

Protein kinase A-mediated septin7 phosphorylation disrupts septin filaments and ciliogenesis

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Research

Keywords: Septin7, septin filament, primary cilium, protein kinase A catalytic subunit

Posted Date: February 13th, 2020

DOI: <https://doi.org/10.21203/rs.2.23438/v1>

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Version of Record: A version of this preprint was published at Cells on February 9th, 2021. See the published version at <https://doi.org/10.3390/cells10020361>.

Abstract

Septins are GTP-binding proteins that form heteromeric filaments for proper cell growth and migration. Among these septins, septin7 (SEPT7) is an important component of all septin filaments. Here we showed that protein kinase A (PKA) phosphorylates SEPT7 at Thr197 thus disrupting septin filament dynamics and ciliogenesis. The PKA targeting site at Thr197 of SEPT7 was conserved among species. Treatment of cAMP or overexpression of PKA catalytic subunit (PKACA2) induced SEPT7 phosphorylation. SEPT7 phosphorylation at Thr197 disrupted septin filament formation by reducing SEPT7-SEPT7 interaction, but not affected SEPT7-SEPT6-SEPT2 or SEPT4 interaction. Besides, overexpression of phosphomimetic SEPT7 mutant (T197E) disrupted endogenous SEPT7 filaments suggesting T197E had dominant negative effect on septin filament polymerization. We also identified SEPT7 interacted with the PKACA2 via the GTP-binding domain. Furthermore, PKA-mediated SEPT7 phosphorylation disrupted primary cilia formation. Thus, our data uncover the novel biological function of SEPT7 phosphorylation in septin filament polymerization and primary cilia formation.

Background

Septins are GTP-binding proteins that polymerize into heterooligomeric core complexes and further assemble into higher-order structure including filaments, ring and cages [1]. The septin filaments play important roles in several biological process in mammalian cells, including cytokinesis, plasma membrane dynamics, and ciliogenesis [2–6]. All septins consist of an amino and carboxyl termini, called the N-terminus and C-terminus, and the guanine nucleotide binding site, called the GTP-binding domain (GBD) [7]. Based on homology of sequences, the mammalian septins are classified into four subgroups: the SEPT2 subgroup (SEPT1, SEPT2, SEPT4, and SEPT5), the SEPT3 subgroup (SEPT3, SEPT9, and SEPT12), the SEPT6 subgroup (SEPT6, SEPT8, SEPT10, SEPT11, and SEPT14), and the SEPT7 subgroup (SEPT7 only) [8–10].

SEPT7 play important roles during cell division, cytokinesis and neuronal differentiation. For example, depletion of SEPT7 leads to microtubule destabilization as well as cytokinesis defect in fibroblasts [11]. SEPT7 is also required for chromosome alignment by affecting the extrusion of the second polar body during meiosis [12]. In mature neurons, depletion of SEPT7 results in decreasing dendritic branching and dendritic-spine morphogenesis [13]. Thus, precise regulation of SEPT7 is crucial for development and differentiation.

SEPT7 is a key component of most types of septin filaments [8]. Septins can hetero-oligomerize into hexamer core complex SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2. SEPT7 interacts with SEPT6 and SEPT7 via the N-C termini and GBD, respectively [14]. Therefore, SEPT7 assemble several septin members into multimeric structure for its biological function.

Phosphorylation is an important mechanism in the regulation of septin function. During epididymal transition of spermatozoa, defective SEPT4 phosphorylation results in the defective membrane diffusion

barrier [15]. In brain, SEPT4 phosphorylation is regulated by the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) [16]. The phosphorylation of SEPT3 by PKG modulates neuronal function [17]. PLK1 interacts and phosphorylates SEPT9 thus affecting cytokinesis [18]. Therefore, phosphorylation of septins is important for various physiological functions.

Primary cilium is a microtubule-based organelle that protrudes from at the surface of most mammalian cells. It plays important roles in development and differentiation. Disruption of cilium structure or function causes many diseases, including obesity, blindness, polycystic kidney disease, mental retardation, polydactyly;[19]. Primary cilium is composed of the central axoneme, which is the extended structure of the mother centriole, and the overlying ciliary membrane [20–22]. Septins have been shown to localize to primary cilium and are involved in the formation of cilia, known as ciliogenesis [23]. For example, SEPT2 functions as diffusion barriers at the base of the ciliary membrane; loss of ciliary membrane protein localization and inhibited ciliogenesis are observed in SEPT2 deficient cells [22]. In *Xenopus* embryos, knockdown of SEPT7 or SEPT2 causes defects in ciliogenesis [23]. In mammals, SEPT2-SEPT7-SEPT9 complex localizes to the axoneme of cilia for ciliogenesis and maintaining ciliary length [21]. Thus, septin complexes are required for maintaining cilia growth and length [24–26] .

