Regulation of primary cilia formation by ceramide

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Running title: Ceramide-regulated ciliogenesis

Key words: sphingolipids, cell polarity, primary cilium, Golgi, centrosome
Abstract

The primary cilium is an important sensory organelle the regulation of which is not fully understood. We found that in polarized Madin-Darby Canine Kidney (MDCK) cells, the sphingolipid ceramide is specifically distributed to a cis-Golgi compartment at the base of the primary cilium. This compartment immunostained for the centrosome marker γ-tubulin, the Rho type GTPase Cdc42, and atypical protein kinase Cζ/λ (aPKC), a kinase activated by ceramide and associated with a polarity protein complex consisting of Par6 and Cdc42. Inhibition of ceramide biosynthesis with Fumonisins B1 prevented co-distribution of aPKC and Cdc42 in the centrosomal/pericentriolar compartment and severely impaired ciliogenesis. Cilium formation and co-distribution of aPKC and Cdc42 were restored by incubation with N-acetyl or N-palmitoyl sphingosine (C2 or C16 ceramide), or the ceramide analog N-oleoyl serinol (S18). Cilium formation was also restored by the GSK-3β inhibitor indirubin-3-monoxime, suggesting that regulation of ciliogenesis depends on the inhibition of GSK-3β by ceramide-activated aPKC. Consistently, inhibition of aPKC with a pseudosubstrate inhibitor prevented restoration of ciliogenesis by C2 ceramide or S18. Our data show for the first time that ceramide is required for primary cilium formation.
Introduction

The primary cilium was first described in 1898, however, its critical function for cell polarity and cell signaling was not discovered until recently (1-6). In contrast to motile cilia that usually have an inner structure of 9+2 microtubules, the primary cilium is non-motile with the two central microtubules missing. Nevertheless, primary cilia are studded with receptors including those for PDGF, Wnt, and sonic hedgehog cell signaling pathways (7-11). This feature makes the primary cilium one of the most important sensory organelles on the apical surface of epithelial cells, suggesting an essential function as a “cellular antenna” linked to cell polarity. As a consequence, loss or impairment of the primary cilium goes in hand with severe disorders of epithelial cell polarity. Disorders linked to impaired ciliogenesis are known as ciliopathies and encompass a variety of diseases such as polycystic kidney disease (PKD), primary ciliary dyskinesia (PCD), retinopathies, combined developmental deficiencies (e.g., Joubert and Meckel syndrome) and other neuronal and sensory disorders (3, 5, 6, 11).

Despite the recent progress in determining the structure and protein composition of primary cilia, it is not fully understood how ciliogenesis is regulated. Immunocytochemistry and electron microscopy studies suggest that the primary cilium originates at the centrosome, usually in close vicinity to the Golgi apparatus (12-14). One of the key factors associated with formation of the primary cilium is γ-tubulin, a cytoskeletal protein that is found in the centriole and pericentriolar material (PCM) at the base of the cilium (2, 15-18). Another factor known to regulate centriole morphogenesis and in turn, cilia formation is the cis Golgi marker protein GM130 (19). Many other factors, in particular intraflagellar transport (IFT) proteins are involved in cilia formation and elongation (5). However, it is still unclear what role lipids play in ciliogenesis.
Most recently, we have found that the sphingolipid ceramide is essential for epithelial cell polarity in the primitive ectoderm and the neuroepithelium (20, 21). We have also shown that its function in cell polarity relies on binding and activation of atypical PKC (aPKC), and the subsequent formation of an aPKC-associated polarity protein complex including the Rho type small GTPase Cdc42 and the polarity protein Par6 (20-22). Therefore, we hypothesized that the ceramide-dependent regulation of the aPKC-associated cell polarity complex is involved in the formation or elongation of the primary cilium. This hypothesis is consistent with reports on the critical function of aPKC, Par6, and Par3 in ciliogenesis (23, 24). Using a pharmacologic inhibitor of ceramide biosynthesis (Fumonisin B1 or FB1) we show here for the first time that ceramide is required for the regulation of ciliogenesis in MDCK cells. We also describe a novel pericentriolar compartment associated with the cis Golgi and enriched in ceramide. Since this compartment is at the base of the primary cilium our results suggest that ceramide in the cis Golgi is critical for ciliogenesis via recruitment and activation of aPKC.

