REPORT

Cell Cycle 12:5, 849–858; March 1, 2013; © 2013 Landes Bioscience

VDAC3 and Mps1 negatively regulate ciliogenesis

Shubhra Majumder and Harold A. Fisk*

Department of Molecular Genetics; The Ohio State University; Columbus, OH USA

Keywords: primary cilia, ciliogenesis, centrosome, basal body, VDAC, Mps1, quiescence

Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; siCon, control siRNA; siVDAC3, VDAC3-siRNA; siMps1, Mps1-siRNA; PACT domain, pericentrin-AKAP450 centrosomal targeting domain

Centrosomes serve to organize new centrioles in cycling cells, whereas in quiescent cells they assemble primary cilia. We have recently shown that the mitochondrial porin VDAC3 is also a centrosomal protein that is predominantly associated with the mother centriole and modulates centriole assembly by recruiting Mps1 to centrosomes. Here, we show that depletion of VDAC3 causes inappropriate ciliogenesis in cycling cells, while expression of GFP-VDAC3 suppresses ciliogenesis in quiescent cells. Mps1 also negatively regulates ciliogenesis, and the inappropriate ciliogenesis caused by VDAC3 depletion can be bypassed by targeting Mps1 to centrosomes independently of VDAC3. Thus, our data show that a VDAC3-Mps1 module at the centrosome promotes ciliary disassembly during cell cycle entry and suppresses cilia assembly in proliferating cells. Our data also suggests that VDAC3 might be a link between mitochondrial dysfunction and ciliopathies in mammalian cells.

Introduction

Centrosomes are microtubule organizing centers (MTOCs) that serve as the poles of mitotic spindles in mitotic cells. The centrosome consists of a pair of centrioles that are precisely duplicated during S-phase. Of the centriole pair, the "mother" is distinguishable from the "daughter" by the presence of distal and subdistal appendages.[1] During cellular quiescence (G_0 phase), the mother centriole is converted to the basal body that assembles a primary cilium, which is then disassembled during cell cycle re-entry.[2] A cilium or flagellum is made of a microtubular axoneme that is ensheathed within a membrane and protrudes out from the cell surface.[3] While motile cilia are found in specific cell types, non-motile primary cilia that transduce physiological and developmental signals are found in almost all mammalian cells at some point during their life cycle.[4,5] During ciliogenesis, the centrosome migrates to the cell surface where the mother centriole is converted to the basal body, which entails it being encapsulated at its distal end by vesicles derived from the Golgi and anchored to the plasma membrane.[6] This is followed by extension of the ciliary axoneme mediated by the intraflagellar transport (IFT) machinery.[7] Recent studies indicate that various molecular machineries that control the temporal switch between basal bodies and centrioles play a crucial role in regulating ciliary assembly-disassembly in co-ordination with the cell cycle.[8-12] Defects in the assembly or function of primary cilia are associated with a series of pathologies broadly known as ciliopathies.[13,14]

We have recently shown voltage-dependent anion channel 3 (VDAC3), a mitochondrial porin, to localize to centrosomes, preferentially to the mother centriole and to interact with the centrosomal protein kinase Mps1, which was also predominantly associated with the mother centriole.[15] Mps1 is required for the spindle assembly checkpoint[16] and is an important regulator of centriole assembly, although it may be dispensable for the canonical centriole duplication cycle.[17-20] Mps1 is required for the recruitment of Centrin 2 (Cetn2) to procentrioles,[15,21] and increasing the level of Mps1 at centrosomes invariably causes centriole re-duplication in human cells.[18,19,22-24] We showed that a centrosomal pool of VDAC3 recruits Mps1 to centrosomes; Mps1 is lost from centrosomes in VDAC3-depleted cells, leading to an inhibition of centriole assembly that can be bypassed by targeting Mps1 to centrosomes independently of VDAC3.[15]

Here, we show that both VDAC3- and Mps1-depleted RPE1 cells inappropriately assemble primary cilia. Tethering Mps1 to the centrosome via the PACT domain can suppress cilia formation in VDAC3-depleted cells, suggesting that VDAC3 negatively regulates ciliogenesis, at least in part by recruiting Mps1 to centrosomes. Thus, our study reveals a novel role for a VDAC3-Mps1 module in controlling ciliogenesis. Moreover, differences in the ciliary phenotypes between VDAC3-depleted and Mps1-depleted cells suggest that VDAC3 may play additional, Mps1-independent roles in ciliogenesis.

Results

VDAC3 depletion leads to primary cilia assembly in non-starved cells. In our previous study, while assessing centriole assembly in asynchronously growing RPE1 cells treated with control (siCon),

*Correspondence to: Harold A. Fisk; Email: fisk.13@osu.edu
Submitted: 01/15/13; Accepted: 01/29/13
http://dx.doi.org/10.4161/cc.23824

VDAC3-(siVDAC3 or siVD3) or Mps1 (siMps1)-specific siRNAs, we noted that only about 10% of siVDAC3 or siMps1 cells incorporated BrdU during a 4 h pulse, compared with roughly 40% of siCon cells (ref. 15; **Fig. S1A**). Because centriole duplication was initiated without delay once siMps1 or siVDAC3 cells entered S-phase, (as judged by proper incorporation of the procentriole marker Sas6 in all BrdU-positive cells), we concluded that these cells had a defect in cell cycle entry rather than cell cycle progression.[15] Here we have further explored that cell cycle defect. Interestingly, we found that a significant fraction of asynchronously grown siVDAC3 or siMps1 cells had assembled primary cilia (**Fig. 1A and B**). Several recent studies demonstrate that the presence of primary cilia can delay the cell cycle,[9,25-28] suggesting that the presence of primary cilia in VDAC3- and Mps1-depleted cells may account for the cell cycle delay. Thus, we set out to determine whether VDAC3 and Mps1 might regulate ciliogenesis.

Generally, the majority of RPE1 cells do not assemble primary cilia during asynchronous growth. Upon cell cycle exit, commonly achieved by prolonged serum starvation, about 80–85% of RPE1 cells form primary cilia.[11,29] Notably, about 70% of non-starved, asynchronously growing RPE1 cells treated with VDAC3-specific siRNA (siVDAC3-1; depletion of VDAC3a and VDAC3b, the two isoforms of VDAC3,[15] were verified by immunoblotting in **Fig. 1C**) assembled primary cilia-like structures as judged by acetylated tubulin (Ac-tub) staining, a well-known marker for the ciliary axoneme (**Fig. 1A and B**), compared with only 15–20% of siCon cells. The Ac-tub-positive axoneme-like structures in siVDAC3 cells are not elongated centrioles,[12] as judged by the absence of any aberrant elongation of various centriolar markers such as Cetn2 (**Fig. S1B**), Cep135 (**Fig. 1D**) and CP110 (**Fig. 2D**). We also observed axoneme-like structures using an antibody against polyglutamylated tubulin,[8] suggesting that this was not an artifact of aberrant tubulin acetylation (**Fig. 1D**), and roughly 75–80% of the Ac-tub stained cilia were positive for the ciliary membrane marker Arl13b (**Fig. 1E**), a small GTPase that regulates Sonic hedgehog signaling in mature cilia.[30] These observations indicate that a majority of the siVDAC3 cells assembled mature primary cilia under non-starved growing conditions. Ectopic expression of a version of VDAC3 resistant to siVDAC3-1 (GFP-sirVDAC3)[15] suppressed cilia formation in VDAC3-depleted cells as compared with GFP alone (**Fig. 1F and G**). RPE1 cells treated with a second siRNA against VDAC3 (siVDAC3-2)[15] showed a similar appearance of primary cilia, although to a lesser extent (about 55–60% in siVDAC3-2 cells compared with 65–75% in siVDAC3-1 and 15–20% in siCon cells; **Fig. S1C and D**). These observations confirm that the aberrant ciliogenesis phenotype in RPE1 cells is an effect of VDAC3 depletion in these cells rather than a non-specific or off-target effect.

