Methods for Measuring Ciliary Calcium Dynamics: Potential Role for the Ciliary Inversin Compartment

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Methods for Measuring Ciliary Calcium Dynamics: Potential role for the Ciliary Inversin Compartment

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Thesis in the Field of Biotechnology
For the degree of Master of Liberal Arts in Extension Studies

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Abstract

The primary cilium is a cellular organelle at the nexus of many human disease syndromes that include polycystic kidney disease (PKD), embryonic developmental defects and retinal degeneration. While the whole organism phenotypes of these “ciliopathy” syndromes are well understood, the cellular and molecular mechanisms remain unclear. The primary cilium has been implicated in a number of cellular signaling pathways, including Sonic Hedgehog, second messengers such as calcium and cyclic AMP, and fluid flow sensing (Berbari, Connor, Haycraft, & Yoder, 2009; Gerdes, Davis, & Katsanis, 2009; Mick et al., 2015; Nachury, 2014; Praetorius & Spring, 2001). However, outside of the Hedgehog signaling pathway few cellular phenotypes have been identified partly due to a lack of available assays for cilium function. In this study I sought to develop an assay to measure calcium ion (Ca\(^{2+}\)) dynamics in the primary cilium of wild-type (WT) cells and use that assay to determine alterations in cells in which the primary cilium protein inversin was deleted by CRISPR/Cas9 gene editing. Ca\(^{2+}\) kinetics were measured by live cell time lapse fluorescence microscopy of the biosensor G-GECO1, that changes fluorescence intensity upon binding of Ca\(^{2+}\), fused to the Ca\(^{2+}\)-insensitive fluorescent protein mCherry and the primary cilium-localized receptor 5HT6 (Su et al., 2013). I used three different methods to generate an abrupt increase in the ciliary calcium concentration: two-photon ablation of the cilium tip with a focused 800nm pulsed laser beam, ATP-mediated calcium release, and illumination of the caged
calcium chelator NP-EGTA in a small area of the cell with a focused 405nm laser beam. The most robust method used NP-EGTA un-caging with the beam positioned in the cytoplasm near the cilium base. Using this method, I found that cells lacking inversin by CRISPR/Cas9 gene editing had longer peak time and emptying time of calcium ions from the primary cilium. This result provides the first cellular phenotype linking a ciliopathy gene and Ca2+ signaling. With this knowledge, detailed molecular pathways can be explored and potential therapeutic interventions developed.
Dedication

To all my mentors and cheerleaders, both in science and out of science, past and present. Without you this would not have been possible.
Acknowledgements

First and foremost, I thank Jagesh Shah for providing such a challenging and interesting problem in a supportive and enabling environment. Your mentorship is second to none, I learned a lot beyond the science and had a great time while I was at it. I am indebted to Peter Czarnecki for his enthusiastic and unwavering guidance on all things invesin and primary cilium as well as contributions of essential reagents. The project would not have been nearly as rigorous without the analysis code generated by David Jakobowitz. I also thank Kelly George for help with cilia cell culture and general discussions, Yinghua Guan for help with the two-photon ablation experiments and helpful discussions, Giada Bianchi for help with single cell sorting, and Patrick Donahue and Michael Porter for contributions to preliminary data analysis. Iain Cheeseman’s enthusiastic support for my work on this project in spite of the slight loss of focus it took from my role at Whitehead was invaluable and inspiring. Edward (Ted) Salmon, my father, has always been my scientific role model and in characteristic fashion he provided excellent and caring advice throughout the thesis process as well as critical review of early drafts of the manuscript. Patrick Donahue and Nancy Salmon provided unwavering support and encouragement throughout my Master’s degree without which the experience would have been much less enjoyable.
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Chapter I

Introduction

Understanding the cellular and molecular mechanisms involved in a disease is critical to efficiently identifying and testing potential therapeutic strategies. A group of human disease syndromes that share polycystic kidney disease (PKD), retinal degeneration and embryonic developmental defects all involve proteins that localize at least in part to a small cellular organelle, the primary cilium. While the whole organism phenotypes of these “ciliopathy” syndromes are well characterized, little is known about the molecular action of the affected proteins at the cellular level. The primary cilium is essential for the Sonic Hedgehog signaling pathway and is implicated in a number of others, including second messengers such as calcium and cyclic AMP and fluid flow sensing (Berbari et al., 2009; Gerdes et al., 2009; Praetorius & Spring, 2001; Mick et al., 2015; Nachury, 2014). How the cilium supports these signaling mechanisms in detail is unclear. Many of the ciliopathy symptoms and the structures of the associated proteins implicate a role for calcium signaling involving ciliary proteins. However, the field lacks a robust assay to define calcium behavior in the primary cilium, preventing systematic evaluation of effects of protein mutation on calcium signaling. Here I outline a set of studies that evaluate methodologies to measure ciliary calcium dynamics and apply the most robust method to cells genetically deleted for a ciliopathy gene.
I.1. Chronic and Polycystic Kidney Diseases

Incidence of chronic kidney disease (CKD) saw a large increase between 1991 and 2004 and since then it has stayed steady (Coresh et al., 2007). CKD renders the kidneys unable to properly filter waste from the blood, resulting in end stage renal disease (ESRD) and death. Treatment of kidney disease requires significant medical intervention such as dialysis or kidney transplant (Coresh et al., 2007). CKD reduces quality of life for patients and puts a substantial burden on the health care system.

The majority of CKD cases arise from complications with obesity, diabetes or hypertension, however a subset of cases (~5%) correlate with genetic mutations (Coresh et al., 2007; Harris, 2009; Lancaster & Gleeson, 2009). The large scale and interconnected causes of diseases like obesity, diabetes and hypertension make laboratory-based systematic studies of organ-specific and cellular effects intractable. Genetic mutations, however, are simple to recreate using laboratory animal models and cell culture, making them powerful tools for systematic study. Effects of genetic modification on specific cellular or organismal processes in the laboratory can provide insight into the cellular mechanisms behind CKD that enables development of new therapies.

Polycystic kidney disease (PKD) is a form of CKD that results from growth of kidney cells into non-cancerous structures called cysts that interrupt function at the location of origin and through damage of surrounding tissue (Coresh et al., 2007). There are three general categories of PKD: autosomal dominant PKD (ADPKD), autosomal recessive PKD (ARPKD) and a group of disease syndromes termed ciliopathies.
ADPKD is the most prevalent genetically-linked PKD, occurring in 1:400-1:1000 people and accounting for 4.4% of all ESRD patients (Harris, 2009; Waters & Beales, 2011). It typically presents symptoms after the third decade of life and has likely been around for many centuries (Torres & Watson, 1998). ADPKD is caused by mutations in the gene \textit{PKD1} or \textit{PKD2}, which encode the proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively. ADPKD is characterized by cysts originating in a variety of areas within the kidney and biliary duct dilation in the liver (Harris, 2009; Waters & Beales, 2011). Mouse models of \textit{PKD2} knockout and some human patients also show defects in left-right determination, a phenomenon known to involve calcium signaling (McGrath, Somlo, Makova, Tian, & Brueckner, 2003; Pennekamp et al., 2002; Yoshiha et al., 2012).

ARPKD occurs in 1:20,000 people and has a childhood onset, progressing more rapidly than ADPKD (Harris, 2009; Waters & Beales, 2011). ARPKD is caused by mutations in \textit{PKHD1} that encodes for fibrocystin. ARPKD is characterized by formation of kidney cysts \textit{in utero} or early childhood and liver fibrosis (Harris, 2009; Waters & Beales, 2011).

Over the last decade, a set of recessive PKDs that often have significant non-renal involvement have been grouped together as syndromic kidney diseases. Many of the gene products involved in these diseases localize to a specific cellular structure, the primary cilium, and are thus referred to as ciliopathies. Some of the diseases currently recognized as ciliopathies include nephronophthisis (NPHP), Senior-Løken Syndrome (SLSN), Joubert syndrome (JBTS), Meckel–Gruber syndrome (MKS), and Bardet–Biedl Syndrome (BBS). These ciliopathies all involve PKD, although specific pathology thereof differs, and can additionally include liver and pancreatic cysts or fibrosis (ARPKD, NPHP, SLSN JBTS, MKS), retinal defects (SRSN, JBTS, MKS, BBS),
problems with neurological development (SLSN, JBTS, MKS, BBS), and skeletal abnormalities such as polydactyly (MKS, BBS) (Harris, 2009; Lancaster & Gleeson, 2009).

Ciliopathies with shared arrays of symptoms are often correlated with proteins reported to associate in complexes within the primary cilium (Czarnecki & Shah, 2012). Little is known about the cellular function of many of the ciliopathy proteins or the associated complexes. Moreover, while some ciliopathy proteins are solely associated with the primary cilium, many such as Nek8 and PC2, are also found in other parts of the cell, further complicating elucidation of their roles (Choi et al., 2013 and Qian et al., 2003, respectively). A more detailed understanding of the normal function of proteins involved in ciliary disease can help identify pathways for therapeutic intervention in cystic kidney diseases.

The apparent simplicity of a single gene mutation to cause ADPKD or ARPKD is provocative for finding a simple solution to treat the disease. However, despite concerted effort little is still known and no preventative treatment has been found. While the effects of the PKD1/2 mutations on the whole organism are clear and consistent in humans and mouse models, the cellular phenotype has yet to be elucidated. Identification of cellular phenotype(s) is key to understanding the mechanism of disease initiation and development of treatment.

Our lab studies the inversin compartment (IC), a protein complex consisting of four proteins (at this time) that localizes to the primary cilium: Inversin, Nek8, ANKS6 and NPHP3. The inversin gene was identified through mouse insertional mutagenesis (Morgan et al., 1998). The inversin mouse (inv) exhibits consistent situs inversus, or
reversal of visceral left-right asymmetry (Morgan et al., 1998). Those mice that survive the situs anomaly go on to phenocopy ADPKD with kidney anomalies and cysts and pancreas abnormalities. Mouse strains with individual mutations for other IC members, Nek8 and Anks6, also heavily phenocopy inv with left-right determination errors and cystic kidneys in those animals that survive to adulthood (Czarnecki et al., 2015).

Knockouts of the PKD2 gene also exhibit situs defects and kidney cysts. Although no molecular association between PC2 (the gene product of PKD2) and the IC has been shown, both PC2 and inversin have been implicated in calcium physiology. The inversin sequence shows two IQ binding domains that suggest the ability to bind the calcium-binding protein calmodulin and PC2 shows strong homology to TRP type ion channels, although direct measurement of activity has yet to be demonstrated (Tsiokas, Kim, & Ong, 2007). The similarities in symptoms that arise from the mutations in ADPKD and the Inversin, Nek8 and ANKS6 mutant mice combined with the potential physiologic functions are intriguing.