Material and Methods

Materials

Madin Darby Canine Kidney (MDCK) II cells were obtained from Dr. Quansheng Du (Medical College of Georgia, Augusta, GA). The anti-GM130 antibody (mouse IgG) was a generous gift from Dr. Richard Cameron (Medical College of Georgia, Augusta, GA). Anti-γ tubulin mouse IgG, anti-acetylated tubulin mouse IgG, and fumonisin B1 (FB1) were purchased from Sigma (St. Louis, MO). Anti-PKCζ (C20) rabbit IgG and anti-Cdc42 mouse IgG was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-ceramide rabbit IgG was generated in our
laboratory as previously described (25). Anti-ceramide mouse IgM (MAS0020) was purchased from Glycobiotech (Kuekels, Germany). Alexa 647-conjugated phalloidin and DMEM medium were obtained from Invitrogen (Carlsbad, CA). All ceramide species were of highest purity and purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). N-oleoyl serinol (S18) was synthesized in our laboratory as previously described (26, 27). Myristoylated pseudo substrate inhibitor of PKCζ (PZI) was obtained from Biomol (Plymouth Meeting, PA). High-performance thin layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). 12-well dishes containing 24 mm diameter polyester filters with pores of 0.4 µm diameter were purchased from Corning, Inc. (Corning, NY). All other reagents and solvents were of analytical quality.

Methods

Cultivation of MDCK II cells

MDCK II cells were maintained in DMEM containing 10% FCS, penicillin and streptomycin in a 5% CO2, 37°C incubator. Cells were seeded onto 24-mm polyester filters (0.4 µm pore size) at a density of 250,000 cells/filter and cultivated for 72 hours to establish apicobasal polarity prior to drug treatment. For further treatment affecting ciliogenesis, cells were incubated for another 72 hours in the presence of various drugs (30 µM FB1, 40 µM S18, 10 µM C2 ceramide; 1 µM C16 ceramide, or 50 µM PZI) and then fixed with 4% p-formaldehyde in phosphate buffered saline (PBS) prior to immunocytochemistry or lipid analysis.
**Immunocytochemistry**

After fixation with 4% p-formaldehyde/PBS, non-specific binding sites were blocked with 3% ovalbumin/10% donkey serum/PBS for 1 hour at 37°C. Cells were then incubated with primary and secondary antibodies at a concentration of 5 or 10 µg/ml in 0.1% ovalbumin as described previously (20-22). Epifluorescence microscopy was performed with an Axiophot microscope (Carl Zeiss MicroImaging, Inc.) equipped with a Spot II CCD camera. Confocal fluorescence microscopy was performed using a Zeiss LSM510 confocal laser scanning microscope equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3), or 633 nm (Cy5, Alexa Fluor 647), respectively.

**Quantitation of cilia formation and statistics**

Cilia length was measured using Zeiss LSM Image Examiner software (Carl Zeiss) (modified from (28). Briefly, captured z-stack confocal images (n >50 cilia from at least 4 separate experiments) were projected at an angle to facilitate visualization and quantitation of the primary cilia. The cilium length was measured by tracing cilia on micrographs using METAMORPH software. Cilia of at least 3 µm length were counted as elongated cilia. Data were expressed as means ± SD. Statistical significance was determined using Student's t test.

**Ceramide analysis**

Total lipids were extracted from cells using a mixture of chloroform:methanol (2:1; v/v) as previously described (20, 21, 29, 30). For analysis of subcellular distribution, ceramide fractions of the cytosol and membranes were dissolved in equal volumes of solvent and the lipids were resolved by high-performance thin-layer chromatography (HPTLC) using the running solvent
chloroform:methanol:acetic acid (95:4.5:0.5; v/v) for the separation of ceramide. Individual bands were visualized by staining with 3% cupric acetate in 8% phosphoric acid and identified by comparing them to the migration distance of standard ceramide. The identity of the ceramide band was confirmed by alkaline hydrolysis of phospholipids followed by silic acid chromatography (20, 30). The amount of ceramide was quantified by densitometric analysis of the band intensities.