A non-mitochondrial pool of VDAC3 negatively regulate ciliogenesis. We have shown that the mitochondrial porin VDAC3 is also localized at the centrosome and regulates centriole assembly.[15] In order to test whether the aberrant ciliogenesis in siVDAC3 cells was an effect of inhibiting a mitochondrial function of VDAC3, we treated RPE1 cells with either Erastin, an inhibitor of the mitochondrial function of VDAC3 (as well as VDAC2),[31,32] or FCCP, an uncoupler of oxidative phosphorylation and therefore an inhibitor of general mitochondrial function.[15] While cytochalasin D, an inhibitor of F-actin dynamics known to induce ciliogenesis,[29] led inappropriate cilia formation in proliferating RPE1 cells, neither Erastin nor FCCP treatment (at concentrations that showed expected effects on cell viability and microtubules),[15] led to any significant increase in cells with cilia (**Fig. 2A**). Therefore, our data suggests that appearance of primary cilia in proliferating siVDAC3 cells does not reflect inhibition of a mitochondrial function of VDAC3 or a general effect of mitochondrial dysfunction, but is likely due to the inhibition of the centrosomal function of VDAC3. Moreover, ectopic expression of GFP-VDAC3, which localizes to the centrosomes in about 60% of RPE1 cells in addition to cytosol and mitochondria (**Fig. 2B** and ref. 15), suppressed ciliogenesis by roughly 2-fold in serum-starved RPE1 cells compared with the expression of GFP alone (**Fig. 2B**).

Because cells that have exited the cell cycle are expected to form cilia, we sought to test if the observed ciliogenesis phenotype of siVDAC3 cells is simply a consequence of cell cycle position by examining siVDAC3 cells that had entered S-phase (as judged by their ability to incorporate BrdU) for the presence of primary cilia. Roughly 30–35% of BrdU-positive siVDAC3 cells (siVDAC3-1 or siVDAC3-2) had a primary cilium, compared with 10–11% of BrdU-positive siCon cells, indicating a roughly 3-fold increase in S-phase siVDAC3 cells that contained cilia (**Fig. 2C**). Moreover, a significant fraction of BrdU-positive cells (roughly 20%) with an intact cilium had just three foci of CP110 (**Fig. 2D**), a centriolar protein that antagonizes cilia formation and is absent from the basal body.[12,33] This indicates that such cells had undergone centriole duplication with an intact primary cilium and a mother centriole/basal body lacking CP110, a phenotype that was very rare in control cells. However, these cells disassembled their cilia before entering to mitosis, since we did not find any cilia-containing mitotic siVDAC3 cell (data not shown). Together, these results demonstrate that the appearance of cilia in siVDAC3 cells is not due to cell cycle exit per se, and suggest that a centrosome associated VDAC3 pool negatively regulates ciliogenesis in proliferating cells.

Mps1 depletion also leads to inappropriate ciliogenesis. The centrosomal pool of VDAC3 binds to Mps1 and regulates the centrosomal level of Mps1, while Mps1 activity controls the recruitment of Cetn2 to the newly formed centrioles.[15] Wondering if Mps1 depletion (siMps1; depletion of Mps1 was verified by immunoblotting in **Fig. 1C**) might induce aberrant ciliogenesis, we examined non-starved siMps1 cells for the presence of primary cilia. Mps1 depletion indeed led to cilia assembly in about 50% of cells, slightly less than that observed in siVDAC3 cells (**Fig. 1B**). The aberrant ciliogenesis in siMps1 or siVDAC3 cells does not appear to reflect the role of Mps1 in recruitment of Cetn2 to centrioles, since only 30–35% of siCetn2 cells assembled cilia (**Fig. S2A**). However, only 14–16% of siMps1 cells that had entered S-phase as judged by BrdU incorporation contained primary cilia (**Fig. 2C**). This result suggests that unlike siVDAC3, most siMps1 cells that assembled primary cilia had exited the cell cycle and were in G_0/G_1 phase.

