applied Biosystems) using the PCR primers. Samples were run and analyzed on an ABI PRISM 3130 (Applied Biosystems). Prediction of the functionality of the identified non-conservative missense mutation c.301A>C (p.Ser101Arg) was performed as described using a multisequence alignment of orthologs and several bioinformatic algorithms [31,32].

Morpholino and mRNA injection of zebrafish embryos

Splice-blocking morpholino (MO) against zebrafish *b9d2* and a control MO (Gene Tools) were injected into one or two cell stage zebrafish embryos. After testing 2ng, 4ng, and 6ng of *b9d2* MO, we selected 2ng for subsequent experiments. We observed similar, but more severe phenotypes with a translation-blocking MO directed against the first ATG of *b9d2*. However, because we were unable to fully rescue the phenotype, we considered the MO unreliable and generated a splice-blocking MO. We transcribed mRNA encoding human *B9D2* WT and p.Ser101Arg from pCS2-wtB9D2 and pCS2-B9D2-S101R (SP6 Message Machine, Ambion). For functional assays in zebrafish, we coinjected 60pg wild type or mutant *B9D2* mRNA with 2ng *b9d2* MO.

Injected zebrafish embryos were classified based on phenotype. Embryos similar to age-matched controls from the same clutch were classified as normal. Embryos displaying mildly shortened body axes, mediolaterally elongated somites and notochord imperfections were categorized as Class I. Embryos displaying severely shortened body axes, mediolaterally elongated somites, and widened and kinked notochords were categorized as Class II. Embryo classifications were based on the generalized classification scheme that has been developed for other ciliopathy loci [33,34]. We compared the proportions of normal and affected embryos resulting from injections of morpholinos or mRNAs, using the chi-square test.

Immunoprecipitation and immunoblot

COS1 cells were grown on 10 cm diameter plates, transfected with pGLAP5- or pEF/V5/Frt-Rer1, Mks1, B9d1, B9d2, or B9d2 p.Ser101Arg plasmids using JetPrime (Polyplus-Transfection) and lysed after 24h in a buffer of 50mM Hepes (pH 7.4), 300mM KCl, 1mM EGTA, 1mM MgCl₂, 10% glycerol, 0.3% Nonidet P-40, 0.5mM DTT, and protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 13,000 G for 10 min, and then incubated with GFP-Trap beads (Chromotek) overnight at 4°C. Beads were washed three times in lysis buffer, resuspended in 3X SDS-PAGE loading buffer, then denatured at 100° for five minutes. Immunocomplexes were resolved on 4-15% TGX gradient gels (Bio-Rad), and transferred to PVDF membrane for immunoblotting (Bio-Rad).

For Gli3 processing, E10.5 embryos were lysed in RIPA with protease and phosphatase inhibitors. Lysates were cleared at 13,000 G for 10 minutes, and protein

concentration was assessed by Bradford assay (Bio-Rad). Proteins were resolved on 7% SDS-PAGE gels, and transferred to PVDF membranes.

For immunoblotting, membranes were blocked with 5% non-fat dry milk (NFDM) in TBS with 0.1% Tween (TBST), then incubated with primary antibodies in 5% NFDM-TBST followed by secondary antibodies in 5% NFDM-TBST. Blots were developed with either Pico enhanced chemiluminescence (ECL, Pierce) or Western lightning ECL (Perkin Elmer). Apparent molecular weights were determined using full range rainbow pre-stained protein standards (Amersham).

Primary antibodies employed were mouse α -GFP (1:1,000, Santa Cruz Biotechnology), chicken α -GFP (1:1000, Aves), goat α -V5 (1:2,000, AbCam), mouse α -Gli3 (1:5,000, gift from Suzie Scales, Genentech), and mouse α -tubulin (1:1,000, Sigma). Horseradish peroxidase conjugated secondary antibodies were donkey α -mouse (1:20,000, Jackson Labs), goat α -chicken (1:5,000, Jackson Labs), and rabbit α -goat (1:5,000, Santa Cruz Biotechnology).

Mass Spectrometry

NIH3T3 cell lines harboring stable, single-integrations of pGLAP5 vector or pGLAP5-B9d1 were created using NIH3T3 Flp-In cells according to the manufacturer's instructions (Invitrogen). Fifty 15cm diameter plates each of pGLAP5 (control) and pGLAP5-B9d1 stable cells were used for LAP purification as described [35], with the exception that GFP trap beads were used in the purification (Chromotek). Purified LAP-complexes were denatured with 3X SDS PAGE sample buffer at 100°C for 10 minutes, and were then resolved on a 4-15% Tris-glycine gradient gel (Bio-Rad).

SYPRO ruby (Invitrogen) stained gel bands were excised and digested using trypsin as reported [36], with the additional use of Protease Max (Promega) for increase peptide recovery and shorter digestion times. The alkylation reagent used was acrylamide, providing a propanamide modification.

Nano reversed phase HPLC was done with an Eksigent 2D nanoLC (Eksigent) with buffer A consisting of 0.1 % formic acid in water and buffer B 0.1 % formic acid in acetonitrile. A fused silica column self packed with duragel C18 matrix (Peeke) was used with a linear gradient from 5 % B to 40 % B over 60 minutes at a flow rate of 600 nL/minute. The nanoHPLC was interfaced with a Michrom Advance source. The mass spectrometer was an LTQ-Orbitrap Velos (Thermo Scientific), which was set in data dependent acquisition mode to perform MS/MS in the ion trap of the top eight most intense ions. A full scan in the Orbitrap of the precursor ion was done at a resolution of 60,000 at 400 m/z . The .RAW files were then converted to mzXML format using msconvert script and searched with a Sorcerer (SageN) processor using Sequest. The database was searched with a precursor mass tolerance of 50 ppm.

All hits with three or more identified peptides in the B9d1LAP purification and zero or one peptide identified in the control purification were reported.

Modeling of the B9D2 C2-domain

A model of the B9D2 C2 domain, based on the calcium-free piccolo C2a domain, was used as a template for electrostatic surface modeling using adaptive poisson-boltzmann solver (APBS) in the visual molecular dynamics software (VMD) [37,38]. A protein data-bank (PDB) file of the modeled B9D2 C2 domain produced by ModBase

was converted to a PQR file with PDB2PQR before applying APBS in VMD [39-41]. Software defaults were used in the electrostatic surface modeling to color positive residues blue and negative residues red.

RESULTS

Mouse *B9d1* mutants display developmental defects similar to those of MKS

Given the importance of *Mks1* and *B9d2*, two genes encoding B9 domain proteins, to ciliary structure, we investigated the function of the third member of this family, *B9d1* [13,14,42]. We obtained a targeted allele of *B9d1* (*B9d1*^{tm1a(EUCOMM)Wtsi}) from the European Conditional Mouse Mutagenesis Program (EUCOMM) that contains a gene trap in the second of six introns (Figure S2A). The resultant mutant allele is predicted to encode a fusion between the first 44 amino acids and a βgeo reporter. Given that the wild-type transcript is almost completely abrogated in homozygous *B9d1* mutant embryos (Figure S2B), the *B9d1* gene trap is likely to create a null or near null allele. In support of this conclusion, we also analyzed mice homozygous for a deletion of *B9d1* exon 3, which encodes a large portion of the B9 domain, and these mutants displayed phenotypes indistinguishable from those homozygous for the gene trap allele (data not shown).

B9d1 mutants die at approximately E14.5 (Table 1). Mutant phenotypes were assessed in the strain of derivation, C57/Bl6, where possible, and in a mixed C57/Bl6-CD1 strain to observe later stages of development, as homozygous mutant embryos on the mixed background die between E17.5 and P1. At E18.5, these *B9d1* mutant mice

displayed kidneys that were larger than wild type and contained multiple cystic lesions in all of the mutants examined (n=5/5; Figure 1A). Dilation and cystic pathology was apparent in multiple nephron segments (Figure S3). Kidney tubules were well ciliated in wild-type animals, whereas the tubules were enlarged and cilia were reduced in number and length in *B9d1* mutants (Figure 1B). During liver development, hepatic ductal plates form as rings around portal veins and are subsequently remodeled, positioning bile ducts centrally adjacent to portal veins by birth [43]. In contrast, ductal plates persisted as peripheral rings in *B9d1* mutant livers at P1, and hyperplastic portal mesenchyme separated enlarged bile ducts from portal veins (Figures 1C-D). Cilia were present and similar in length in wild type and mutant bile ducts despite the occurrence of ductal plate malformation (Figure 1E).

B9d1 mutants also developed preaxial polydactyly with either one or two extra digits, with 92% (n=23/25) penetrance in hindlimbs and 24% (n=6/25) penetrance in forelimbs (Figure 1F). We also observed occasional exencephaly, although that phenotype showed incomplete penetrance (n=3/49; Figure 1G). Thus, *B9d1* mouse mutants display renal cystic dysplasia, the defect most commonly associated with MKS, as well as ductal plate malformation of the liver and polydactyly, other prominent characteristics of MKS.

In addition to displaying hallmarks of MKS, *B9d1* mutants display dextrocardia with a frequency of 44% (n=20/45), suggesting that heart looping is randomized in the mutant embryos (Figure 1H). We observed additional phenotypes associated with human ciliopathies in *B9d1* mutants at varying frequencies, including holoprosencephaly (Figure

1H), microphthalmia (n=32/37; Figure 1G), cleft palate (n=9/19; Figure S4), ventricular septal defect (VSD), and thinning of the myocardial wall (Figure S5).

Randomized heart looping can be due to disruptions in fluid flow in the embryonic node [44]. Because beating cilia propel nodal flow leftward, mutations in ciliogenic genes such as *Kif3a* and *Ift88* disrupt left-right patterning [45,46]. To assess whether defects in ciliogenesis might underlie the randomized heart looping observed in *B9d1* mutant mice, we examined nodes using both immunofluorescence and scanning electron microscopy (SEM). *B9d1* mutants exhibit normal node morphology, but lack almost all nodal cilia (Figures 2A-D); the few cilia that do form were short and displayed swollen tips (Figure 2D). In addition to analyzing nodal cilia, we examined the expression pattern of *Lefty* genes, early molecular markers, and genes implicated in the establishment of left-right patterning [28,47-49]. In response to nodal flow *Lefty1/2* are expressed in the floorplate and left lateral plate mesoderm (LPM) of E8.0 embryos. Expression of *Lefty1/2* was absent in *B9d1* mutant embryos at E8.0 (n=2/2; Figure 2E), which is similar to other mutants in which L-R patterning is disrupted at an early stage of axis specification [44,50-54]. We surmise that the absence of nodal cilia is the likely cause of the laterality and heart looping defects of *B9d1* mutants.

Mouse B9d1 is required for Hh signaling and ciliary protein localization

Cilia are essential for the transduction of several intercellular signals, most notably Hh during neural tube development [23,24,55-59]. *B9d1* mutants lack cilia in the neural tube (Figure 2F) and exhibit holoprosencephaly (Figure 1H), a phenotype associated with abrogated Hh signaling [60]. Further analysis of the neural tube by

transmission electron microscopy (TEM) revealed that basal bodies in *B9d1* mutant embryos dock properly at the apical surface of the cell yet do not form axonemes (Figures 2G and 2H).

To test whether Hh signaling is affected in *B9d1* mutants, we examined patterning of the ventral neural tube [61]. During neural tube development, high levels and durations of Hh signaling specify the ventral most cell fates, including the floor plate, while moderate Hh signaling specifies more dorsal neural subtypes, including motor neurons [62,63].

Molecular analysis of FoxA2 expression, a marker of the floor plate, indicated that the floor plate is reduced in *B9d1* mutants (Figure 3A). Similar to the floor plate, high levels of Hh signaling are required for the induction of adjoining V3 interneuron precursors [64,65]. Analysis of Nkx2.2, a marker of V3 interneuron precursors, revealed reduced expression in *B9d1* mutants (Figure 3B). Additionally, *B9d1* mutants showed a variable reduction in the number of motor neurons, as indicated by Islet1/2 expression (Figure 3C). The loss or reduction of ventral neural marker expression in *B9d1* mutants was accompanied by the ventral expansion of Pax6 expression (Figures 3A-C), normally restricted to the dorsal neural tube by Hh signaling [66]. Together, these results indicate that *B9d1* is required for high and moderate level Hh signaling in the neural tube.

In addition to their roles in promoting activation of the Hh transcriptional program, cilia are essential for formation of the transcriptional repressor form of Gli3 (Gli3R) [22,59]. Unlike the neural tube, Shh controls anteroposterior limb bud patterning principally by inhibiting the formation of Gli3R. Shh is secreted from the zone of polarizing activity in the posterior of the limb bud, creating an opposing gradient of

Gli3R decreasing from anterior to posterior [67] Loss of Gli3 results in polydactyly, similar to Shh gain of function [68,69]. Mice with defects in ciliogenesis can display preaxial polydactyly, attributable to defects in Gli3R formation [22,58,59,70].

The presence of preaxial polydactyly in *B9d1* mutants (Figure 1F) was consistent with defective Gli3 processing. *B9d1* mutants typically developed one or two supernumary preaxial digits on hindlimbs and occasionally on forelimbs, a limb phenotype more pronounced than that reported for *Mks1* mutants [13,15]. To assess whether B9d1 is required for Gli3 processing, we analyzed Gli3 in *B9d1* heterozygote and mutant mouse embryo lysates. Full length Gli3 was stabilized in mutant embryos compared to wild type (Figure 4A), consistent with reduced Hh responsiveness.

In response to Hh ligand, Smoothened (Smo), a positive-regulator of Hh signaling, translocates into primary cilia and activates the pathway [20]. To examine further the role of B9d1 in Hh signal transduction, we analyzed the localization of Smo to cilia in response to Smo AGonist (SAG). Similar to the cilia status of bile ducts, mouse embryonic fibroblasts (MEFs) derived from *B9d1* mutant embryos were ciliated (Fig. 4B). In response to SAG treatment, Smo localized to cilia in WT MEFs but not *B9d1* mutant MEFs (Figure 4B and Figure S6), consistent with reduced Hh responsiveness. We also quantified the change in mRNA levels of *Gli1* and *Ptc1*, general transcriptional targets of Hh signaling, in MEFs treated with SAG. *B9d1* mutant MEFs treated with SAG showed blunted increases in *Gli1* and *Ptc1* activation as compared to WT SAG-treated MEFs (Figures 4C and 4D). Together, these results indicate that *B9d1* is required for robust responsiveness to Hh signals even in cells that do not require B9d1 for ciliogenesis.