**Results**

*Loss of ceramide prevents ciliogenesis while ceramide analogs promote cilia elongation*

To determine the role of ceramide for ciliogenesis we used the pharmacologic ceramide synthase inhibitor fumonisin B1 (B1) to deplete MDCK cells of ceramide, and replenishment with ceramide and ceramide analogs to rescue the phenotype. FB1 was added to the medium 72 hours after plating of the cells to deplete the entire cell culture of ceramide (31, 32). Using high-performance thin-layer chromatography (HPTLC) and densitometric analysis of the band intensities we found that FB1 reduced the ceramide level to less than 50% of untreated controls (Fig. 1A, lane 3, lane 2 is control). Cells incubated with FB1 and S18 showed reduction of ceramide levels, but they were enriched with S18 (Fig. 1A, lane 4). Subcellular fractionation of homogenized MDCK cells after incubation with S18 and HPTLC analysis of the individual subcellular fractions showed that S18 was distributed to cellular membranes (Fig. 1B). This result suggested that S18 was able to replace ceramide in the plasma membrane or intracellular membranes of FB1-treated cells.
To confirm that potential effects of ceramide depletion are specific for ciliogenesis we determined the establishment of apicobasal polarity in FB1-treated cells using immunocytochemistry for apical and lateral marker proteins. Fig. 2A shows that the distribution of F-actin (apical), ZO-1 (apicolateral), and E-cadherin (lateral) of FB1-treated MDCK cells was equivalent to that of control cells suggesting that the effect of ceramide depletion was specific for ciliogenesis. Primary cilia were visualized using an antibody against acetylated tubulin, a marker for primary cilia (14, 18, 23). Immunocytochemistry for acetylated tubulin showed that FB1 reduced the number and length of primary cilia by 60% or 78%, respectively (Figs. 2C and F, 3A and B). The number and length of cilia was restored by incubation of FB1-treated cells with C2 ceramide, C16 ceramide, or S18 (Figs. 2D-F, 3A and B), indicating that ceramide and not its derivatives were involved in cilia formation. This assumption was also supported by the observation that S18 (without FB1) promoted elongation of primary cilia by 1.5-fold (Fig. 3B). Incubation with sphingosine-1-phosphate had no effect on cilia formation or elongation (not shown). There was no impairment of cilia formation by incubation with sphingosine or dihydrosphingosine, suggesting that FB1 did not prevent cilia formation due to increased levels of these two ceramide precursors. Taken together, this data indicated that ceramide is critical to initiate the formation and promote the elongation of primary cilia in MDCK cells.

**Golgi-resident ceramide co-distributes with γ-tubulin at the base of the primary cilium**

The necessity of ceramide for ciliogenesis suggested that it is functionally involved at subcellular sites that participate in cillum formation. To detect ceramide we used two anti-ceramide antibodies (MAS0020 and rabbit IgG) for immunocytochemistry. The cilium base is located in close vicinity to the centrosome and the PCM (15). In these subcellular compartments,
γ-tubulin participates in cillum formation by priming the 9+0 microtubule structure which originates at the basal body (centriole) and protrudes from the apical cell membrane (15-17). Fig. 4A-F shows that ceramide formed a distinct structure or compartment at the base of the cillum (arrows), which was detected by immunocytochemistry for acetylated tubulin and ceramide. Co-staining for F-actin with Alexa 647-phalloidin showed that ceramide was localized at the apical pole of the cell on top of the nucleus (Fig. 4E). At this site, ceramide was co-distributed with γ-tubulin in a perinuclear compartment reminiscent of the centrosome and the PCM or the cis Golgi ribbon associated with the centrosome (Fig. 4F).

Since the centrosome is localized close to the cis Golgi we stained for GM130, a cis Golgi matrix protein that regulates centrosome morphology and function (19). Fig. 4G shows that ceramide and GM130 were co-distributed on top of the nucleus in polarized MDCK cells. Co-localization with GM130 suggested that ceramide was enriched in the cis Golgi, one of the first target compartments to which ceramide is transported once its biosynthesis has been accomplished in the ER (33-35). The trans Golgi is another compartment to which ceramide is transported from the ER. However, wheat germ agglutinin (WGA) staining of the trans Golgi did not indicate enrichment of ceramide in this compartment (Fig. 4H). Therefore, our data suggested that cis Golgi-derived ceramide is involved in the centriolar or pericentriolar initiation or regulation of primary cillum formation.
Ceramide activation of aPKC is critical for ciliogenesis

In previous studies we have shown that ceramide activates aPKC and that this activation is essential for cell polarity in primitive ectoderm cells and neural progenitors (20, 21). Since the aPKC-associated protein complex in cell polarity is similar to that involved in ciliogenesis (23) we hypothesized that ceramide-mediated activation of aPKC will regulate cilia formation. To test this hypothesis we incubated MDCK cells with a myristoylated pseudosubstrate inhibitor of aPKC (PZI). PZI is specific for aPKC and has been used to disrupt polarity-related processes in MDCK cells (36). Treatment with PZI by itself reduced the number of primary cilia to 20% of the control value (Fig. 5). PZI also reduced the number of cilia restored by C2 ceramide or S18 in ceramide-depleted cells. These results suggested that ceramide-mediated activation of aPKC is essential for ciliogenesis (Fig. 5).