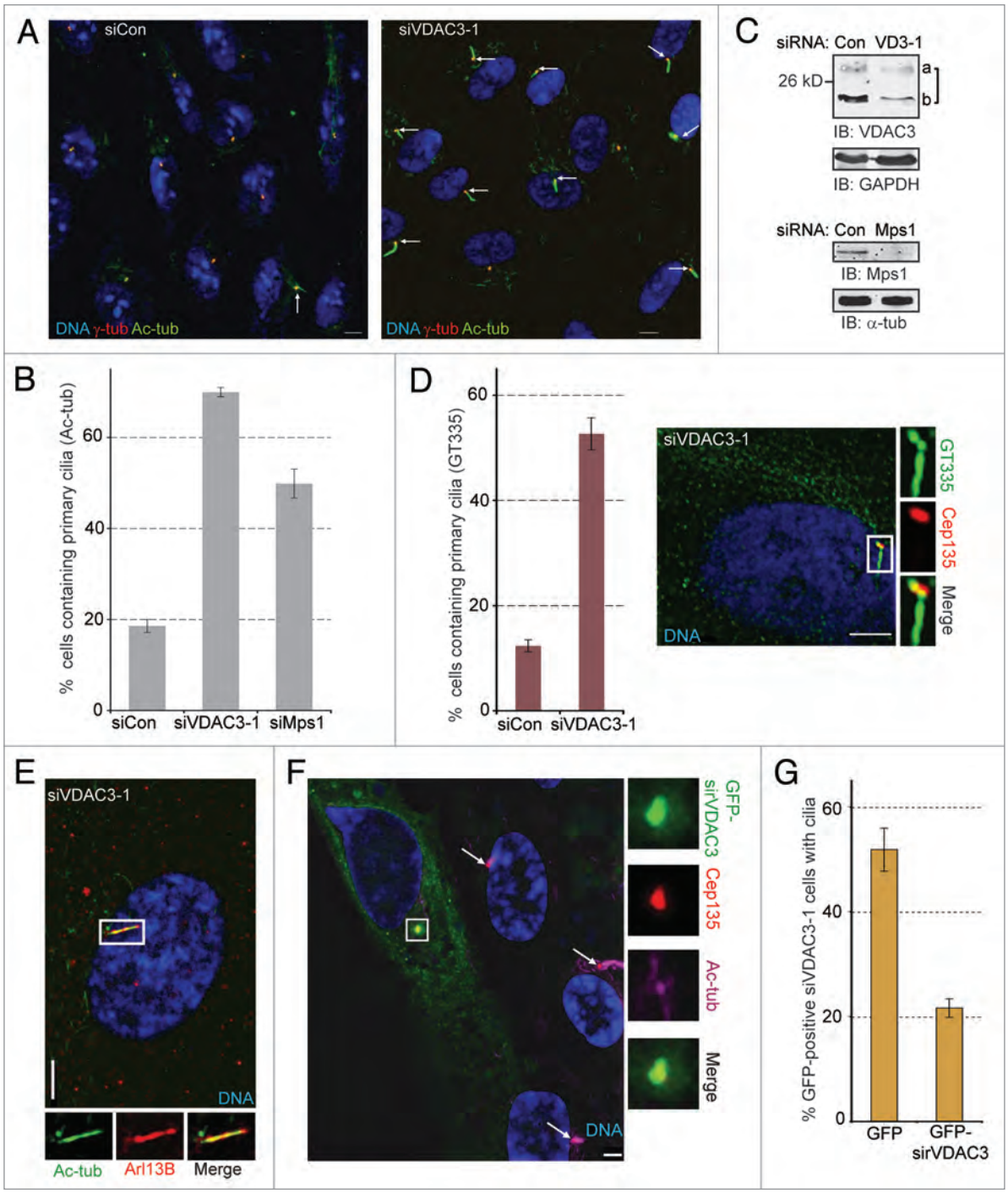

Figure 1. VDAC3 depletion led to primary cilia assembly in non-starved cells. (**A and B**) Asynchronously growing RPE1 cells treated with siRNAs against Lamin A/C (siCon), VDAC3 (siVDAC3-1) and Mps1 (siMps1) were stained for γ-tub and Ac-tub to identify centrosome and primary cilia respectively. (**A**) Shown are representative images of random fields of siCon and siVDAC3-1 cells stained for γ-tub (red) and Ac-tub (green). Arrows mark cilia. (**B**) Percentage of cells with cilia were plotted as bars where values represent mean ± SD for three independent experiments, 400–500 cells were counted per replicate. (**C**) Immunoblots show the depletion of VDAC3 (VDAC3a and VDAC3b were decreased by roughly 40% and by 75%) in siVDAC3-1 cells (siVD3-1) and Mps1 (by roughly 85%) in siMps1 cells compared with siCon cells prepared similarly as (**B**). GAPDH or α-tub was used as loading control. (**D**) Asynchronously growing siCon and siVDAC3-1 cells were stained for polyglutamylated tubulin (GT335, green) and Cep135 (red). Percentage of cells with cilia were plotted where values represent mean ± SD for three independent experiments, roughly 200 cells were counted per replicate. In this and all other images, panels show digitally magnified images of a region surrounded by the box. (**E**) Representative image of a siVDAC3-1 cells prepared as in (**A**) stained for Arl13B (red) and Ac-tub (green). (**F and G**) siVDAC3-1 cells were transfected with plasmids expressing GFP or GFP-sirVDAC3. (**F**) Shown is a representative field of siVDAC3-1 cells stained for Cep135 (red) and Ac-tub (magenta). The cell that expressed GFP-sirVDAC3 (green) did not contain a primary cilium while the other untransfected cells had cilia as marked by arrows. DNA is blue and bar is 5 μm in (**A–G**). (**G**) The percentage of GFP-positive cells containing cilia were plotted as bars. Values represent mean ± SD for three independent experiments, 50–70 GFP-positive cells counted per replicate.

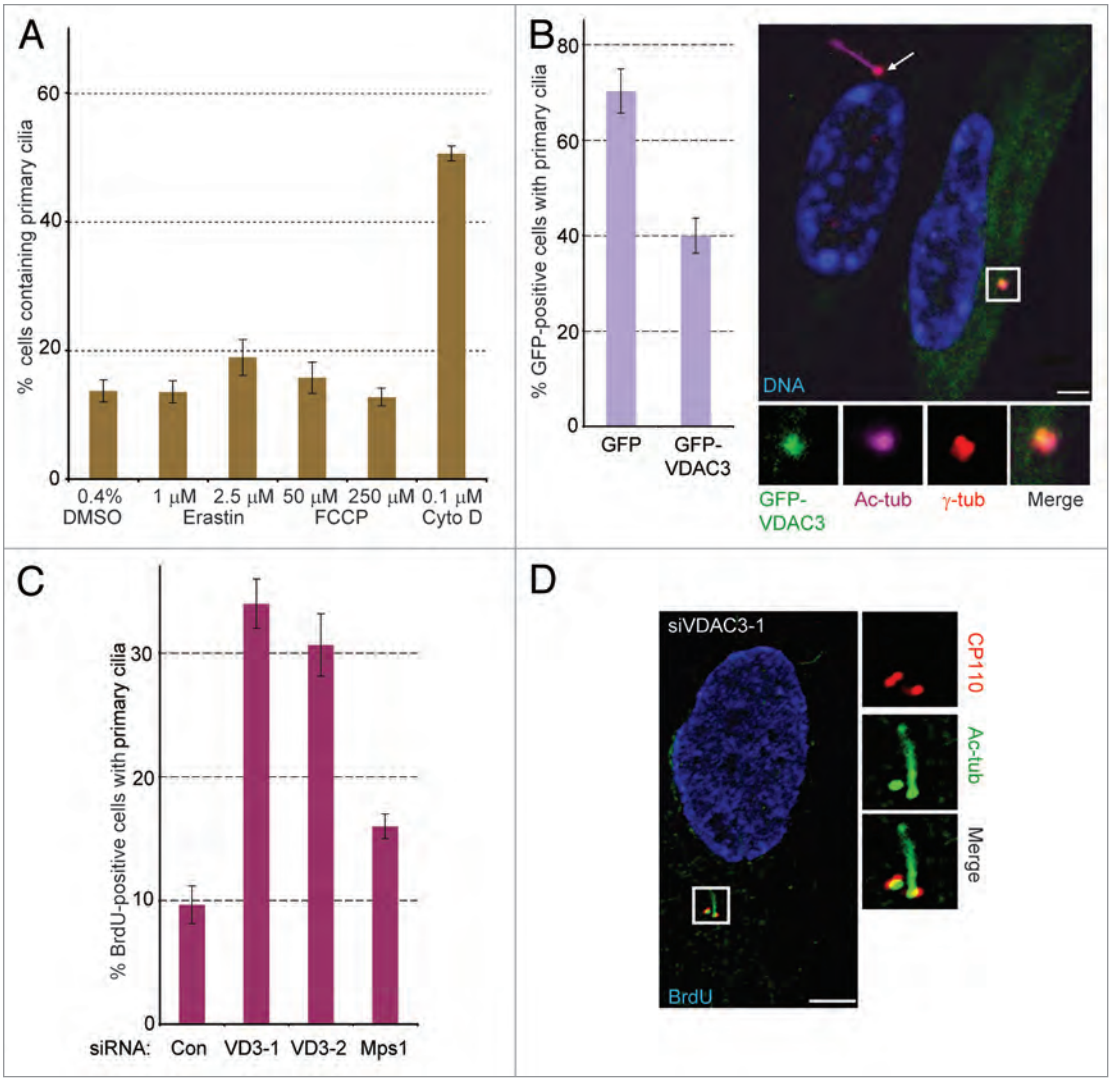

Figure 2. A non-mitochondrial pool of VDAC3 negatively regulate ciliogenesis. (**A**) Asynchronously growing RPE1 cells were treated with DMSO (16 h), 1 or 2 μM Erastin (16 h), 0.1 μM Cytochalasin D (Cyto D; 16 h), 50 μM FCCP (16 h) and 250 μM FCCP (for 4 h), fixed and stained for Ac-tub to identify cilia. Percentage of cells containing cilia were plotted as bars and values represent mean ± SD for three independent experiments, where at least 100 cells were counted per replicate. (**B**) RPE1 cells expressing GFP or GFP-VDAC3 (green) were serum starved for 48 h and stained for Ac-tub. Values represent the mean ± SD for three independent experiments, where 50–75 GFP-positive cells were counted per replicate. Shown is a random field containing GFP-VDAC3 expressing (green) and non-expressing cells stained for Ac-tub (magenta), γ-tub (red) and DNA (blue). Arrow marks the cilium. (**C and D**) Cells prepared as in **Figure 1A** were labeled with BrdU for 4 h. Bar graph shows the percentage of BrdU-positive cells with Ac-tub stained cilia. Values represent the mean ± SD for three independent experiments, where at least 100 cells were counted per replicate. (**D**) Representative image of a siVDAC3-1 cell stained for CP110 (red), Ac-tub (green) and BrdU (blue). Bar is 5 μm in (**B and D**).

