Characterization of an apical ceramide-enriched compartment regulating ciliogenesis

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ABSTRACT **We show that in Madin–Darby canine kidney (MDCK) cells, an apical ceramide-enriched compartment (ACEC) at the base of primary cilia is colocalized with Rab11a. Ceramide and Rab11a vesicles isolated by magnetic sorting contain a highly similar profile of proteins (atypical protein kinase C [aPKC], Cdc42, Sec8, Rab11a, and Rab8) and ceramide species, suggesting the presence of a ciliogenic protein complex associated with ceramide at the ACEC. It is intriguing that C16 and C18 ceramide, although less abundant ceramide species in MDCK cells, are highly enriched in ceramide and Rab11a vesicles. Expression of a ceramide-binding but dominant-negative mutant of aPKC suppresses ciliogenesis, indicating that the association of ceramide with aPKC is critical for the formation of this complex. Our results indicate that ciliogenic ceramide is derived from apical sphingomyelin (SM) that is endocytosed and then converted to the ACEC. Consistently, inhibition of acid sphingomyelinase with imipramine disrupts ACEC formation, association of ciliogenic proteins with Rab11a vesicles, and cilium formation. Ciliogenesis is rescued by the histone deacetylase (HDAC) inhibitor trichostatin A, indicating that ceramide promotes tubulin acetylation in cilia. Taken together, our results suggest that the ACEC is a novel compartment in which SM-derived ceramide induces formation of a ciliogenic lipid–protein complex that sustains primary cilia by preventing deacetylation of microtubules.**

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INTRODUCTION

The primary cilium is a unique membrane protrusion in mammalian cells that serves as an "antenna" for signaling factors and mechanical impacts. It was first described in 1898, although its critical function for cell polarity and cell signaling was not discovered until recently (Singla and Reiter, 2006; Pan and Snell, 2007; Gerdes *et al.*, 2009; Satir *et al.*, 2010; Seeley and Nachury, 2010). It has now been recognized that primary cilia are essential for cell migration and differentiation in a vast variety of cell types. Primary cilia are studded with growth factor receptors, including those for platelet-derived growth factor, Wnt, and sonic hedgehog cell signaling pathways (Singla and Reiter, 2006; Eggenschwiler and Anderson, 2007; Rohatgi *et al.*, 2007). In addition, they serve as mechanical sensors measuring urinary flow in the kidney (Michaud and Yoder, 2006). Pathological alterations of primary cilia are known to lead to severe disease phenotypes called ciliopathies. Among these are polycystic kidney disease, Bardet–Biedl syndrome, von Hippel–Lindau syndrome, retinopathies, and obesity (Pan *et al.*, 2005; Satir, 2007; Satir *et al.*, 2010). Many of the ciliopathies show pathological phenotypes in multiple tissues, underlining the importance of understanding key factors regulating ciliogenesis.

Central to the primary cilium is the axoneme consisting of a 9 + 0 microtubule structure. Cilium initiation, elongation, and maintenance are regulated by centriole positioning, microtubule nucleation and elongation, and intraflagellar transport (IFT). Despite the recent progress in determining the structure and protein composition of primary cilia, it is not fully understood how ciliogenesis is regulated. The primary cilium originates at the centrosome, usually in close vicinity to the Golgi apparatus. A presumably Golgi-derived centriolar vesicle (CV) engulfs the distal end of the mother centriole, from which the axoneme—a structure of nine peripheral microtubules—begins to emerge (Sorokin, 1962; Cohen *et al.*, 1988). The CV fuses

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Abbreviations used: ACEC, apical ceramide-enriched compartment; ASMase, acid sphingomyelinase; FB1, fumonisin B1; nSMase, neutral sphingomyelinase; SM, sphingomyelin; S18, N-oleyl serinol; TSA, trichostatin A.

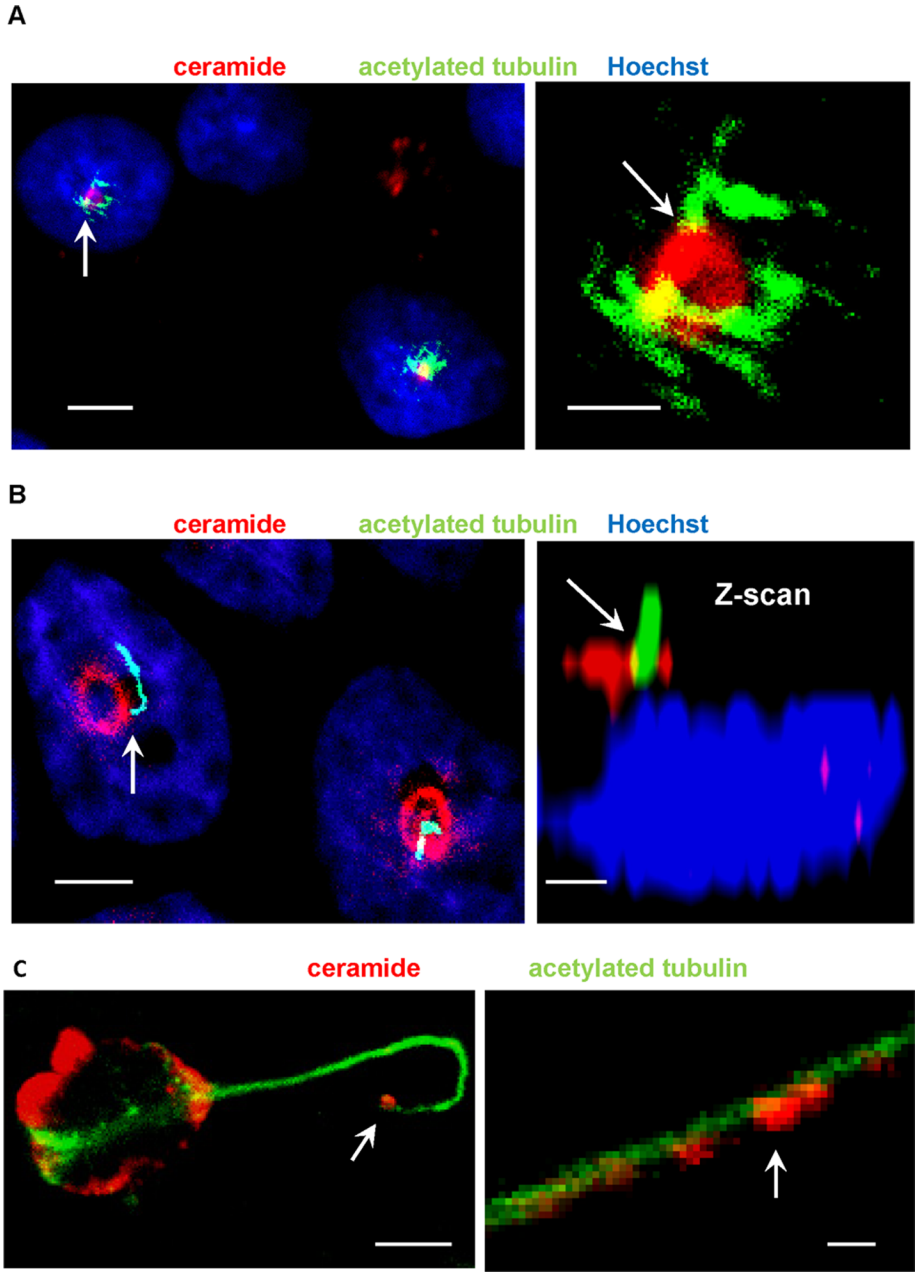

FIGURE 1: Ceramide is associated with the initiation of the primary cilium at the ACEC. MDCK cells were serum deprived, and ceramide (red) and acetylated tubulin (green) were visualized by immunocytochemistry. (A) At 24 h after serum deprivation. Right, arrow points at ACEC shown at higher magnification. Bar, 5 (left), 1 μm (right). (B) At 72 h after serum deprivation. Right, *Z*-scan of the ciliated cell. Arrows point at the cilium attachment site at the ACEC. Bar, 2 μm (left), 1 μm (right). (C) Primary cilium with ceramide distribution to the tip and along the cilium shaft (right). Arrows point at ceramide. Bar, 0.5 μm (left), 0.1 μm (right).

first with secondary vesicles and then with the plasma membrane at the ciliary assembly site. The centriole forming the cilium base is now termed the basal body, a structure at which the axoneme nucleates. This process of early ciliogenesis leads to the procilium, a short stub detectable on the cell surface by staining for acetylated tubulin. Elongation of the procilium relies on the traffic of cargo proteins mediated by transport proteins along the axoneme to the tip of the cilium. Impairment of these processes leads to loss of the primary cilium and development of severe cellular defects such as aberrant wound migration, kidney cyst formation, and even cancer.