We then tested whether other components of a ceramide/aPKC-associated polarity complex were co-distributed with ceramide in the centrosome or the PCM. In our previous studies we found that ceramide-induced association of aPKC with Par6 and the small GTPase Cdc42 phosphorylates and inactivates GSK-3β and regulates cell polarity in primitive ectoderm cells and neuroprogenitors (20, 21). Likewise immunocytochemistry showed apical co-distribution of ceramide, aPKC, and Cdc42 in polarized MDCK cells (Fig. 6A and D; and Supplemental Fig. 2). This distribution was equivalent to the ceramide-rich Golgi or PCM compartment (Fig. 4). Ceramide depletion with FB1 obliterated the apical co-distribution of aPKC and Cdc42 concurrent with the lack of cilia formation (Fig. 6B and D; and Supplemental Fig. 2). However, the apicolateral distribution of aPKC and Cdc42 was still visible after incubation with FB1, which was consistent with the establishment of apicobasal polarity prior to cilia formation (see
also ZO-1 and E-cadherin in Fig. 2A). C2 ceramide and S18 restored the apical co-distribution of aPKC and Cdc42 in FB1-treated cells (Fig. 6C and D, and Supplemental Fig. 2). S18 also restored phosphorylation of GSK-3β, which was almost completely obliterated in FB1-treated cells (Fig. 7A). Consistent with a critical function of S18- or ceramide-dependent phosphorylation of GSK-3β in ciliogenesis, the GSK-3β inhibitor indirubin-3’-monoxime restored ciliation of ceramide-depleted MDCK cells (Fig. 7B). Therefore, our results suggest that the centrosomal/pericentriolar ceramide compartment associates with aPKC and Cdc42, which induces phosphorylation of GSK-3β and promotes cilia formation and elongation.

Discussion

The sphingolipid ceramide has for long been implicated in the induction of apoptosis. Most recently, our group has shown that ceramide has additional, non-apoptotic functions (20, 21). In particular, the regulation of cell polarity in primitive ectoderm and neural progenitor cells critically relies on ceramide (20, 21). We have found that this regulation is determined by the interaction of ceramide with aPKC and its associated polarity complex(es) (22, 26). Since aPKC is also involved in the regulation of ciliogenesis (23) we tested the effect of ceramide on this process. We show here, for the first time, that ceramide is a key regulatory factor for the formation and elongation of primary cilia.

To define a specific role of ceramide for ciliogenesis it is indispensable to confirm that apicobasal polarity was not affected during cilia formation in FB1-treated cells. This was achieved by showing the apical, apicolateral, or lateral distribution of F-actin, ZO-1, and E-cadherin, respectively. ZO-1 is a tight junction protein that is regulated by aPKC independent of
ciliogenesis (37-39). Hence, the apicolateral distribution of ZO-1 indicates that apicobasal polarity was established before FB1 treatment and then maintained in ceramide-depleted cells.

Several lines of evidence support the hypothesis that ceramide is essential for ciliogenesis:

1) FB1, an inhibitor of ceramide biosynthesis, dramatically reduces the number of ciliated cells and the length of primary cilia.
2) C2 ceramide, C16 ceramide, and the novel ceramide analog S18 restore cilia formation in FB1-treated cells. In particular, restoration of primary cilia by S18 is important since this ceramide analog is not converted to any metabolic derivative of ceramide (20, 21). The rescue effect of S18 may be due to enrichment of this ceramide analog in the membranous ceramide compartment and activation of aPKC. Taken together, impairment of cilia formation by ceramide depletion and restoration of primary cilia by C2 ceramide, C16 ceramide and S18 strongly suggests an essential function of ceramide in ciliogenesis.