Centrosomal levels of Mps1 and VDAC3 were markedly reduced in starved cells and increased after serum addition. Since the depletion of VDAC3 or Mps1 facilitated ciliogenesis, we wondered if the centrosomal level of these two proteins remains low during quiescence, when most cells form cilia. Notably, several studies have demonstrated that the expression of Mps1 is very low in resting cells and tissues with a low proliferative index,[34,35] while Mps1 level is upregulated in intensely proliferating cells.[36,37] Similarly, we observed that upon serum starvation, which leads to quiescence in RPE1 cells, the total Mps1 level was greatly reduced, but that Mps1 level was elevated after re-addition of serum to those cells (**Fig. 3A**). Consistent with these observations, centrosomal Mps1 was either completely lost or substantially diminished in the majority of starved RPE1 cells (**Fig. 3B and C**). By 20 h after serum stimulation, centrosomal Mps1 was significantly increased in roughly 55–65% of cells, the bulk of which were BrdU-positive and was largely associated with a single centriole (**Fig. 3B and C**), presumably the mother centriole as seen previously.[15] Moreover, roughly 50% of serum-stimulated RPE1 cells had lost their primary cilia by 20 h after serum re-addition, and roughly 70–75% of non-ciliated RPE1 cells showed intense Mps1 signal at the centrosome (**Fig. 3C**). Thus, we observed a strong correlation between the increase in the abundance of Mps1 at the centrosome, re-entry of the starved

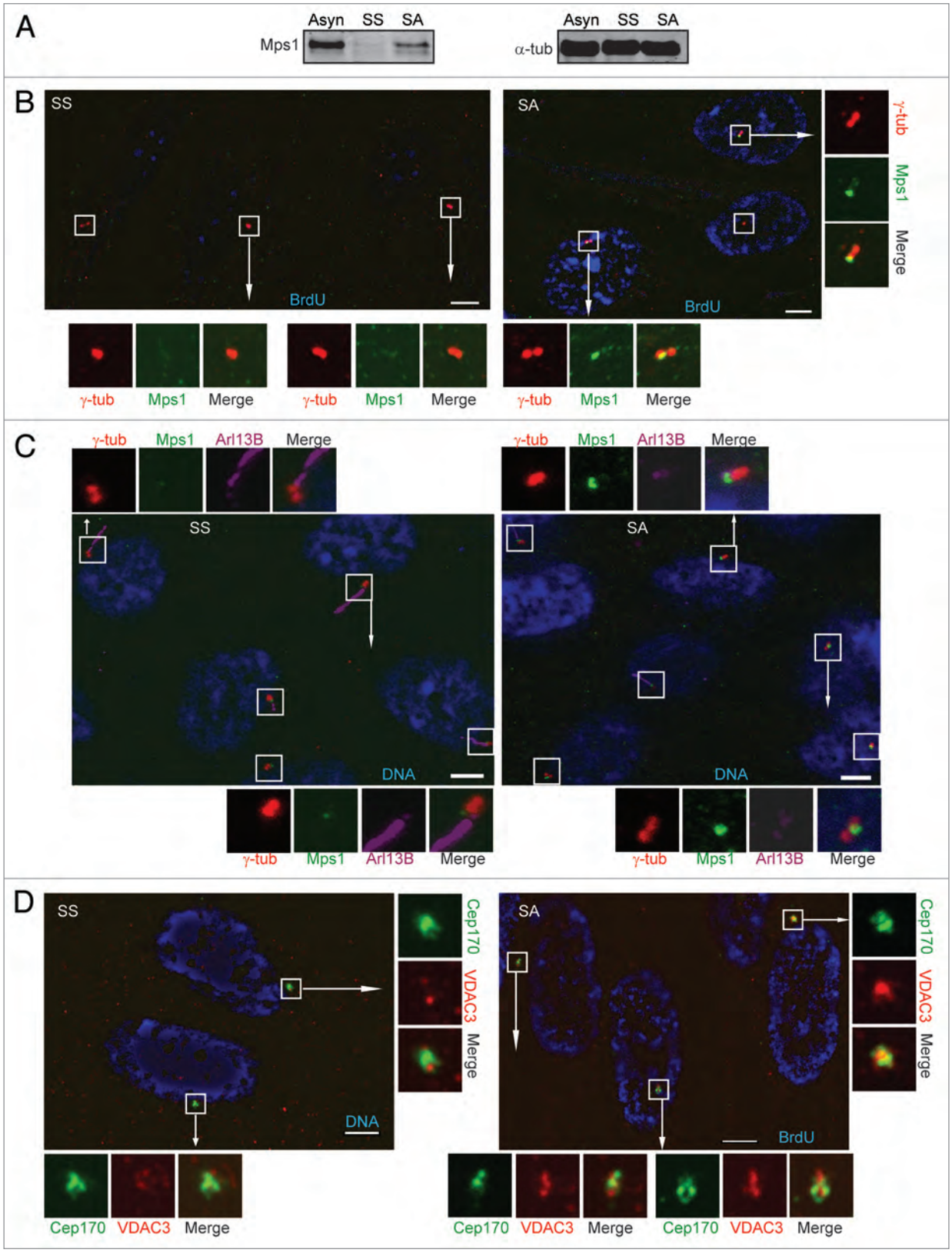

Figure 3. Centrosomal levels of Mps1 and VDAC3 were reduced in starved cells and increased after serum addition. (**A**) Immunoblots show the cellular level of Mps1 in RPE1 lystes from cells that were asynchronously growing (Asyn), serum starved (SS), are starved and stimulated with serum for 20 h (SA). α-tub was used as loading control. (**B–D**) Shown are representative fields of RPE1 cells that were serum starved (SS) or serum stimulated for 20 h (SA) and; (**B**) labeled with BrdU (blue) and stained for Mps1 (green) and γ-tub (red); (**C**) stained for Mps1 (green), Arl13B (magenta), γ-tub (red) and DNA (blue), or (**D**) stained for VDAC3 (red) and Cep170 (green). SS cells were stained for DNA (blue) and SA cells were labeled with BrdU (blue) in (**D**). The centrosome and/or cilia of every cell are indicated by a box, magnified panels are shown for cells marked by arrows. Bar = 5 μm in (**B–D**).

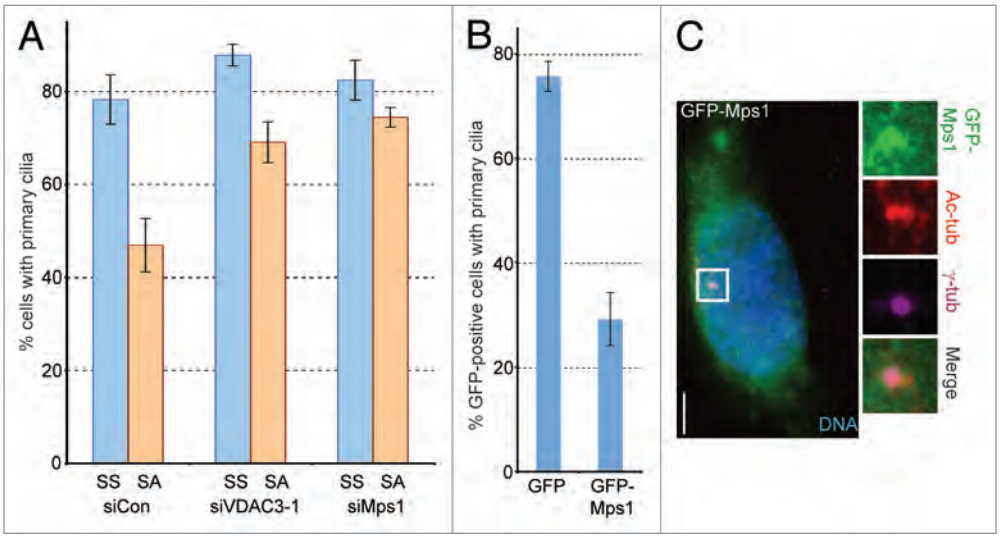

Figure 4. Mps1 controls ciliary diassembly during serum stimulation. (**A**) siCon, siVDAC3-1 and siMps1 cells were serum starved (SS), stimulated with serum for 20 h (SA) and fixed. Cells were stained for Ac-tub and the percentage of cells with primary cilia were plotted as bars. Values represent mean ± SD for three independent experiments, where at least 200 cells were counted per replicate. (**B and C**) RPE1 cells expressing GFP or GFP-Mps1 were serum starved for 48 h, stained for Ac-tub and the GFP-positive cells containing primary cilia were counted. (**B**) Values represent the mean ± SD for three independent experiments, where 60–75 cells were counted per replicate. (**C**) Representative image of a serum starved GFP-Mps1 (green) expressing RPE1 cell stained for γ-tub (magenta), Ac-tub (red) and DNA (blue). Bar is 5 μm.

cells into S-phase and disassembly of primary cilia. It is therefore tempting to speculate that the growing cells that are depleted of centrosomal Mps1 do not progress to S-phase phase, partly because of their inability to disassemble primary cilia.