Interestingly, many of the proteins involved in PKD-associated ciliopathies are confined to a specific sub-region of the cilium called the transition zone. The correlation of cellular localization of PKD associated proteins and syndromic symptoms implies an important general role of this portion of the cilium, though specifically what that role is remains unknown.

I.2. Cilia Architecture and Molecular Constituents

There are two main types of cilia in the body: Motile cilia and primary cilia. Motile cilia are typically found on differentiated cells in areas of the body that
generate fluid flow, such as the lungs and oviducts, and there are typically multiple motile cilia per cell (Czarnecki & Shah, 2012). The sperm is a noted exception, where it has a single motile flagella, with the same architecture as other motile cilia (Czarnecki & Shah, 2012). Primary cilia, on the other hand, are thought to be on almost every cell in the body, occur at one per cell and serve primarily as signaling structures (Czarnecki & Shah, 2012). Primary cilia have been reported to act as a cellular sensory organ for hormones, fluid flow, and other extracellular signaling mechanisms (Czarnecki & Shah, 2012; Drummond, 2012).

Cilia are long, thin projections from the surface of the cell that are approximately 200nm in diameter and have a characteristic architecture. The dominant, central structure is the axoneme, a ring of microtubule doublets that grow out of the basal body (defined below) and protrudes beyond the cell periphery, forming an antenna-like structure. There are typically nine doublets in the outer ring, and motile cilia have an additional pair of single microtubules in the center of the doublet ring. Primary cilia lack central microtubules, leading to an alternate nomenclature with motile cilia referred to as 9+2 cilia and primary cilia referred to as 9+0 cilia. However, as with many biological categories, there are exceptions to this simplified nomenclature (Drummond, 2012). Current models implicate the role of motile 9+0 cilia of the mouse embryonic node in generating a leftward flow in the embryonic left-right organizer that is detected by non-motile cilia for proper organization of left-right asymmetry, a process known to require calcium signaling (Yoshiba et al., 2012).

The ciliary axoneme grows out of the proximal-most component of the cilium, the basal body. The basal body is derived from one centriole of the cell’s centrosome and is
embedded in the actin cytoskeleton just below the plasma membrane (Battle, Ott, Burnette, Lippincott-Schwartz, & Schmidt, 2015). The portion of the cilium immediately distal to the basal body is termed the transition zone (TZ). The TZ was initially characterized by distinct structures visible by electron microscopy (EM). Transmission EM reveals dense protein structures that connect the microtubule doublets to the plasma membrane, termed transition fibers and Y-linkers (Czarnecki & Shah, 2012; Gerdes et al., 2009). Freeze-fracture scanning EM shows small protuberances on the outside of the plasma membrane, termed the ciliary necklace, located approximately at the position of the transition zone (Czarnecki & Shah, 2012; Gerdes et al., 2009). It is not yet known precisely what molecules comprise these physical features of the cilium. However, in recent years it has come to light that many protein complexes, including those associated with ciliopathies, localize near or at the TZ (Czarnecki & Shah, 2012). Additionally, studies support a model where the transition zone acts as a gatekeeper for movement of molecules into and out of the cilium (reviewed in Czarnecki & Shah, 2012).

The portion of the axoneme distal to the transition zone is called the doublet zone. Here, molecular motors use the microtubules as tracks to move cargo to and from the ciliary tip. This movement, called intraflagellar transport (IFT), has a characteristic speed and is required for maintenance of the cilia structure (Drummond, 2012). Primary cilia from different tissue and species origins have different typical lengths (Ishikawa & Marshall, 2011). Changes in the velocity of IFT have been shown to affect cilia length (Besschetnova et al., 2010). Additionally, experimental perturbation of cAMP and calcium lead to changes in cilia length and IFT anterograde velocity (Besschetnova et al.,
The distal end of the cilium consists of the singlet zone, where one of each MT doublet extends beyond the partner, and the cilium tip.

The membrane covering the primary cilium has been shown to contain an array of proteins that is distinct from that found on the rest of the plasma membrane (Hu et al., 2010). The separation of the membrane between the cilium and cell body (plasma membrane) results from directed secretion and a diffusion barrier at the transition zone that prevents ciliary and plasma membrane components from mixing (Hu et al., 2010). This compartmentalization of membrane proteins provides one basis for the idea that the primary cilium has a special function in the cell.

Within the primary cilium there are several sub-ciliary protein complexes, including the BBSome, MKS/B9 complex, NPHP 1-4-8 complex, NPHP 5-6 complex and the IC (Czarnecki & Shah, 2012). Protein content in each of these complexes is somewhat distinct and purification of protein complexes reveal strong intermolecular interactions (Sang et al., 2011). Some of the genes that produce these proteins are mutated in human disease and the protein complex within the cilium where a mutated gene product is located seems to influence which symptoms are involved (Czarnecki & Shah, 2012; Lancaster & Gleeson, 2009). For example, the symptoms classified as Bardet-Biedl Syndrome (BBS; polydactyly, cognitive disability, obesity and retinitis pigmentosa) are associated with mutations in one of several of the proteins that comprise the BBSome (Ishikawa & Marshall, 2011). Mutations in or deletion of IC proteins, however, result in left-right determination errors and PKD in mice, similar to the symptoms of ADPKD mouse models and human patients (Choi et al., 2013; Czarnecki et al., 2015; Yokoyama et al., 1993).
The correlation between protein complex and phenotypic symptoms point to possible cellular function of the proteins and/or complexes involved. Ultrastructural analysis and immunofluorescence-based optical microscopy show that localization of most of these ciliary complexes are constrained to an area near the TZ of the primary cilium (Sang et al., 2011; reviewed in Czarnecki & Shah, 2012). This localization at the boundary between the cell body and cilium suggests that some of the TZ localized proteins serve a gatekeeping function between the cilioplasm and cytoplasm or between the cilium and plasma membranes.

I.3. The Primary Cilium and Signaling

For many years the primary cilium was assumed to be vestigial. However, mounting evidence suggests that it acts as a signaling center (Praetorius & Spring, 2001), (Nauli et al., 2003), (Berbari et al., 2009; Lienkamp, Ganner, & Walz, 2012; Drummond, 2012). The Hedgehog signaling pathway is likely the best characterized signaling pathway that is reliant on the primary cilium (Drummond, 2012; Nachury, 2014). Briefly, in the absence of the signaling molecule Hedgehog (Hh), the protein Patched (Ptch) is located at the base of the primary cilium and the protein Smoothed (Smo) is inactivated and does not accumulate in the primary cilium (Gerdes et al., 2009). In this inactivated state, the transcription factor Gli enters and exits the primary cilium in the repressive form, which enters the nucleus and represses expression of specific genes (Gerdes et al., 2009). When Hh ligand binds to Ptch, Ptch is removed from the base of the cilium and activated Smo is concentrated in the membrane of the primary cilium. The cilium-localized activated Smo indirectly enables cleavage of the full-length transcription
factor Gli into a shorter, activated form (Gerdes et al., 2009). This cleaved Gli moves from the primary cilium to the nucleus and turns on a host of genes (Drummond, 2012). In this light, it is interesting to consider the role of the cilium as a "conditional" compartment that can switch between gli transport and gli processing functions and that such a conditional compartment function could provide a basis for the action of other signaling pathways in the cilium.

I.4. Calcium Signaling and Fluid Flow

The connection between the primary cilium and calcium was one of the first proposed purposes of the primary cilium; however, the detailed mechanisms still evade consensus (Delling et al., 2016; Nauli et al., 2003; Praetorius & Spring, 2001). The hypothesis for the link between the primary cilium and calcium was drawn from studies showing that left-right asymmetry is set up early in development as a consequence of asymmetric calcium signaling in the node, and embryos without primary cilia have left-right determination errors (Wright, 2001; Nonaka, et al., 2002; Watanabe et al., 2003). The calcium asymmetry has been shown to depend on directional flow of fluid within the node, although the mechanism for sensing that asymmetric flow was unknown and is still debated (Nonaka, Shiratori, Saijoh, & Hamada, 2002). One hypothesis is that flow in the node provides mechanical bending of primary cilia that opens mechanically-gated calcium channels (Jin et al., 2014; Praetorius & Spring, 2001; Watanabe et al., 2003). A recent study where flow on the cilium was isolated from flow on the cellular surface showed no immediate calcium entry after cilium bending, suggesting that flow sensing by
the primary cilium is unlikely due to acute calcium entry by mechanically gated channels (Delling et al., 2016).

The involvement of calcium signaling in the primary cilium as a contributor to PKD is evocative for several reasons: PC2, one of the causative proteins of ADPKD, has homology to TRP family ion channels (Tsiokas et al., 2007) mouse models and human diseases that have knockout of or mutations in cilium-localized proteins have errors in left-right determination, a process known to be dependent on calcium signaling (Nonaka et al., 2002; Wright, 2001) on a cellular level, changes to intracellular calcium have been shown to change intraflagellar transport (IFT), an integral mechanism for the creation and maintenance of primary cilia (Besschetnova et al., 2010) and primary cilia are known to be involved in signaling in the retina and olfactory epithelium (Tsiokas et al., 2007). However, it remains unclear if calcium signaling in the primary cilium is robust across all cell types and how exactly it works. Earlier studies showing fluid-flow induced calcium effects in bending primary cilium, while quite sophisticated and compelling, do not unequivocally rule out alternative mechanisms of fluid-sensing calcium signaling since flow on the cilium was not isolated from flow on the cell surface (Jin et al., 2014; Nauli et al., 2003). The recent data from Delling, et al. (2016) that create an isolated ciliary bending are consistent with a model where flow-mediated calcium entry into the primary cilium is indirect, involving flow sensing in other areas of the cell membrane and/or through non-calcium pathways.
I.5. Primary Cilium as Conditional Calcium Compartment

Recently, the resting concentration of calcium was reported to be higher in the primary cilium than in the cytoplasm (Delling, DeCaen, Doerner, Febvay, & Clapham, 2013). Additionally, single cilium patch clamping experiments showed voltage in both attached and detached cilia that was higher than that of the parent cells (DeCaen, Liu, Abiria, & Clapham, 2016). Voltage across a membrane indicates difference in ion concentrations across the membrane, with higher ion concentration generating higher voltage. These findings imply the primary cilium has a mechanism to control and maintain the movement of ions and small molecules between the cilium and cytoplasm. The localization of the IC at the base of the cilium, with known association with the basal body and having many calcium binding domain, is well positioned to participate in this role.