Previous studies from our and other laboratories have shown that ceramide and its analogs associate with and activate aPKC (22, 26, 40-44). This association takes place in the cell membrane, a perinuclear ER and/or mitochondria-associated compartment, and the Golgi apparatus (21, 22). Our previous studies are consistent with the observation that S18 distributes to the membrane fraction after subcellular fractionation. We have also shown that S18 activates aPKC in in vitro assays and in living cells (26). It is thus likely that ceramide or S18-induced activation of aPKC at a membranous compartment is critical for the function of ceramide in ciliogenesis. This hypothesis is supported by two observations: 1) inhibition of aPKC prevents restoration of primary cilia by S18, C2 or C16 ceramide in ceramide-depleted cells; and 2)
immunocytochemistry shows co-distribution of aPKC and aPKC-associated proteins with ceramide in a membranous compartment at the base of the cilium.

The co-distribution of ceramide with GM130 suggests that this membranous compartment is derived from the cis Golgi. Ceramide enrichment in this compartment is consistent with the de novo sphingolipid biosynthesis pathway, which involves ceramide transport from the endoplasmic reticulum (ER) to the cis Golgi (34, 45). Another transport pathway using the ceramide transport protein CERT mediates traffic of ceramide from the ER to the trans Golgi compartment (46). Since the ceramide signal is not co-distributed with the trans Golgi marker WGA it is rather unlikely that ceramide in the trans Golgi is involved in ciliogenesis.

GM130 is not only a cis Golgi marker, but has also been found to actively regulate the biogenesis and morphology of the centrosome (19). For a long time, it has been known that there is a close vicinity and interaction between the centrosome and the Golgi in polarized cells (12). In epithelial cells with apicobasal polarity, the centrosome and PCM are located on top of the nucleus and linked to an apical cis Golgi ribbon. GM130 and γ-tubulin are involved in generating a microtubule-associated network that physically links the centrosome to this apical cis Golgi ribbon (12-14, 19). γ-tubulin is also essential for nucleating the primary cilium at its basal body within the region of centrosome and PCM. Therefore, it appears reasonable to speculate that ceramide in the cis Golgi may regulate ciliogenesis via the interactive network between the centrosome and the Golgi ribbon. At present, we cannot exclude that other organelles, such as pericentrosomal recycling endosomes, participate in the ceramide-rich compartment. Further characterization of this compartment will be part of our future studies.
Fig. 8 depicts the structural arrangement by which the regulation of ciliogenesis may be mediated after recruitment and activation of aPKC to the ceramide compartment. Ceramide depletion by treatment with FB1 obliterates co-distribution of aPKC with Cdc42, which is restored by S18. This result clearly indicates that ceramide in the cis Golgi (ribbon) associates with aPKC and Cdc42, potentially leading to a vectorial activation of cilia formation or elongation. It remains to be elucidated how ceramide regulates aPKC at the Golgi and how this regulation promotes ciliogenesis. It has been suggested that aPKC phosphorylates microtubule-associated proteins or that aPKC-associated polarity proteins such as Par3 or Par6 are involved in cilia formation/elongation (23). Our group has shown that in primitive ectoderm and neural progenitor cells, ceramide is essential for the formation of an aPKC-Par6-Cdc42 complex, which could sustain ciliogenesis in a similar fashion as it regulates cell polarity (20-22). Another regulatory factor involved in ciliogenesis is glycogen synthase kinase-3β (GSK-3β), a protein kinase phosphorylated by aPKC. Inactivation of GSK-3β by phosphorylation of serine 9 has been shown to be critical for cilium formation sustained by van Hippel-Lindau protein (pVHL) (47). Consistent with this role of GSK-3β inactivation, we have found that ceramide depletion reduces and S18 treatment restores GSK-3β phosphorylation, concurrent with loss and restoration of primary cilia, respectively. Therefore, we assume that a ceramide-activated complex of aPKC with Par6 and Cdc42 phosphorylates GSK-3β, which induces or sustains primary cilia formation or maintenance (Fig. 8).

This is the first report showing that ceramide is critical for the regulation of primary cilia formation and elongation. We will now investigate how ceramide promotes ciliogenesis via aPKC and whether lack or excess of ceramide may disturb this process in ciliopathies. In the
case of a lack of ceramide, analogs such as S18 may pave way to treat ciliopathies. To further define the role of ceramide in ciliogenesis is thus a novel path to understand the regulation of ciliogenesis and to treat disorders related to it.