Although numerous studies have elegantly shown that many proteins, including those for vesicle transport (such as Rab11a and Rab8), fusion (exocyst proteins such as Sec8 and Sec10), and cargo transport/IFT (such as BBS1 in the BBsome), are essential for ciliogenesis (Nachury *et al.*, 2007, 2010; Loktev *et al.*, 2008; Babbey *et al.*, 2010; Knodler *et al.*, 2010; Das and Guo, 2011; Westlake *et al.*, 2011; Zuo *et al.*, 2011), it is not clear how membrane lipids participate in this process. The function of lipids in the initiation and elongation of primary cilia is inevitable since membrane expansion demands the net transport of membrane lipids in form of lipid vesicle trafficking and fusion. Moreover, membrane vesicle trafficking and fusion regulated by lipids offers an attractive means for the controlled redistribution of growth factor receptors and other cargo proteins from the cell surface to the primary cilium.

In previous studies, we found that the sphingolipid ceramide is essential for the formation, elongation, or maintenance of primary cilia (Wang *et al.*, 2009a). We also found that atypical protein kinase C (aPKC) is bound and activated by ceramide (Bieberich *et al.*, 2000; Bieberich, 2011; Wang *et al.*, 2005, 2009b). However, the source of ceramide and its interaction with other proteins in a ciliogenic complex remained unclear. Our new results suggest that the apical ceramide enriched compartment (ACEC) is derived from endolysosomal degradation of sphingomyelin (SM) by acid sphingomyelinase (ASMase). We also show evidence that ceramide-associated aPKC forms a lipid vesicle–bound protein complex with Cdc42, Sec8, Rab11a, and Rab8, which regulates cilium maintenance by its interaction with the BBsome and histone deacetylase 6 (HDAC6). Our results point at a novel function of ceramide in vesicular membrane transport or fusion, and assembly of a lipid–protein complex regulating ciliogenesis.

RESULTS

The ACEC nucleates the primary cilium and is formed by ceramide derived from SM

Previously we showed that a ceramide-enriched compartment is located at the base of the primary cilium in Madin–Darby canine kidney (MDCK) cells (Wang *et al.*, 2009a). To further elucidate the distribution and function of ceramide, we performed immunocytochemistry with two anti-ceramide antibodies (mouse immunoglobulin M [IgM] and rabbit immunoglobulin G [IgG]) at different stages of ciliogenesis induced by serum deprivation of MDCK cells grown to confluence. Figure 1 shows that before formation of the procilium, the cilium marker acetylated tubulin is colocalized with the ACEC. Of these cells, 75% were ciliated within 48 h after serum deprivation.

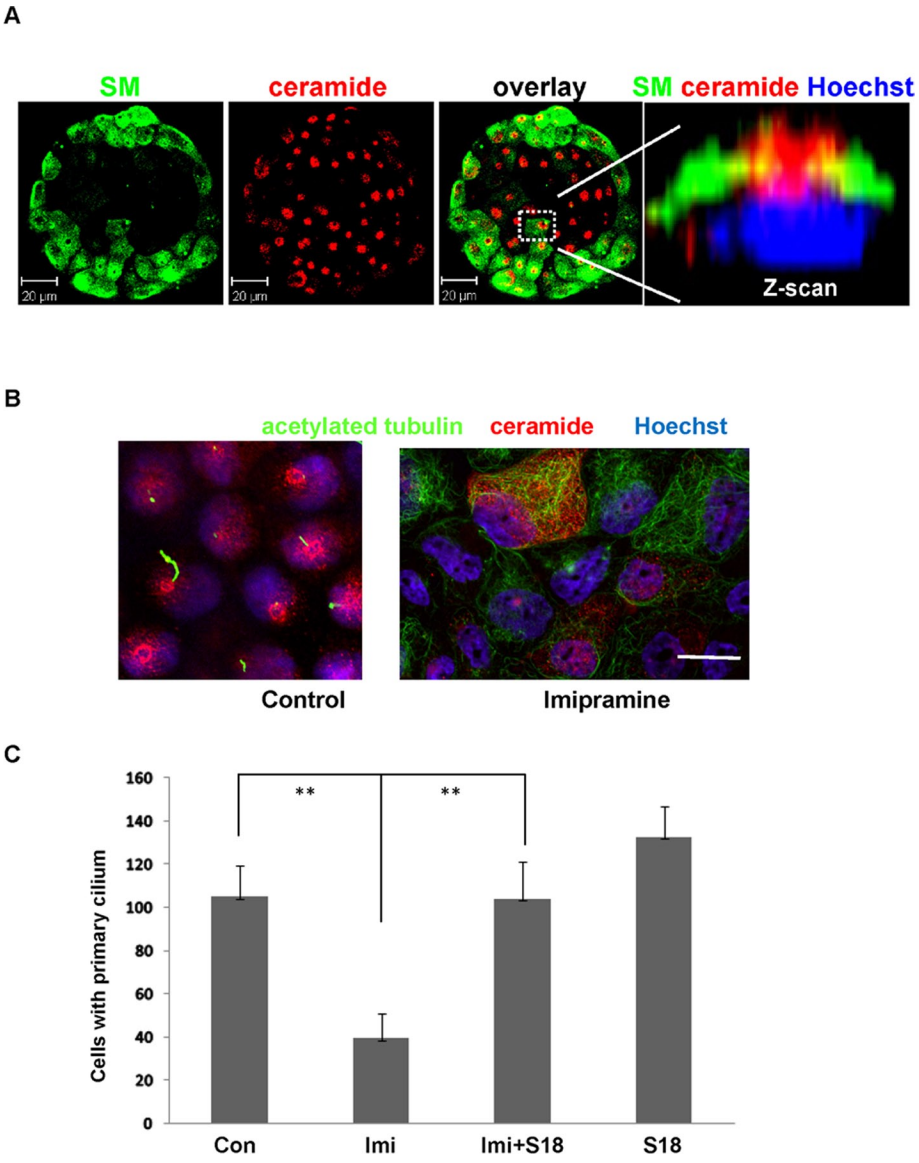

FIGURE 2: The ACEC is derived from apical SM hydrolyzed by ASMase. (A) Colony of MDCK cells serum deprived for 72 h, followed by immunocytochemistry for SM (green) and ceramide (red). Right, the image of a *Z*-scan. Consistent with the assumption that apical SM was gradually converted to ceramide during ACEC formation, the fluorescence signal for SM was reduced, whereas that of ceramide was increased. (B) Incubation of MDCK cells with the ASMase inhibitor imipramine (40 μM) led to delocalization of ceramide and acetylated tubulin, and prevented formation of primary cilia. Bar, 10 μm. (C) Quantification of data shown in B (number of cilia in 150 cells). Cilium formation was restored by incubation of imipramine (Imi)-treated cells with the novel ceramide analogue S18. $n = 5$, $^{**}p < 0.01$.

In 10% of the ciliated cells, no ACEC was detectable. In the initial phase of ciliogenesis, the acetylated tubulin filaments appeared to originate from the ACEC (Figure 1A, arrows). At a later time point (72–96 h after serum deprivation), the base of the cilium codistributed with one site at the ring-shaped ACEC (Figure 1B). In the elongated cilium, ceramide was often concentrated at the tip of the cilium and in particulate clusters along the cilium itself (Figure 1C, arrows).

We then characterized the origin of ceramide in the ACEC. In the plasma membrane, ceramide is derived from SM, which is replenished by ceramide that is first generated in the endoplasmic reticulum (ER) and then converted to SM in the Golgi before its transport to the membrane. We wanted to determine whether ceramide in the ACEC is directly derived from the endoplasmic reticulum (ER) or Golgi or, alternatively, is generated by hydrolysis of SM in the apical plasma membrane and then transported to the ACEC via endocytotic vesicle trafficking. Figure 2A shows that during ciliogenesis, cells lost immunostaining for SM at the apical plasma membrane, whereas ceramide staining increased. This result suggested that ceramide is generated in the endolysosomal degradation pathway, which is known to be initiated by ASMase-mediated hydrolysis of membrane-bound and endocytosed SM (Kitatani *et al.*, 2008; Rozenova *et al.*, 2010). A detailed analysis showed that already 6 h after serum deprivation, SM was reduced, whereas the ceramide compartment appeared at the apical membrane (Supplemental Figure S1). During this time period, acetylated tubulin colocalized with the ACEC, although a primary cilium was not yet detectable. Formation of the procilium began in the next 18 h, concurrent with further reduction of SM and concentration of ceramide at the base of the cilium (Supplemental Figure S1). The greatest increase in the number of cilia was observed between 24 and 48 h after serum deprivation, whereas a period of elongation followed between 72 and 96 h (data not shown).