Given the failure of Smo to move to MEF cilia in response to SAG, we asked whether other ciliary proteins might fail to localize properly to the cilia of *B9d1* mutant MEFs. Whereas the intraflagellar transport protein Ift88 localized to both WT and *B9d1* mutant MEF cilia, the small GTPase Arl13b and the adenylate cyclase ACIII were only found in WT MEF cilia (Figures 4E-G and Figure S6) [22,55,71]. This protein localization defect is similar to that seen upon disruption of *Tctn1*, *Tctn2*, *Tmem67*, or *Cc2d2a*, components of a larger transition zone complex that also contains B9 proteins (Table S3) [72]. Thus, *B9d1* is required for the ciliary localization of a discrete subset of proteins, including Smo, Arl13b, and ACIII.

***B9D2* is a candidate MKS locus**

B9d2, a B9 protein related to *B9d1*, localizes to the basal body and the ciliary axoneme, and loss of *B9d2*, either in vivo or in cultured cells, results in ciliogenic defects [11,14,42]. In addition, conditional inactivation of *B9d2* in the kidney produces cysts similar to those of *Mks1* and *B9d1* mutant mice [13-15]. The phenotypic overlap observed between *Mks1*, *B9d1* and *B9d2* mutant mice, and the evolutionarily co-occurrence of these genes, suggested that human *B9D1* and *B9D2* variants could cause MKS.

To test this hypothesis, we used a combination of homozygosity mapping and candidate gene sequencing to screen *B9D1* and *B9D2* in 96 unrelated MKS patients. No regions of homozygosity overlapping with *B9D1* segregated with MKS phenotypes in our patient cohort, and sequencing analysis without bias for linkage or homozygosity did not identify any non-synonymous changes that would be consistent with a pathogenic role of

this gene in our cohort of MKS patients. We note, however, that a recent study reported a *B9D1* mutation in an individual with MKS [73]. We identified a fifth-degree consanguineous multiplex family from Surinam with Indian-Pakistani background (Figure 5A), in which two affected fetuses displayed overlapping phenotypes including cystic kidneys, ductal plate malformation, polydactyly and occipital encephalocele (Figures 5B-M). Linkage analysis demonstrated identical homozygous haplotypes in both patients (Figure 5A) that were distinct from the haplotypes of the unaffected son (Figure 5A), but no regions of homozygosity in other known MKS disease genes. Sequencing revealed a homozygous, non-conservative missense mutation (c.301A>C) in *B9D2* resulting in a serine to arginine substitution (p.Ser101Arg) in both affected individuals (Figure 5N). This change segregated with the MKS phenotype in this family, has not been described in all accessible databases of human genome variation, and was not present among 300 ethnically matched control chromosomes or 388 control chromosomes from other ethnic backgrounds.

Residue 101 is invariantly serine in chordate B9D2 proteins, and is either a serine or threonine in the B9D2 orthologs of ciliated protists *Chlamydomonas*, *Naegleria* and *Batrachochytrium* (Figure 5O). This high degree of conservation suggests that serine 101 is important for B9D2 function, and that substituting this small, polar, non-ionizable residue with a large, basic residue such as arginine may not be tolerated (Figure S7). Similarly, *in silico* analysis predicted that this mutation is pathogenic (Table S1).

The candidate MKS p.Ser101Arg mutation causes loss of B9D2 function

We have shown previously that morpholino (MO) suppression of *mks1* in zebrafish results in developmental defects that include shortened body axis, broad notochords, long, thin somites, and failure of tail extension [34]. Further, we and others have demonstrated the tractability of *in vivo* rescue in this system to determine the pathogenic effect of alleles found in ciliary genes with high specificity and sensitivity [74-76]. To test the pathogenicity of the *b9d2* MKS allele, we generated a splice-blocking MO against zebrafish *b9d2* and attempted rescue with control or mutant human *B9D2* mRNA. Injection of increasing amounts (2, 4, 6 ng) of *b9d2* MO resulted in dosage dependent phenotypes that can be grouped into three classes based on previously reported criteria (normal, class I and class II) [34]. Class I embryos displayed mildly shortened body axes, mediolaterally elongated somites and notochord imperfections; class II embryos displayed severely shortened body axes, mediolaterally elongated somites, and widened and kinked notochords (Figure 6A and Figure S8).

The specificity of the MO-induced phenotypes was confirmed by co-injection of human *B9D2* mRNA. Upon masked scoring of 50-150 embryos per injection, we observed that whereas 29% of the *b9d2* morphants were of class I and 21% were of class II, co-injection of *b9d2* morpholino with 60 pg human *B9D2* mRNA reduced the prevalence of class I to 14% and of class II to 4%. In contrast, co-injection of *b9d2* morpholino with 60 pg of human *B9D2* c.301A>C mRNA yielded morphants of which 33% were of class I and 14% were of class II. The failure of *B9D2* c.301A>C to reduce the frequency of *b9d2* morpholino phenotypes suggests that the MKS-associated mutation is a strong hypomorphic or functional null allele (Figure 6B).

Effect of the *B9D2* mutation on protein function

To investigate the pathogenicity of the *B9D2* mutant allele and the mechanisms by which it may result in ciliary dysfunction and human disease, we explored the potential consequences of the p.Ser101Arg amino acid substitution in mammalian cells. *B9D2* has been shown to localize primarily to the basal body and, under certain conditions, to the axoneme of the primary cilium [11]. We therefore tested whether introduction of the Arg101 residue affects *B9D2* localization. Upon expression of GFP-*B9D2* and GFP-*B9D2* S101R in hTERT-RPE1 cells, we found that both localize to the basal body (Figure 7), rendering mislocalization an unlikely cause of disease and pointing instead to potential biochemical causes.

The phenotypic overlap caused by loss of any of the three mouse B9 proteins raised the possibility that the B9 proteins promote ciliogenesis by acting together in a non-redundant fashion. Similarly, the presence of MKS-associated mutations in *B9D1*, *B9D2* and *MKS1* suggests that these proteins may function cooperatively in humans, reminiscent of the related ciliopathy, Bardet-Biedl syndrome (BBS [MIM 209900]), in which the products of several causative genes can form a complex [77]. To investigate whether B9 proteins might similarly form a MKS-related complex, we assessed whether tagged B9 proteins coimmunoprecipitated. GFP-tagged versions of Mks1, B9d1, and B9d2 coimmunoprecipitated V5-tagged versions of the other B9 proteins (Figure 8). GFP tagged B9 proteins did not coimmunoprecipitate with the V5 vector or a V5-tagged control protein, Rer1, indicating that the interaction of the B9 proteins with each other is specific.

To confirm further the observed interactions, we created NIH-3T3 cell lines harboring stable, single integrations of a control plasmid expressing the localization and affinity purification (LAP) tag (pGLAP5) or a plasmid expressing B9d1 with a carboxy-terminally fused LAP (pGLAP5-B9d1). We tandem affinity purified LAP proteins from the control and B9d1-LAP-expressing cells, separated them by gel electrophoresis, sectioned the gel lanes and subjected them to mass spectrometry [29,35]. Mass spectrometric analysis detected abundant peptides of Mks1 (50 peptides with 37% coverage) and B9d2 (32 peptides with 42% coverage) among the proteins that co-purified with B9d1-LAP. We also identified a number of interacting proteins that have been suggested recently to be part of a larger transition zone complex functioning within the cilium (Table S3) [72].

To determine how the p.Ser101Arg mutation affects B9D2 activity, we asked whether this mutation alters B9 protein complex formation. Reciprocal coimmunoprecipitations indicated that B9d2 p.Ser101Arg failed to interact with Mks1, without compromising its interaction with B9d1 (Figure 8). These results indicate that the B9 proteins interact physically and that the MKS-associated mutant form of B9d2 disrupts its interaction with Mks1, thus compromising the integrity of this ciliogenic complex. Therefore, we propose that mutations that disrupt the interactions among components of the B9 complex result in human MKS.

DISCUSSION

In the last decade, our understanding of the role of primary cilia in human disorders has benefited from the identification of a burgeoning group of ciliopathy-associated genes. In this study, we showed that disruption of *B9d1* in the mouse perturbs ciliogenesis and ciliary protein localization, abrogates Hh signaling and results in phenotypes that fall on the severe end of the ciliopathy clinical spectrum, phenotypes similar to defects associated with MKS. In addition, we identified a non-conservative missense mutation in *B9D2* in individuals affected with MKS. Finally, we demonstrated that the B9 domain containing proteins physically interact, and that the MKS-associated mutant form of B9d2 has diminished function and reduced capacity to interact with Mks1, previously implicated in MKS [2,78]. Together, these results suggest that a complex of B9 proteins cooperatively supports mammalian ciliogenesis and ciliary protein localization, and abrogated complex function results in MKS.

B9d1 is essential for ciliogenesis, ciliary protein localization and Hh signal transduction

Ciliary defects compromise the response to Hh signals in vertebrates by abrogating both the ability of Smo to activate Gli2, and Gli3R formation [59,79]. The Hh-related phenotypes of *B9d1* mutants include dorsalization of the neural tube consistent with decreased Gli2 activity, as well as limb bud patterning defects associated with disrupted Gli3 processing. In MEFs, B9d1 is dispensable for ciliogenesis, but is required for robust response to the Hh pathway activator SAG. This likely reflects the requirement for B9d1 for the movement of Smo in response to pathway activation, and

supports the contention that some MKS phenotypes, such as polydactyly, are due to disruptions in Hh signal transduction [15].

As cilia may participate in multiple signaling pathways, other manifestations of MKS may be due to defects in intercellular communication apart from Hh signaling [33,80-82]. In addition to Smo, B9d1 is essential for the ciliary localization of Arl13b and ACIII. Similar defects in ciliary membrane composition are observed in mouse mutants affecting B9d1-interacting proteins [72]. It is likely that alterations in ciliary membrane composition affect diverse ciliary functions, resulting in some of the pleiotropic phenotypes associated with MKS.

Mutation of *B9D2* is associated with MKS

We detected a homozygous missense mutation affecting a highly conserved residue of B9D2 in the affected MKS individuals of a single pedigree, suggesting that B9D2 is essential for human development. In addition to the human genetic data, the failure of B9D2 bearing the p.Ser101Arg mutation to rescue zebrafish *b9d2* morphants or to interact with Mks1 indicates that this mutation abrogates function.

A significant fraction of cases of other genetically heterogeneous ciliopathies, such as nephronophthisis and BBS, are caused by private mutations [83-85]. Our work indicates that this may be true for MKS as well. Human genetic studies with limited pedigrees are insufficient to prove the causality of alleles and, by extension, the causal role of specific genes to the phenotype. However, testing the allele in a physiologically relevant system provides insight into its function. In the case of the MKS-associated

allele of *B9D2*, we used three approaches to provide evidence of causality: human genetics, *in vivo* rescue and biochemical interaction data.

Although we did not detect *B9D1* mutations in our cohort of 96 MKS patients, a recent study identified *B9D1* mutations as a cause of MKS [73]. If *B9D1* mutations result in early gestational lethality, as exhibited by the *B9d1* mutant mice, *B9D1* mutations in postnatal MKS cases may not reflect the prevalence of the alleles.

The B9 domain of B9D2 promotes interaction with MKS1

It is striking the B9 domain containing proteins are found only in organisms that form cilia, and that all B9 proteins are involved in ciliary functions in both *C. elegans* and vertebrates [11-15]. The p.Ser101Arg mutation of B9D2 identified in MKS individuals occurs within the B9 domain, suggesting that it is critical to protein function. The B9 domain is related to the C2 domain involved in calcium-dependent lipid binding present in several other MKS proteins, including MKS5 (RPGRIP1L) and MKS6 (CC2D2A) [86]. Despite the possibility that MKS1, B9D1, and B9D2 suggests share biochemical activities with C2 domain proteins, the overlapping ciliary defects exhibited by the mouse B9 mutants suggest that they are not functionally redundant either with each other or with C2 domain proteins [13,15].

The MKS-associated mutation in the B9 domain of B9D2 does not destabilize the protein, interfere with its localization to the ciliary base, or compromise its interaction with B9d1. Instead, the inability of the mutant form of B9d2 to physically interact with Mks1 reveals that the mutation compromises formation of the B9 protein complex. These

results suggest that B9 domains may support protein-protein interactions, and that disruption of the B9 protein complex may underlie some forms of MKS.

B9 proteins have nonredundant functions in development

In support of the contention that B9 proteins function together in a complex, *B9d1* mutant mice display phenotypes associated with the MKS1 variant of MKS, including cleft palate, holoprosencephaly, microphthalmia, and polydactyly [2,16-18]. Furthermore, the two MKS fetuses harboring *B9D2* mutations displayed polydactyly. The late gestational MKS fetus, in addition to occipital encephalocele, exhibited anencephaly and a sacral dimple, reflecting neural tube closure defects.

Mutations in each B9 gene in humans can be associated with MKS, and loss of each B9 protein in mice causes phenotypes consistent with MKS [2,13-15,73]. However, the descriptions of the three B9 mouse mutant phenotypes are not identical. The *B9d2* null phenotype has not been described, but conditional deletion of *B9d2* in the nervous system and kidneys caused kidney cysts, hydrocephalus, and a loss or stunting of cilia in the CNS [14].

Two distinct *Mks1* mutations cause cystic kidneys, polydactyly, neural tube defects, reduced skull ossification, other skeletal abnormalities, and hepatic defects. *Mks1^{krc}* is a splice-site mutation that results in deletion of the B9 domain, and *Mks1^{del64-323}* is an in frame deletion of exons 3-10 that removes the first nine amino acids of the B9 domain [13,15]. Despite extensive phenotypic similarities, the two mutants are not indistinguishable. At E10.5 *Mks1^{krc}* mutants did not display gross abnormalities, while *Mks1^{del64-323}* mutants displayed expansion of the mesencephalic flexure, reduced

telencephalon size and microphthalmia, similar to *B9dl* mutants [13,15]. Moreover, *MksI^{del64-323}* mutants exhibited neural tube patterning defects similar to those of *B9dl* mutants, and more profound than those of *MksI^{krc}* mutants [13,15]. As *MksI^{krc}* phenotypes were assessed on a C3H or mixed C3H/CD1 background, and *MksI^{del64-323}* phenotypes were assessed on a C3H or C57Bl/6 background (C. Lo, personal communication), the phenotypic differences may reflect differences in allele strength or modification by background-dependent loci [13,15].