It is unlikely that entry of calcium into the cytoplasm through the cilium is the cause of calcium dependent signaling. Due to the very small volume of the cilium, a small number of calcium ions can cause a large change in ciliary concentration (Delling et al., 2013; Nachury, 2014). If this small number of ions were transferred to the relatively enormous volume of the cytoplasm, there would be no effect on the total calcium concentration (Delling et al., 2013; Nachury, 2014). I propose a mechanism whereby the locally high calcium concentration within the cilium generates modification of one or more proteins being transported through the cilium that, when released to the cytoplasm, affect change within the cell. This is similar to the role of the cilium in Sonic Hedgehog signaling where the Gli transcription factor is proteolytically processed when binding of Sonic Hedgehog ligand to its cellular receptor localizes the protein.
Smoothened to the primary cilium but otherwise cycles through the cilium unchanged because Smoothened is excluded from the primary cilium. Identifying the potential “gatekeeper” for maintaining a ciliary calcium gradient is a powerful step towards understanding the role of ciliary calcium. Moreover, factors that change how long ciliary calcium can be maintained may also modify cilium-dependent calcium signaling.

I.6. Polycystins, PC1 and PC2

PC1 and PC2, the proteins mutated in ADPKD, have been shown to localize to the endoplasmic reticulum (ER), plasma membrane and primary cilium (Waters & Beales, 2011). PC1 has 11 transmembrane (TM) domains and a long N-terminal tail that extends into the extracellular space; some have categorized it as a possible GPCR, however a canonical GPCR has only 7 TM domains (Nauli & Zhou, 2004; DeCaen et al., 2016). PC2 shows highest homology with the TRP family of voltage-gated calcium channel (Mochizuki et al., 1996). The PC1/2 heterodimer has been reported to be a calcium channel (Nauli et al., 2003). Recent work in the Clapham lab showed that the related proteins PC1-like1 and PC2-like1 (PC1l1 and PC2l1, respectively, and products of *PKD1l1* and *PKD2l1*, respectively) act as a heteromer ion channel in the primary cilium (DeCaen et al., 2016). PC1l1 and PC2l1 have sequence homology to PC1 and PC2, respectively, but have not yet been reported to be involved in ADPKD nor other PKD syndromes. This could be due to an essential role of these proteins, wherein mutations would be incompatible with early development.
I.7. Inversin Compartment

All current members of the IC – Inversin, Anks6, Nek8 and NPHP3 – localize distal to the transition zone of the primary cilium, as shown by fluorescent protein fusions, immunofluorescence and immunogold electron microscopy (Czarnecki et al., 2015; Shiba et al., 2009; Watanabe et al., 2003). This localization suggests that the IC participates in gate-keeping of molecules between the cilium and cytoplasm, though direct evidence for this has yet to be presented.

The sequence of inversin, the parent member of the IC and the focus of this study, contains several predicted structural domains: 16 ankyrin repeats, two conserved IQ domains each with an adjacent basic region, and two possible nuclear localization sequences (Morgan et al., 1998; Yasuhiko et al., 2001). While these domains provide tantalizing hypotheses as to the function of inversin, how they generate the localization of inversin in the cell or how the protein functions within the cell is still unknown.

Ankyrin repeats are involved in a variety of protein-protein interactions and are found in proteins with a wide range of functions (Morgan et al., 1998; Sedgwick & Smerdon, 1999). The binding partner(s) of these repeats in inversin are currently unknown. However, the broad possibilities of the ankyrin repeats combined with the putative nuclear localization signals provides an intriguing possibility for inversin to have an as yet undetermined function in the nucleus.

IQ domains are reported to be involved in calmodulin binding, and indeed two groups independently showed that inversin binds calmodulin (Morgan et al., 2002; Yasuhiko et al., 2001). Additionally, the inversin-calmodulin interaction appears to occur when calmodulin is in the calcium-free state, as binding was seen in the presence of
the calcium chelator EGTA but not Ca$^{2+}$ (Yasuhiko et al., 2001). Calmodulin has not been visualized in the primary cilium either by immunofluorescence or the recent proximity biotin labeling (Mick et al., 2015; Plotnikova et al., 2012; Watanabe et al., 2003). However, calmodulin’s small size, likely low abundance in the primary cilium and/or possible chemical incompatibilities with the techniques could explain the lack of detection. Given that PKD2 mutations implicate calcium signaling in situs determination, the role of calcium in inversin compartment function may provide a common pathway for their overlapping phenotypes.

I.8. Measuring Calcium dynamics in the primary cilium

With so many aspects of PKD and associated proteins consistent with known calcium protein motifs and processes, measurement of calcium dynamics in the primary cilium could shed light onto the true function of these proteins. Genetically encoded calcium indicators (GECI) change their fluorescence properties upon binding of Ca$^{2+}$, enabling visualization of Ca$^{2+}$ behavior by live fluorescence microscopy. However, expressing a GECI in both the cytoplasm and cilium causes the signal from the very small cilium to be overwhelmed by the signal from the very large cytoplasm. Tethering a GECI to an exclusively ciliary protein increases contrast for the cilium signal by eliminating the signal of the large cytoplasm. Past studies have shown entry of calcium into the cilium including ATP stimulation of the cell (Su et al., 2013), release of calcium from caged EGTA in the cytoplasm adjacent to the cilium base (Delling et al., 2013), from the media outside the cell upon cilium tip ablation (Delling et al., 2013) and detection of changes in ciliary calcium ions in response to flow (Jin et al., 2014).
However, there is no publication that directly compares different methods for generating and measuring calcium dynamics in primary cilia nor that systematically defines characteristic behaviors of the calcium dynamics.

I.9. Methods Developed and Hypothesis Tested in This Thesis

Based on the above information, I propose a model in which the primary cilium is a conditional compartment for calcium and that the calcium residence time is an important control point for generating signals that will be transmitted to the cytoplasm. I further propose that the IC is the gatekeeper for calcium. If this is true, mutations in or deletion of IC proteins would result in a change in calcium dynamics in the primary cilium. My null hypothesis is that the genetic deletion of inversin will have no effect on the behavior of calcium in the cilium.

To test this hypothesis, I sought to develop an assay to define calcium dynamics in the primary cilium of wild-type cells and to use that assay to determine whether those dynamics are altered in cells lacking the IC due to genetic knockout of INVS. Four assays were tested, each utilizing live cell fluorescence microscopy of a cilium-targeted version of the GECI G-GECO1 but differing in the methodology to generate an abrupt localized calcium transient. The most robust method was to release calcium in the cytoplasm adjacent to the cilium base by localized un-caging of NP-EGTA. Kinetic changes in calcium ion concentration were quantified using custom MATLAB code.

I find that the absence of inversin increases the measures of peak duration and emptying time of calcium ions from the primary cilium. My data refutes the null
hypothesis and shows that residence time of Ca$^{2+}$ is longer in cells genetically null for Inversin.
Chapter II
Materials and Methods

To study the dynamics of Ca\textsuperscript{2+} in primary cilium, several cell lines with stable genetic alteration were created. I used live cell fluorescence microscopy to monitor the intensity change of calcium-sensitive fluorescent proteins after abrupt Ca\textsuperscript{2+} change and quantified the dynamics using custom MATLAB code. I tested four previously published techniques for generating an abrupt change in Ca\textsuperscript{2+} concentration and used the most robust method to compare the dynamics of wild type cells to those of cells lacking inversin by CRISPR/Cas-9 gene editing.

II.1. Reagents and Genetic Constructs

The cell line 3T3 (mouse fibroblast) was maintained in culture using complete DMEM (DMEM with high glucose, no Hepes, 10% fetal bovine serum, 1% penicillin and streptomycin, 1% glutamine or GlutaMax). Stable expression of transgenic proteins was maintained with constant presence of the appropriate selection antibiotic (2\(\mu\)g/ml blasticidin, 3 \(\mu\)g/ml puromycin).

Transgenic cell lines used are detailed in Table 1. For a subset of experiments, the calcium biosensor GCaMP3 was expressed in the cytoplasm of wild-type (WT) 3T3 cells using the pBABE retroviral vector (Morgenstern & Land, 1990; Shah et al., 2004; L. Tian et al., 2009). Calcium dynamics in the primary cilium were visualized using the previously published reporter 5HT6-mCherry- G-GECHO1 generously provided by T. Inoue (Su et al., 2013). Lentiviral particles harboring this construct were generated in with the vectors pCDH-UbC-MCS-EF1-Puro (used in conjunction with GCaMP3 in wild-
type cells) and pCDH-UbC-MCS-EF1-blasticidin (used in wild-type and CRISPR knockout cell lines; pCDH-UbC-MCS-EF1-Puro lenti vector from System Biosciences, modified for blasticidin in Shah laboratory; Prentice-Mott et al., 2016).

CRISPR knockout (KO) of INVS was accomplished using the vector pSpCas9(BB)-P2A-EGFP and the small guide RNA sequence of 5’-UGAGUAGCUUCCGUGCUCG-3’ to target a Cas9 protospacer adjacent motif (PAM) at nucleotide 483 in the INVS cDNA (NM_010569). Inversin add-back (INVS+) used the full cDNA sequence (NM_010569) with a Flag-tag at the N-terminus in either the lentiviral vector pCDH-UbC-MCS-EF1-Puro or retroviral vector pBABE-puro.

Table 1. Transgenic 3T3 cell lines used in this study.

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<th>Recipient cell line</th>
<th>Added Viral Vector</th>
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<td>0</td>
<td>3T3 IF</td>
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<tr>
<td>*3T3 GG</td>
<td>3T3</td>
<td>(a) 5HT6-mCherry- G-GECO1 in pCDH-UbC-MCS-EF1-Puro (b) GCaMP3 in pBABE-Blast</td>
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<tr>
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<td>3T3 INVS KO 6A3</td>
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<td>1</td>
<td>3T3 ATP, NP-EGTA</td>
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<tr>
<td>3T3 INVS+</td>
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<td>Full length INVS-Flag in pCDH-UbC-MCS-EF1-Puro</td>
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<td>3T3 IF</td>
</tr>
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<td>3T3 INVS+</td>
<td>5HT6-mCherry- G-GECO1 in pCDH-UbC-MCS-EF1-Blast</td>
<td>2</td>
<td>3T3 ATP, NP-EGTA</td>
</tr>
</tbody>
</table>

* indicates monoclonal cell lines were used.
The G-GECO1 biosensor has a $K_d$ of 750nM, similar to the reported calcium concentration in the primary cilium of 450-750nM, and GCaMP3 has a $K_d$ of 405nM, slightly higher than the cytosolic calcium concentration of approximately 100nM (L. Tian et al., 2009; Zhao et al., 2011; Delling et al., 2013; Akerboom et al., 2012). Matching the G-GECO1 sensor $K_d$ to the concentration of $\text{Ca}^{2+}$ allows for maximal dynamic range both above and below the basal $\text{Ca}^{2+}$ concentration.