To test the significance of the endolysosomal degradation pathway for ACEC formation and ciliogenesis, we incubated cells with imipramine, a tricyclic antidepressant known to induce proteolytic degradation of ASMase (Albouz *et al.*, 1986). Figure 2, B and C, clearly shows that incubation of MDCK cells with imipramine reduced the number of primary cilia, which was restored by incubation with the ceramide analogue S18. A similar result was observed with the ceramide synthase inhibitor fumonisin B1 (FB1), which prevented ciliogenesis that was also restored with S18 (Wang *et al.*, 2009a). A detailed analysis showed that disruption of ciliogenesis by the two inhibitors was dose dependent (Supplemental Figure S2). Imipramine was more effective than FB1 in inhibiting cilium formation, suggesting that there is a differential sensitivity of MDCK cells to the two inhibitors. Imipramine and FB1 are known to disrupt the salvage pathway of ceramide biosynthesis in the ER, which is critical to replenish SM from ceramide degraded in the endolysosomal pathway (Kitatani *et al.*, 2008). We tested whether FB1 or imipramine disturbed the level and distribution of SM and ceramide at the apical membrane. Figure 3A shows that incubation of MDCK cells with these inhibitors dramatically reduced the level of ceramide at the apical cell membrane. Of interest, FB1 and imipramine also reduced immunostaining of SM, indicating that endolysosomal degradation of SM and regeneration of ceramide and SM in the salvage pathway are critical for the distribution of SM and ceramide to the apical cell membrane.

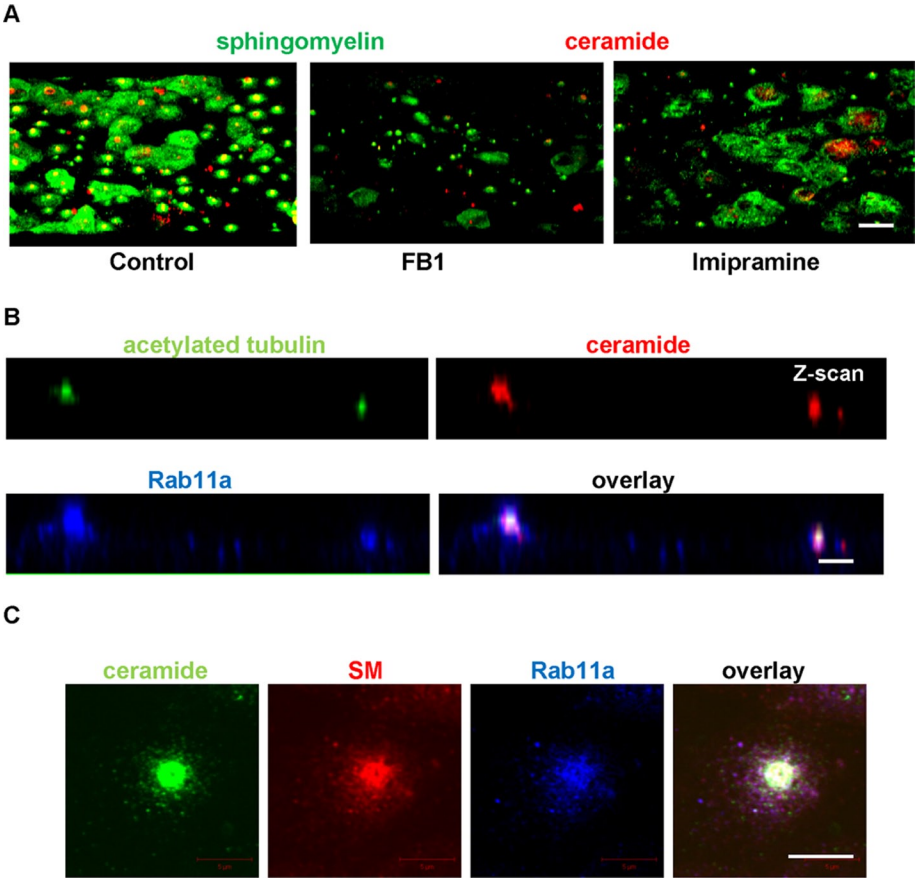

FIGURE 3: ACEC formation relies on endolysosomal processing of SM involving Rab11a(+) compartments. (A) Projection view from top onto the apical plane of control cells and cells treated with two inhibitors in the salvage pathway for endolysosomal processing and remodeling of SM and ceramide (imipramine and FB1). Immunocytochemistry for SM (green) and ceramide (red) shows that the two lipids disappear from the apical plane when SM and ceramide processing is inhibited. Bar, 10 μm. (B) *Z*-scan of serum-deprived MDCK cells shows colocalization of ceramide (red) with Rab11a (blue) at the base of primary cilia (acetylated tubulin, green). Bar, 5 μm. (C) Colocalization of ceramide, SM, and Rab11a at the ACEC. Bar, 5 μm.

Rab11a codistribution with the ACEC is critical for ciliogenesis

So far, our data suggested that endocytosis of SM and then ASMase-catalyzed hydrolysis of SM to ceramide is the first step in ceramide-dependent regulation of ciliogenesis. Previous studies provided evidence that Rab11a is involved in the endocytosis of SM/cholesterol-containing lipid vesicles, mediates vesicle transport in ciliogenesis, and is highly enriched at the base of the primary cilium (Takahashi *et al.*, 2007; Nachury *et al.*, 2010). Because the generation or enrichment of ciliogenic ceramide in the ACEC was likely to involve endolysosomal degradation of SM by ASMase and metabolic recycling via the salvage ceramide biosynthesis pathway, we postulated that there is a codistribution and association of SM and ceramide with Rab11a. Using immunocytochemistry, we found that ceramide was colocalized with Rab11a at the base of the cilium (Figure 3B), suggesting that Rab11a is associated with the ACEC. Rab11a was highly enriched in the center of the ACEC, and it was associated with SM-containing vesicles in its periphery (Figures 3C and 4A). The profile analysis in Figure 4A (right) shows a striking colocalization of Rab11a, ceramide, and SM as indicated by the three peaks of overlapping pixel intensities. This colocalization was consistent with our hypothesis that there is an endocytotic transport of SM toward the ACEC before the conversion of SM to ceramide. Before formation of the ACEC, ceramide and Rab11a were not colocalized (Figure 4B). Colocalization occurred in >80% of the MDCK cells within 15 h of cultivation in serum-free medium, suggesting that serum deprivation activated ASMase-catalyzed hydrolysis of SM to ceramide and ACEC formation.

When MDCK cells were incubated with FB1 or imipramine, the apical level of ceramide dropped concurrently with dispersion and redistribution of Rab11a to the cytosol (Figure 4C and Supplemental Figure S3). We found that the number of Rab11a(+)/ACEC(+) cells dropped from 80% (controls) to 13% (FB1-treated cells) or <1% (imipramine-treated cells), suggesting that the salvage pathway of ceramide biosynthesis was critical for the codistribution of apical ceramide with Rab11a. The novel ceramide analogue S18 restored the apical distribution of Rab11a in imipramine-treated cells, suggesting that it replaced apical ceramide for the codistribution process (Figure 4C). Of interest, in cells showing either apical ceramide or Rab11a staining (<20% of serum-deprived MDCK cells), cilium formation was not observed (Supplemental Figure S3). Staining for acetylated tubulin in primary cilia or procilia was only found when the ACEC was costained with Rab11a, indicating that ceramide and Rab11a act synergistically on ciliogenesis.

Rab11a is directly associated with lipid vesicles enriched with C16 and C18 ceramide

The synergistic activity of ceramide and Rab11a suggested that these two factors were physically associated in ceramide vesicles at the ACEC. We used magnetic sorting to isolate ceramide or Rab11 vesicles and to determine their association with other proteins regulating ciliogenesis and to analyze their lipid composition (Casanova *et al.*, 1999; Wang *et al.*, 2005; Bieberich, 2011). MDCK cells were grown to confluence and serum starved until >80% of the cells showed codistribution of ceramide and Rab11a to the ACEC and formed primary cilia. Cells were homogenized by mechanical fragmentation without detergent, and a postmitochondrial fraction was isolated by centrifugation. Ceramide- or Rab11a-containing vesicles were then purified from the cell homogenate by binding to anti-ceramide or anti-Rab11a rabbit IgG-coated magnetic beads, and the copurified protein and lipid fraction was analyzed by SDS–PAGE/immunoblotting and mass spectrometry (lipidomics), respectively. Unrelated rabbit IgG was used as the negative control. To avoid any cross-reactivity with the rabbit antibodies used for vesicle purification, all of the candidate proteins were detected on the immunoblots using antibodies raised in mouse.