We reported previously that the VDAC3 antibody weakly stained mitochondria but strongly stained centrosomes in the majority of RPE1 cells, where the bulk of VDAC3 lay within the region defined by Cep170, a mother centriolar protein that is associated with the appendages.[15] In serum-starved cells, this centrosomal VDAC3 signal was low and, like Mps1, increased considerably after serum addition, particularly in those cells that entered S-phase (**Fig. 3D**). Moreover, in starved cells the distribution of centrosomal VDAC3 no longer overlapped that of Cep170 and closely resembled the residual centrosome staining we previously observed in siVDAC3 cells.[15] Thus, our results support the hypothesis that a centrosomal function of VDAC3 and Mps1 negatively regulates cilia assembly and cells regulate the level of these proteins at the basal body to favor ciliogenesis during cellular quiescence.

Mps1 and VDAC3 control ciliary disassembly during serum stimulation of the starved cells. In order to further verify the hypotheses that the centrosomal level of VDAC3 and Mps1 correlate with cilia assembly and disassembly, we examined whether RPE1 cells could resorb cilia during release from starvation in Mps1- and VDAC3-depleted cells. Indeed, we observed a significant decrease in siVDAC3 cells that resorbed their primary cilia after 20 h of serum re-addition and an even greater reduction in siMps1 cells, as compared with siCon cells (**Fig. 4A**). This suggests the possibility that certain thresholds of VDAC3 and Mps1 at the centrosome are crucial to facilitate disassembly of primary cilia during serum-stimulated release from quiescence, which is coordinated with cell cycle re-entry.

Consistent with the idea that Mps1 may negatively regulate cilia assembly, Mps1 is barely detectable in quiescent cells and increases dramatically as cells re-enter the cell cycle and lose their primary cilia (see above). To further test the hypothesis that Mps1 negatively regulates ciliogenesis, we asked if overexpressing Mps1 could inhibit the formation of primary cilia during serum starvation. RPE1 cells expressing either GFP or GFP-Mps1 were serum starved and examined for the presence of cilia. **Figure 4B** shows that the ectopic expression of GFP-Mps1 suppressed cilia assembly, and to an even greater degree than GFP-VDAC3 (**Fig. 2B**), suggesting that the reduction of Mps1 level in quiescent cells may play a key role in facilitating cilia formation.

Targeting Mps1 to centrosomes independently of VDAC3 suppresses aberrant ciliogenesis in siVDAC3 cells. Because centrosomal Mps1 levels are greatly reduced in VDAC3-depleted cells,[15] we next asked if the aberrant ciliogenesis in siVDAC3 cells is due to the failure to recruit Mps1 to centrosomes. GFP-Mps1-PACT is an exclusively centrosomal version of Mps1 that is tethered to centrosomes independently of VDAC3 via the PACT domain.[23,38] GFP-Mps1-PACT is functional in centriole assembly[23] and, like GFP-Mps1, can suppress ciliogenesis in serum-starved cells (**Fig. S2B**). We found that GFP-Mps1-PACT caused a 2-fold decrease in the percentage of siVDAC3 cells containing primary cilia compared with GFP-PACT alone (**Fig. 5**). Moreover, ciliary lengths were shortened in roughly 20% of siVDAC3 cells expressing GFP-Mps1-PACT that still had cilia (data not shown). This observation indicates that tethering Mps1 to centrosomes independently of VDAC3 largely bypassed the aberrant assembly of primary cilia in non-starved siVDAC3 cells, and further supports the hypothesis that a centrosomal function of a VDAC3-Mps1 module prevents cilia formation during proliferation.

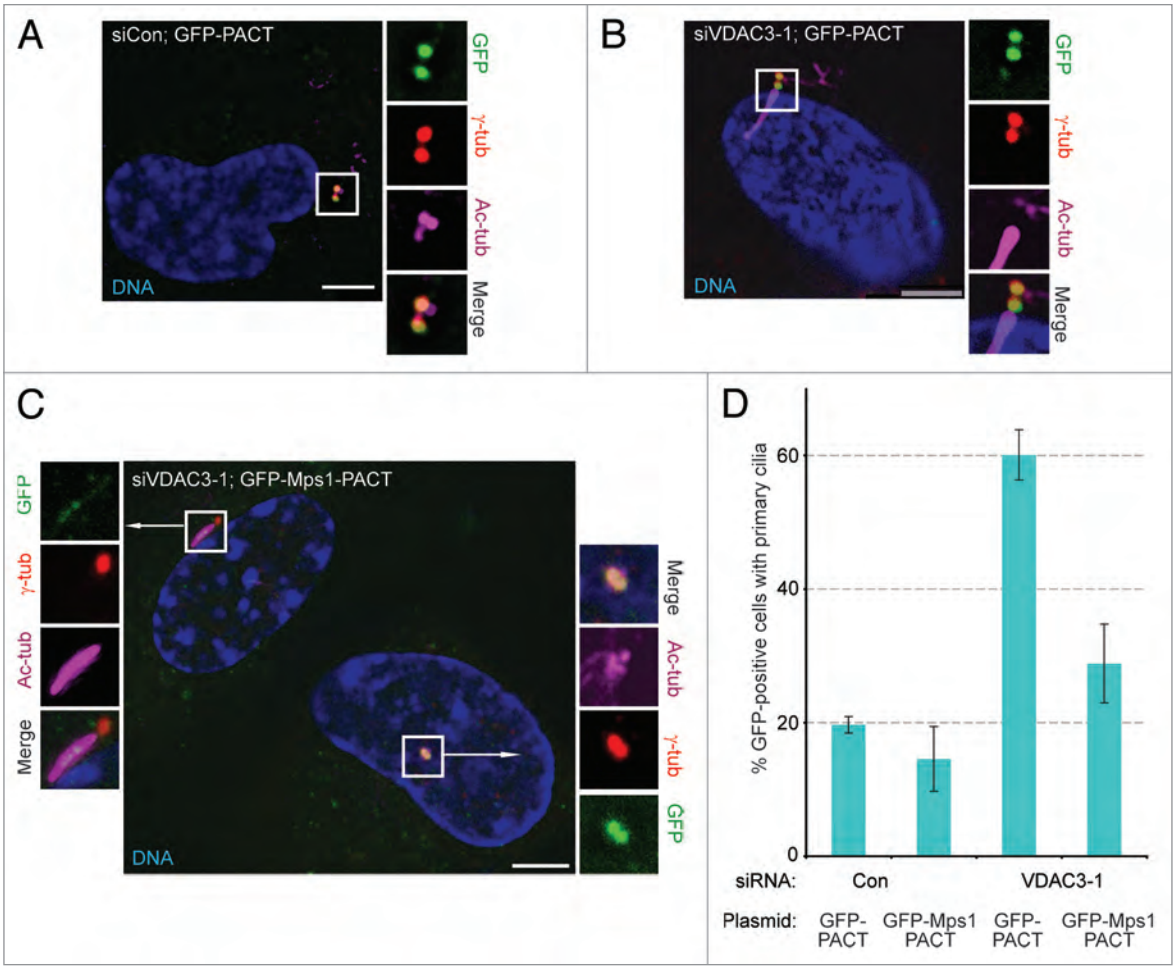

Figure 5. Targeting Mps1 to centrosomes independently of VDAC3 suppresses aberrant ciliogenesis in siVDAC3 cells. (**A and D**) Asynchronously growing siCon or siVDAC3-1 cells expressing GFP-PACT (green) or GFP-Mps1-PACT (green) were stained for Ac-tub to examine the presence of primary cilia. (**A and B**) Shown are representative images of indicated cells stained for γ-tub (red) and Ac-tub (magenta). (**C**) An image of a random field of siVDAC3-1 population, where the cell in the right was expressing GFP-Mps1-PACT but the cell in the left was untransfected, stained for γ-tub (red) and Ac-tub (magenta). The GFP-Mps1-PACT expressing cell does not contain a cilium while the non-expressing one contains a cilium. DNA is blue and bar = 5 μm in (**A–C**). (**D**) Percentage of GFP-positive cells with cilia, values represent the mean ± SD for three independent experiments, 100 cells counted per replicate.