**Abbreviations:**

aPKC, atypical PKC (PKCζ, λ/ι); Cdc42, cell division cycle 42; FB1, fumonisin B1; HPTLC, high-performance thin-layer chromatography; GM130, Golgi matrix 130; MDCK, Madin Darby Canine Kidney; Par3 and 6, partitioning defective 3 and 6; PCM, pericentriolar material; PZI, pseudosubstrate inhibitor of PKCζ (aPKC); S18, N-oleoyl serinol; SPT, serine palmitoyltransferase; WGA, wheat germ agglutinin.

**Acknowledgments**

This work was supported, in part, by NIH grant R01NS046835 and a March of Dimes grant 6-FY08-322. The authors are grateful to Drs. Richard Cameron and Somsankar Dasgupta, Medical College of Georgia, Augusta, GA, for their assistance with cell culture and ceramide analysis. We thank the imaging core facility under supervision of Drs. Paul McNeil and Katsuya Miyake, Medical College of Georgia, Augusta, GA, for assistance. The authors also thank Dr. Robert K. Yu for institutional support.
References


Figure legends

**Figure 1** Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18 – Lipid analysis

A. HPTLC analysis of ceramide. MDCK cells were incubated with FB1 and/or S18 for 48 hours, the lipids extracted, and ceramide and S18 separated HPTLC. Lane 1, ceramide standard; lane 2, untreated cells; lane 3, FB1-treated cells; lane 4, FB1 and S18-treated cells. B. Subcellular fractionation of MDCK cells to determine the distribution of S18. Cells were treated with S18 and the lipids in the membrane and cytosol analyzed by subcellular fractionation and HPTLC. Note that S18 is exclusively recovered from the membrane fraction. Lane 1, ceramide standard; lane 2, S18 standard; lane 3, cytosolic fraction; lane 4, membrane fraction.

**Figure 2** Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18 – Immunocytochemistry

A. Confocal immunofluorescence microscopy for ZO-1 (Cy2, green) and E-cadherin (Cy3, red), and staining with Alexa 647-phallodin (Cy5, blue) of control and FB1-treated cells. The figure shows the X/Z plane after z-scan reconstruction. Arrowheads point at the apicolateral distribution of ZO-1 (white in overlay due to partial co-distribution with F-actin (apical) and E-cadherin (lateral)), indicating establishment and maintenance of apicobasal polarity in FB1-treated cells. B-E. Projections reconstructed from z-scans as described in C. Secondary antibodies were different from those used in A (acetylated tubulin, Cy2, green; ceramide, Cy3, red). Bar = 50 µm. F. Immunocytochemistry as in B-E, however, top view on apical plane. Ciliogenesis was rescued with C16 ceramide. Bar = 2 µm.
Figure 3 Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18 -

Quantitative evaluation

A. Immunocytochemistry for acetylated tubulin (Cy2, green), ceramide (Cy3, red) and Topro-3 staining of nuclear DNA (blue) shows that FB1 greatly diminishes the number of elongated cilia. Incubation with C16 ceramide rescues ciliogenesis. B and C. Counting of primary cilia with length >3 µm (A) and determination of the average cilium length (quantitation from N=4 independent experiments using micrographic images with >800 cells/image). The number of ciliated cells in the control was set to 100% (*, p<0.05; **, p<0.01).

Figure 4 Ceramide is enriched in a cis Golgi compartment associated with the centrosome at the base of the primary cilium

A-D. Epifluorescence (A) and confocal (B-E) immunofluorescence microscopy using antibodies against acetylated tubulin (Cy3, red) and ceramide (Cy2, green). A, B, X/Y plane; C, X/Z-plane; D, 3D projection, view from top front. Note that the cilium originates on top of the ceramide-rich compartment (arrows). E. X/Z-plane reconstruction using confocal immunofluorescence microscopy for ceramide (Cy2, green), F-actin (Alexa 488-phalloidin, red), and Topro 3 (blue). F. Epifluorescence microscopy after immunocytochemistry for ceramide (Cy2, green) and γ-tubulin (Cy3, red). G, H. Confocal immunofluorescence microscopy for ceramide (Cy2, ceramide) and the cis Golgi marker GM130 (Cy3, red in left panel) or the trans Golgi marker WGA (Cy3, red in right panel). Note that only the distribution of GM130 overlapped with that of ceramide (yellow). Images show view from top onto apical (X/Y) confocal plane. Bars = 2 µm.
**Figure 5 Ciliogenesis requires ceramide-dependent activation of aPKC**