In this study, we aimed to explore whether SEPT7 phosphorylation influences septin filament assembly and ciliogenesis. We found PKA increased SEPT7 phosphorylation at the Thr197. Over-expression of phosphomimetic SEPT7 reduced filaments assembly, suggesting that SEPT7 phosphorylation at the Thr197 is important for the assembly of SEPT7 filament. We also found PKA interacted with SEPT7 through GTP binding domain. Furthermore, we found PKA-mediated SEPT7 reduced the frequency of ciliated cells and reduced the ciliary length. These findings suggest that SEPT7 phosphorylation disrupts the septin filament assembly and ciliogenesis.

Methods

DNA constructs, cell culture, and transfection

Human SEPT7 and PKA were amplified from a human RNA panel (Clontech, Mountain View, CA,USA) and cloned into pFLAG-CMV-2, pEGFP- C3 and pCDNA3-HA vectors, as described previously [27]. All constructs were verified by DNA sequencing. For transient transfection, malignant human testis pluripotent embryonic carcinoma NT2/D1 cells and human embryonic kidney 293 T cells were incubated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Cells were transfected with plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h post-transfection, the cells were subjected to immunofluorescence staining or immunoblotting (IB). Alternatively, cells were treatment cAMP for analysis of protein expression.

Immunoprecipitation Assay And Western Blot Analysis

For immunoprecipitation analysis, 2 µg of an anti-Flag antibody was incubated with Dynabeads protein G (Thermo Fisher Scientific) at room temperature for 15 min on a rotator, and the cell lysates were immunoprecipitated with the bead-antibody complex at 4°C overnight on a rotator. The samples were washed three times with wash buffer (100 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.5% Tween-20, and 0.01% NP-40). The precipitates mixed with SDS sample buffer then boiled for 10 min. For western blotting, the proteins were adjusted to an equal amount of protein, electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membranes (Millipore). The blots were then incubated with an anti-Flag (1:5000), anti-Myc (1:5000), anti-GFP (1:4000), anti-HA (1:5000), anti-PKA (1:1000) or anti-phospho-Threonine (1:1000) antibody.

Immunofluorescence Analysis Of Filament Formation

For immunofluorescence analysis, the Flag-SEPTIN7 plasmids were transfected into NT2/D1 cells or 293T cells using Lipofectamine 2000 (Invitrogen). After 24 hours NT2/D1 cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) that permeabilized with 0.1% triton X-100 in PBS, and blocked with the antibody diluent (Dako) 1 hours. NT2/D1 cells or 293T cells were washed three times with PBS. The filament can be visualized using fluorescence microscopy (Olympus BX60).

ClustalW Multiple Sequence Alignment

The human SEPT7 orthologous proteins in various species used multiple sequence alignment by the ClustalW2 program. (<http://www.ebi.ac.uk/>). The accession numbers for SEPT7 proteins of different species were as follows: Homo sapiens (NP_001779.3 isoform 1, NP_001011553.2 isoform 2), Sus scrofa (pig) (XP_003134838.1 isoform X1), Canis lupus familiaris (dog) (XP_022283282.1), Felis catus (domestic cat) (XP_003982994.1), Mus musculus (house mouse) (NP_033989.2), Rattus norvegicus (Norway rat) (NP_072138.2), Bos taurus (cattle) (NP_001001168.1), Pongo abelii (Sumatran orangutan) (NP_001126872.1), Xenopus laevis (African clawed frog) (NP_001086183.1), Xenopus tropicalis (tropical clawed frog) (XP_002939459.1), Danio rerio (zebrafish) (NP_001242958.1). The accession numbers for SEPTIN groups of Homo sapiens were as follows: SEPT1 (NP_443070.1), SEPT2 (NP_001008491.1), SEPT3 (isoform A: NP_663786.2; isoform B: NP_061979.3), SEPT4 (isoform 1: NP_004565.1; isoform 2: NP_536340.1; isoform 3: NP_536341.1), SEPT5 (NP_002679.2), SEPT6 (isoform A: NP_665798.1; isoform B: NP_055944.2; isoform D: NP_665801.1), SEPT7 (isoform 1: NP_001779.3; isoform 2: NP_001011553.2), SEPT8 (isoform A: NP_001092281.1; isoform B: NP_055961.1; isoform C: NP_001092282.1; isoform D: NP_001092283.1), SEPT9 (isoform A: NP_001106963.1; isoform B: NP_001106965.1; isoform C: NP_006631.2; isoform D: NP_001106967.1; isoform E: NP_001106964.1; isoform F: NP_001106968.1), SEPT10 (isoform 1: NP_653311.1; isoform 2: NP_848699.1), SEPT11 (NP_060713.1) and SEPT14 (NP_997249.2). The above information provided by the National Center for Biotechnology Information (NCBI) database. (<http://www.ncbi.nlm.nih.gov/>).