Discussion

We have demonstrated novel roles of VDAC3 and Mps1 in regulating ciliogenesis. Both proteins were largely associated with the mother centriole/basal body, had very low level at the centrosome during quiescence, and overexpression of both either inhibited cilia assembly or promoted ciliary disassembly. Since VDAC3 appears to be the centrosomal receptor for Mps1,[15] it seems logical to suggest that VDAC3 and Mps1 cooperate in a common mechanism for ciliary resorption and the regulatory switch from basal body to centriole. However, the phenotypes associated with modulation of the two proteins do not completely overlap. GFP-Mps1 suppressed ciliogenesis to a greater degree than GFP-VDAC3, and fewer Mps1-depleted cells were able to disassemble their cilia upon serum stimulation. In contrast, the aberrant assembly of cilia was more pronounced upon VDAC3 depletion due to the presence of cilia in S-phase cells. Together, these observations suggest that VDAC3 and Mps1 cooperate to promote ciliary disassembly, while VDAC3 might have an additional function that inhibits cilia assembly in cycling cells. Thus, our data supports the presence of a pool of VDAC3 at the centrosome that executes its function in part through recruiting Mps1 and in part independently of Mps1. Nonetheless, this study raises important questions about the downstream mechanism of Mps1 and VDAC3 in the ciliary assembly-disassembly process and its coordination with the cell cycle. A few mechanistic pathways were recently described that negatively regulate ciliogenesis.[4,8,11,25,26,29,39] VDAC3 and Mps1 might contribute to one or more of these pathways or function in ciliogenesis through a novel pathway, which we look forward to characterizing in future studies.

While the observation that a mitochondrial porin regulates ciliogenesis may be striking, perhaps it should not be surprising. Both membrane-associated and mitochondrial proteins have recently been found at the basal body and transition zone, with either proven or suggested roles in ciliogenesis.[14,40-42] Both

VDAC3 and VDAC2, which was recently found as part of the NPHP2 complex at the base of primary cilia,[14,42] are present in the sperm outer dense fiber.[43] The cytoplasmic dynein Tctex-1, a VDAC1-interacting protein,[44] is also localized at the ciliary transition zone and negatively regulates ciliogenesis,[25] suggesting that the VDAC family as a whole may have broad roles in ciliogenesis. Similarly, trichoplein, a keratin binding protein,[45] localizes both to mitochondria, where it regulates endoplasmic reticulum-mitochondrial juxtaposition,[46] and to centrioles,[47] where it negatively regulates primary cilia assembly and promotes cell cycle progression by activating Aurora A kinase.[9,11] Interestingly, an earlier study demonstrated ultra-structural abnormalities in the epididymal axoneme causing reduced sperm motility in two-thirds of mice lacking the VDAC3 gene.[48] This might suggest a role for VDAC3 in the proper assembly and maintenance of sperm flagella, contrasting with our conclusion that VDAC3 negatively regulates ciliogenesis. However, the axonemal defect in that study was associated with compromised mitochondrial function, whereas our data indicates that it is a centrosomal function of VDAC3 that negatively regulates ciliogenesis. Nevertheless, we cannot completely rule out the possibility that a mitochondrial function of VDAC3 influences ciliogenesis through some unknown mechanism. It was recently shown that a mutation in the X-prolyl aminopeptidase 3 (XPNPEP3) gene, which encodes a mitochondrial enzyme, leads to a NPHP-like ciliopathy,[49] suggesting that mitochondrial dysfunction influences ciliogenesis. It is possible that VDAC3, or the VDAC family more broadly, might be a link between mitochondrial dysfunction and ciliopathies, which is beyond the scope of this current study but will be the subject of future experiments. In conclusion, we have demonstrated that a centriolar function of VDAC3 and Mps1 are important to regulate ciliogenesis in human cells, and are looking forward to identifying the precise mechanism of this novel pathway in future studies.

Materials and Methods

Plasmids. Previously described plasmids used for this study- pHF7 (GFP) and pHF36 (GFP-Mps1), pHF279 (GFP-VDAC3), pHF283 (GFP-sirVDAC3), pHF284 (GFP-PACT), pHF285 (GFP-Mps1-PACT).[15,17,18,23] Plasmids created for this study are as follows: GFP-tagged mammalian expression constructs- pHF286 (GFP) and pHF287 (GFP-Mps1) were created by inserting a 492 bp DNA fragment encoding the β globin intron downstream of the SV40 promoter in pHF7 (GFP) and pHF36 (GFP-Mps1). The sequences of PCR primers are available upon request. The identity of all constructs was verified by sequence analysis.

Cell culture. hTERT-RPE1 cells were cultured in DME/F-12 (1:1) (Hyclone) supplemented with 10% FBS (Atlanta Biologicals), 100 U/ml penicillinG and 100 μg/ml streptomycin (Hyclone) at 37°C in a humidified chamber in the presence of 5% CO_2. For serum starvation, RPE1 cells were incubated in serum and antibiotic free DME/F12 (1:1) for 48 h. To identify the cells in S-phase, cells were either incubated with EdU (10 μM; Invitrogen) or with BrdU (40 μM; Sigma) for 4 h before fixation.

DNA and siRNA transfections. Mammalian constructs were transfected using FuGENE 6 (Promega). Stealth siRNAs directed against VDAC3 (siVDAC3-1: nucleotides 330–354; siVDAC3-2: nucleotides 625–649)[15] and Mps1 (nucleotides 1360–1384)[21] were obtained from Invitrogen and siGLO Lamin A/C siRNA (siControl) obtained from Dharmacon were used at a final concentration of 40 nM for transfection using Lipofectamine RNAiMAX (Invitrogen).

Cytology. Antibodies and working dilutions for indirect immunofluorescence (IIF) were as follows: GTU-88 mouse anti-γ-tubulin, 1:200 (Sigma); rabbit anti-γ-tubulin, 1:200 (Sigma); goat anti-γ-tubulin, 1:50 (Santa cruz); rabbit anti-Cetn2, 1:4,000,[21] 4G9 mouse anti-Mps1, 1:200 (H00007272-M02, Novus Biologicals); rabbit anti-CP110, 1:500 (a kind gift from Dr Brian Dynlacht, New York University School of Medicine); rat anti-BrdU, 1:250 (Abcam); mouse anti-acetylated tubulin, 1:1,000 (Sigma); GT335 mouse anti-glutamylated tubulin, 1:1,000 (Sigma); rabbit anti-VDAC3, 1:50 (Aviva Systems Biology); rabbit anti-Arl13B, 1:200 (Proteintech); 3E6 mouse anti-GFP, 1:200 (Invitrogen), rabbit anti-Cep135, 1:500 (Abcam). Secondary antibodies for IIF were goat or donkey anti-rabbit, anti-mouse, or goat anti-rat conjugated to Alexa 350 (1:200), Alexa 488 (1:1,000), Alexa 594 (1:1000), or Alexa 750 (1:200) (all from Invitrogen) and DNA was stained with Hoechst 33342 (Sigma). Cells were fixed with either PBS containing 4% formaldehyde (Ted Pella) and 0.2% Triton X-100 for 10 min at room temperature, or in methanol at -20°C for 10 min. For visualizing BrdU, cells fixed in methanol were stained with primary and secondary antibodies to cellular antigens, fixed again in methanol, treated with 2 N HCl for 30 min, followed by staining with anti-BrdU antibody.[50] Click-iT EdU Cell Proliferation kit (Invitrogen) was used according to manufacturer's instruction to visualize EdU-positive cells. All images were acquired at ambient temperature using an Olympus IX-81 microscope, with a 63× or 100× Plan Apo oil immersion objective (1.4 numerical aperture) and a QCAM Retiga Exi FAST 1394 camera, and analyzed using the Slidebook software package (Intelligent Imaging Innovations).