The relationship of ciliary dysfunction and ciliopathies

In addition to MKS, defects in the function of primary cilia are thought to underlie related disorders including BBS, Leber congenital amaurosis (LCA [MIM 204000]), and Senior-Loken syndrome (SLSN [MIM 266900]). Several of these disorders share retinal degeneration as a feature, but include other characteristics such as obesity, polydactyly and renal abnormalities (BBS), midface hypoplasia (LCA), or nephronophthisis, a cystic kidney disorder (SLSN). Further examples of ciliopathies include Joubert syndrome (JBTS [MIM 213300]), characterized by hypoplasia of the cerebellar vermis, and Orofaciodigital syndrome (OFD [MIM 311200]), characterized by malformation of the face, mouth and digits. It is not clear how ciliary dysfunction results in such diverse phenotypes [87].

Moreover, some ciliopathy genes display extensive allelism. For example, mutations in *CEP290* are associated with MKS, JBTS, SLSN, LCA, and BBS [88]. Although some *CEP290* mutations may affect discrete functions or compromise activity to different degrees, other *CEP290* mutations are associated with more than one

ciliopathy, suggesting that modifier effects may account for the distinct resultant phenotypes [88,89].

In support of the possibility that ciliary modifier loci have strong effects on expressivity, we observed that mouse *B9d1* mutants with C57/Bl6 or mixed CD1-C57/Bl6 backgrounds displayed distinct phenotypes. It will be interesting to assess whether other *B9D1* or *B9D2* mutations underlie ciliopathies related to MKS, such as JBTS, and whether weaker alleles of *B9* genes modify other ciliopathies.

A second possible explanation for the origins of different ciliopathy phenotypes is that different ciliogenic protein complexes have distinct functions, affecting ciliogenesis to different extents or in different tissues. In support of this possibility, *B9* mutants display tissue-specific defects in ciliogenesis and ciliary protein mislocalization. For example, in mouse *B9d1* mutants, cilia are absent in the node and neural tube, reduced in number and length in cystic kidneys, and present in bile ducts and MEFs. Similarly, in mouse *Mks1* mutants, cilia are reduced in number and length in the node, limb buds, and neural tube, but not in lung airways and bile ducts [15].

As in the mammalian bile duct, B9 proteins are not required for ciliogenesis in *C. elegans*, as nematodes mutant for the orthologs of *Mks1*, *B9d1*, *B9d2*, or a combination of all three do not display overt structural ciliary defects [11,12]. The *C. elegans* orthologs of B9 proteins are, however, required for cilia-dependent sensory functions [12].

Thus, B9 proteins are required for the formation of cilia in some tissues and organisms, and are required for ciliary function in others. As B9d1 is essential for the localization of a subset of ciliary proteins, defects in ciliary protein composition are likely to underlie some intercellular signaling abnormalities in mouse *B9* mutants. The

similarity of mouse *Mks1* and *B9d1* mutant phenotypes to human MKS suggests that MKS may not be caused by the absence of cilia, but by tissue-specific ciliary loss and dysfunction.

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WEB RESOURCES

Online Mendelian Inheritance in Man (OMIM) <http://www.omim.org/>

HUGO Gene Nomenclature Committee (HGNC) <http://www.genenames.org/>

Genome Database (GDB) <http://www.gdb.org/>

ModBase <http://modbase.compbio.ucsf.edu>

PDB2PQR Program <http://kryptonite.nbcn.net/pdb2pqr/>

SIFT http://sift.jcvi.org/www/SIFT_seq_submit2.html

AlignGVGD <http://agvgd.iarc.fr/index.php>

Mutation Taster <http://www.mutationtaster.org>

European Conditional Mouse Mutagenesis Program (EUCOMM)

<http://www.eucomm.org/>

Figure 1. *B9dl* mutants display defects similar to those of MKS. (A) Coronal sections of E18.5 kidneys stained with H&E. Mutant kidneys are larger and contain multiple cysts. Scale bar, 500µm. (B) P1 kidney tubules stained with a marker of cilia, acetylated tubulin (AcTub, red), and nuclei (DAPI, blue). *B9dl* mutant tubules possess fewer cilia than wild type tubules. Dotted lines mark the borders of tubules. Scale bar, 25µm. (C) P1 liver sections stained with H&E. Scale bar, 100µm. (D) Liver sections stained with a marker of bile ducts, Sox9 (red), E-cadherin (green), and nuclei (DAPI, blue). The ductal plate (arrowheads) fails to remodel in mutant livers, resulting in an abundance of portal mesenchyme (arrow), and separation of bile ducts (asterisks) from the central portal vein (pv). Scale bar, 50µm. (E) Bile ducts stained for cilia (AcTub, red) and nuclei (DAPI, blue). Cilia are present in mutant bile ducts. Scale bar, 10µm. (F) E13.5 forelimbs (top) and hindlimbs (bottom) stained with alcian blue, showing preaxial polydactyly. (G) Left lateral view of E11.5 embryos. *B9dl* mutants exhibit reduced telencephalons, microphthalmia and occasional exencephaly (arrow). (H) Frontal view of E10.5 embryos. *B9dl* mutants display midline facial defects, randomized heart looping and holoprosencephaly. Scale bars for F-H, 1mm.

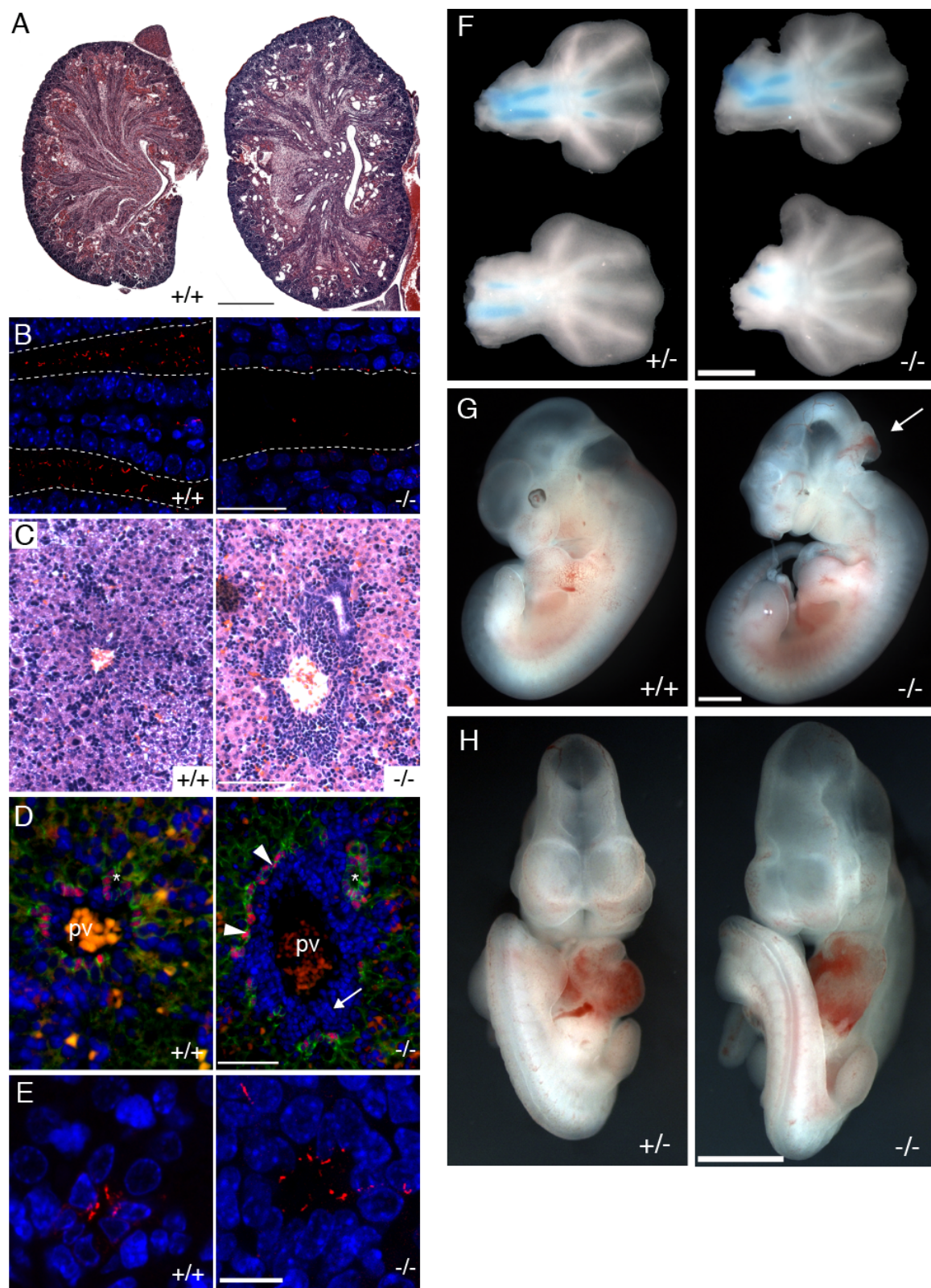


Figure 2. *B9dl* mutants have few and abnormal cilia and do not express an early marker of LR patterning. (A) Ventral view of E8.0 nodes with anterior oriented upward, depicting cilia (AcTub, green), basal bodies (Ninein, red) and nuclei (DAPI, blue). Scale bar, 50µm. Few cilia are present in *B9dl* mutant nodes, while the number of basal bodies is unaffected. (B-D) Scanning electron micrographs of E8.0 nodes reveal a reduction in the number of cilia in *B9dl* mutants. (B) The morphology of the node is normal in *B9dl* mutants. Scale bar, 20µm. (C) There is a dramatic reduction in the number of cilia in *B9dl* mutant nodes. Scale bar, 5µm. (D) The few cilia that form in *B9dl* mutant nodes are short and can display bulges at their distal tips. Scale bar, 2µm. (E) Frontal view of E8.0 embryos stained for expression of *Lefty1/2*. Expression is absent in the midline and left lateral plate mesoderm of *B9dl* mutant embryos. Scale bar 500µm. (F) Transverse sections of E10.5 embryos showing the ventral neural tube and perineural mesenchyme stained with a marker of cilia (Arl13b, green) and nuclei (DAPI, blue). Scale bar, 50µm. *B9dl* mutants show a reduction in the number of cilia in both tissues. (G,H) Transmission electron micrographs of cilia in E9.5 neural tubes. Scale bar, 500nm. (G) Serial sections through a *B9dl* heterozygous E9.5 neural tube cilium. (H) Serial sections through a *B9dl* mutant E9.5 neural tube cilium reveal a docked basal body, a disrupted axoneme, and a bulging ciliary membrane.

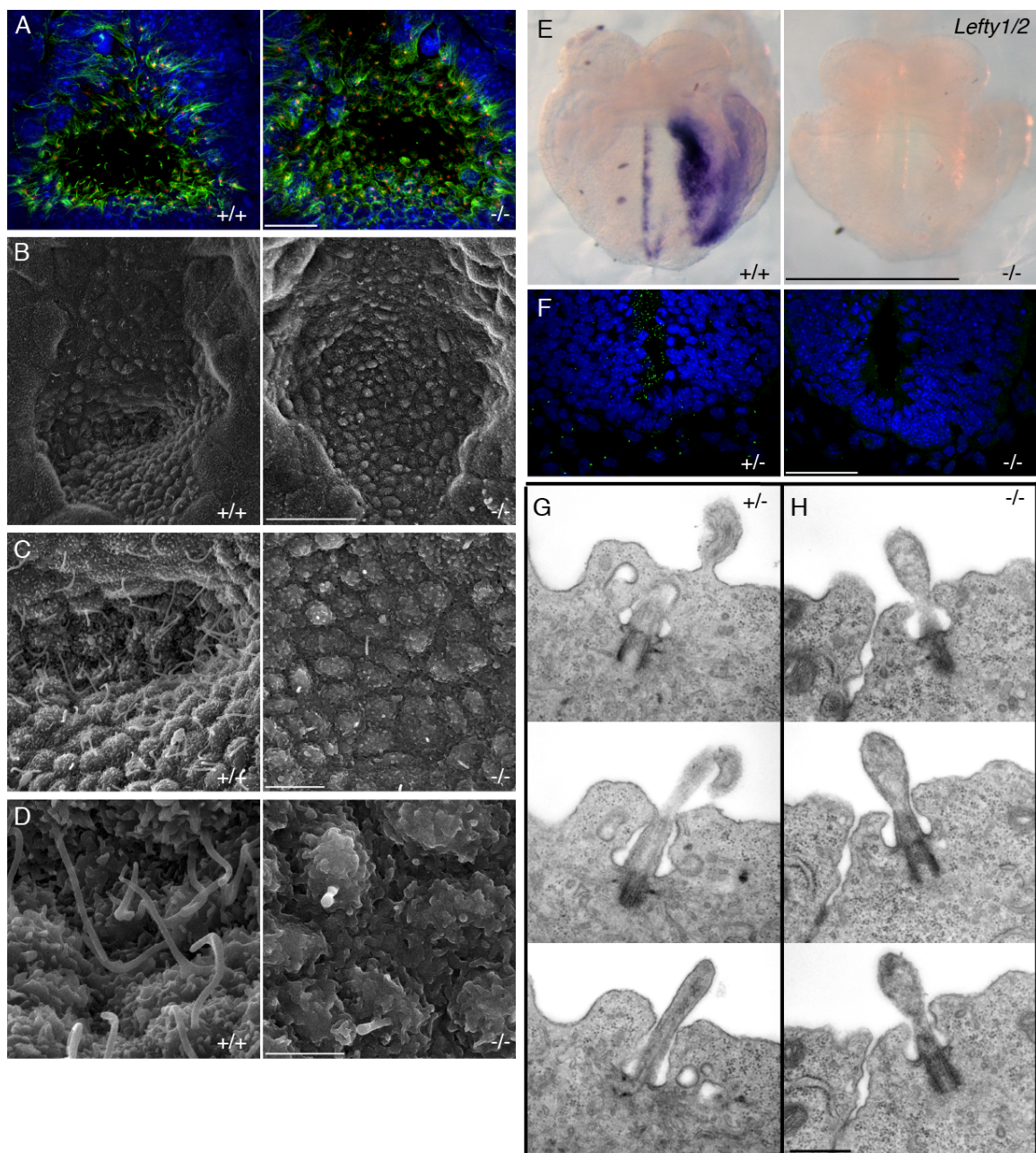


Figure 3. *B9dl* is required for Hh-dependent neural tube patterning. (A-C)

Transverse sections of E9.5 embryos stained for expression of Pax6 (red) and, in green,

FoxA2 (A), Nkx2.2 (B), or Islet1/2 (C). Nuclei are visualized with DAPI (blue). **(A)**

B9dl mutants show reduction of the floorplate marker FoxA2 and ventrally expanded

Pax6 expression. **(B)** *B9dl* mutants have reduced expression of the V3 interneuron

precursor marker Nkx2.2. **(C)** Expression of motor neuron marker Islet1/2 is reduced and

ventrally shifted in *B9dl* mutants. Scale bar, 100µm.