Figure 5A shows that the negative controls contained only trace amounts of nonspecifically bound protein, whereas vesicles purified with anti-ceramide antibodies contained Rab11a. This result demonstrated the specificity and efficacy of the purification procedure and was consistent with the data from immunocytochemistry

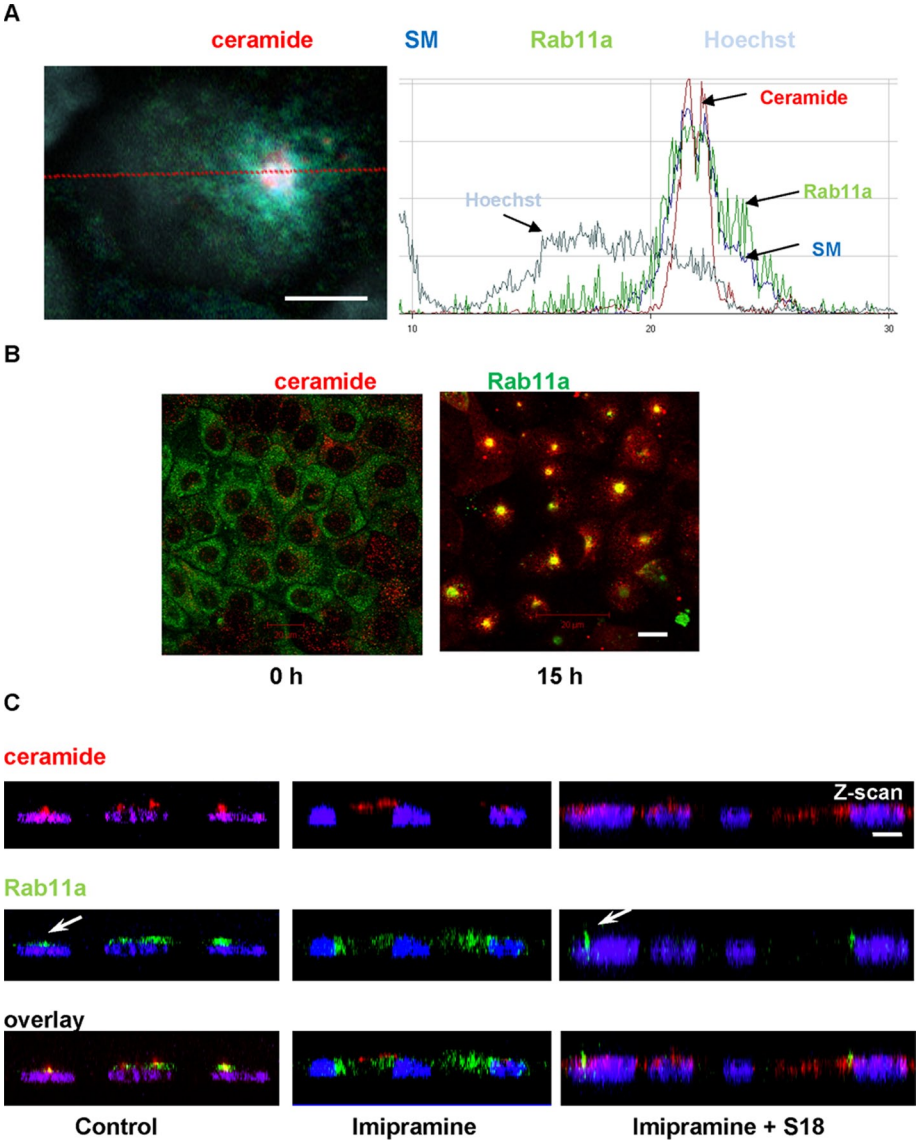

FIGURE 4: Serum deprivation induces codistribution of SM, ceramide, and Rab11a at the ACEC. (A) Immunocytochemistry for ceramide (red), SM (blue), and Rab11a (green) showing colocalization with the ACEC. Right, shows a pixel profile along the axis indicated at the left. Bar, 5 μm. (B) Immunocytochemistry for ceramide (red) and Rab11a (green) before and 15 h after serum deprivation. Bar, 10 μm. (C) Codistribution of ceramide (red) and Rab11a (green) by serum deprivation is prevented in the presence of imipramine. It can be rescued by incubation with a ceramide analogue (S18). Arrow points at primary cilium. Bar, 5 μm.

showing codistribution of ceramide and Rab11a in ciliated MDCK cells (Figures 3C and 4A). In conjunction with the observation that ceramide and Rab11a were colocalized at the ACEC, our data suggested that the physical interaction of ceramide with Rab11 is involved in the formation of primary cilia.

To determine which ceramide species were enriched in ceramide vesicles, we performed sphingolipidomics analysis using mass spectrometry of lipid extracts from vesicles purified by magnetic sorting with rabbit anti-ceramide or Rab11a antibodies. Figure 5B (middle) shows that C16:0, C18:0, C24:0, and C24:1 ceramide were the predominant species (25–40% each) in ceramide vesicles. This observation is interesting, in that C16 and C18 ceramide were less abundant species in the total lipid pool of serum-deprived MDCK cells, whereas the majority of ceramide was of the C24:0 species (Figure 5B, top). In fact, a quantitative analysis based on lipid-bound phosphate indicated that all of the intracellular C16 and C18 ceramide was recovered in ceramide vesicles (Figure 5B). Therefore our results suggested that C16 or C18 ceramide may play a role in the formation or ciliogenic function of the ACEC. Rab11a vesicles contained ceramide species highly similar to those found in ceramide vesicles, strongly suggesting that the two vesicle populations were, at least in part, identical and derived from the same compartment (Figure 5B, bottom). In summary, these results suggested that there is a specific association of proteins and ceramide species in ceramide vesicles that appeared to be functionally involved in ACEC/Rab11a formation and its role in ciliogenesis.

Ceramide-bound aPKC is critical for ciliogenesis

So far, our results showed that ceramide interacts with Rab11a, but they did not yet clarify whether ceramide bound directly to Rab11 or if additional proteins were involved. Our previous results showed that ceramide bound and activated aPKC (Wang *et al.*, 2005, 2009b). It is also known that aPKC is associated with Cdc42 via binding to Par6 and that a Rab11a-Rabin8-Rab8 network recruits Cdc42 and exocyst proteins (e.g., Sec8, Sec10, and Sec15) to the apical membrane of MDCK cells (Etienne-Manneville and Hall, 2003; Cau and Hall, 2005; Bryant *et al.*, 2010; Bieberich, 2011). Recently it was found that the association of aPKC-Par6–bound Cdc42 with the exocyst (via Sec10) is critical for the formation of primary cilia (Zuo *et al.*, 2011). Therefore we hypothesized that ACEC-bound aPKC might mediate the association of ceramide with a protein network involved in ciliogenesis.

First, we determined the significance of ceramide binding to aPKC for primary cilium formation. We expressed a dominant-negative mutant of aPKC (C20ζ–green fluorescent protein [GFP]) that was previously shown to prevent binding of endogenous aPKC to ceramide in MDCK cells (Wang *et al.*, 2009b). C20ζ-GFP encompasses the GFP-tagged C-terminus of aPKC, which binds to ceramide. However, this aPKC mutant cannot bind to and phosphorylate its target proteins or interact with other aPKC-associated proteins such as Par6 and Cdc42. Figure 6A shows that C20ζ-GFP was colocalized with ceramide, but cells expressing C20ζ-GFP did not show formation of primary cilia. In fact, the aPKC mutant even prevented the polarized distribution of ceramide to the ACEC, suggesting that the association of ceramide with functional aPKC is critical not only for ciliogenesis, but also for ACEC formation.