Primary antibodies used were rabbit polyclonal anti-ANKS6 (1:1000, Sigma Aldrich HPA008355), mouse monoclonal IgG1 anti-polyglutamylated tubulin (clone GT335, 1:1000, Adipogen), and mouse monoclonal IgG2b anti-acetylated tubulin (clone 6-11B-1, 1:10,000, Sigma Aldrich). Diluted working stocks of all antibodies, except acetylated tubulin, were kept at 4°C and reused for up to 6 months to conserve antibody. Secondary antibodies used were Alexa Flour 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG1, Alexa Fluor 674 goat anti-mouse IgG, and Alexa Fluor 647 goat anti-mouse IgG2b (ThermoFisher).

II.2. Generation of CRISPR Cell Lines

Generation of CRISPR knockout (KO) in IMCD and 3T3 cells was performed by Peter Czarnecki in the Shah laboratory according to the protocol by Ran, et al. (2013) using the constructs detailed above. Monoclonal CRISPR KO cell lines were generated by single cell sorting (based on side scatter size) for GFP-positive cells that excluded propidium iodide into 96-well plates containing DMEM with 50% 3T3-conditioned DMEM and a final concentration of 20% FBS. Successfully grown clones were screened for successful loss of INVS by absence of ANKS6 immunostaining (details below).
Subsequent restoration of INVS (add-back; INVS+) in CRISPR KO cell lines was achieved by lentiviral transduction into the monoclonal CRISPR KO line using the constructs detailed above and screened for return of ANKS6 immunostaining.

II.3. Generation of Stable

3T3 Cell Lines Expressing Calcium Sensors

Lentivirus preparation, transduction and initial selection were performed by Peter Czarnecki in the Shah laboratory. Briefly, 293T cells were transiently co-transfected with lentiviral plasmid and packaging plasmids and grown for 2 days, the virus-containing media were filtered through a 0.45μm syringe filter and applied to the desired recipient cell line for two days after which the transduced cells were placed under strong selection based on the selection marker (5μg/ml puromycin and/or 4μg/ml blasticidin). Prior to single cell cloning, polyclonal cell populations were evaluated for sensor concentration(s) and cilium morphology by widefield fluorescence microscopy (details below) for GCaMP3 and/or mCherry at 40x. Single cell cloning was carried out by dilution: A T25 flask at 70% confluence (approximately 1x10^6 cells) was trypsinized and re-suspended with complete media to a final volume of 2 ml followed by two-step dilution to generate 1:4.5x10^5 and 1: 9x10^4 final dilutions to generate on average one cell in every 3 or 10 wells, respectively, when distributed in 90ul to each well of 2-4 96-well plastic bottom plates. Plates were incubated at 37°C with 5% CO₂ until clones grew to
confluence, at which time they were distributed to a larger vessel for expansion and then to a glass bottom dish for evaluation of sensor expression level and cilium morphology.

II.4. Immunofluorescence

Trypsinized and re-suspended cells were plated in duplicate into wells of a 24-well plate that each contained one squeekie-clean 12mm round #1.5 glass coverslips (ThermoFisher) in 500μl complete DMEM or into 12-well chamber slides (ibidi GmbH) in 250μl complete DMEM per well (Waterman Storer, 2001). Cell were grown for 2-3 days to 80-90% confluence then incubated in low-serum DMEM (DMEM, 0.25% fetal bovine serum, 1% penicillin and streptomycin, 1% glutamine) for 1-2 days. Cells were rinsed 1-2 times in MTSB (MicroTubule Stabilizing Buffer - 55mM Pipes, 25mM potassium chloride, 5mM magnesium sulfate, 5mM EGTA, pH7.2) to remove debris, fixed with 3.7% formaldehyde (37% formaldehyde solution diluted fresh in MTSB) for 15min, washed 2 times for 5 minutes in MTSB plus 100mM glycine to quench autofluorescence generated by the aldehyde fixation, and washed once for 5 minutes with MTSB. Non-specific binding of proteins was blocked by incubation in 2% BSA plus 0.1% Triton X-100 for 30 min at room temperature (RT) or at 4°C overnight. Primary antibodies other than that for acetylated-alpha-tubulin were diluted as described above in staining buffer (50% blocking solution in MTSB) and incubated for 20-30min at room temperature or 4°C overnight. Cells were then washed three times for five minutes each in excess volume (0.5-1.0 ml) of MTSB plus 0.1% Triton-X 100 (MTSB-T) followed by incubation with anti-acetylated-alpha-tubulin (fresh 1:10,000 dilution in staining buffer) for 20min at RT. Cells were again washed three times for five minutes each in excess
volume of MTSB-T, followed by incubation with combined secondary antibodies for 30 min at RT. Cells were washed three times in excess volume MTSB-T for 5 min each, incubated with Hoescht (1:10,000 in MTSB) for 30-60 sec and washed once for 5 min with MTSB prior to mounting with Vectashield H-1000 mounting media and being sealed with nail polish. Coverslips (12mm) were mounted onto 1x3 inch glass slides and 12-well chamber slides were mounted with a single 24x60mm #1.5 glass coverslips (Fisher Scientific). Immunofluorescence samples were imaged by widefield epifluorescence microscopy for ANKS6 and GT335 using a 20x/0.8 Plan Apochromat Ph2 objective lens or for Poly-E using a Plan Apochromat 60X 1.4NA oil immersion objective lens.

II.5. Widefield Epifluorescence Microscopy

Widefield epifluorescence microscopy was performed on a Nikon Ti inverted epifluorescence microscope with incubation enclosure maintained at 37°C using a 20x/0.8NA Plan Apochromatic Ph2, 40X/1.3NA Plan Apochromat oil immersion, or Plan 60X/1.4NA Apochromat oil immersion objective lens, a Photometrics CoolSnap CCD camera and automated stage and shutters (LUDL) that were all controlled with Nikon Elements software (version 3.22.14; Nikon Instruments, Melville, NY). Epifluorescence illumination was generated by a mercury arc lamp on the microscope and individual microscope filter sets used for visualization of mCherry (excitation 520-550nm, beam splitter long pass (LP) 570nm, emission 590-620nm), Alexa Fluor 647 (ex. 590-650nm, LP660nm, em 663-738nm), Alexa Fluor 488 (ex. 455-485, LP495nm, em 500-545nm), and Hoescht (3340-380nm, LP400nm, 435-485nm). Filters were selected using the
software control of the microscope. A halogen light source and phase optics generated a phase contrast image.

II.6. Live Imaging of 3T3 Cells

Re-suspended 3T3 cells were added to 35mm MatTek dishes (MatTeck Corp) containing 1.5-2 ml complete DMEM or added to 12-well chamber coverslips (Fisher Scientific 22x60mm #1.5 coverslip with ibidi silicon chamber walls) with 200μl complete DMEM. After two days, with cells at a density of 60-70% for cilia tip experiments or 85-95% for all other conditions, media was changed to low serum (DMEM, 0.25% FBS, 1% pen/strep, 1% GlutaMax) for an additional 2 days. Experiments perturbing the cillum tip (two-photo ablation and NP-EGTA) required selection of cells during data acquisition whose cillum extended beyond the perimeter of the cell so as to avoid ablation or activation within the cytoplasm of the cell.

II.7. Two-Photon Cillum Tip Ablation

Cells in MatTek dishes were imaged on the previously described widefield epifluorescence microscope into which a Ti:Sapphire laser (MaiTai, SpectraPhysics) beam was focused at the specimen plane using a 60X NA1.4 objective. The wavelength was set at 800nm and the laser power was set at 40mW exiting the laser as measured by a laser power meter (Newport). The XY position of the focused laser beam in the image plane was saved in the software and the stage was moved to position the cillum tip in the path of the laser. Preliminary images were collected at 3 second intervals prior to a 50ms
pulse of the Ti:Sapphire laser and up to 100 images collected thereafter. Laser ablation timing was controlled with a LUDL shutter and the system-integrated Nikon Elements software.

II.8. ATP-mediated Calcium Release in 3T3 Cells

Cells in 12-well coverslips or MatTek dishes were imaged either on the widefield fluorescence microscope at 40X or on a spinning disk confocal (SDC). On the ADC, cells were imaged with 60x 1.4NA Plan Apochromat objective on a Nikon Ti microscope attached to an Andor Revolution Yokogawa X1 SDC head and Andor iXon 897 EM-CCD camera. All components were controlled by the MetaMorph software (v7.8.9; Molecular Devices, Sunnyvale, CA, USA). The MultiDimensional Acquisition function was used to collect one image each for the brightfield and mCherry (excited 561nm, emission 607/36nm) channels and a time series of the G-GECO1 channel (excitation 488nm, emission 525/40nm). After ~30 time points, a volume of ATP solution equal to 50% or 100% of the imaging media was added for a final concentration of 100μM ATP. Focus was maintained using the Perfect Focus System on the Nikon microscope. Full frame images were acquired so as to collect as many cilia as possible. Image interval was set at 1 second for widefield imaging and as fast as possible for SDC imaging.
II.9. Calcium Release by NP-EGTA Un-caging in 3T3 Cells

On the day of imaging, one 50μg tube of NP-EGTA, AM (a-nitrophenyl EGTA, AM, Thermo Fisher Scientific) was resuspended in 63.3μl DMSO (1mM). Cells in MatTek dishes were loaded for 40min in 1μM NP-EGTA, AM at 37°C and 5% CO2. Prior to imaging, media was exchanged for fresh pH-equilibrated 0.25% FBS DMEM supplemented with 10mM Hepes pH 7.3 and without NP-EGTA. Cells were kept warm for imaging using a LiveCell stage-top incubation system (Pathology Devices). Cells were imaged at 60X on the SDC described above that also includes the Andor FRAPPA point photomanipulation system. Exposure time for the G-GECO1 channel was set at 150-200 ms with EM Gain of 20, 5 MHz readout speed, 5.1X pre-amp gain and only the center quadrant was collected to increase frame rate. One set of images of the brightfield, mCherry and G-GECO1 signals was collected using the MultiDimensional Acquisition function before use of the Targeted Illumination function for NP-EGTA un-caging and associated image collection. Targeted Illumination was set up to collect six images, illuminate the sample with 20 pulses of 405nm laser light (100mW at the source) each lasting 200-300μs at a position designated by a 3x3 pixel circle region, and continuing to collect images for 96 time points thereafter as fast as possible. Each dish was imaged no more than 20 minutes after addition of fresh media to minimize effects of medium pH changes. Final values were generated from 9-25 cilia in 2 independent experiments.
III.10. Image Analysis and Figure Preparation

Image data was stored on and accessed from a locally managed OMERO server (Allan et al., 2012). Image analysis was performed using the Fiji package of ImageJ with Bio-Formats plugin (Schindelin et al., 2012; Linkert et al., 2010). Images were prepared for figures using Fiji, graphs were generated in Prism (GraphPad, Version 7.0a), MATLAB (The Mathworx Inc, vR2016b) and Microsoft Excel 2011 (version 14.6.0), and figures were assembled in Adobe Illustrator (version CC2014).