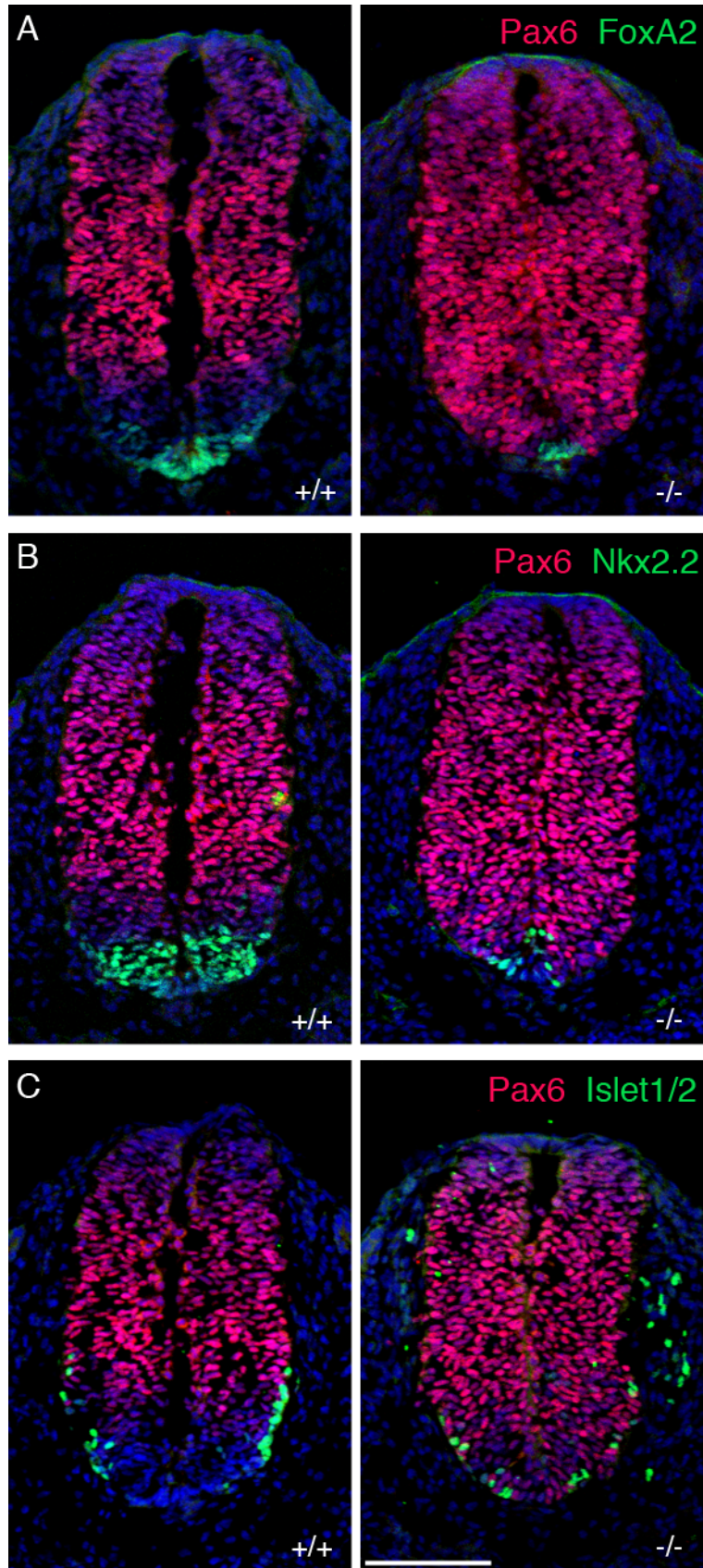


Figure 4. Hh signaling and protein localization defects in *B9d1* mutants. (A) Gli3 processing was assessed in E10.5 *B9d1* heterozygote or mutant embryo lysates. Full length *Gli3* is stabilized in mutant lysates compared to the heterozygotes. (B) *B9d1* wild type and mutant MEFs stained for cilia (AcTub, blue), basal bodies (γ -Tubulin, green) and Smo (red) in response to SAG treatment. Smo fails to localize to *B9d1* mutant MEFs treated with SAG. Scale bar, 2.5 μ m. (C,D) *Gli1* and *Ptc1* mRNA levels of *B9d1* wild type and mutant MEFs stimulated with SAG. The transcriptional response to SAG treatment is abrogated in *B9d1* mutant MEFs. Y-axis values represent the fold change of mRNA compared to wild type MEFs treated with vehicle only. Error bars represent the standard deviation. Asterisk indicates $p < 0.0001$. (E-G) *B9d1* wild type and mutant MEFs stained for cilia (AcTub, blue), basal bodies (γ -Tubulin, green) and the ciliary proteins (red) Ift88 (E), Arl13b (F), and ACIII (G). Arl13b and ACIII fail to localize to *B9d1* mutant cilia. Scale bar, 2.5 μ m.

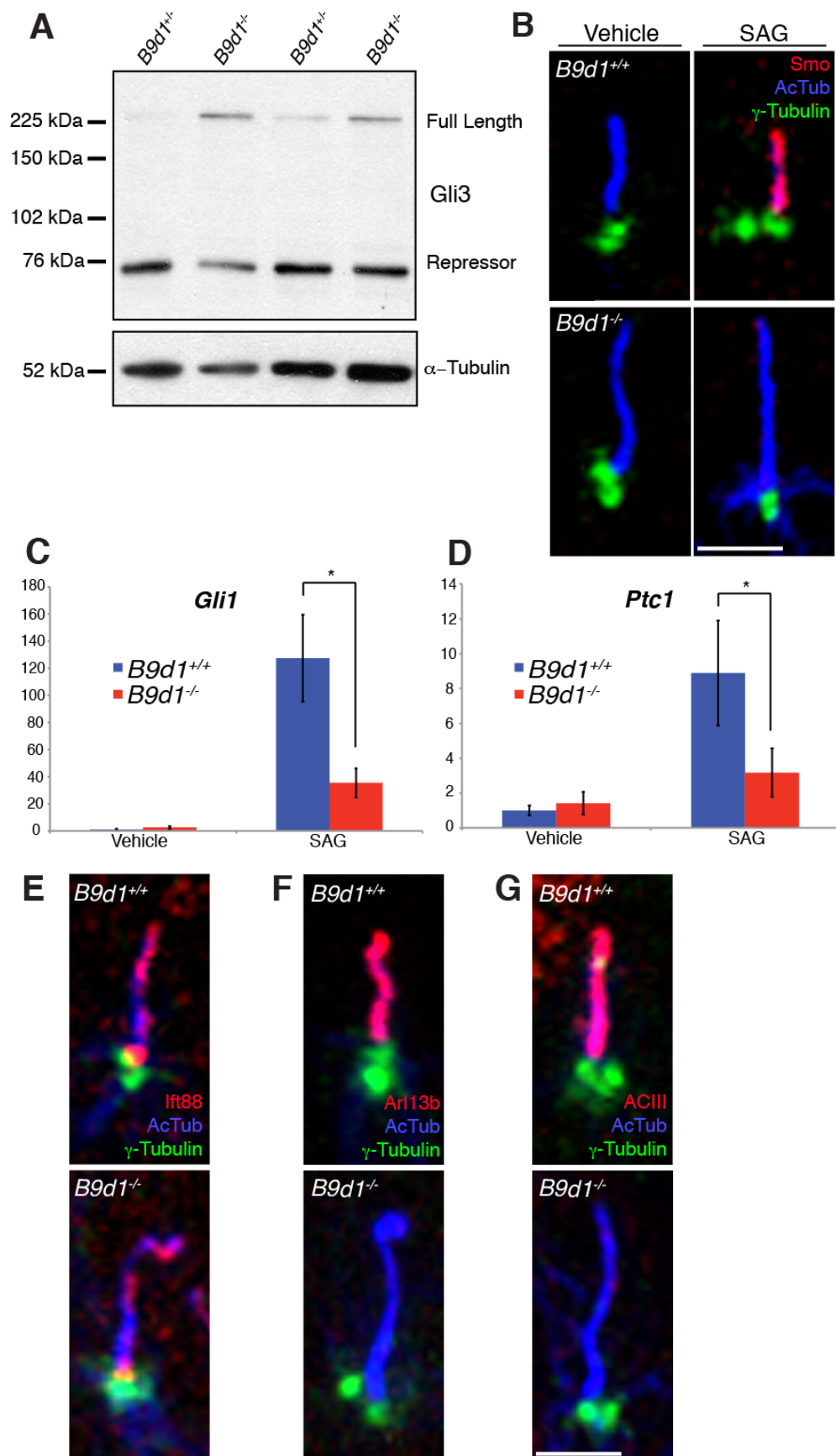


Figure 5. A *B9D2* mutation is associated with MKS. (A) Pedigree structure of a fifth-degree consanguineous multiplex family, which includes two affected MKS individuals harboring a homozygous point mutation in *B9D2* (c.301A>C). (B,C) Frontal and lateral views of an affected fetus aborted at the 17th gestational week due to occipital encephalocele. (D,E) Polydactyly of the hands and feet. (F) H&E stained kidney section shows renal cysts. (G) H&E stained liver section reveals ductal plate malformation. (H) Cytokeratin-19 staining shows defective modeling and persistence of bile duct remnants, confirming the ductal plate malformation (asterisks). (I,J) Affected fetus aborted at the 29th gestational week. Postmortem analysis confirmed anencephaly, with occipital encephalocele, sacral dimple, and postaxial polydactyly of both hands and feet. (K) H&E stained cystic kidney section shows reduction of glomeruli and tubuli. (L) H&E stained kidney section at the level of the medulla showing some small cysts (arrows). Compared to normal fetal kidney control samples, the affected renal tissue showed less glomeruli. The Bowman's capsule was not clearly dilated and the proximal renal tubuli seemed normally developed. Immunohistochemistry with epithelial membrane antigen (EMA) and CD15 antibodies (data not shown) demonstrated that cyst formations mainly affected the distal tubuli, while some small renal cysts were also seen at the medullary level. (M) H&E stained liver section reveals fetal hematopoiesis and ductal plate malformation (asterisks). (N) *B9D2* sequence traces are shown for an affected fetus (lower panel) and one of the parents (upper panel). Arrow indicates position of homozygous mutation (c.301A>C). (O) ClustalW alignment of *B9D2* protein sequences from diverse ciliated eukaryotes reveals conservation of either serine or threonine at the position corresponding to Ser101 of the human sequence (highlighted). Asterisks indicate

identical residues, colons indicate conserved residues, and periods indicate semi-conserved residues.

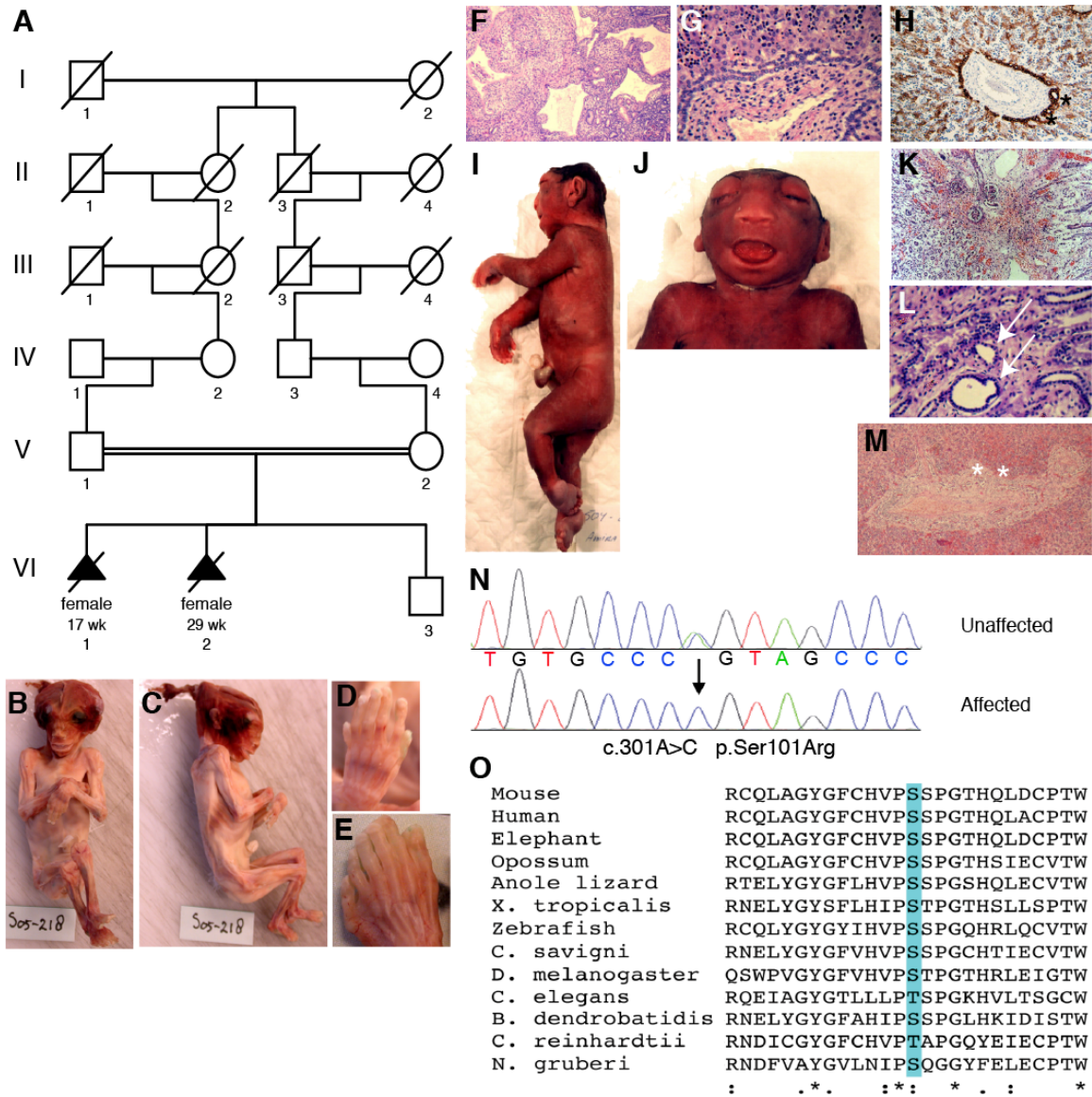


Figure 6. B9D2 p.Ser101Arg fails to rescue zebrafish phenotypes caused by suppression of *b9d2*. (A) Injection of MOs against *b9d2* produces defects in zebrafish development including shortened body axis, widened and kinked notochord (asterisk), and broadened somites (arrow). (B) Graph of the frequency of class I and II phenotypes among zebrafish embryos. Whereas coinjection of wild type human *B9D2* RNA (WT RNA) reduces the frequency of developmental defects caused by 2ng *b9d2* MO, *B9D2* S101R RNA does not rescue the phenotype (*b9d2* MO + S101R).