Statistical analysis

Data were expressed as the means \pm SEM. Statistical significance analysis was determined by analyzed through one-way analysis of variance (ANOVA), combined with the Tukey's Multiple Comparison Test for posterior comparisons. P-values were considered significant at *P < 0.05; **P < 0.01; ***P < 0.001

Results

Conservation of the SEPT7 phosphorylation site among species

Our previous study shows that PKA phosphorylates SEPT12 at Ser196, which locates at the GTP-binding domain (GBD), thus disrupting septin filaments in the sperm annulus [28]. GBD is conserved among septin family, we thus screened the PKA recognition motif, [R/K]-X-X-[pS/T], using amino acid alignments of 28 selected septin sequences among all septin subgroup. This PKA recognition motif was identified in most septins, except for SEPT1, SEPT5, SEPT6 and SEPT8 (Fig. 1A). Here we were interested in SEPT7 as it was the irreplaceable component of all septin filaments [7]. The putative PKA phosphorylation site of human SEPT7 located on the Thr197, we thus checked whether this PKA phosphorylation site was conserved among different species. From zebrafish to homosapien, the predicted phosphorylation site is highly conserved (Fig. 1B). Thus, Thr197 of SEPT7 was a putative phosphorylation site that targeted by PKA.

SEPT7 Is Phosphorylated By PKA

Next, we checked whether PKA was responsible for SEPT7 phosphorylation at Thr197. Due to lack of antibody specifically against phosphorylated SEPT7 on Thr197, alternatively, FLAG-tagged SEPT7 was transfected into cells and the cell lysates were analyzed by immunoblotting with antibody against phospho-Threonine following immunoprecipitation with FLAG antibody. Treatment of 8-Br-cAMP, the activator of PKA, increased the phosphorylation on all Thr sites of SEPT7 in 293T (Fig. 2A) and NT2/D1 (Fig. 2B) in a dose dependent manner. This phosphorylation signal was reduced when cells were transfected with phospho-deficient SEPT7 (T197A) mutant. These results suggested that activation of PKA increased SEPT7 phosphorylation at Thr197 site. To further confirm this, the PKA catalytic subunit, PKACA2, was co-transfected with wild-type SEPT7 or T197A mutant and the phosphorylation status was observed. As expected, overexpression of PKACA2 increased SEPT7 phosphorylation at Thr and this signal was reduced in T197A mutant of 293T and NT2/D1 cell lines (Fig. 2C-D). Thus, PKA phosphorylates SEPT7 on Thr197.

SEPT7 Interacts With PKA Via The GTP-binding Domain

Then we checked whether SEPT7 interacted with PKACA2. FLAG-tagged SEPT7 and HA-tagged PKACA2 were co-transfected into 293T and NT2/D1 cell lines followed by performing immune-precipitation assay. PKACA2 was detected in the precipitant of FLAG-SEPT7 and vice versa (Fig. 3A), suggesting that SEPT7 interacted with PKACA2. Next, the interacting domain of SEPT7 was examined. The SEPT7 was dissected into N-terminus (N'), GTP-binding domain (GBD), and the C-terminus (C'). GFP-tagged GBD, N', or C' were co-transfected with HA-tagged PKACA2 into 293T or NT2/D1 cell lines followed by performing immune-precipitation assay. PKACA2 was detected in the precipitant of GFP-tagged GBD (Fig. 3B), but not in N' or C' precipitant (Fig. 3C-D), and vice versa. Thus, SEPT7 interacted with PKACA2 via the GBD.

SEPT7 Phosphorylation Disrupts Septin Filament Formation

SEPT7 is crucial for septin filament oligomerization, we thus checked whether SEPT7 phosphorylation affected septin filament formation. Two human SEPT7 mutant constructs, the T197E and T197A, which mimetic the constitutive phosphorylated and de-phosphorylated status, respectively, were generated and their effects on septin filaments was observed. The SEPT7 filaments were observed throughout the cytoplasm when cells were transfected with wild-type SEPT7 (Fig. 4A, upper panel, and 4B). This phenotype was also observed in cells transfected with the T197A (Fig. 4A, middle panel, and 4B). However, when cells were transfected with T197E, the septin filaments were hardly to be detected and smear signal was shown throughout the cytoplasm (Fig. 4A, lower panel; and 4B), suggesting that phospho-mimetic SEPT7 did not form filaments. Next, cells were co-transfected with wild-type and T197E constructs and the septin filaments were examined. Wild-type SEPT7 showed filamentous structures in the cells. However, when cells were co-transfected with T197E, the population of cells with septin filaments was decreased in a dose dependent manner (Fig. 4C), suggesting T197E had dominant-negative effect in SEPT7 filament formation. Thus, phosphorylation of SEPT7 disrupts septin filament formation.