MDCK cells were cultivated in the presence of the reagents as indicated in the figure and the number of primary cilia counted as described in the legend for Fig. 3A. The number of ciliated cells was set to 100% (FB1 (30 µM), aPKC inhibitor PZI (50 µM), S18 (40 µM), C2 (C2 ceramide, 10 µM). Note that S18 and C2 ceramide restored cilium formation, while PZI blocked ceramide or S18-mediated cilium restoration (*, p<0.05; **, p<0.01).

**Figure 6 Ceramide co-distributes with aPKC and Cdc42**

A-D. Confocal immunofluorescence microscopy for ceramide (Cy2, green), Cdc42 (Cy3, red), and aPKC (Cy5, blue). Bar (right panel) = 5 µm. B, C. Immunocytochemistry as in A, however, after incubation with FB1 or FB1+C2 ceramide. D. X/Z plain reconstruction from z-scan. Note that the apical co-distribution of aPKC and Cdc42 (white in overlay due to co-distribution with ceramide) was obliterated by incubation with FB1, and restored by C2 ceramide and S18. Also note that FB1 did not prevent apicolateral co-distribution of aPKC and Cdc42, indicating that apicobasal polarity was established and sustained throughout the incubation reaction. Bar = 10 µm. See Supplemental Fig. 2 for top view on apical and apicolateral plane.

**Figure 7 Ceramide biosynthesis inhibition reduces GSK-3β phosphorylation inactivation, which is critical for ciliogenesis**

A. MDCK cells were cultivated for 3 days on filter membranes and then for another 2 days incubated with FB1. The immunoblot shows that the phosphorylation of serine 9 in GSK-3β was dramatically reduced. Incubation with S18 restored phosphorylation and inactivation of GSK-3β.
B. Treatment with FB1 reduced primary cilium formation, which was restored by incubation with the GSK-3β inhibitor indirubin-3’-monoxime.

**Figure 8** *Model for ceramide-dependent regulation of ciliogenesis*

Ceramide is enriched in a centrosomal/pericentriolar compartment derived from the cis Golgi underneath of the apical membrane and on top of the nucleus of polarized epithelial cells. The compartment recruits and/or activates aPKC and Cdc42, which promotes elongation of the primary cilium, most likely mediated by a polarity complex acting on microtubule or γ-tubulin-associated proteins. Ceramide depletion by incubation with FB1 prevents apical co-distribution of aPKC and Cdc42, which impairs ciliogenesis.
Figure 1

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Figure 2

A

![Diagram showing Z, Y, and X axes with Con and FB1 labels](image)

B

- acetylated tubulin
- ceramide
- Topro-3

C

Control vs. FB1

D

FB1 + S18

E

FB1 + C2 ceramide

F

- acetylated tubulin
- ceramide
- Topro-3

Control vs. FB1 vs. FB1 + C16 ceramide
Figure 3

A

Relative percentage of cells with elongated cilium (%)

Con  FB1  FB1+S18  FB1+C2  FB1+C16

N=4

B

Cilium length

Average cilium length (μm)

Con  FB1  FB1+S18  S18

N=4
Figure 4

A
Ceramide acetylated tubulin Hoechst

B
Ceramide acetylated tubulin

C
Apical
Basal

D
Ceramide ac. tubulin

E
Ceramide F-actin Topro-3

F
Ceramide γ-tubulin Hoechst

G
Ceramide GM130

H
Ceramide WGA
Figure 6

A Control

B FB1

C FB1 + C2 ceramide

D Ceramide Cdc42 aPKC

Control FB1

FB1 + C2 ceramide FB1 + S18
Figure 7

A

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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tGSK-3α/β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Relative percentage of cells with elongated cilium (%)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FB1</th>
<th>FB1+Ind</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120</td>
<td>40</td>
<td>150</td>
</tr>
</tbody>
</table>
Figure 8

Primary cilium (acetylated tubulin(+))

γ-tubulin

F-actin

cis-Golgi (GM130(+))

trans-Golgi (WGA(+))
aPKC/Cdc42/GSK-3β

Ceramide (↑)

Centrosomal compartment

Nucleus