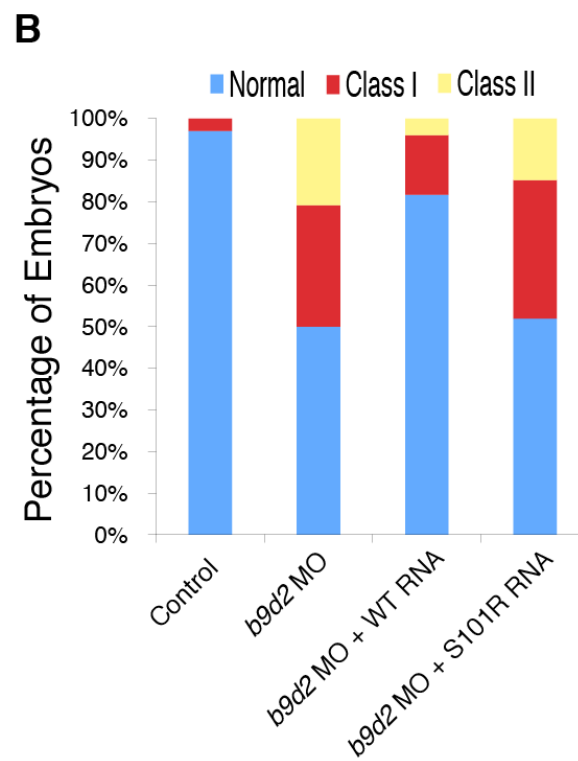
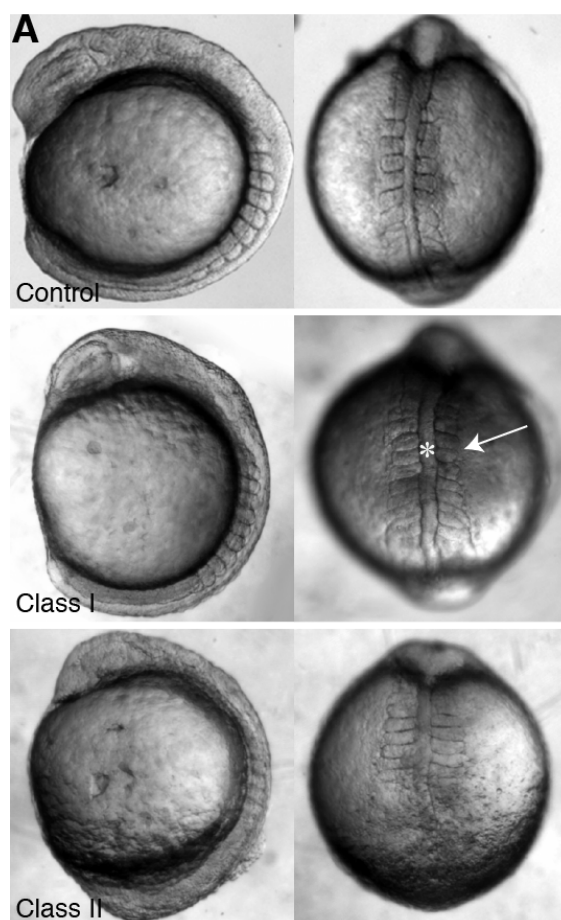


Figure 7. Localization of wild type B9D2 and B9D2 S101R. hTERT-RPE1 cells expressing GFP-tagged wild type B9D2 or B9D2 S101R (green). Expressions of both are enriched at the base of cilia (AcTub, red, arrows). Nuclei are visualized with DAPI (blue). Scale bar, 10µm.

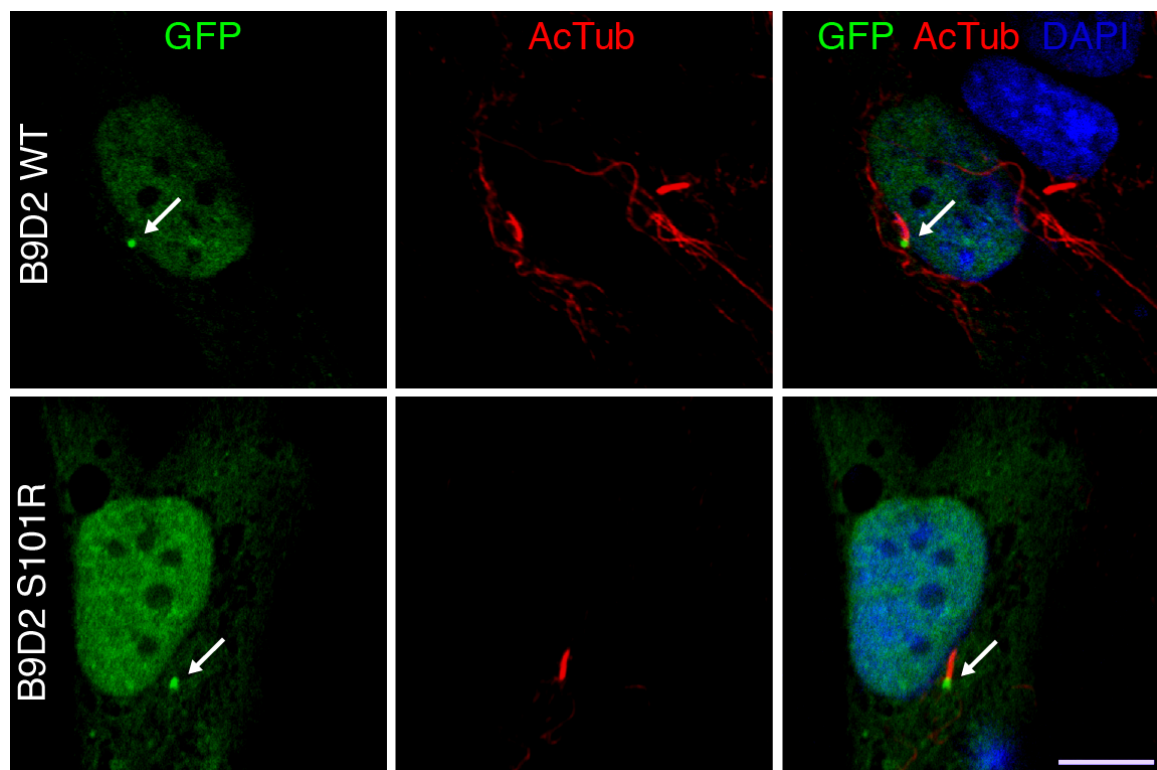


Figure 8. B9 proteins form a complex and B9d2 S101R fails to interact with Mks1.

Coimmunoprecipitation of epitope tagged B9 proteins. Immunoprecipitation of GFP-tagged Mks1 (**A**), B9d1 (**B**), B9d2 and B9d2 S101R (**C**) revealed interactions with V5-tagged Mks1, B9d1, B9d2, and B9d2S101R but not vector alone or a non-specific (NS) control protein, Rer1. In contrast to all other B9 protein combinations, B9d2 S101R did not interact with Mks1.

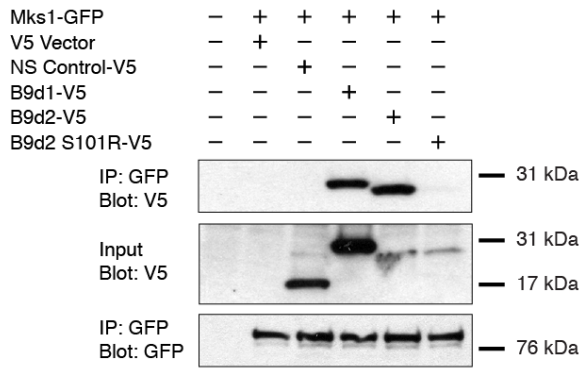
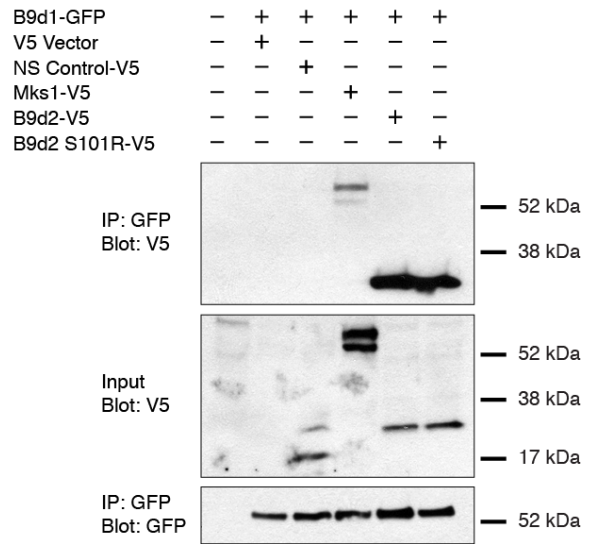
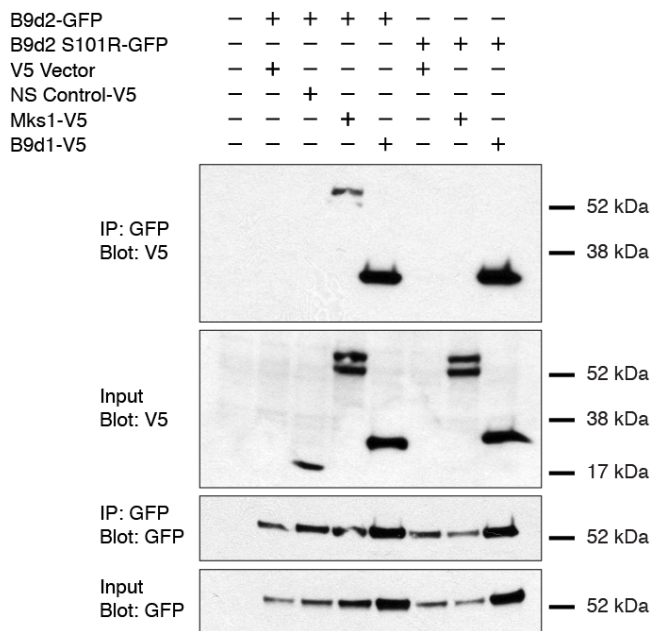
A**B****C**

Table 1. Survival of *B9d1* embryos

Age	Genotype		
	<i>B9d1</i> ^{+/+}	<i>B9d1</i> ^{+/-}	<i>B9d1</i> ^{-/-}
E8.0	10/47 (21)	24/47 (51)	13/47 (28)
E8.5	2/15 (13)	9/15 (60)	4/15 (27)
E9.5	22/95 (23)	48/95 (51)	25/95 (26)
E10.5	35/112 (31)	57/112 (51)	20/112 (18)
E11.5	15/55 (27)	28/55 (51)	12/55 (22)
E12.5	19/67 (28)	33/67 (49)	15/67 (23)
E13.5	28/93 (30)	41/93 (44)	24/93 (26)
E14.5	5/12 (42)	7/12 (58)	0/12 (0)
Embryonic survival scored at the ages listed. Shown is the number of surviving embryos among the total examined, with percentages in parentheses.			

Figure S1. Evolutionary conservation of B9 proteins. Phylogenetic tree of B9 proteins demonstrates conservation of *MKSI*, *B9D1*, and *B9D2* in diverse ciliated organisms.

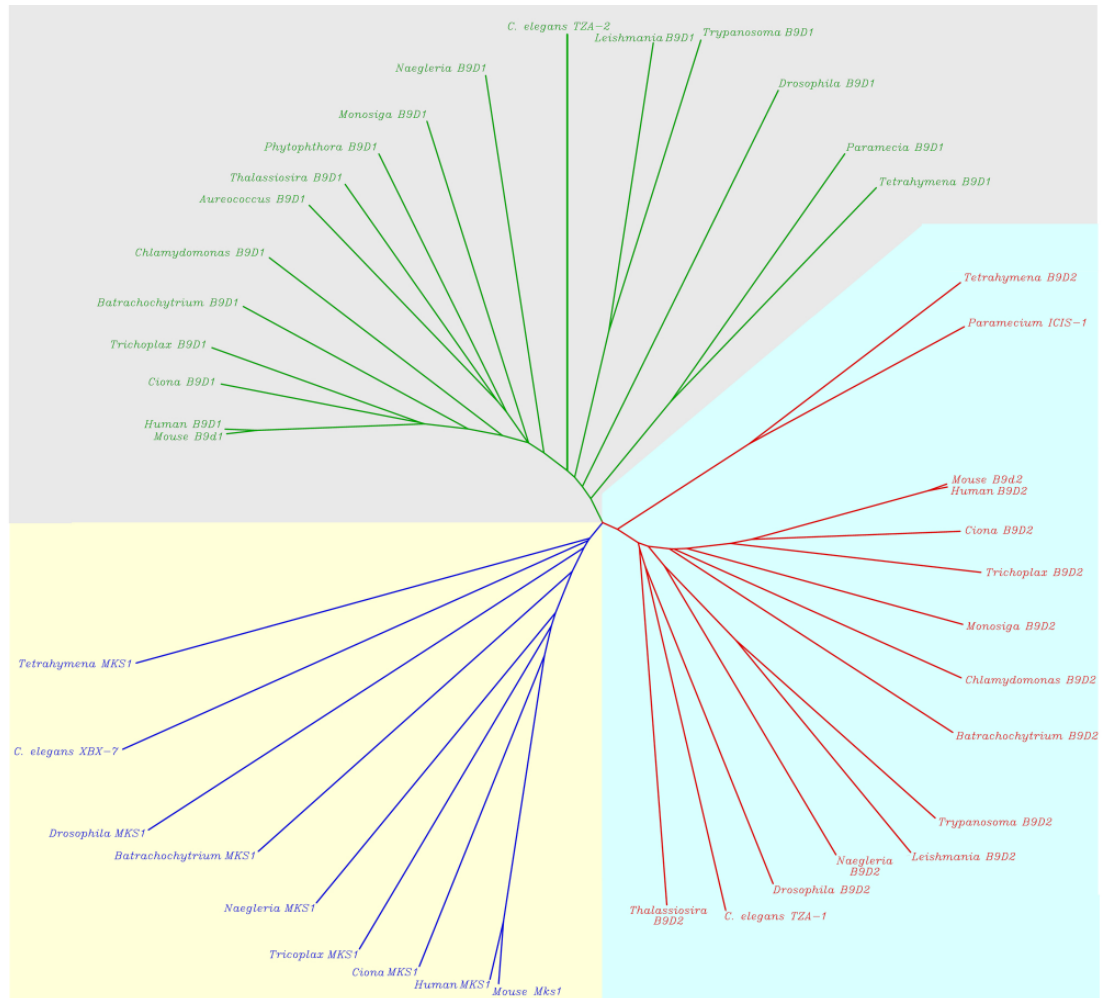


Figure S2. *B9d1*^{tm1a(EUCOMM)Wtsi} is a strong hypomorphic or null allele. (A) Schematic of the targeting strategy to generate *B9d1*^{tm1a(EUCOMM)Wtsi}. A splice acceptor of an *Engrailed2* intron (En2) generates a fusion between the first 44 amino acids of *B9d1* and a β geo reporter. (B) RT-PCR of E9.5 embryo mRNA utilizing two separate primer pairs, with a sense primer homologous to a region of exon 1 and an antisense primer homologous to a region of exon 2 (left), or a sense primer homologous to a region spanning exon 3 and 4 and an antisense primer homologous to a region within exon 7 (right), revealing that *B9d1* mRNA is strongly reduced in embryos homozygous for the *B9d1* mutation.