We then tested whether ceramide was critical for the association of aPKC with Rab11a vesicles. Using the magnetic isolation procedure, we found that aPKC was present in the ceramide vesicle fraction but not in the negative control purified with preimmune serum rabbit IgG (Figure 6B). Of interest, acetylated tubulin was also found

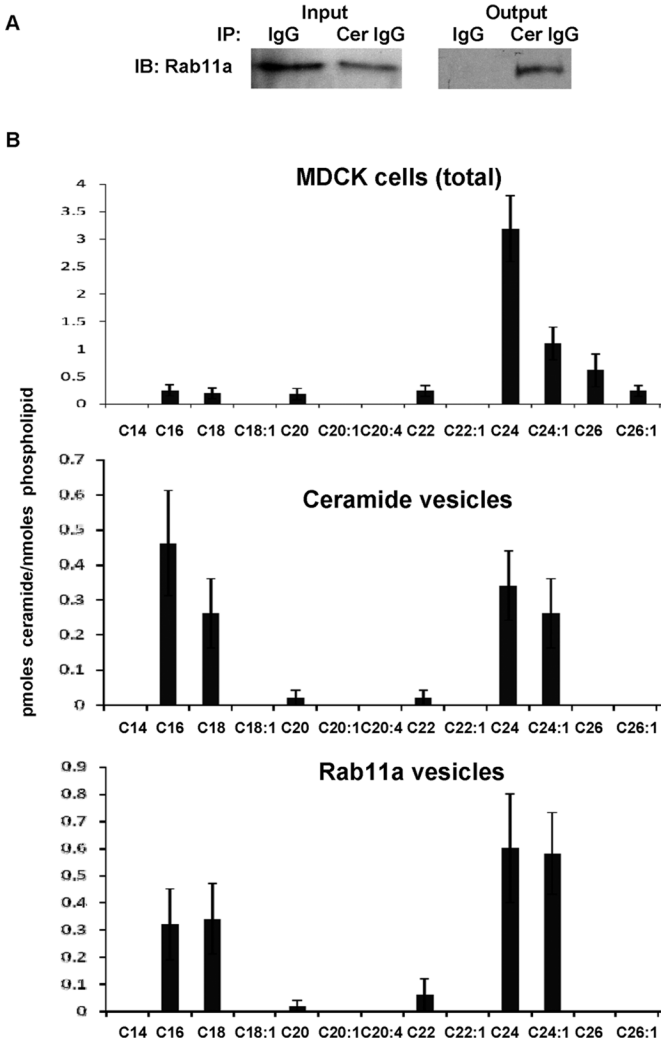

FIGURE 5: Ceramide and Rab11a vesicles contain almost identical ceramide pools enriched with C16 and C18 ceramide. Analysis of ceramide and Rab11a vesicles isolated from ciliated MDCK cells using anti-ceramide rabbit IgG or anti-Rab11a rabbit IgG immobilized on magnetic beads. Eluted vesicles were analyzed for the presence of Rab11a using immunoblotting (A) and for different ceramide species using sphingolipidomics/mass spectrometry (B). (A) Left, input control (corresponding to 50% of cell homogenate used for the isolation procedure). Right, output. (B) Sphingolipidomics (liquid chromatography–tandem mass spectrometry) analysis of ceramide species from ciliated MDCK cells (72 h after serum deprivation) and ceramide or Rab11a vesicles. Note that the ceramide profile of ceramide and Rab11a vesicles is almost identical and encompasses the entire pool of C16 and C18 ceramide in ciliated MDCK cells (as quantified by the amount of ceramide/lipid-bound phosphate). $n = 3$.

in ceramide vesicles, suggesting that there is an interaction between ceramide and acetylated tubulin. For the analysis of the ceramide-dependent aPKC association with Rab11a, we tested whether ceramide depletion of MDCK cells by incubation with FB1 or imipramine prevented copurification of aPKC with Rab11a vesicles. Loading of each sample (Rab11a vesicles from untreated or inhibitor-treated MDCK cells) was normalized to equal amounts of Rab11a as quantified by the signal obtained with a mouse anti-Rab11a antibody. Input controls confirmed that treatment with FB1 or imipramine did not affect the total amount of protein before copurification with Rab11a vesicles. Immunoblot (Figure 6C) and densitometric analysis (data not shown) revealed that inhibition of ceramide generation with FB1 or imipramine reduced copurification of aPKC with Rab11a vesicles by 90 or 70%, respectively. In summary, these results suggested that ceramide enrichment in the ACEC/Rab11a compartment is essential for its interaction with aPKC and might also mediate the association of other ciliogenic proteins such as Par6 and Cdc42 with Rab11a vesicles.

Ceramide is critical for the association of Rab 11a vesicles with ciliogenic proteins

To test the significance of ceramide for the interaction of ciliogenic proteins with Rab11a, we isolated ceramide and Rab11a vesicles and tested for copurification of these proteins with the vesicles. On the basis of the discussion in the preceding section, we focused our analysis on Cdc42, Sec8, and Rab8 since these proteins can potentially form a molecular network with ceramide-bound aPKC. We also tested whether ceramide depletion with FB1 or impramine affects binding of Cdc42, Sec8, and Rab8 to Rab11a vesicles.

Figure 6, B and C, shows that Cdc42, Sec8, and Rab8 copurified with ceramide and Rab11a vesicles. Association of Rab11a vesicles with Cdc42, Sec8, and Rab8 was significantly reduced when MDCK cells were depleted of ceramide by incubation with FB1 (Figure 6C). Of interest, imipramine also compromised binding of Rab8 to Rab11a vesicles; however, it only partially prevented association with Cdc42 and Sec8. This observation suggested the existence of distinct transport or fusion pathways for the association of ceramide/Rab11a vesicles with Rab8, or Cdc42 and Sec8, or the involvement of other ceramide-generating enzymes such as neutral sphingomyelinases (nSMases). Indeed, immunocytochemistry analyses showed that at the center of the ACEC, Rab8 and Sec8 were codistributed with ceramide and Rab11a, whereas in its periphery, Rab8 or Sec8 and Rab11a vesicles did not colocalize (Figure 6, D and E) and thus seem to belong to different trafficking pathways (Figure 6, D and E). About 70% of the ceramide vesicles were colocalized with Rab11a and Rab8, whereas the remaining ceramide vesicles were only Rab11a(+) or not associated with Rab proteins. Therefore it is likely that this portion of ceramide or Rab11a(+) vesicles might serve other functions such as endosomal transport independent of ciliogenesis. At this point, it remains to be determined whether the different trafficking pathways engaged in ciliogenesis encompass other sphingolipids or ceramide-generating enzymes such as nSMases. However, our results strongly suggest that vesicular trafficking and generation of ceramide from SM regulates the interaction of key factors that regulate vesicle transport and fusion in ciliogenesis.

Inhibition of HDAC6 rescues ciliogenesis in ceramide-depleted cells: evidence for the role of the ACEC in the stabilization of primary cilia by tubulin acetylation

The observation that acetylated tubulin originated from the ACEC (Figure 1A) and was pulled down with ceramide vesicles (Figure 6B) suggested that ceramide regulates tubulin stabilization by acetylation. We tested this assumption by incubating imipramine-treated MDCK cells with trichostatin A (TSA), an inhibitor for several isoforms of HDAC. Figure 7A shows that TSA restored the number of primary cilia in imipramine-treated cells, suggesting that ceramide release by ASMase down-regulates deacetylation of tubulin in primary cilia. It is known that deacetylation in primary cilia is down-regulated by the interaction of the BBsome with HDAC6 (Loktev *et al.*, 2008). A potential effect of ceramide on this interaction would imply a physical association of ceramide, the BBsome, and HDAC6.