II.10.a. For Fixed Cell Immunofluorescence.

Nuclei, ANKS6 and GT335 punctae and cilia (delineated by anti-acetylated-tubulin) were tallied using the Count Cells function in Fiji. Approximately 1000 cells from each cell line for each of three experimental repeats were measured. Calculations were performed in Microsoft Excel and Prism was used to generate plots and calculate statistics.

II.10.b. For Live Cell Calcium Dynamics.

Data from the cilia tip ablation and ciliary un-caging were analyzed by drawing a segmented line along the center of the cilium using the mCherry channel, generating a copy of the line and placing it in the background near the cilium, copying both to the G-GECO1 channel and measuring the intensity for all time points using the MultiMeasure function in the Regions Tools of Fiji.
For calcium dynamics generated by ATP and cytoplasmic NP-EGTA photo-un-caging, three sets of measurements were generated from the raw data using custom Fiji macros: The starting mCherry intensity, the time course G-GECO1 intensities and the actual elapsed time (Appendix C). Briefly: A threshold was applied to the mCherry channel to highlight the cilia. A region of interest (ROI) was generated around one cilium using the magic wand tool and a duplicate ROI was placed adjacent to the cilium for background measurement. The average intensity for both ROIs were measured for mCherry and G-GECO1 using the MultiMeasure function. The same regions were transferred to the time series file and intensities for both regions were measured for all time points. Files where the cilium did not remain in the region for the entire time series were excluded from analysis. The true elapsed time was extracted from the image file metadata using a separate macro (Appendix C) or by copying and pasting the time series information from the OMERO viewer.

Dynamics information was generated using a set of custom MatLab scripts (Appendix C). Briefly: For each cilium, the cilium G-GECO1 mean intensity was background subtracted using the mean intensity of the background region at each time point, the intensity-time trace was mean-filtered (window size of 3) and fit to a spline and the time point at peak (ATP) or prior to activation (NP-EGTA) was determined. To quantify the intensity traces, I identified 4 “signpost” time points along the rise and fall of G-GECO1 intensity, each defined by percentage of maximum intensity (20% and 80% or 35% and 65%). I defined the signposts as fill start (the time point before the maximum at which the intensity was 20% or 35% of the maximum intensity), peak start (the time point before the maximum at which the intensity was 65% or 80% of the maximum
intensity), peak end (the time point after the maximum at which the intensity was 65% or 80% of the maximum intensity) and end time (the time point after the maximum at which the intensity was 20% or 35% of the maximum intensity; Figure 1). These signposts were then used to calculate the residence time values of filling time (time between fill start and peak start), peak duration (time between peak start and peak end) and emptying time (between peak end and end time; Figure 1). If the final G-GECO1 intensity for a cilium did not return below the lower threshold (20% or 35%), all data from that trace was excluded. The data was consolidated by genotype and output parameter in MATLAB. Further consolidation and calculation of maximum fold change were performed in Excel. Prism was used to calculate statistical comparisons between KO and WT using a two-tailed student t-test and the correlation between mCherry intensity (sensor levels) and measured values.

Figure 1. Schematic of calcium dynamics quantitation. Four “signpost” times, defined by 35% (green dots) and 65% (red dots) of the maximum intensity, were used to calculate filling time (ft), peak duration (pd) and emptying time (et).
Chapter III
Results

In this study I evaluated ciliary calcium dynamics in multiple cell lines expressing fluorescent genetically encoded ciliary calcium ion sensors and in some cases genetically modified by CRISPR/Cas-9 genome editing. Four assays were evaluated to define calcium dynamics in the primary cilium of WT cells, and used the most robust assay was used to determine whether calcium dynamics are altered in cells lacking the IC via the genetic deletion of INVS. Kinetic changes in calcium ion concentration were recorded using digital imaging microscopy of living cells and quantified using custom MATLAB scripts.

III.1. Generation of Stable Cell Lines With a Ciliary Calcium Indicator

I used a construct for the calcium sensor that contained both the calcium sensor (G-GECO1) for calcium ion concentration measurements and mCherry fluorophore for estimation of the calcium sensor concentration. The initial transduction of 3T3 cell populations with the cilium-targeted calcium sensor 5HT6-mCherry-G-GECO1 (in puromycin-resistance lentivirus vector) and the cytoplasm-localized GCaMP3 (cell line 3T3 GG) generated significantly heterogeneity in per-cell sensor concentrations, cilium sensor localization and cilium morphology. A second WT sensor line was generated with
only the cilium-targeted calcium sensor 5HT6-mCherry- G-GECO1 (in blasticidin-resistance lentivirus vector) that produced a much higher percentage of positive cells though still some heterogeneous cilia morphology. Cilia exhibiting high levels of the 5HT6-mCherry- G-GECO1 sensor had very long and sometimes bulbous morphologies indicating a potential perturbation of the cilium under high sensor expression. To improve experimental throughput for live cell imaging, all sensor cell lines underwent single cell cloning and visual selection for normal cilium morphology and moderate sensor expression. The INVS KO cell line 6A3 and the polyclonal KO INVS+ lines expressing the cilium sensor derived therefrom were infected with the cilium-targeted sensor 5HT6-mCherry-G-GECO1 in lentivirus blasticidin-resistance vector followed by single cell. One clone was isolated for each of the KO and INVS+ lines that had the desired sensor level and cilia morphology. The KO with sensor line 7F7 was one out of 17 clones and the INVS+ with sensor line 8E3 was one out of 20 clones. Unfortunately, the INVS add-back line with sensor, 8E3, was negative for ANKS6 staining, indicating failure of INVS add-back. Thus add-back data was unavailable for this study.

III.2. Ciliary Calcium Perturbations

Four strategies were tested to determine the best way to measure calcium dynamics in the primary cilium, as illustrated in Figure 2.
Figure 2. Schematics of abrupt calcium ion release strategies. (A) Two-photon ablation of cilium tip, (B) NP-EGTA un-caging in the cilium tip, (C) NP-EGTA un-caging in the cytoplasm and (D) ATP-mediated calcium release.

III.2.a. 2-photon Cilium Tip Ablation

Ablation of the cilium tip using a focused Ti:Sapphire laser tuned to 800nm (Figure 2A) generated an immediate and strong increase in G-GECO1 signal within the cilium, bleaching of the sensor and mCherry near the ablation site and no sustained change in the cytoplasm intensity of dual sensor cells (Figure 3A,C). Extended the imaging time after ablation revealed that the cilia maintain high G-GECO1 intensity while retracting toward the cell body (Figure 3A). This indicates that the technique likely creates massive disruption to the cilium structure and physiology. Additionally, it is impossible to know if or when the cilium membrane closes. These challenges pose many possible confounding factors, therefore I chose not to pursue this method further.
Figure 3. Calcium dynamics in the cilium tip. (A) Ablation of cilium tip with 800nm 2P illumination results in prolonged high G-GECO1 intensity and partial retraction of the cilium. (B) Illumination with 405nm light in cilium tip of NP-EGTA loaded cells generates extremely fast calcium pulse. (C) and (D) Graph of average G-GECO intensity (thick line) over time for A and B, respectively. Thin line in C is intensity of a region in the cytoplasm adjacent to the cilium. Note difference in time scales. Arrowheads and squares indicate illumination time and location, respectively.

III.2.b. NP-EGTA Un-caging in the Cilium Tip

The caged EGTA reagent NP-EGTA, AM works by binding calcium ions in the cellular medium with the EGTA portion, diffusing into the cell then getting trapped inside the cell by cleavage of the AM ester bond by native cellular esterases (Tsien, 1981). Illumination of NP-EGTA with a modest intensity of 405nm laser light at a level that does not damage the cell causes a conformational change in the NP-EGTA molecule.
that prevents calcium binding. This releases the Ca\(^{2+}\) from the illuminated NP-EGTA molecules, thereby generating a spatially controlled source of calcium ions.

Cilia that extend away from the cell body with the cilium tip beyond the cell periphery also enabled generation of a local calcium bolus in the cilium tip by un-caging of NP-EGTA (Figure 2B) by using a focused 405nm laser beam for illumination, preventing possible contribution of calcium release in the cytoplasm. NP-EGTA un-caging in the cilium tip of 3T3 WT GG cells generated a flash from the calcium sensor G-GECO in the cilium that dissipated approximately one second after activation (Figure 3B,D). This time scale was too short to accurately measure in our microscopy system, therefore alternate methods for measuring calcium dynamics were explored.

III.2.c. NP-EGTA Un-caging in the Cytoplasm Adjacent to the Cilium

Photo-un-caging of NP-EGTA with 405nm light in the cell cytoplasm (Figure 2C) generated a fast moving wave of calcium within the cytoplasm, as evidenced by cytoplasmic GCaMP3 fluorescence (Figure 4A). Un-caging in the cytoplasm far from the cilium base resulted in little or no measurable intensity change within the cilium, suggesting insufficient additional calcium ions entering the cilium to be detected. Un-caging within 1-2μm of the cilium base typically generated a robust G-GECO1 signal in cilia of WT cells (fold change of 3.4+/−2.3 between pre-activation minimum and peak maximum). This final technique was used for further experiments. As shown in Su, et al., (2013) with ATP, occasionally the calcium signal was seen flowing into the cilium from base to tip, though usually there was insufficient time resolution. The signal appears to dissipate evenly across the area of the cilium, though more careful study is needed for more an accurate description (Figure 4B,C). The pattern of intensity over
time had a general shape of rapid increase followed by slower decrease. The precise shape varied from cilium to cilium (Figure 4D).