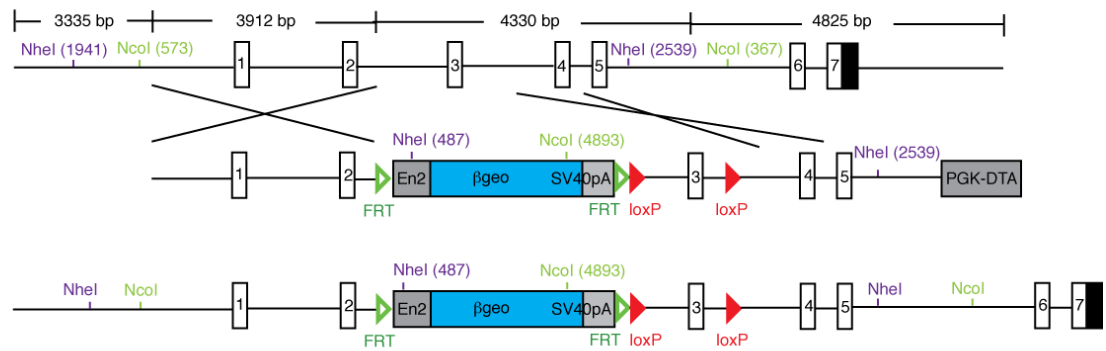
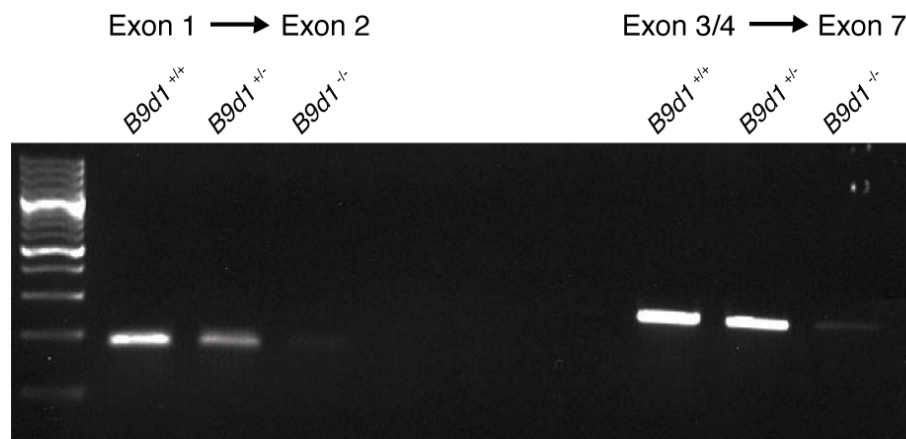
A**B**

Figure S3. Multiple origins of *B9d1* mutant renal dilations and cystic lesions. (A)

Hematoxylin and eosin-stained coronal section of P1 kidneys shows dilation of the Bowman's capsule (asterisk) surrounding the glomerulus in a *B9d1* mutant kidney. Scale bar, 50µm. **(B)** Kidney sections stained with a marker of collecting ducts (*Dolichous biflorus* lectin (DBA), green) and nuclei (DAPI, blue) or **(C)** a marker of proximal tubules (*Lotus tetragonolobus* lectin (LTL), green) and nuclei (DAPI, blue). **(B-C)** Large cysts (asterisks) are found in both regions of the *B9d1* mutant kidneys. Scale bar, 50µm.

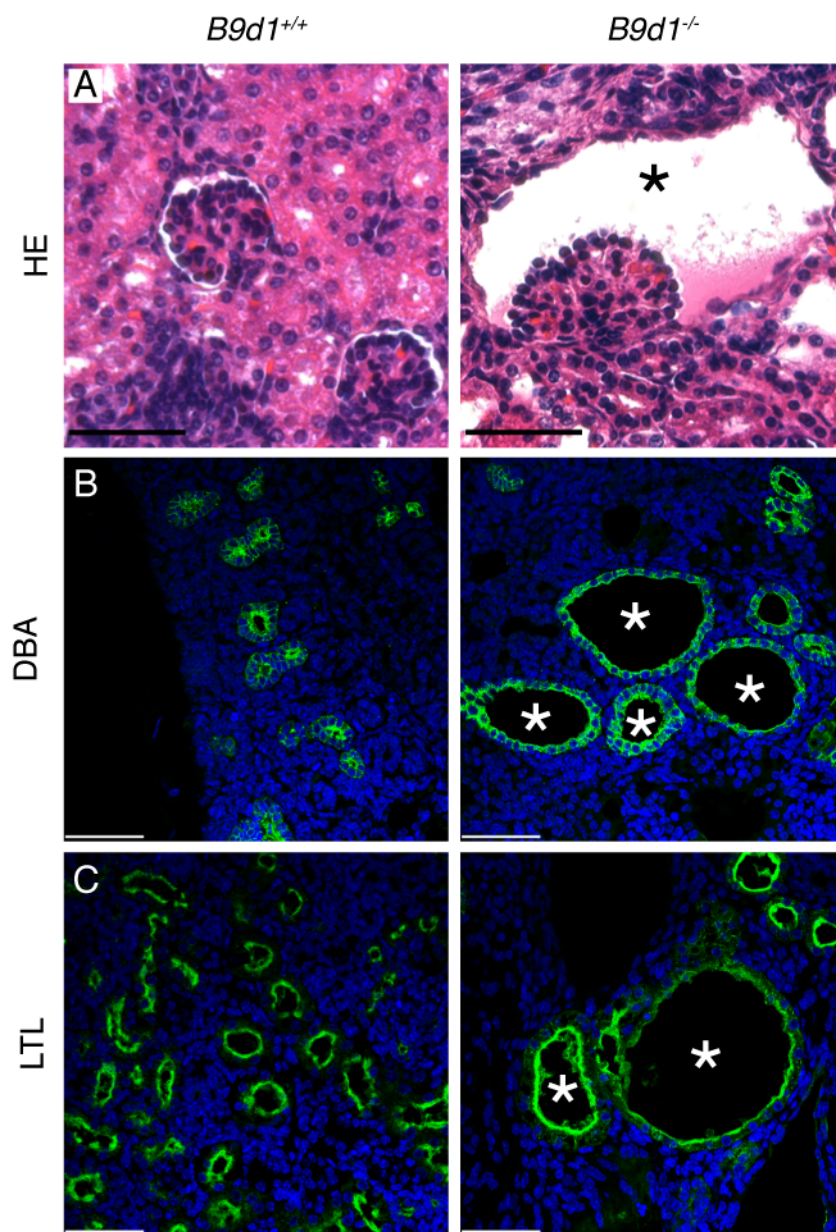
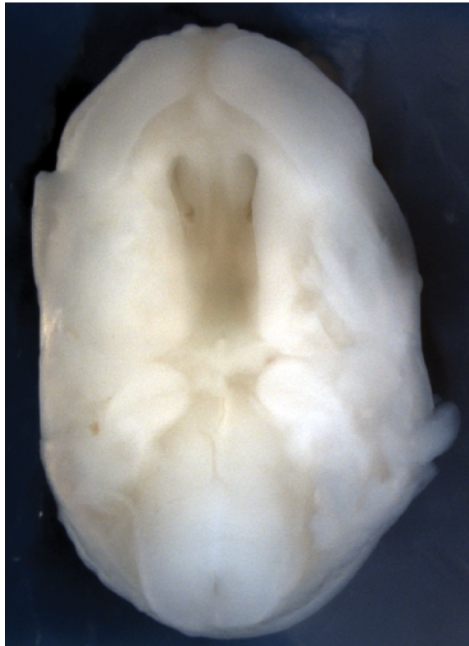


Figure S4. *B9d1* mutants develop cleft palate. Ventral view of E13.5 embryo palates reveals clefting of the primary palate (lip) in *B9d1* mutants (arrow).

B9d1^{+/+}

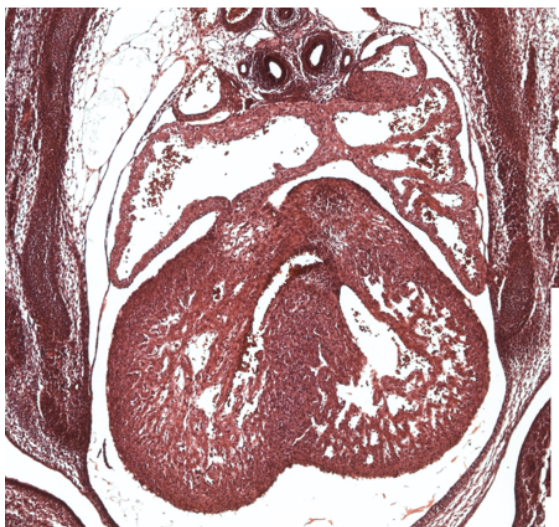


B9d1^{-/-}



Figure S5. *B9dl* mutants display ventricular septal defect. H&E stained transverse section of E13.5 hearts. *B9dl* mutants fail to complete the membranous ventricular septum.

B9d1^{+/+}



B9d1^{-/-}

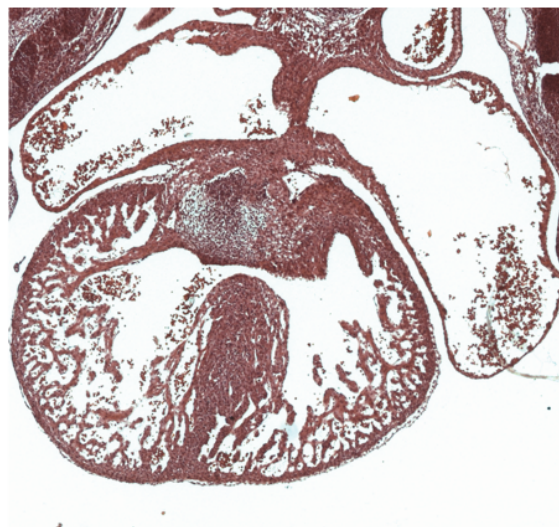


Figure S6. Protein localization in MEF cilia. Full panel view of protein localization depicted in Figures 4B and 4E-G. Arrows point to cilia. Scale bars, 25 μ m.

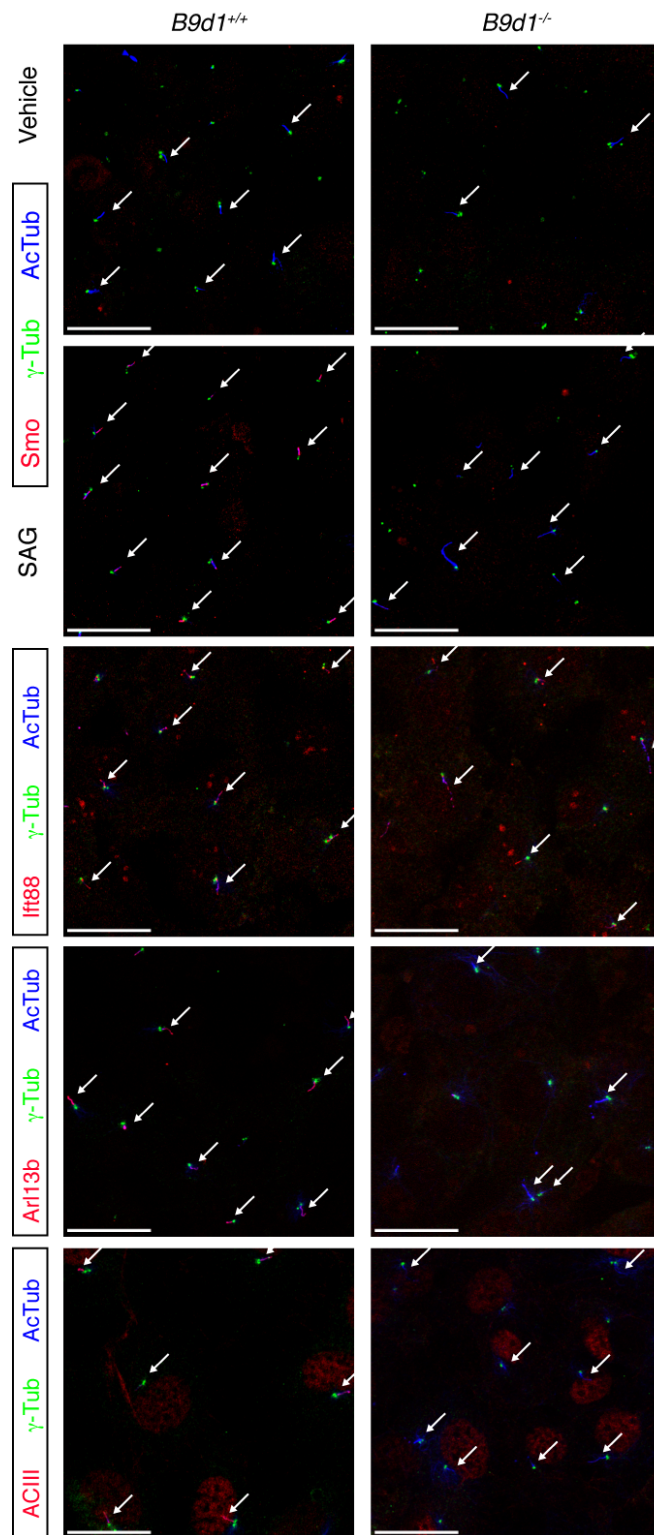


Figure S7. Modeling of B9D2 C2 domain. Electrostatic surface and cartoon modeling of the human B9D2 domain based on the structure of the calcium-free piccolo C2a domain. Electrostatic potential is colored in surface diagrams with positively charged residues colored in blue, and negatively charged residues in red. Conserved residues involved in membrane lipid binding are highlighted in licorice drawing with basic residues (K and R) in blue, aromatic residues (F, W and Y) in orange, and Ser101 in red. Residues 3-121 of human B9D2 were modeled, β - sheets are shown in yellow, and α -helixes are not shown.