Immunoblotting. Human cells were lysed in either HeLa lysis buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl and 1% NP-40) or RIPA buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl and 1% TritonX-100, 0.25% Na-Deoxycholate, 0.1% SDS). Antibodies for immunoblot analysis were: 1:2,000 rabbit anti-GFP (Sigma), 1:1,000 N1 mouse anti-Mps1 (Invitrogen): 1:20,000 DM1A mouse anti-α-Tubulin (Sigma), 1:1,000 rabbit anti-VDAC3 (Aviva Systems Biology), 1:20,000 mouse anti-GAPDH (Sigma), 1:750 rabbit anti-Cetn2 (Biolegend). Secondary antibodies were Alexa680-conjugated donkey anti-mouse/rabbit (Invitrogen) and IRDye800-conjugated donkey anti-mouse/rabbit (Rockland), both used at 1:10,000 dilution for all primaries that were analyzed by Odyssey imaging system (Li-Cor) except anti-Cetn2, which was hybridized with a HRP-conjugated sheep anti-rabbit IgG (GE Healthcare) and detected with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). The background-corrected intensities of the bands were calculated using Odyssey imaging system (Li-Cor).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by a National Institutes of Health grant (GM77311) to H.A.F., and S.M. was partially supported by an Up on the Roof fellowship from the Human Cancer Genetics Program of The Ohio State University Comprehensive Cancer Center.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/23824

References

1. Piel M, Meyer P, Khodjakov A, Rieder CL, Bornens M. The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. J Cell Biol 2000; 149:317-30; PMID:10769025; http://dx.doi.org/10.1083/jcb.149.2.317
2. Kobayashi T, Dynlacht BD. Regulating the transition from centriole to basal body. J Cell Biol 2011; 193:435-44; PMID:21536747; http://dx.doi.org/10.1083/jcb.201101005
3. Afzelius BA. Ciliary structure in health and disease. Acta Otorhinolaryngol Belg 2000; 54:287-91; PMID:11082764
4. Avasthi P, Marshall WF. Stages of ciliogenesis and regulation of ciliary length. Differentiation 2012; 83:S30-42; PMID:22178116
5. Afzelius BA. Cilia-related diseases. J Pathol 2004; 204:470-7; PMID:15495266; http://dx.doi.org/10.1002/path.1652
6. Seeley ES, Nachury MV. The perennial organelle: assembly and disassembly of the primary cilium. J Cell Sci 2010; 123:511-8; PMID:20144999; http://dx.doi.org/10.1242/jcs.061093
7. Rosenbaum JL, Witman GB. Intraflagellar transport. Nat Rev Mol Cell Biol 2002; 3:813-25; PMID:12415299; http://dx.doi.org/10.1038/nrm952
8. Kobayashi T, Tsang WY, Li J, Lane W, Dynlacht BD. Centriolar kinesin Kif24 interacts with CP110 to remodel microtubules and regulate ciliogenesis. Cell 2011; 145:914-25; PMID:21620453; http://dx.doi.org/10.1016/j.cell.2011.04.028
9. Inoko A, Matsuyama M, Goto H, Ohmuro-Matsuyama Y, Hayashi Y, Enomoto M, et al. Trichoplein and Aurora A block aberrant primary cilia assembly in proliferating cells. J Cell Biol 2012; 197:391-405; PMID:22529102; http://dx.doi.org/10.1083/jcb.201106101
10. Plotnikova OV, Nikonova AS, Loskutov YV, Kozyulina PY, Pugacheva EN, Golemis EA. Calmodulin activation of Aurora-A kinase (AURKA) is required during ciliary disassembly and in mitosis. Mol Biol Cell 2012; 23:2658-70; PMID:22621899; http://dx.doi.org/10.1091/mbc.E11-12-1056
11. Pugacheva EN, Jablonski SA, Hartman TR, Henske EP, Golemis EA. HEF1-dependent Aurora A activation induces disassembly of the primary cilium. Cell 2007; 129:1351-63; PMID:17604723; http://dx.doi.org/10.1016/j.cell.2007.04.035
12. Spektor A, Tsang WY, Khoo D, Dynlacht BD. Cep97 and CP110 suppress a cilia assembly program. Cell 2007; 130:678-90; PMID:17719545; http://dx.doi.org/10.1016/j.cell.2007.06.027
13. Egorova AD, van der Heiden K, Poelmann RE, Hierck BP. Primary cilia as biomechanical sensors in regulating endothelial function. Differentiation 2012; 83:S56-61; PMID:22169885
14. Sang L, Miller JJ, Corbit KC, Giles RH, Brauer MJ, Otto EA, et al. Mapping the NPHP-JBTS-MKS protein network reveals ciliopathy disease genes and pathways. Cell 2011; 145:513-28; PMID:21565611; http://dx.doi.org/10.1016/j.cell.2011.04.019
15. Majumder S, Slabodnick M, Pike A, Marquardt J, Fisk HA. VDAC3 regulates centriole assembly by targeting Mps1 to centrosomes. Cell Cycle 2012; 11:3666-78; PMID:22935710; http://dx.doi.org/10.4161/cc.21927
16. Kang J, Chen Y, Zhao Y, Yu H. Autophosphorylation-dependent activation of human Mps1 is required for the spindle checkpoint. Proc Natl Acad Sci USA 2007; 104:20232-7; PMID:18083840; http://dx.doi.org/10.1073/pnas.0710519105
17. Fisk HA, Mattison CP, Winey M. Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression. Proc Natl Acad Sci USA 2003; 100:14875-80; PMID:14657364; http://dx.doi.org/10.1073/pnas.2434156100
18. Kasbek C, Yang CH, Fisk HA. Antizyme restrains centrosome amplification by regulating the accumulation of Mps1 at centrosomes. Mol Biol Cell 2010; 21:3878-89; PMID:20861309; http://dx.doi.org/10.1091/mbc.E10-04-0281
19. Fisk HA, Winey M. The mouse Mps1p-like kinase regulates centrosome duplication. Cell 2001; 106:95-104; PMID:11461705; http://dx.doi.org/10.1016/S0092-8674(01)00411-1
20. Pike AN, Fisk HA. Centriole assembly and the role of Mps1: defensible or dispensable? Cell Div 2011; 6:9; PMID:21492451; http://dx.doi.org/10.1186/1747-1028-6-9
21. Yang CH, Kasbek C, Majumder S, Yusof AM, Fisk HA. Mps1 phosphorylation sites regulate the function of centrin 2 in centriole assembly. Mol Biol Cell 2010; 21:4361-72; PMID:20980622; http://dx.doi.org/10.1091/mbc.E10-04-0298
22. Kasbek C, Yang CH, Fisk HA. Mps1 as a link between centrosomes and genomic instability. Environ Mol Mutagen 2009; 50:654-65; PMID:19274768; http://dx.doi.org/10.1002/em.20476
23. Kasbek C, Yang CH, Yusof AM, Chapman HM, Winey M, Fisk HA. Preventing the degradation of mps1 at centrosomes is sufficient to cause centrosome reduplication in human cells. Mol Biol Cell 2007; 18:4457-69; PMID:17804818; http://dx.doi.org/10.1091/mbc.E07-03-0283
24. Liu J, Cheng X, Zhang Y, Li S, Cui H, Zhang L, et al. Phosphorylation of Mps1 by BRAF(V600E) prevents Mps1 degradation and contributes to chromosome instability in melanoma. Oncogene 2012; PMID:22430208
25. Li A, Saito M, Chuang JZ, Tseng YY, Dedesma C, Tomizawa K, et al. Ciliary transition zone activation of phosphorylated Tctex-1 controls ciliary resorption, S-phase entry and fate of neural progenitors. Nat Cell Biol 2011; 13:402-11; PMID:21394082; http://dx.doi.org/10.1038/ncb2218
26. Kim S, Zaghloul NA, Bubenshchikova E, Oh EC, Rankin S, Katsanis N, et al. Nde1-mediated inhibition of ciliogenesis affects cell cycle re-entry. Nat Cell Biol 2011; 13:351-60; PMID:21394081; http://dx.doi.org/10.1038/ncb2183
27. Jackson PK. Do cilia put brakes on the cell cycle? Nat Cell Biol 2011; 13:340-2; PMID:21460803; http://dx.doi.org/10.1038/ncb0411-340
28. Sung CH, Li A. Ciliary resorption modulates G1 length and cell cycle progression. Cell Cycle 2011; 10:2825-6; PMID:21964298; http://dx.doi.org/10.4161/cc.10.17.16943
29. Kim J, Lee JE, Heynen-Genel S, Suyama E, Ono K, Lee K, et al. Functional genomic screen for modulators of ciliogenesis and cilium length. Nature 2010; 464:1048-51; PMID:20393563; http://dx.doi.org/10.1038/nature08895
30. Larkins CE, Aviles GD, East MP, Kahn RA, Caspary T. Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins. Mol Biol Cell 2011; 22:4694-703; PMID:21976698; http://dx.doi.org/10.1091/mbc.E10-12-0994
31. Yagoda N, von Rechenberg M, Zaganjor E, Bauer AJ, Yang WS, Fridman DJ, et al. RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. Nature 2007; 447:864-8; PMID:17568748; http://dx.doi.org/10.1038/nature05859
32. Yang WS, Stockwell BR. Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. Chem Biol 2008; 15:234-45; PMID:18355723; http://dx.doi.org/10.1016/j.chembiol.2008.02.010
33. Tsang WY, Bossard C, Khanna H, Peränen J, Swaroop A, Malhotra V, et al. CP110 suppresses primary cilia formation through its interaction with CEP290, a protein deficient in human ciliary disease. Dev Cell 2008; 15:187-97; PMID:18694559; http://dx.doi.org/10.1016/j.devcel.2008.07.004
34. Mills GB, Schmandt R, McGill M, Amendola A, Hill M, Jacobs K, et al. Expression of TTK, a novel human protein kinase, is associated with cell proliferation. J Biol Chem 1992; 267:16000-6; PMID:1639825
35. Hogg D, Guidos C, Bailey D, Amendola A, Groves T, Davidson J, et al. Cell cycle dependent regulation of the protein kinase TTK. Oncogene 1994; 9:89-96; PMID:8302607
36. Poss KD, Nechiporuk A, Hillam AM, Johnson SL, Keating MT. Mps1 defines a proximal blastemal proliferative compartment essential for zebrafish fin regeneration. Development 2002; 129:5141-9; PMID:12399306
37. Qin Z, Barthel LK, Raymond PA. Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish. Proc Natl Acad Sci USA 2009; 106:9310-5; PMID:19474300; http://dx.doi.org/10.1073/pnas.0811186106
38. Gillingham AK, Munro S. The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. EMBO Rep 2000; 1:524-9; PMID:11263498
39. Cao J, Shen Y, Zhu L, Xu Y, Zhou Y, Wu Z, et al. miR-129-3p controls cilia assembly by regulating CP110 and actin dynamics. Nat Cell Biol 2012; 14:697-706; PMID:22684256; http://dx.doi.org/10.1038/ncb2512
40. Garcia-Gonzalo FR, Corbit KC, Sirerol-Piquer MS, Ramaswami G, Otto EA, Noriega TR, et al. A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. Nat Genet 2011; 43:776-84; PMID:21725307; http://dx.doi.org/10.1038/ng.891
41. Williams CL, Li C, Kida K, Inglis PN, Mohan S, Semenec L, et al. MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis. J Cell Biol 2011; 192:1023-41; PMID:21422230; http://dx.doi.org/10.1083/jcb.201012116
42. Dowdle WE, Robinson JF, Kneist A, Sirerol-Piquer MS, Frints SG, Corbit KC, et al. Disruption of a ciliary B9 protein complex causes Meckel syndrome. Am J Hum Genet 2011; 89:94-110; PMID:21763481; http://dx.doi.org/10.1016/j.ajhg.2011.06.003