III.2.d. ATP-mediated Calcium Release

Stimulation of 3T3 GG cells with 100μM ATP (Figure 2D) generated a strong calcium signal in the cytoplasm (Figure 5A, circles) and the primary cilium (Figure 5A, 1 and 2), as previously shown by Su, et al. (2013). Additionally consistent with observations by Su, et al. (2013), the calcium signal appeared to start in the cytoplasm and progress up the cilium in a wave (Figure 5B). The pattern of intensity over time in response to ATP was quite variable. Sometimes with a sharp increase and slow decrease (Figure 5C, left), multiple peaks (Figure 5C, center) or with a gradual increase and decrease (Figure 5C, right). The time scale of Ca\(^{2+}\) residence in the primary cilium was typically in the 10s of seconds (Figure 5C).
Figure 4. NP-EGTA un-caging in the cytoplasm. (A) Un-caging NP-EGTA in cells expressing both 5HT6-mCherry- G-GECO1 and GCaMP3 generates a fast moving wave of calcium in the cytoplasm. The image is pseudocolored as indicated on the right to highlight the change in GCaMP3 intensity in the cytoplasm of the cell. Within the one second between pre- and post- activation with 405nm light the calcium has increased in the entire cytoplasm. Scale bar is 10μm. Box indicates location of 405nm illumination. (B) Cilium morphology and location of 405nm illumination in two representative cells (box). Same scale as A. (C) Kymograph of G-GECO1 intensity along each cilium (Y axis) over time (X axis) for cilia shown in B. Dashes in B indicate boundary of line used. Scale bar for Y axis (at far left) is 5μm. (D) Graph of average intensity over time for cilia shown in B and C.
Figure 5: ATP activation. (A) Calcium ion behavior in cytoplasm (GCaMP3) and cilium (5HT6-mCherry-G-GECO1) after ATP activation and imaged in widefield fluorescence. Cilia 1 and 2 were defined by the mCherry localization. Intensity values are plotted as indicated. Background regions have camera offset subtracted. Cilia regions have their respective cytoplasm region (designated by circle) subtracted. Scale bar 10μm. (B) Calcium wave seen entering the base of the cilium and filling to the tip. Widefield imaging, cilium sensor only. Scale bar 5μm. (C) Normalized intensity over time traces from three representative cilia imaged by spinning disk confocal. Because the start of calcium entry is not precisely known with ATP activation, the G-GECO1 signal peak is defined as time zero.
III.3 Validation of Sensor

After establishing the NP-EGTA activation protocol using the monoclonal cell line, experiments were performed in two polyclonal WT cell lines (puromycin- or blasticidin-resistant versions) to determine what effect sensor concentration, as measured by mCherry intensity, might have on measured dynamics. Cilia were visually selected to have normal physiology and sufficient mCherry intensity for clear identification of the cilium. Final cilium intensity and maximum peak intensity showed positive correlation with mCherry intensity in both WT and KO (r = 0.45-0.69; Table 2, Figure 6A). The dynamic measurements (peak duration and emptying time) had extremely low correlation with mCherry over a 10-fold range of mCherry intensities (r = -0.25 - 0.05; Table 2, Figure 6B). When sensor level increases we see an increase in intensity-based readout but do not see a change in time-based measurements. Therefore, when the cilia have normal morphology and sensor intensities within our system’s detectable range, the dynamics measurements are likely not influenced by sensor concentration.

Table 2. Correlation of calcium dynamics measurements with mCherry intensity.

<table>
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<tr>
<th>Cell line</th>
<th>#</th>
<th>vs. Baseline Intensity</th>
<th>vs. Final intensity</th>
<th>vs. Max intensity</th>
<th>vs. Duration 65-65%</th>
<th>vs. Emptying 35-65%</th>
<th>vs. Emptying 20-80%</th>
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<tr>
<td>3T3 wt</td>
<td>47</td>
<td>0.59*</td>
<td>0.6331*</td>
<td>0.68*</td>
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<td>-0.11</td>
<td>-0.16 (n=46)</td>
</tr>
<tr>
<td>3T3 KO 7F7</td>
<td>32</td>
<td>0.69*</td>
<td>0.4541*</td>
<td>0.59*</td>
<td>-0.11</td>
<td>-0.25</td>
<td>-0.03 (n=30)</td>
</tr>
</tbody>
</table>

* indicates $\alpha = 0.5$
Figure 6. Correlation of mCherry and calcium dynamics measurements. (A) Relationship between mCherry intensity and final intensity (black open square) or maximum intensity (red triangle). (B) Relationship between sensor concentration as indicated by mCherry intensity and peak duration (black circle) or emptying time (red cross).

III.4. Ciliary Calcium Measurements and Analysis

To quantify the intensity traces, I defined 4 “signpost” time points along the rise and fall of calcium ion concentration based on percentage of maximum intensity (two points each at 20% and 80% or 35% and 65% of peak intensity). In order from the start of the time course, these time points were fill start, peak start, peak end and end time (as defined in the Methods section). These signpost values were then used to calculate the calcium residence times of fill time, peak duration and emptying time that I used to characterize the calcium dynamics.
III.5. Calcium Dynamics in the Primary Cilium After NP-EGTA Activation and ATP Stimulation In WT Cells

The mean filling time (fill start to peak start, as defined above) was 318±133ms with NP-EGTA for the more inclusive 20%-80% measurement (Table 3). This represents less than two measured time points, thereby making the measurement highly susceptible to spline fitting bias and window averaging and hence a poor quantitative measure. Therefore it was not used for comparisons. With either the 20%-80% or 35%-65% cutoff, less than 10% of cells with NP-EGTA un-caging in the cytoplasm failed to empty to baseline (Table 3).

For ATP stimulation, the traces were much less consistent (Figure 5D). Almost half the cells (42%) failed to return to the 20% cutoff by the end of the experiment though only 13% failed to return to the higher 35% cutoff (Table 3). The time scale of the dynamics was much longer with ATP than with NP-EGTA. The filling time (20-80% cutoff) was 2127±535ms, 6.6 times longer than for NP-EGTA (Table 3). The peak duration (peak start to peak end, as defined above) and emptying time (peak end to end time, as defined above) were also over 6 times longer than with NP-EGTA (6987±3388 ATP vs. 1022±1271 NP-EGTA and 22627±9888 ATP vs. 3736±4998 NP-EGTA respectively; Table 3). The spread of the values with ATP were more evenly distributed than the NP-EGTA values as seen in; the median and mean filling times; they were similar for ATP (2100 and 2127, respectively) while for NP-EGTA the median filling time was lower than the mean (280 and 318, respectively), indicating a bulk of the NP-EGTA values were below the mean (Table 3). The large difference in time scale and high variability in intensity pattern make ATP-mediated calcium release a less robust
method for measuring calcium residence time as compared with NP-EGTA un-caging in the cytoplasm.

Table 3. Ciliary calcium dynamics.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>#</th>
<th># Fail to empty to cutoff of a</th>
<th>Mean Filling time b 20-80% (ms)</th>
<th>Median Filling time 20-80% (ms)</th>
<th>Max intensity (AU)c</th>
<th>Peak Duration b 80-80% (ms)</th>
<th>Median Filling time 65-65%* (ms)</th>
<th>Peak Duration b 80-20%* (ms)</th>
<th>Emptying Time b 65-35%* (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 wt ATP</td>
<td>26</td>
<td>11 (42%)</td>
<td>2127 ± 535</td>
<td>2100</td>
<td>96.25 ± 88.09</td>
<td>6987 ± 3388</td>
<td>13748 ± 8064</td>
<td>22627 ± 9888</td>
<td>11200 ± 6547</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3 wt NP-EGTA</td>
<td>49</td>
<td>3 (6%)</td>
<td>318 ± 133</td>
<td>280</td>
<td>75 ± 67</td>
<td>1022 ± 1271</td>
<td>2111 ± 2907</td>
<td>3736 ± 4998</td>
<td>2044 ± 2557</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (4%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3 KO 7F7 NP-EGTA</td>
<td>32</td>
<td>6 (2%)</td>
<td>388 ± 160</td>
<td>320</td>
<td>348 ± 136</td>
<td>1550± 1071 [0.064]</td>
<td>3462 ± 2688 [0.040]</td>
<td>6334 ± 3653</td>
<td>2444 ± [0.017]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# = number of cilia measured, a = Parenthesis indicate percentage, b = Mean filling time, peak duration and emptying time are mean +/- SD, c = Intensity values are background subtracted, * = p<0.05 between WT and KO with NP-EGTA, brackets are p value for difference from NP-EGTA WT.

III.6. Characterization of Ciliary Calcium Dynamics in INVS KO Genotype

Confirmation of protein knockout by CRISPR is best done with genotyping but quick screening by immunofluorescence (IF) is highly desirable. There are no reliable INVS antibodies, however the IC protein ANKS6 is known to indirectly depend on INVS via Nek8, has a robust antibody available, and localization by IF proved to mirror the INVS genotype (Figure P; Shiba, Manning, Koga, Beier, & Yokoyama, 2010). Single cell sorting of the INVS KO CRISPR transfection produced 12 clones, of which two clones were positive for ANKS6 by immunofluorescence, indicating presence of the IC
and failure of the CRISPR, and were not propagated. Two additional clones did not grow, leaving 8 ANKS6-negative lines. INVS add-back (INVS+) by lentiviral plasmid transduction was performed with two INVS KO clones and the clone with higher ANKS6 recovery was selected for further study. For the chosen INVS KO cell line (3T3 INVS KO 6A3, “KO”), the percent of ciliated cells with ANKS6 in KO was 0.1% +/- 0.1% compared to 60.8% +/- 7% in the WT and 61.5% +/- 10.7 for the INVS+, respectively (Figure 7A,B). The greater-than-zero value for ANKS6 incidence the INVS KO cells likely represents random localization of background signal with cilia. The percent of cilia-positive cells was similar for all three cell lines (WT 80% +/- 3%, KO 67% +/- 7% and INVS+ 66% +/-0.4%; Figure 7A-D), indicating ciliation is not affected by INVS KO. The glutamylated tubulin antibody GT335 maintains high incidence per cilium regardless of INVS genetic background (WT 97%, KO 95% and INVS+ 97%; Figure 7C,D).