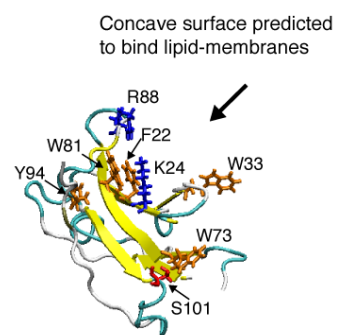
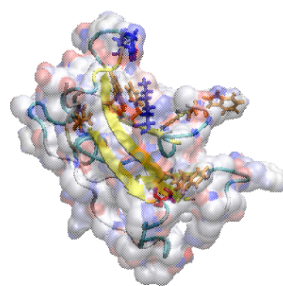
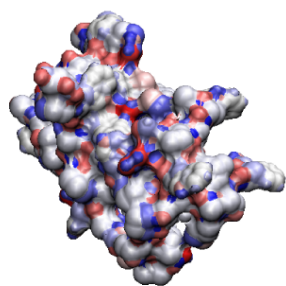


Figure S8. Titration curve of *b9d2* MOs. Injection of a *b9d2* splice blocker MO at concentrations of 0ng, 2ng, 4ng, and 6ng resulted in an increase in the number of embryos with early developmental defects (3%, 50%, 55% and 79%, respectively). A concentration of 2ng was used for all subsequent MO experiments.

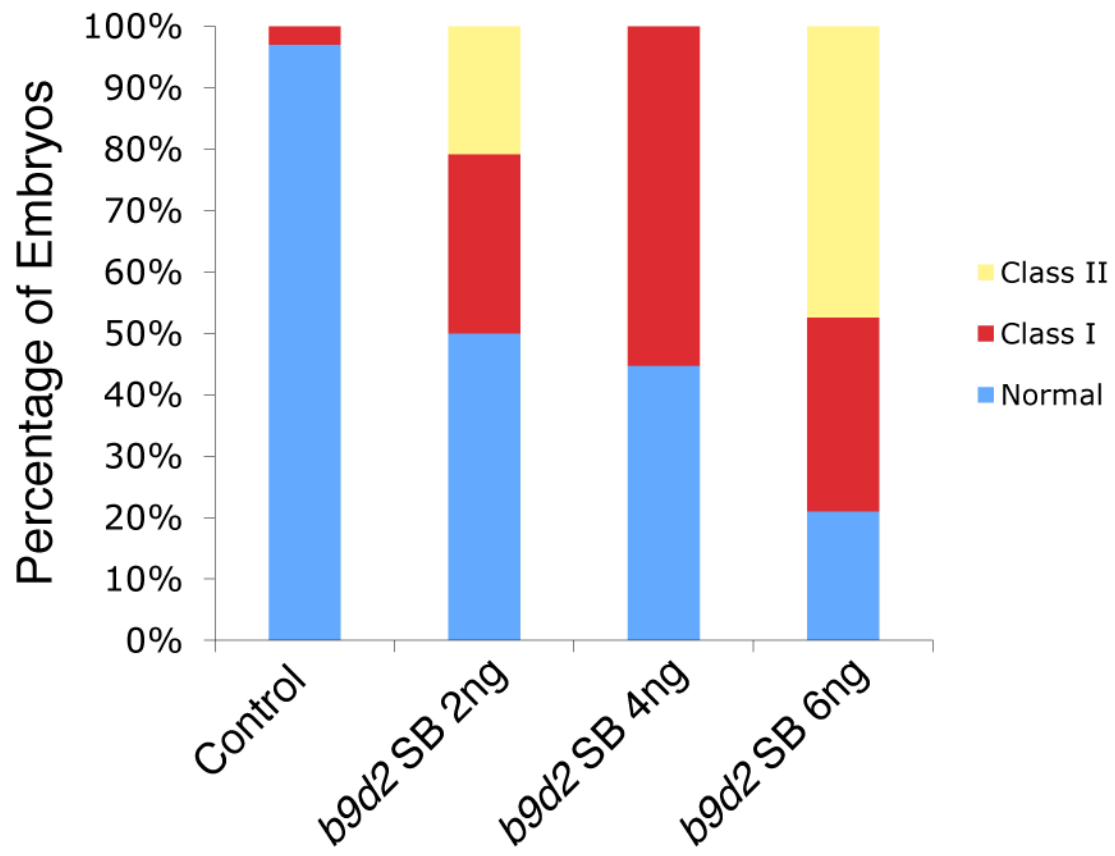


Table S1. Scoring of the missense B9D2 mutation c.301A>C (p.Ser101Arg) by bioinformatic algorithms

Mutation	GD ¹		SIFT ²		Align GVGD ³		Mutation Taster ⁴		Consensus
	VS	MG	VS	MG	VS	MG	VS	MG	MG
p.Ser101Arg	109	HLP	0.06	I	C65	HLP	0.99	HLP	LP

VS, Variant score; MG, Mutation Group; HLP, highly likely pathogenic; LP, likely pathogenic; I, indeterminate; N, neutral.

¹GD (Grantham Distance): Measurement of chemical differences between the two amino acids under consideration.

²SIFT (Sorting Intolerant From Tolerant): Multisequence alignment of orthologs.

³AlignGVGD: Multisequence alignment of orthologs.

⁴Mutation Taster (Schwarz JM et al.: Mutation Taster evaluates disease-causing potential of sequence alterations. Nat Methods 7: 575-6, 2010).

Interpretation of variant scores (VS) and deduced mutation group (MG):

Grantham distance: The Grantham score for chemical differences is a measurement for chemical differences between amino acids that combines residue side chain composition, polarity and volume. Similar pairs of amino acids score in the range of 5 to 60, whereas strongly dissimilar pairs score >100 (Grantham, 1974).

SIFT: 0.0 (HLP), 0.01-0.04 (LP), 0.05-0.09 (I), >0.1 (N).

AlignGVGD: C55-C65 (HLP), C35-45 (LP), C15-25 (I), C00 (N).

Mutation Taster: Predicted to be disease causing; the p value is the probability of the prediction, i.e. a value close to 1 indicates a high 'security' of the prediction. Please note that the p value used here is not the probability of error as used in t-test statistics.

Table S2. Primer sequences

Application	Name	Sequence
qRT-PCR	Ptc1F	5'-TGATTGTGGAAGCCACAGAAAA-3'
qRT-PCR	Ptc1R	5'-TGTCTGGAGTCCGGATGGA-3'
qRT-PCR	Gli1F	5'-CTTCACCCTGCCATGAAACT-3'
qRT-PCR	Gli1R	5'-TCCAGCTGAGTGTTGTCCAG-3'
qRT-PCR	β -actinF	5'-CACAGCTTCTTTGCAGCTCCTT-3'
qRT-PCR	β -actinR	5'-CGTCATCCATGGCGAACTG-3'
RT-PCR	B9d1Ex1F	5'-AAGACAACCGAGAGCCTCCT-3'
RT-PCR	B9d1Ex2R	5'-CAGTCCTGGCCATACACAAA-3'
RT-PCR	B9d1Ex3Ex4F	5'-GGCTGGCCACAGATTGTACT-3'
RT-PCR	B9d1Ex7R	5'-TCCTTGGTCACCACATTGAA-3'
Microsatellite analysis	ComF	5'-TACGCATCCCAGTTTGAGACG-3'
<i>B9D2</i> sequencing exon 1	1F	5'-TTTGCCGTAGCTGGAGAGAC-3'
<i>B9D2</i> sequencing exon 1	1R	5'-CCCCAGCAAGCTCCATAAG-3'
<i>B9D2</i> sequencing exon 2	2F	5'-CATGGGTTGGCAATTAGGA-3'
<i>B9D2</i> sequencing exon 2	2R	5'-CTGGGCCTGGAGTACTGAGA-3'
<i>B9D2</i> sequencing exon 3	3F	5'-GGTTCAGAGTCCATGCTTC-3'
<i>B9D2</i> sequencing exon 3	3R	5'-CAGGGACAGGTCTTGGTGTT-3'
<i>B9D2</i> sequencing exon 4	4F	5'-CCTGGGGTCTCCAGTGAGTA-3'
<i>B9D2</i> sequencing exon 4	4R	5'-GACCCCGAGGTCCTAGAAAG-3'

Table S3. Mass Spectrometry of B9d1-LAP

Gene	MW	B9D1 Spectra	LAP Spectra	B9d1 coverage	LAP coverage
B9d1	23 kDa	42	0	44%	0%
B9d2	19 kDa	32	0	42%	0%
Hspa8	71 kDa	29	0	39%	0%
Mks1	64 kDa	50	0	37%	0%
Tctn1	63 kDa	45	0	33%	0%
Atp5b	56 kDa	26	0	32%	0%
Tmem231	36 kDa	12	0	31%	0%
Tubb2b	50 kDa	3	0	26%	0%
Cc2d2a	182 kDa	70	0	25%	0%
Tuba4a	50 kDa	3	0	24%	0%
Atp5a1	55 kDa	16	1	22%	2%
Ybx1	36 kDa	10	0	20%	0%
Tctn2	77 kDa	24	0	18%	0%
Slc25a5	33 kDa	12	0	17%	0%
Ldha	36 kDa	5	0	17%	0%
Krt34	45 kDa	3	0	17%	0%
Hnrnph1	49 kDa	7	0	15%	0%
Gm6180	18 kDa	4	0	15%	0%
Hba-a1	15 kDa	13	1	23%	8.50%
Hnrnpu	88 kDa	16	1	16%	1.50%
Tmem107	16 kDa	3	0	13%	0%
Park2	12 kDa	3	0	13%	0%
Tctn3	65 kDa	12	0	12%	0%
Vdac2	32 kDa	3	0	11%	0%
Tmem218	12 kDa	3	0	10.00%	0%
Tuft1	45 kDa	3	0	9.70%	0%
Gm2574	36 kDa	13	1	12.00%	2.40%
Tpm3	29 kDa	5	1	14%	4.80%
Impa1	30 kDa	3	0	8.75%	0%
Psma2	28 kDa	4	0	8.50%	0%
Mdh2	36 kDa	3	0	8.30%	0%
Slc25a3	40 kDa	3	0	7.30%	0%
Cct7	60 kDa	5	0	7.20%	0%
Hdac10	72 kDa	4	0	6.80%	0%
Ahi1	120 kDa	10	1	7.90%	1.60%
Gm8910	29 kDa	3	0	6.30%	0%
Akr1c18	37 kDa	5	0	6.20%	0%
Olfir653	39 kDa	3	0	5.90%	0%
D1Pas1	73 kDa	5	0	5.00%	0%
Kif23	109 kDa	3	0	4.90%	0%
Dhx9	150 kDa	8	0	4.80%	0%
Cct3	61 kDa	3	0	4.60%	0%

Fbln2	126 kDa	6	0	4.40%	0%
Lmna	65 kDa	3	0	4.40%	0%
Tcp10b	47 kDa	3	0	4.10%	0%
Hnrnpc	37 kDa	4	0	3.90%	0%
Zfp668	68 kDa	5	1	4.80%	1.50%
Mavs	53 kDa	3	0	3.20%	0%
Zscan4f	58 kDa	3	0	3.20%	0%
Usp12	43 kDa	8	0	3.00%	0%
Actn1	103 kDa	6	1	4%	1.20%
Ccdc60	63 kDa	3	0	2.80%	0%
Lamp1	44 kDa	4	0	2.70%	0%
Cyp4a14	59 kDa	3	0	2.60%	0%
Vmn2r24	96 kDa	4	1	3.60%	1.40%
Olfm4	57 kDa	4	0	2.20%	0%
Ano1	110 kDa	3	0	2.10%	0%
Atp1a4	115 kDa	4	1	3.70%	1.70%
Tmem209	70 kDa	3	0	1.90%	0%
Trip12	68 kDa	7	0	1.70%	0%
C2	142 kDa	4	0	1.60%	0%
Syvn1	67 kDa	5	0	1.50%	0%
Flnb	278 kDa	6	0	1.40%	0%
Scn7a	192 kDa	3	0	1.20%	0%
Nkrf	78 kDa	3	1	2.50%	1.40%
Fam75d3	145 kDa	8	0	0.67%	0%
Abca7	238 kDa	3	0	0.46%	0%
4932438A13Rik	555 kDa	3	0	0.38%	0%
Snrpf	10 kDa	3	1	26%	26%
Ccdc84	38 kDa	3	1	8.70%	8.70%
Aipl1	38 kDa	3	1	5.80%	5.80%
4933413G19Rik	35 kDa	3	1	5.50%	5.50%
Kcnj16	48 kDa	4	1	4.50%	4.50%
Fgf14	28 kDa	3	1	4.50%	4.50%
Sftpd	38 kDa	3	1	3.50%	3.50%
Lnp	48 kDa	3	1	3.30%	3.30%
Ggnbp2	84 kDa	4	1	3%	3%
Leprel1	80 kDa	3	1	2.40%	2.40%
Cyp2j8-ps	57 kDa	3	1	2.20%	2.20%
Depdc5	178 kDa	3	1	1.90%	1.90%
9930013L23Rik	156 kDa	3	1	1.70%	1.70%
Tdrd9	68 kDa	3	1	1.60%	1.60%
Sbno1	154 kDa	3	1	1%	1%
Clip2	112 kDa	3	1	0.79%	0.79%
Rsb1	89 kDa	3	1	1.30%	1.40%
Map3k9	129 kDa	3	1	1.30%	2.20%

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Chapter 4: Unanswered Questions

The regulation of Wnt signaling by Kif3a

We have characterized the role of primary cilia in the regulation of canonical Wnt signaling. In particular, we have shown that the kinesin II component Kif3a regulates Wnt signaling through dual ciliary and non-ciliary mechanisms. Specifically, the phosphorylation status of Dvl2 and Dvl3 is regulated by Kif3a through Casein kinase I (CK1). Interestingly, two recent reports have identified TMEM237 and STK11 (cilia associated proteins) as regulators of Dvl phosphorylation, further supporting the link between cilia or cilia proteins and the regulation of Wnt signaling [1,2]. *TMEM237* is particularly interesting, as mutations in this gene are associated with Joubert syndrome, which is characterized by cerebellar vermis hypoplasia, a defect caused by altered Wnt signaling [3]. The molecular mechanism governing the CK1 phosphorylation of Dvl proteins remains unclear.