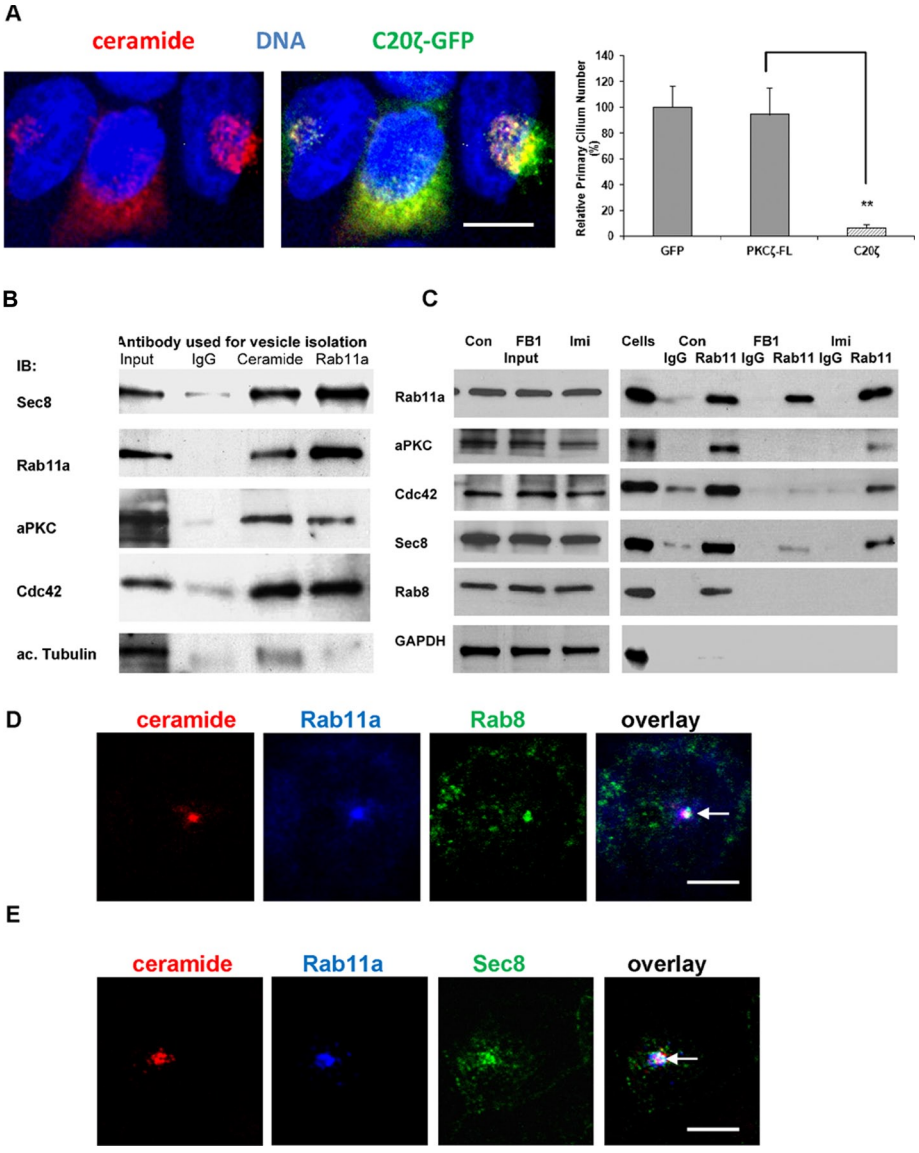

FIGURE 6: Ceramide is critical for association of Rab11a vesicles with aPKC and other ciliogenic proteins. (A) Expression of the ceramide-binding, dominant-negative PKCζ mutant C20ζ-GFP in MDCK cells. Left, immunocytochemistry for ceramide shows codistribution with C20ζ-GFP; right, relative number of cilia in GFP-expressing cells. $n = 5$, $p < 0.01$. Bar, 5 μm. (B) Magnetic isolation of ceramide and Rab11a vesicles, followed by immunoblotting for various proteins involved in ciliogenesis. Note that the protein profile of ceramide and Rab11a vesicles is almost identical, although acetylated tubulin was only recovered from ceramide vesicles. Input corresponds to 20% of cell homogenate used for the isolation procedure. (C) As in B, however, MDCK cells were serum deprived in the presence of inhibitors for SM and ceramide processing (FB1 and imipramine). GAPDH was used as a negative control not involved in Rab11a vesicle binding or ciliogenesis. (D) Immunocytochemistry for ceramide, Rab11a, and Rab8 shows colocalization at the ACEC. (E) As in D, except using an antibody against Sec8. Arrows point at location of primary cilium as determined by the colocalization of ceramide and acetylated tubulin in the same sample (data not shown). Bars, 5 μm.

Indeed, immunocytochemistry for ceramide, the BBsome-resident protein BBS1, and HDAC6 clearly showed that they were colocalized at the ACEC and that this colocalization was disrupted by imipramine (Figure 7, B–D). Given that Rab8 interacts with the BBsome (Nachury *et al.*, 2007, 2010; Westlake *et al.*, 2011), the ACEC might be a hub for a larger ciliogenic lipid–protein complex stabilizing primary cilia by promoting acetylation (or preventing deacetylation) of tubulin at the cilium base.

DISCUSSION

The formation, elongation, and maintenance of the primary cilium are regulated by vesicular transport and fusion, which is likely to bring together a complex of lipid vesicle–associated proteins that are critical for ciliogenesis. Although it is well known that a multitude of transport and fusion proteins participate in this regulation, little information is available on the function of lipids in ciliogenesis. It is important to fill this gap since lipids are a major physical component mediating formation and fusion of membrane vesicles. We showed for the first time that the sphingolipid ceramide is a key regulatory lipid in cell polarity–related processes, including ciliogenesis (Wang *et al.*, 2009a). Using an anti-ceramide antibody generated in our laboratory, we discovered the presence of an apical ceramide-enriched compartment at the base of the primary cilium. Furthermore, by depleting cells of ceramide and restoring ciliogenesis with a ceramide analogue, we showed that ceramide is essential for cilium formation.

Our present data show that apical SM disappears and ceramide appears in the ACEC during the initial phase of cilium formation, suggesting that conversion of apical SM to ceramide is instrumental for ciliogenesis. SM is synthesized from ceramide in the Golgi apparatus and then transported to the cell membrane. Cell membrane–resident SM is then either directly converted to ceramide by nSMase or, alternatively, internalized by endocytosis and then hydrolyzed by ASMase to ceramide in endosomes and lysosomes. Ceramide can be further degraded to sphingosine, which is used in the ER to regenerate ceramide and, eventually, SM in the Golgi. This recycling turnover of SM and ceramide allows for the rapid remodeling of the ceramide and SM composition with respect to the fatty acids linked to the sphingosine residue in these two lipid species. It is believed that different fatty acids in ceramide determine the function of distinct ceramide species.

Although the salvage pathway remodels the type of fatty acid within distinct ceramide and SM species, the immediate release of these ceramide species at the cell membrane or the endolysosomal trafficking pathway is mediated by nSMases or ASMase, respectively. Our loss-of-function experiments showing impairment of primary cilium formation by the ASMase inhibitor imipramine and the ceramide synthase inhibitor FB1 strongly suggest that the endolysosomal salvage pathway is important for the generation of ceramide for ciliogenesis. Consistent with this hypothesis, immunocytochemistry shows that in addition to enrichment in the ACEC, SM or ceramide is also abundant in Rab11(+) endocytotic vesicles.