43. Hinsch KD, De Pinto V, Aires VA, Schneider X, Messina A, Hinsch E. Voltage-dependent anion-selective channels VDAC2 and VDAC3 are abundant proteins in bovine outer dense fibers, a cytoskeletal component of the sperm flagellum. J Biol Chem 2004; 279:15281-8; PMID:14739283; http://dx.doi.org/10.1074/jbc.M313433200
44. Schwarzer C, Barnikol-Watanabe S, Thinnes FP, Hilschmann N. Voltage-dependent anion-selective channel (VDAC) interacts with the dynein light chain Tctex1 and the heat-shock protein PBP74. Int J Biochem Cell Biol 2002; 34:1059-70; PMID:12009301; http://dx.doi.org/10.1016/S1357-2725(02)00026-2
45. Nishizawa M, Izawa I, Inoko A, Hayashi Y, Nagata K, Yokoyama T, et al. Identification of trichoplein, a novel keratin filament-binding protein. J Cell Sci 2005; 118:1081-90; PMID:15731013; http://dx.doi.org/10.1242/jcs.01667
46. Cerqua C, Anesti V, Pyakurel A, Liu D, Naon D, Wiche G, et al. Trichoplein/mitostatin regulates endoplasmic reticulum-mitochondria juxtaposition. EMBO Rep 2010; 11:854-60; PMID:20930847; http://dx.doi.org/10.1038/embor.2010.151
47. Ibi M, Zou P, Inoko A, Shiromizu T, Matsuyama M, Hayashi Y, et al. Trichoplein controls microtubule anchoring at the centrosome by binding to Odf2 and ninein. J Cell Sci 2011; 124:857-64; PMID:21325031; http://dx.doi.org/10.1242/jcs.075705
48. Sampson MJ, Decker WK, Beaudet AL, Ruitenbeek W, Armstrong D, Hicks MJ, et al. Immotile sperm and infertility in mice lacking mitochondrial voltage-dependent anion channel type 3. J Biol Chem 2001; 276:39206-12; PMID:11507092; http://dx.doi.org/10.1074/jbc.M104724200
49. O'Toole JF, Liu Y, Davis EE, Westlake CJ, Attanasio M, Otto EA, et al. Individuals with mutations in XPNPEP3, which encodes a mitochondrial protein, develop a nephronophthisis-like nephropathy. J Clin Invest 2010; 120:791-802; PMID:20179356; http://dx.doi.org/10.1172/JCI40076
50. Tsou MF, Wang WJ, George KA, Uryu K, Stearns T, Jallepalli PV. Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. Dev Cell 2009; 17:344-54; PMID:19758559; http://dx.doi.org/10.1016/j.devcel.2009.07.015