It would be interesting to further characterize the role of CK1 in this process, as it is not clear whether KIF3A acts directly or indirectly upon CK1. Mammals contain two CK1 genes implicated in Wnt signaling, *CK1δ* and *CK1ε*, which share overlapping functions [4]. The Ptacek lab at UCSF has both *Cklδ* and *Cklε* KO mice, and it would be possible to derive MEFs from the double knock-out (DKO) animals. These cells would make it possible to test whether the regulation of Wnt signaling by Kif3a is dependent on CK1. The SuperTOPFlash Wnt reporter plasmid could be transfected into Ck1 DKO cells in combination with siRNAs against Kif3a. If loss of Kif3a resulted in increased pathway activation in control, but not CK1 DKO cells, it would argue for a direct requirement of

CK1 in this process. We have found that CK1 kinase activity is not altered in Kif3a KO cells by an *in vitro* kinase assay.

One possibility is that CK1, but not its kinase activity is required for the Kif3a mediated regulation of Dvl phosphorylation. The CK1 DKO MEFs could be utilized to test this possibility. Wild-type or kinase-dead CK1 expression constructs could be introduced into the CK1 DKO MEFs in parallel with a Wnt pathway reporter. If kinase-dead CK1 also restores Wnt signaling to DKO cells, CK1 is likely to regulate Wnt signaling in a kinase-independent manner. Together, these studies would determine how CK1 and cilia interact to control canonical Wnt signaling.

As previous studies have also shown that non-canonical Wnt signaling is regulated through the cilium, it would also be interesting to test the requirement of CK1 in this process. Cilia deficient zebrafish display convergent extension defects consistent with suppressed non-canonical Wnt signaling [5]. Like its role in canonical signaling, the primary cilium might modulate non-canonical Wnt signaling by restraining CK1 function. This could be tested through the use of chemical inhibitors and morpholinos (MOs) to deplete CK1 activity in unciliated zebrafish embryos. Loss of cilia in zebrafish results in convergence and extension defects (elongated somites, broadened notochord, and shortened body axis). Thus, if chemical or MO suppression of CK1 were able to partially or totally suppress the non-canonical Wnt phenotypes of unciliated embryos, it would strongly suggest that CK1 contributes to the ciliary regulation of non-canonical Wnt signaling. If CK1 suppression does not reverse these phenotypes in unciliated embryos, then cilia likely regulate this pathway in a CK1 independent way.

The function of the transition zone

We have described a functional role for the transition zone complex at the base of the cilium. Specifically, we have identified a role for the transition zone in the localization of membrane proteins to the cilium. In some tissue contexts, this results in a drastic reduction in the number of cilia, while in most tissues cilia and IFT remain intact. Phenotypes associated with disruption of the transition zone might all be related to the loss of ciliary membrane protein localization. The regulation of membrane protein localization by the transition zone remains enigmatic.

It is likely that disruption of the transition zone severs the link between the axoneme and ciliary membrane. Y-linked structures have been observed at the transition zone of cilia in diverse species, and they likely serve to link the membrane to the axoneme. These structures may comprise the experimentally observed “diffusion barrier” between the plasma and ciliary membrane [6-12]. We described the failed localization of ciliary membrane proteins like Arl13b and AC3 in MEFs devoid of the transition zone components Tctn1, B9d1, Mks3, and Cc2d2a. As we used a static endpoint assay to assess the localization of these membrane proteins, it remains unclear whether Arl13b and AC3 fail to enter the cilium, or whether they fail to be retained within the cilium. A dynamic live-cell imaging assay would be useful in addressing this question. Chih et al. showed that the fluorescent recovery after photobleaching (FRAP) dynamics of ciliary localized GFP-Htr6 and GFP-Sstr3 was altered when TZ components were targeted with small interfering RNAs (siRNAs) [13]. The recovery of membrane protein fluorescence was significantly faster when TZ components were knocked down, suggesting that a diffusion barrier at the base of the cilium had been disrupted [13]. While this result does

seem to suggest that the movement rate of cytoplasmic, or plasma membrane localized receptors into the cilium is increased, it is unclear whether this rate is affected in the opposite direction. To address this, it would be useful to perform a similar experiment in which the plasma membrane pool of fluorescently tagged receptor is photobleached and the ciliary pool is not. Measurement of the recovery rate of the membrane fluorescence would address whether the retention of ciliary localized receptor is affected.

In *Caenorhabditis elegans* (*C. elegans*), mutation of transition zone components results in the disruption of the membrane-axoneme linking Y-fibers mentioned above [6]. In collaboration with Stefan Geimer, we have attempted a similar analysis of the integrity of Y-fibers in mammalian cells (*Tctn1* MEFs), and have not observed a similar disruption of these Y-shaped fibers (unpublished data). Detection of these Y-fibers in mammalian cells however is difficult in comparison to the relatively larger sized flagella and cilia of *Chlamydomonas reinhardtii* or *C. elegans*. In addition to this mutational analysis, we have attempted to assess the subcellular localization of individual transition zone components via immunological labeling and TEM. We have not had great success labeling cells with antibodies against the endogenous proteins, as the fixation conditions required for TEM often denature or mask the epitopes detected by these antibodies. We have also tried Immuno-TEM in NIH3T3s stably expressing GFP-B9d1, using secondary labeling with chicken anti GFP antibodies, without much success. Correlated light and electron microscopy with the recently developed dual light and EM miniSOG tag might be another useful approach. In collaboration with Xiaokun Shu at UCSF, Francisco Garcia-Gonzalo in our lab has begun constructing a vector expressing B9d1-miniSOG to create cell lines stably expressing this protein.

We are also interested in establishing a hierarchy of the localization of the transition zone proteins to the base of the cilium. We have observed that in the absence of *Tctn1*, *B9d1*, *Tmem231*, or *Cc2d2a*, the other components of the transition zone fail to localize properly. Chih et al. described a similar phenomenon and showed by cell fractionation, that the transition zone protein complex fails to assemble into a large macromolecular complex in the absence of *B9d1* or *Tmem231* [13]. So far, nearly every TZ component we have tested is required to localize all of the other TZ components, and for localization of membrane proteins to the cilium. Thus, despite the homology between many of these proteins, they remain functionally non-redundant. *Tmem67* (*Mks3*) and *Cep290* also both localize to the transition zone of cilia, and can cause Meckel syndrome in humans. Targeted knock-out of either of the genes encoding these proteins however results in phenotypes less severe than that of *Tctn1*, *B9d1*, *Tmem231* or *Cc2d2a* mutants [3,13-16]. Furthermore, deletion of *Tmem67* does not lead to mislocalization of the ciliary membrane protein *Arl13b* [15]. We recently discovered that TZ components do not mislocalize in *Tmem67* mutant MEFs (unpublished data); thus this protein is not required to build the transition zone. *Tmem67* was not identified as an interactor of *B9d1*, *B9d2*, *Tmem231*, *Tctn1* or *Mks1*, yet it does co-immunoprecipitate with *Tctn1* when both are overexpressed [13,15-17]. *Tmem67* itself is mislocalized in *B9d1*, *Tctn1*, *Tmem231*, and *Cc2d2a* mutant cells. It is possible that *Tmem67* functions downstream of the transition zone, and that its function is disrupted in the absence of core transition zone components. It would be interesting to characterize proteins that co-immunoprecipitate with *Tmem67* as they may suggest its function.

In attempt to identify other components required to build the transition zone, we have analyzed the localization of several TZ components in the absence of the IFT proteins Ift88 and Kif3a and found that the TZ still assembles in their absence (unpublished data). Given that the most severe ciliary phenotypes are associated with mutation of the IFT components, and that the TZ can still assemble in their absence, we can conclude that the TZ does not function in the absence of a cilium or IFT despite its proper localization. Now that we have a good idea of what is required for the transition zone to assemble, but how it assembles remains unclear. Our results have not suggested a hierarchy such as sequential assembly of transition zone components at the base of the cilium, thus it remains a possibility that the transition zone is pre-assembled before it is localized to the base of the cilium. In order to address this, Jeremy Reiter has constructed an FKBP-tagged TCTN1 construct to force controlled retention of the Tectonic1 protein in the endoplasmic reticulum. If components of the transition zone accumulate at the endoplasmic reticulum when this FKBP-TCTN1 fusion protein is expressed, this would suggest that Tctn1 is sufficient to seed assembly of the complex and that the complex can assemble outside of the cilium. The small molecule Rapamycin can be used to release the FKBP-fusion protein from the endoplasmic reticulum in living cells, and thus the localization of the complex to the base of the cilium could be visualized in real-time with live-cell imaging.

An analysis of the molecular function of individual TZ components has not been completed, but should be informative. A domain analysis of the primary amino acid sequence of these proteins suggests that many of them are likely to interact with membranes or lipids. Tectonic proteins, TMEM67, TMEM231 and TMEM17 (another

likely TZ component) are predicted to contain transmembrane domains. CC2D2A possesses a calcium dependent lipid binding domain (C2) and B9D1, B9D2, and MKS1 contain C2-like B9 domains. Using lipid overlay blots, we have tested the lipid binding capacity of recombinant B9-domain containing MKS1 and B9D2. We found that both proteins share an affinity for PI(3)P, PI(4)P, and PI(5)P, but not other phosphoinositides or membrane components (Figure 4.1). It is possible that the B9-domain containing components of the TZ complex function to interact with the plasma or ciliary membrane directly, or they may selectively bind distinct domains in these membranes. Given that MKS1 and B9D2 had the highest affinity for PI(3)P, they might also serve to intercept incoming vesicles, which are highly enriched in PI(3)P.

The transition zone in adult tissue homeostasis

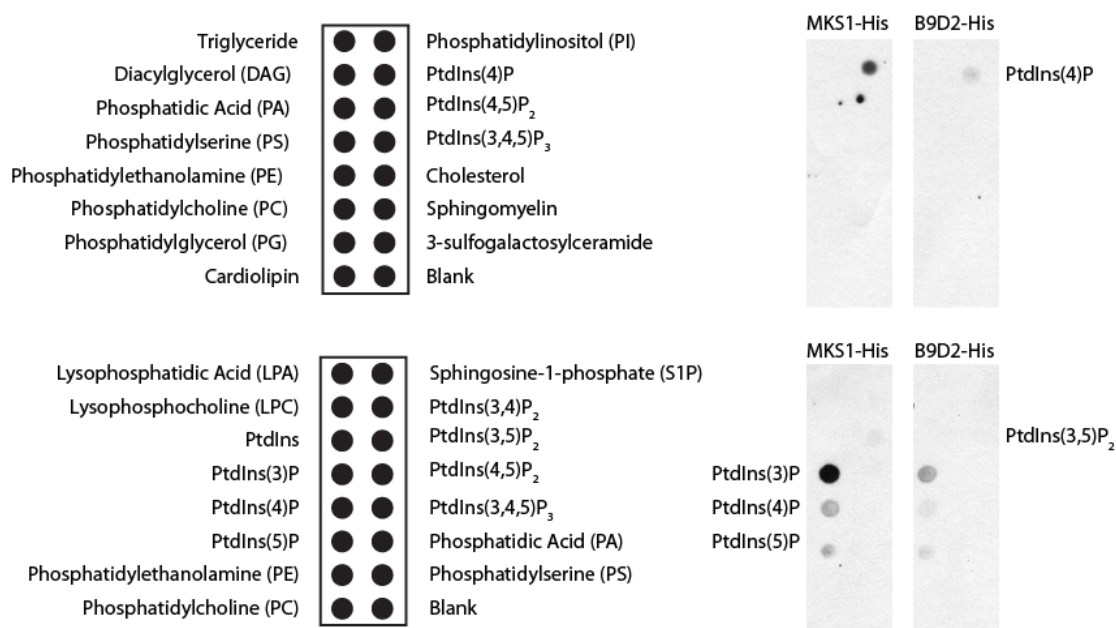
An extensive analysis of the transition zone proteins and the phenotypes associated with their targeted deletion in the mouse has been completed. Numerous developmental phenotypes have been linked to the disruption of the transition zone and cilia in general, yet nearly nothing is known about the role of the ciliary transition zone in adult tissue homeostasis or adult onset diseases. To address this, I have utilized the conditional allele of *B9d1* to disrupt transition zone function postnatally. At one month of age, I activated a ubiquitous CAGGCreER by injecting tamoxifen, at a dose sufficient to achieve efficient recombination in all tissues, into mice homozygous for a conditional allele of *Bd91* to disrupt transition zone function [18]. At six months following Cre activation, the retina and kidneys were analyzed. No obvious phenotypes were observed

(not shown), suggesting the transition zone plays a limited role in the adult, or that phenotypes take longer than six months to appear in these mice.

In *C. elegans*, experiments have suggested a genetic interaction between transition zone and NPHP genes, including an interaction between *b9d1* and *nphp4* [6,19]. We have utilized the *B9d1* conditional allele to test for a genetic interaction between *B9d1* and *Nphp4* in the adult mouse. In *B9d1^{flox/flox};Nphp4^{-/-};CAGGCreER* animals, I activated the ubiquitous Cre described above at one month post birth. At six months post Cre activation, I harvested tissues and performed a histological analysis to determine the presence of obvious phenotypes. The retinas of *Nphp4* null mice degenerate rapidly postnatally, and this was observed in all mice null for *Nphp4* in our hands [20]. No observable retinal degeneration was observed in *CAGGCreER;B9d1^{flox/+};Nphp4^{+/-}* animals. No disease was observed in the kidneys in any of these animals including *B9d1;Nphp4* trans homozygotes, suggesting that no genetic interaction between *B9d1* and *Nphp4* exists in adult mice. We have not yet obtained animals of the genotype: *Bd9I^{flox/flox};CAGGCreER;Nphp4^{+/-}* though analysis of these animals would be highly informative. Analysis of endpoints longer than six-months post tamoxifen injection might also be in formative.

Figure 4.1 B9 proteins bind phospholipids

Recombinant His tagged MKS1 and B9D2 bound PtdIns(4)P, but not other membrane lipids (upper panel). Recombinant His tagged MKS1 and B9D2 bound PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)P₂ in order of decreasing affinity (bottom panel).



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