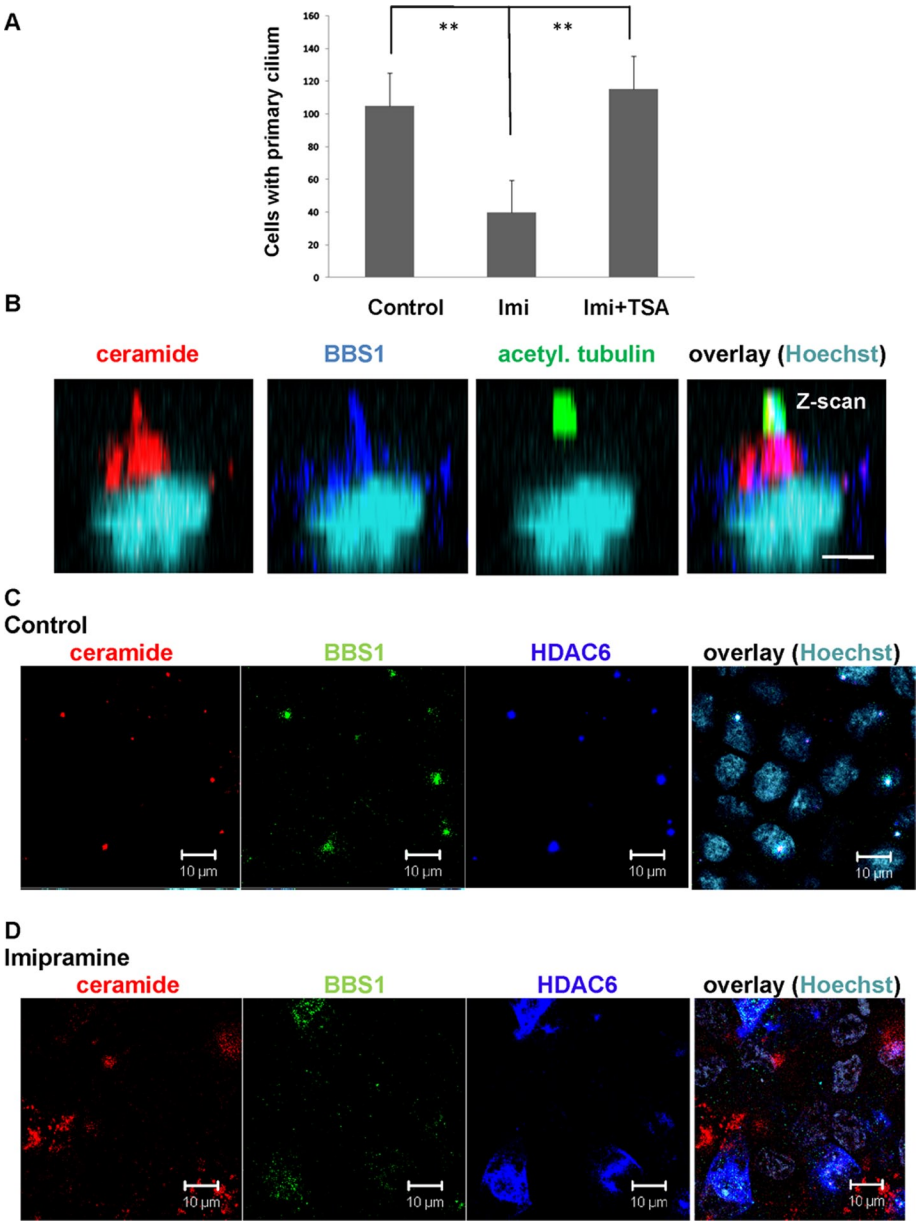

FIGURE 7: The ACEC inhibits deacetylation of acetylated tubulin: a potential mechanism for the initiation and stabilization of the primary cilium. (A) Incubation of imipramine-treated MDCK cells with the HDAC inhibitor TSA (0.5 µM) rescued ciliogenesis (figure shows number of cilia in 150 cells). $n = 5$; $p < 0.01$. (B) Codistribution of BBS1 (blue) with ceramide (red) and acetylated tubulin (green) as determined by immunocytochemistry (figure shows *Z*-scan). Bar, 5 µm. (C, D) In ciliated control MDCK cells (C), ceramide (red), BBS1 (green), and HDAC6 (blue) are codistributed at the ACEC, which is inhibited by imipramine (D).

Of interest, the peripheral SM vesicle array, the ceramide-enriched center compartment, or the colocalization of SM and ceramide vesicles with Rab11a is not visible before serum deprivation of MDCK cells. This suggests that the ACEC is a newly formed compartment, most likely as part of a specialized endosome the formation of which requires Rab11a-dependent endocytosis and activation of ASMase. Either the ACEC is directly assembled from ceramide vesicles or ceramide is first transported through the endolysosomal salvage pathway of ceramide biosynthesis. SM derived from the cell membrane is then transported via Rab11a vesicles, converted to ceramide, and fused with the ACEC. The disruption of the ACEC by imipramine and FB1 indicates that the salvage pathway is the source for the generation of ceramide necessary for ACEC formation. This assumption is also consistent with our previous study showing that the ceramide-enriched compartment at the base of the cilium contains a Golgi marker (GM130) and indicates that ceramide is remodeled in the salvage pathway before its conversion to SM in the Golgi. Given that GM130 is a *cis*-Golgi marker, it is possible that there are additional vesicle-trafficking pathways from the Golgi to the ACEC, which may complement transport of Golgi-derived SM to the cell membrane. Alternatively, distinct SM species may be differently endocytosed and transported from the plasma membrane to the ACEC. For example, C18 SM/C18 ceramide may be retained and shunted to the ACEC, whereas longer-chain SM may be degraded in lysosomes. In this case, the SM cycle could enrich for a pool of ciliogenic ceramide at the membrane that is sensitive to imipramine but not necessarily to FB1. On the other hand, the observation that the association of ciliogenic proteins to Rab11a was prevented by FB1 suggests that the salvage pathway is critical for remodeling of ceramide and SM species in ciliogenesis.

The role of Rab11a in the ceramide-dependent regulation of ciliogenesis is of particular importance. Rab11a is a small, Ras superfamily–type GTPase that is essential for trafficking of recycling endosomes. Recently, Rab11a was shown to bind to Rabin8 and Rab8 (Rab activation complex), which is a critical step in the assembly of the ciliary membrane (Knodler *et al.*, 2010; Nachury *et al.*, 2010; Das and Guo, 2011; Westlake *et al.*, 2011). We found that a major portion of SM and ceramide vesicles surrounding and within the ACEC are Rab11a(+)/Rab8(+). Moreover, ceramide depletion with FB1 or imipramine perturbs binding of ciliogenic proteins such as aPKC or Rab8 to Rab11a vesicles. Therefore it is likely that ceramide regulates the function of Rab11a and, potentially, its interaction with Rab8 in ciliogenesis.

Rab11a is also interesting as interaction partner for other proteins in ciliogenesis. A ciliogenic protein complex associated with Rab11a is the exocyst, which is composed of several proteins that mediate vesicular membrane fusion. One of these proteins, Sec15, has been shown to directly interact with Rab11a (Oztan *et al.*, 2007). Our results show that Sec8 colocalizes with Rab11a in the ACEC and that ceramide depletion prevents this distribution. Therefore the ACEC is likely to be a hub for the codistribution and, potentially, interaction of proteins that are critical for vesicular transport and fusion in ciliogenesis. In this regard, it is interesting to note that a number of proteins involved in the formation or function of cilia, such as aPKC, Arl6/BBS3, patched, or retinitis pigmentosa 2, have been described to localize to a ring- or disk-shaped compartment at the transition zone at the cilium base (Rohatgi

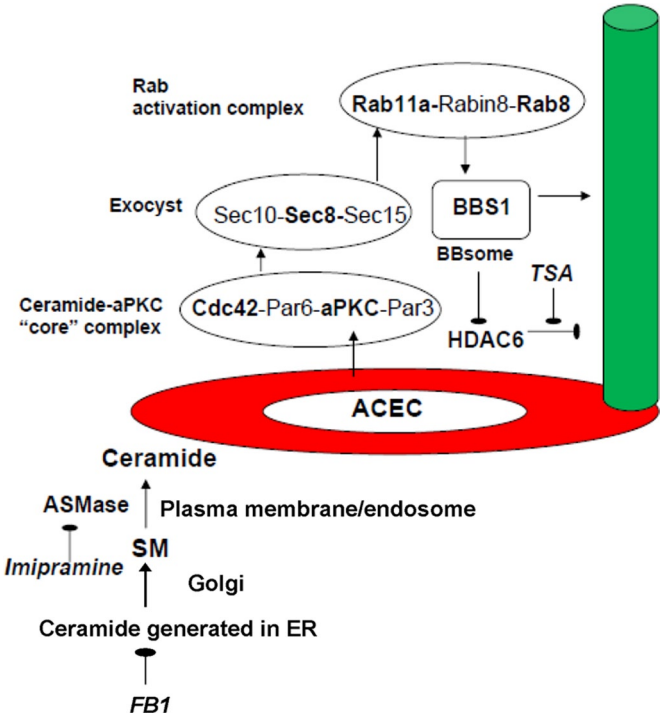

FIGURE 8: Model for the ACEC-mediated stabilization of the primary cilium. SM from the cell membrane or Golgi is converted to ceramide, which binds to an aPKC-Par6-Cdc42 core complex. This core complex is associated with the exocyst (via Cdc42/Par6 binding to Sec10-Sec8-Sec15) and may regulate fusion of ceramide vesicles with the ACEC. The exocyst also mediates association of the ceramide–aPKC core complex with the Rab activation complex (Rab11a-Rabin8-Rab8), which is critical for primary cilium formation. Recruitment of the BBsome to the Rab activation complex (via binding of Rabin8 to BBS1) inhibits HDAC6 and initiates or stabilizes the cilium by promoting acetylation of tubulin at the ACEC. In bold are proteins we have shown to colocalize or copurify with ceramide in the ACEC or lipid vesicles.

et al., 2007; Evans *et al.*, 2010; Hurd *et al.*, 2010; Wiens *et al.*, 2010; Pruliere *et al.*, 2011). It is not clear whether this distribution is related to the ACEC. However, it is likely that vesicular transport and fusion is a prerequisite for the formation of the ACEC and that the ACEC contributes to the assembly of the ciliary vesicle, pocket, or membrane.