In the dynamics experiments, peak duration (peak start to peak end) was slightly, but not significantly, longer in KO compared to WT when set to an 80% cutoff (1022±1271ms WT and 1550±1071ms KO, p=0.06) but showed a statistically significant 1.6x difference when set at the more inclusive 65% (2111 ±2907ms WT and 3462±2688ms KO, p=0.02; Table 3). For KO compared to WT there was a 1.7x difference in emptying time (peak end to end time) for both the 80-20% cutoff (3736±4998ms for WT and 6334±3653ms for KO, p=0.017), and 65-35% cutoff (2044±2557ms for WT and 3446±2444ms for KO, p=0.01; Table 3).
Figure 7. ANKS6 and GT335 in confluent 3T3 cells. (A) Immunofluorescence for DAPI, acetylated-tubulin and ANKS6 or GT335 in 3T3 WT, INVS KO and INVS+ cells. Scale bar is 10μm. (B) Quantitation of ANKS6 incidence with cilia. WT (black), INVS KO (mid grey) and INVS+ cells (light gray). 60% of cilia have ANKS6 in WT cells, no cilia have ANKS6 in the KO and 61% of INVS+ cilia are ANKS6 positive, similar to WT. (C) Quantitation of ANKS6 incidence with cilia.
In this study I sought to develop an assay to define calcium dynamics in the primary cilium of wild-type cells and to use that assay to determine whether those dynamics are altered in cells lacking the IC due to genetic knockout of INVS.

IV.1. Reagent Development

To understand the dynamics of calcium within the primary cilium I used the genetically encoded calcium biosensor G-GECO1 fused to the primary cilium localized protein 5HT6 and the calcium-independent fluorescent protein mCherry (5HT6-mCherry-G-GECO1; (Su et al., 2013)). The G-GECO1 increases fluorescence intensity upon binding Ca\(^{2+}\), therefore this construct enabled visualization of Ca\(^{2+}\) dynamics within the primary cilium without interference from a cytoplasmic signal. Although not used in the studies presented here, the Ca\(^{2+}\)-insensitive mCherry can also act as a volume marker for ratiometric measurements of Ca\(^{2+}\) concentration. Initial transduction by lentivirus lead to highly variable expression levels and cilia morphologies, requiring the generation of clonal lines from single or small numbers of cells for experimental consistency and efficiency. Excessively high cilium-localized protein expression has been shown to generate abnormal cilium morphology and was also seen in our hands (Su et al., 2013).
One concern with vast excess of a biosensor such as G-GECO1 is that the sensor will act as a sink for the detected ion and thereby bias measurements to be longer values. Our data indicate that the criterion of normal cilium morphology is sufficient to limit the cilia sensor concentration to within a range that leads to unbiased dynamics measurements at a gross level (Table 2, Figure 6). However, further study is needed to define the relationship of sensor concentration and calcium dynamics more precisely.

To help define a cellular phenotype for INVS KO, I relied on the powerful CRISPR/Cas-9 gene editing technique to remove INVS from 3T3 cells and combined it with the primary-cilium localized G-GECO1 for visualization of calcium dynamics by live cell imaging. CRISPR/Cas-9 is a transformative tool for studying protein function by enabling consistent population-wide genomic changes in tissue culture cells. The clean genetic background generated by CRISPR/Cas-9 mutation will further enable addition of exogenous plasmids to not only confirm KO effects but also investigate how specific mutations in INVS, when added back to the KO background, affect IC formation and calcium behavior in primary cilia.

In our hands, the generation of a single cell clone with good cilium morphology and sensor expression was inefficient. Our genetic add-backs, without a sensor, were highly successful, as evidenced by equivalent prevalence of ANKS6 positive cilia in the add-back and WT despite total loss in the KO (Figure 7A,B). Therefore, I recommend first generating a stable monoclonal CRISPR KO cell line with the cilium sensor that has appropriate cilium morphology into which add-back plasmids are transduced or transfected. Rather than, for instance, selecting for the add-back prior to adding the sensor, or selecting for the add-back and sensor simultaneously. If total expression level
of the add-back plasmid is likely to influence results, further single cell cloning is also recommended to improve experimental consistency.

IV.2. Calcium Dynamics Assay Development

The 5HT6-mCherry- G-GECO1 biosensor cell lines allowed me to evaluate several strategies for measuring the dynamics of calcium in the primary cilium. My initial inclination was to generate calcium in the cilium and monitor removal. Cilium tip ablation had been used previously as a proof-of-principle for the cilium sensor activity (Delling et al., 2013). While I also saw robust calcium response after ablation with a pulsed Ti:Sapphire laser, the resulting prolonged elevated G-GECO1 signal and eventual retraction of the cilium indicated to me that there was substantial damage to the cilium structure. Since this damage is likely to introduce many confounding factors in measuring calcium dynamics, cilium tip ablation was abandoned as a strategy for measuring calcium dynamics. However, it is a very straightforward method of testing the sensor as it generated a massive local calcium bolus.

My second strategy to generate a calcium burst within the cilium was to un-cage the caged calcium reagent NP-EGTA at the cilium tip using a brief pulse from a focused 405 nm laser beam. This generated a fluorescent burst that did not persist but was instead too short to accurately measure with our 150-200ms image delay. Faster image collection was attempted on a point scanning confocal with Ga-As based detectors but sufficient signal to noise was not attainable with the sensor expression levels used. It would be interesting to see if the higher efficiency GaAsP or hybrid PMT-APD detectors
could generate sufficient SNR. Since both strategies for generating calcium in the cilium tip were not amenable to the equipment available, I moved to strategies that filled the cilium with calcium prior to emptying.

Su, et al.,(2013) showed that ATP activation of the cell generates a wave of calcium into the cilium from base to tip (Figure 5). Also, DeCaen, et al. (2013) showed that un-caging NP-EGTA in the cytoplasm immediately adjacent to the primary cilium base resulted in movement of calcium into the primary cilium. I found that ATP activation is by far the simplest of the four tested strategies to implement since no special lasers or laser manipulation equipment are required. Additionally, since the manipulation applies to all cells in the field of view, it is the highest throughput option. However, ATP-mediated calcium release generated very long dynamics parameters as compared to diffusion and the NP-EGTA un-caging data. ATP-mediated calcium release is known to work through P2X and P2Y receptors to release internal calcium stores and likely activates a range of signaling pathways (Su et al., 2013). The 1.5s filling time measured for ATP activation, 6-7 times longer dynamics for other parameters and frequent occurrence of multiple pulses of Ca\(^{2+}\) after stimulation indicate additional modes of action beyond initial Ca\(^{2+}\) release. This complicates dissection of the behavior of single calcium transients and therefore was not pursued further in this study. A high concentration of ATP (100μM) was used in this study to maximize effect. A more detailed investigation into the dynamics generated by lower concentrations of ATP (1-10μM) are needed to determine if a better balance between ease of implementation and complexity of calcium response can be found.
Un-caging of NP-EGTA in the cytoplasm adjacent to the cilium opening was the best assay. It was relatively consistent from day to day. The calcium movement time scales were similar to those expected for diffusion of very small ions and were within the ranges I could accurately measure. The downsides to this method are the need for somewhat specialized instrumentation, specifically point photo-manipulation in line with the confocal imaging to allow for sufficiently fast change from un-caging to imaging, and the low throughput of one cilium at a time. Fortunately, such instrumentation was available for my study. As stated for the NP-EGTA in the cilium tip, a point scanning confocal with high efficiency detector may be a possible alternate for the Andor Revolution spinning disk confocal system used in this study but needs to be tested.

IV.3. Calcium Dynamics in the Cilium of WT and INVS KO Cells

Our data with NP-EGTA un-caging in the cytoplasm near the cilium base shows that residence time of calcium in the primary cilium is different in the absence of the IC in the INVS KO cells as compared to WT. Specifically, calcium residence time was longer for the INVS KO. I propose several possible explanations for the longer calcium residence time in the absence of IC. One could be disruption of the normal protein profile in the cilium, with an increase in proteins that bind calcium thereby generating a sink to keep calcium within the cilium. This could be investigated by staining for known calcium binding proteins. Another is that the amount of or functions of calcium channels in the primary cilium are altered in the absence of the IC resulting in either increased entry of calcium and/or decreased calcium removal. This could be investigated by
repeating the NP-EGTA protocol from this paper while the cells are in media with varying concentrations of calcium outside the cell to change the concentration gradient and availability of external Ca\(^{2+}\).

Several additional experiments using the cytoplasmic NP-EGTA assay could shed light onto the molecular function of INVS and the cellular role of the IC. Measuring calcium dynamics in cells lacking other IC components would distinguish between effects caused by loss of the entire IC complex and by the individual proteins directly. Additionally, measurement of the calcium dynamics in primary cilium of cells with different constructs added-back into the KO line, such as full length, partial constructs or constructs containing clinically relevant mutations, could provide insight into the molecular mechanisms of ciliopathies.

IV.4. Conclusions

The diameter for the cilium is 200nm, 30 fold smaller than the diameter of a round 3T3 cell and ciliary volume is orders of magnitude smaller than the cell volume. Therefore it seems unlikely that the primary cilium could supply sufficient calcium molecules to the cytoplasm to generate a functional signal. In support of this, in cells expressing both the 5HT6-mCherry-G-GECO cilium sensor and the cytoplasmic GCaMP3 calcium sensor, ablation of the cilium tip generated an extremely robust and sustained G-GECO1 signal in the cilium but no detectable sustained change to the GCaMP3 signal in the cytoplasm (Figure 3C). Therefore, a calcium-dependent signal
from the cilium to the rest of the cell is likely indirect, through second messenger(s) and/or protein modifications.

This study does not provide insight into what the second messenger(s) could be, but there are several tantalizing options. One hypothesis is that elevated calcium in the cilium generates a covalent modification to a protein and this modified protein then goes into the cell body to affect change; analogous to the cleavage of Gli in the Sonic Hedgehog pathway. The use of the cilium-targeted APEX for intracellular protein biotinylation combined with mass spectrometry could provide insight into this by comparing the cilium-resident proteins in cells with differing IC genotypes (Mick et al., 2015).

Alternately, calcium is known to affect cyclic-AMP (cAMP) signaling in the cilium (Besschetnova et al., 2010). cAMP is also known and postulated to have a host of direct effects in the primary cilium (Pietrobon et al., 2011). The effect on cAMP levels due to calcium entry into the cilium could be investigated using a similar strategy as that used in this study. Specifically, target a cAMP biosensor to the primary cilium and generate calcium entry via the NP-EGTA cytoplasmic un-caging experiments described herein.

I have compared a variety of assays for quantifying the dynamics of calcium in the primary cilium and identified one that is most robust, photo-un-caging NP-EGTA in the cytoplasm adjacent to the cilium base. The alternate assays could well prove helpful in different experimental circumstances or with different instrumentation. I used this assay to assess the calcium dynamics in the primary cilium under genetic knockout of the inversin protein by CRISPR/Cas9 (INVS KO) and discovered that calcium stays in the
primary cilia of INVS KO longer than in the primary cilia of WT cells. Residence time of calcium in the primary cilium provides a defined cellular phenotype for genetic situations that have heretofore been limited to organ- and organism-scale phenotypes. This cellular phenotype can then be used to investigate the molecular functions and possible therapeutic interventions for ciliopathy associated protein mutations.
IMCD cells are derived from mouse kidney inner medullary collecting ducts, presumably making them physiologically similar to cells in an intact kidney and therefore optimal for studying renal cell biological phenomenon. IMCD cells generate long primary cilia, however the primary cilium is on the apical side and protrudes vertically from the cell surface. This orientation makes it difficult to study behavior along the cilium length by light microscopy because the cilium is perpendicular to the image plane. I tried two described techniques to generate side-facing cells that would have their apical cilium aligned with the image plane (Delling et al., 2013; Roth, Rieder, & Bowser, 1988).

A.1. Methods

All IMCD cell lines are described in Table A.1 using the same constructs, cell maintenance protocol and widefield epifluorescence microscopy as described in the main text. For live imaging, trypsinized and resuspended IMCD cells were plated onto ethanol and UV sterilized ACLAR UltRx 2000 (Honeywell) or Cyclopore Track etched membrane (25mm 0.1um pore, Whatman Inc.) membrane placed at the bottom of a 6-well tissue culture plate and grown to full confluence (approximately two days) before switching to 0.25% DMEM for another two days (Delling et al., 2013; Roth et al., 1988). Membranes were mounted between a large 24x50mm #1.5 glass coverslip (Fisher
Scientific) and a cross-oriented 22x30mm coverslip (Fisher Scientific) that were separated by parallel strips of double stick tape (3M) to form a channel into which the membrane was folded cell-side outward to generate a horizontal orientation of the apical-basal axis at the fold apex (Figure A1A; (Waterman Storer, 2001)). The channel was filled with DMEM by capillary action between the two coverslips. For ATP experiments, the ends were kept open and during imaging 200μl of DMEM containing 100μM ATP was perfused through the channel by pipetting the solution onto one open end and using a small triangle of filter paper on the other open end to pull the fluid through. For two-photon ablation, the open ends were sealed with VALAP (1:1:1 by weight Vaseline, lanolin and paraffin wax; Sluder, Nordberg, Miller, & Hinchcliffe, 2007) to prevent evaporation.

Table A1. Transgenic IMCD cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell lines used</th>
<th>Recipient cell line</th>
<th>Added Viral Vector</th>
<th>Total genes added</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMCD</td>
<td>IMCD</td>
<td>--</td>
<td>0</td>
<td>IMCD IF</td>
</tr>
<tr>
<td>IMCD cilia sensor</td>
<td>IMCD</td>
<td>5HT6-mCherry- G-GECO1 in pCDH-UbC-MCS-EF1-Puro</td>
<td>1</td>
<td>IMCD live</td>
</tr>
<tr>
<td>IMCD INVS KO</td>
<td>IMCD</td>
<td>CRISPR INVS KO</td>
<td>0</td>
<td>IMCD IF</td>
</tr>
<tr>
<td>IMCD INVS KO cilia sensor</td>
<td>IMCD KO</td>
<td>5HT6-mCherry- G-GECO1 in pCDH-UbC-MCS-EF1-Blast</td>
<td>1</td>
<td>IMCD live</td>
</tr>
</tbody>
</table>

A.2. Results and Discussion

The ACLAR membrane was too thick such that, when folded, the horizontally oriented cells at the fold apex were too far from the coverslip for the objective to focus.
Additionally, the stiffness of the ACLAR made generating the folded structure quite difficult. The Cyclopore membrane was thinner, enabling the horizontal cells to be within the objective working distance (Figure A1B, C). The Cyclopore membrane mounting technique was compatible with the two-photon ablation experiments. An optimal example, where the cilium remains, is shown, in Figure A1C, however this happened less frequently than total disappearance of the cilium or movement of the cilium away from the laser beam. Although an improvement from ACLAR, the time consuming mounting technique still usually resulted in torn cell sheets with few or highly damaged cells in the desired orientation. Additionally, maintaining cell focus and location during ATP perfusion in this sample preparation was quite difficult. These many challenges were likely to introduce confounding variables and were low throughput; therefore I chose not to pursue IMCD cells beyond preliminary experiments.
Figure A1. IMCD cells (A) Schematic of membrane mounting from top view (XY) and side view (YZ). Light gray rectangles indicate double-stick tape connecting the upper and lower coverslips (thin outline) and generating a channel in which the circular membrane (thick outline) is folded in half. Not to scale. (B) Optimal view of cells at the membrane apex. Arrows in A and B indicate membrane-cell interface. (C) Successful ablation of an IMCD cilium. This represents the optimal case and was very rare. Most often the cilium disappeared after ablation. Arrowhead and square indicate time and location of ablation, respectively. Scale bars are 10μm.
Appendix B

Poly-glutamylation of the Cilium Proximal Axoneme

In the course of this study, another member of the lab noticed that loss of IC components in IMCD cells reduced prevalence of poly-glutamylation (poly-E) at the proximal area of the primary cilium. Using the immunofluorescence protocol described for ANKS6, I investigated this in 3T3 cells. Antibodies used were rabbit anti-Poly-E (1:1000, generously provided by J. Gaertig) primary and Alexa Fluor 488 anti-rabbit secondary and image was performed at 60X. Scoring of cilia positive for poly-E in WT, INVS KO and INVS+ cells showed reduction of poly-E in INVS KO (30%) compared to WT (74%) and partial recovery of poly-E incidence in INV+ cells (60%; Figure B1A,B). This experiment was performed with a higher passage number of the INV+ cell line that was later found to have lower ANKS6 occurrence. Therefore, it is not immediately clear if the partial recovery is due to lower INVS+ incidence or reflects only partial recovery. While not completely conclusive, the modification of poly-E by INVS genotype is intriguing and would be beneficial to investigate further with a variety of partial and/or mutated inversin add-back constructs to determine a more specific relationship.

Figure B1 (next page). Poly-glutamylation (poly-E) in confluent 3T3 cells. (A) Confluent 3T3 cells of WT, KO and INVS+ genotypes were stained for nuclei (DAPI), acetylated tubulin to highlight the primary cilium and poly-E. Scale bar is 10μm. (B) Quantitation of poly-E incidence with acetylated tubulin. WT (black), INVS KO (mid grey) and INVS+ cells (light gray). INVS KO results in significant loss of Poly-E whereas INVS+ has levels close to WT.
Appendix C

Custom Software Code

The custom Fiji/ImageJ macros and MATLAB analysis programs presented here enabled the efficient quantification of calcium dynamics in the primary cilium. All macros for Fiji/ImageJ and MATLAB code used in this thesis and described here are available at the github repository https://github.com/wcsalmon/ciliary_calcium

C.1. Macros for Fiji/ImageJ

C.1.a. NP-EGTA data

**Andor Dynamics + mCherry NP-EGTA.ijm** This macro has two parts. The first measures the starting mCherry and G-GECO1 signals in a multichannel image by thresholding the mCherry channel, having the user select the cilium to generate a region of interest (ROI), duplicating the ROI in a background location selected by the user, measuring the mean intensity of both regions in all channels of the image, and saving the region information and intensity information to locations designated in the macro. The second part transfers the two ROIs to a single-channel time series file, measures the average intensity of both ROIs for each time point and saves the data to a folder designated in the macro. Steps requiring manual input during analysis are prompted with dialog boxes. Before running the macro, folder locations for saving the various output
files (described above) must be modified. This macro works with two files: One file that has a single time point and multiple wavelengths (taken at start) and one file with a single wavelength over time. The macro can be run before or after opening the files.

**Pull Elapsed Time.ijm.** This macro uses the deltaT value from the Bio-Formats metadata to generate a single column table of elapsed time from a time series file with a single wavelength. The macro must be modified to update the locations for saving the output files prior to running the macro. The macro will prompt file selection. Modified from the macro planeTimings.txt by Curtis Rueden (github page URL is included within the macro).

C.1.b. ATP data

**Andor Dynamics + mCherry ATP.ijm.** This performs the same functions as the NP-EGTA version, however it expects a single file that includes the time series and multiple channels. The macro must be modified to update the locations for saving the output files and for labeling individual cilia from a single field of view prior to running the macro. Steps requiring manual input during the analysis are prompted with dialog boxes. Macro can be run before or after opening the files. The output must be modified to remove duplicate entries generated from the multiple wavelengths.

**Pull Elapsed Time ATP.ijm.** This macro uses the deltaT value from the Bio-Formats metadata to generate a single column table of elapsed time from a time series file with multiple wavelengths. The macro must be modified to update the locations for saving the output files and designating which channel to use for time measurement. Run the macro
prior to selecting the file. Modified from the macro planeTimings.txt by Curtis Rueden (github page URL is included within the macro).

C.2. MATLAB Code

All MATLAB code was generated by David Jacobowitz, a rotation graduate student in the Shah laboratory.

There are separate sets of analysis codes for NP-EGTA and ATP data due to the difference in data files generated from the macros above and the difference in determination of the calcium peak. Each data type has a pre-processing program to convert the output files from the Fiji macros described above to optimal files for the dynamics measurements code. The functions within the analysis programs are described in the Methods section, but briefly the data is spline fit, mean filtered with a window of 3 time points and the intensity peak found. The intensity peak value and time point are then used to define the four “signpost” values: the time point prior to the peak at which intensity is 20% or 35% of the peak is the fill start time, the time point prior to the peak at which intensity is 80% or 65% the peak intensity is the peak start, the time point after the peak at which the intensity is 80% or 65% of the peak is the peak end and the time point after the peak at which the intensity is 20% or 35% is the end time.

The main difference between the NP-EGTA and ATP analysis is how the intensity peak is defined. For NP-EGTA, the timing of calcium release is known from when the laser illumination was triggered by the software. Since the laser illumination was set to occur at the same time point in all experiments, the time point prior to laser
illumination is defined as baseline for all files, the peak intensity is found mathematically
and the “signpost” values defined relative to those two anchors. Calcium release due to
ATP-stimulation is less tightly controlled in time, therefore analysis of the ATP data
relies only on finding the initial intensity peak to define the “signpost” values. This is less
robust, particularly with noisy data and the multiple intensity peaks seen in the ATP data
collected.
References


determination and organ patterning. *Nature Communications*, 6, 6023. http://doi.org/10.1038/ncomms7023