Figure 8 illustrates the hypothesis that the ACEC is a hub for the formation of the ciliary membrane and other processes during ciliogenesis. This model emerges from data in our laboratory showing that ceramide physically interacts with aPKC, a family of three PKC members: PKCζ, λ, and ι. In addition, a number of previous studies in other laboratories also showed that aPKC is instrumental for ciliogenesis of both primary and motile cilia, although the role of ceramide was not investigated (Fan *et al.*, 2004; Pruliere *et al.*, 2011). Consistent with a ceramide–aPKC complex, aPKC colocalizes with Rab11a at the ACEC, which is compromised when cells are depleted of ceramide. More strikingly, C20ζ-EGFP, a ceramide-binding but inactive mutant of PKCζ, is dominant negative for ciliogenesis when expressed in MDCK cells. These data strongly suggest that ceramide binding to aPKC in the ACEC is critical for cilium formation. Furthermore, our data indicate that ceramide binding is required for the aPKC-mediated interaction with other proteins in ciliogenesis, potentially as part of a larger lipid–protein complex with Rab11a. Most recently, Cdc42 was found to regulate ciliogenesis and mediate binding of aPKC to the exocyst, presumably by binding to Sec10. In the exocyst, Sec10 interacts with Sec8 and Sec15, a protein binding to Rab11a in the Rab activation complex (Figure 8; Zuo *et al.*, 2011). We showed that ceramide depletion with imipramine or FB1 perturbed the association of Cdc42 to Rab11a vesicles. Moreover, our previous study showed that ceramide depletion disrupts the distribution of Cdc42 to the apical membrane of MDCK cells (Wang *et al.*, 2009a). Therefore we hypothesize that a ceramide-associated aPKC-Par6-Cdc42 "core complex" binds to the exocyst and the Rab activation complex as depicted in Figure 8. Because ciliogenesis can be restored with the HDAC inhibitor TSA, it is likely that this ACEC-associated molecular network of "core complex," exocyst, and Rab activation complex affects the acetylation of tubulin at the cilium base.

Indeed, Rabin 8 has been found to bind to BBS1, a protein subunit of the BBsome (Knodler *et al.*, 2010). The BBsome is critical for many processes at the cilium base, among which is also inhibition of HDAC6 (Loktev *et al.*, 2008). Hence the ACEC-associated molecular network can be extended to the BBsome, ultimately linking ceramide generation by ASMase to the inhibition of HDAC6 and nucleation of acetylated tubulin for ciliogenesis. Consistent with this model, we found that acetylated tubulin can be copurified with ceramide vesicles and that the first detectable filaments of acetylated tubulin appear to emerge from the ACEC. Because the basal body is the initial base for acetylated tubulin extending into primary cilia, we hypothesize that the ACEC functionally interacts with the basal body to sustain acetylation and, therefore, initiation, elongation, and maintenance of primary cilia. In initial studies, we found that primary cultured astrocytes deprived of serum will also grow primary cilia that colocalize with ceramide (Supplemental Figure S4). Therefore it is likely that ceramide-regulated ciliogenesis can be found in a variety of mammalian cell types.

In summary, our study shows for the first time that ceramide enriched in a novel compartment—the ACEC—is involved in the regulation of a lipid–protein molecular network that is critical for ciliogenesis. In future studies, we will further elucidate this mechanism and how cilium formation is regulated by ceramide.

MATERIALS AND METHODS

MDCK II cells were obtained from Quansheng Du (Georgia Health Sciences University, Augusta, GA). Anti-ceramide antibodies were purchased from Glycobiotech (Kuekels, Germany; mouse IgM clone 0020) or generated in our laboratory (anti-ceramide rabbit IgG) as previously described (Krishnamurthy *et al.*, 2007). Anti-PKCζ (C20, sc-216) rabbit IgG, anti-BBS1 rabbit IgG (sc-134455), and anti-Cdc42 mouse IgG (B-8, sc-8401) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Rab11a (47, ab78337) was from Abcam (Cambridge, MA). Anti-Rab11a rabbit IgG (71-5300) and anti-Rab8 mouse IgG (610844) and DMEM were obtained from Invitrogen (Carlsbad, CA). All ceramide species were of the highest purity and purchased from Avanti Polar Lipids (Alabaster, AL). S18 was synthesized in our laboratory as previously described (Bieberich *et al.*, 2000, 2002). TSA, imipramine, FB1, anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse IgG, and anti–acetylated tubulin mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG magnetic beads were from New England BioLabs (Ipswich, MA) or Miltenyi (Auburn, CA). Anti-rSec8 mouse IgG (14G1, VAM-SV016) was from Enzo Life Sciences (Plymouth Meeting, PA). Anti-HDAC6 mouse IgG (GTX 84377) was obtained from GeneTex (Irvine, CA). Antibodies were validated to recognize the respective proteins in MDCK cells based on prior studies and peptide competition experiments. All reagents and solvents were of analytical quality.

Cultivation of MDCK II cells and expression of C20ζ-EGFP

Cells were maintained in DMEM containing 10% fetal calf serum, penicillin, and streptomycin in a 5% CO_2 atmosphere at 37°C until reaching confluency. Serum was withdrawn from the medium and the cells further cultivated in serum-free medium for 72 h to establish apicobasal polarity before treatment with various reagents affecting ceramide biosynthesis or ciliogenesis (20 µM FB1, 40 µM imipramine, 40 µM S18, 0.5 µM TSA, or combinations of these drugs). The dominant-negative PKCζ mutant C20ζ-EGFP was expressed as previously described (Wang *et al.*, 2009b). After incubation with reagents or expression of the mutant aPKC, cells were harvested and used for vesicle isolation and protein or lipid analysis or fixed with 4% *p*-formaldehyde in phosphate-buffered saline (PBS) for immunocytochemistry. The C20ζ-EGFP fragment should not be confused with the anti-aPKC antibody that is also termed C20 and recognizes a C-terminal fragment of full-length aPKC.

Immunocytochemistry and image acquisition

After fixation with 4% *p*-formaldehyde/PBS, nonspecific binding sites were blocked with 3% ovalbumin/10% donkey serum/PBS for 1 h 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 (Wang *et al.*, 2009a). Confocal fluorescence microscopy was performed using a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Jena, Germany) equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3), or 633 nm (Cy5, Alexa Fluor 647), respectively. LSM 510 Meta 3.2 software was used for image acquisition. Adobe Photoshop CS2 software (Adobe, San Jose, CA) was used for background reduction, pseudocolorizing, and overlaying of pseudocolorized grayscale images. Images obtained with secondary antibody only were used as negative controls representing the background intensity in a particular laser channel. Antigen-specific immunostaining was quantified by counting cells that showed signals twofold or more above background fluorescence.

Statistical analysis

Counting of cells with particular fluorescence signals for cilium formation or distribution of other antigens was performed by three individuals in a blinded assay using at least five fields on each section or slide from four independent samples with at least 50 cells in each field. Means and SDs were determined for counts of single signals and the signal distributions analyzed using a two-tailed, equal-variance Student's *t* test in Excel 2007 (Microsoft, Redmond, WA). $p < 0.05$ was considered statistically significant. For codistribution/colocalization analysis, a chi-square test was used as described previously (Bieberich *et al.*, 2003).

Isolation and analysis of ceramide and Rab11a vesicles

MDCK cells were plated at 75% confluence on 150 mm dishes. The serum-containing cell culture medium was changed to serum-free medium once cells reached 100% confluence. After treatment with various reagents for 72 h, cells were washed three times with PBS. Then, cells were scraped off, pelleted, and stored at −80°C for later use. A total amount of 20 g of MDCK cells was used for the isolation and analysis of ceramide and Rab11a vesicles. To isolate the vesicles, cell pellets of 0.2 g (per incubation of vesicles with one specific antibody or nonspecific control IgG) were homogenized by using a rotating Teflon homogenizer in 1 ml of cold MACS buffer (provided by Miltenyi) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The cell homogenate was centrifuged at 3000 rpm for 5 min at 4°C, and then the supernatant was centrifuged at 10,000 rpm for 20 min at 4°C. A 30-µl amount of anti–rabbit IgG-conjugated magnetic beads (Miltenyi or New England BioLabs) was preblocked with MACS buffer for 1 h at 4°C, and aliquots corresponding to 10 µl of beads were bound for 2 h at 4°C to 1.5 µg of nonspecific rabbit IgG, anti-ceramide rabbit IgG, or anti-Rab11a rabbit IgG, respectively. After washing with MACS buffer, the antibody-bound beads were incubated with the cell homogenates at 4°C overnight. Beads were collected using a magnetic column (Miltenyi) or stand (New England BioLabs) and washed six times with PBS. After the final wash, beads were centrifuged and then boiled in 100 µl of SDS sample buffer for protein analysis by SDS–PAGE and immunoblotting, or the lipids were extracted with 1 ml of $CHCl_3$/MeOH (2:1) for lipid analysis. Different ceramide species were quantified in the sphingolipidomics (liquid chromatography–tandem mass spectrometry) analysis core facility at the Medical University of South Carolina (Charleston, SC; under the supervision of Jacek Bielawski). The lipid concentration was normalized to protein and lipid phosphate when analyzing organic cell extracts and to lipid phosphate when analyzing vesicle extracts.

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