SEPT7 Phosphorylation Disrupts SEPT7/ SEPT7 Interaction

Septin filament was orchestrated by the order of the septin hexamer core complex arranged as SEPT2-6-7-7-6-2 or SEPT4-6-7-7-6-4 [14] (Fig. 5A). SEPT7 phosphorylation disrupted septin filaments, thus, the interactions of SEPT7 with different septins were examined. Different septin constructs were transfected into NT2/D1 cells followed by performing immunoprecipitation assay. The interactions of FLAG-tagged SEPT7, including wild-type, T197A, or T197E, with Myc-tagged SEPT6, HA-tagged SEPT4, or HA-tagged SEPT2 were then examined by immunoblotting assay. First, we checked whether the interaction between SEPT7, which was important for polymerization of the septin complex, was affected by SEPT7 phosphorylation. FLAG-tagged SEPT7 and GFP-tagged SEPT7 were co-transfected into cells, the FLAG-SEPT7 was then precipitated by anti-FLAG antibody. GFP-SEPT7 could be detected in either wild-type or T197A precipitant (Fig. 5B). However, this interaction was hardly to be detected in the precipitant of T197E mutant (Fig. 5B), suggesting that SEPT7 phosphorylation disrupted SEPT7-SEPT7 interaction.

Next, the SEPT2-6-7 and SEPT4-6-7 complex were checked. The SEPT2 and SEPT6 were detected in wild-type SEPT7 or T197A precipitant or T197E mutants, suggesting that SEPT7 phosphorylation did not affect the orchestration of SEPT2-6-7 hetero-complex (Fig. 5C). SEPT7 phosphorylation status also did not affect SEPT4-6-7 hetero-complex (Fig. 5D), suggesting that SEPT7 phosphorylation affected SEPT7-SEPT7 interaction. Our immunofluorescence data suggested that SEPT7 T197E was a dominant-negative mutant on septin filament polymerization. To further confirm the dominant-negative effect of T197E, cells were co-transfected with equal amount of wild-type SEPT7 (GFP-tagged SEPT7 or HA-tagged SEPT7) and different amounts of FLAG-tagged SEPT7 T197E, and the interaction between GFP-SEPT7 and HA-SEPT7 was examined. In the absence of T197E, GFP-SEPT7 interacted with HA-SEPT7 (Fig. 5E). However, with the increasing amount of T197E, the interaction between GFP-tagged SEPT7 and HA-tagged SEPT7 was reduced, supporting that T197E was a dominant negative mutant on the assembly of septin filament. Thus, SEPT7 phosphorylation blocks SEPT7-SEPT7 interaction, but not affects SEPT2-6-7 or SEPT4-6-7 complex interaction.

Then the effect of SEPT7 phosphorylation on different septin filaments were examined by immunofluorescent staining. Wild-type SEPT7 and T197A mutant formed filaments, and these SEPT7 filaments were co-localized with SEPT2, SEPT4, SEPT6, and SEPT7, respectively (Fig. 6A-D, upper and middle panels). However, the T197E mutant showed dispersed signal in the cytoplasm, and no SEPT2, SEPT4, SEPT6, and SEPT7 filaments were observed (Fig. 6A-D, lower panel), suggesting that T197E mutant disrupted septin filaments. Taken together, SEPT7 phosphorylation blocks SEPT7-SEPT7 interaction thus disrupting septin filament formation.

Overexpression Of PKA Disrupts SEPT7 Filament Formation

Our data showed that SEPT7 was phosphorylated by PKA and SEPT7 phosphorylation disrupted septin filaments. Then we checked whether overexpression of PKA affects septin filaments. SEPT7 showed filamentous structures in 293T or NT2/D1 cells, however, these SEPT7 filaments became faint and were disrupted when PKACA2 was overexpressed (Fig. 7A). Then, we checked whether overexpression of PKACA2 affected SEPT7-SEPT7 interaction. In the absence of PKACA2, SEPT7 interacted with SEPT7. However, this interaction was reduced when PKACA2 was overexpressed (Fig. 7B), supporting that PKACA2 blocked SEPT7-SEPT7 interaction. Then, the SEPT2- or SEPT4-SEPT6-SEPT7 complexes were examined. SEPT7 interacted with SEPT6, SEPT2 and SEPT4, and these complexes were not affected when PKACA2 was overexpressed (Fig. 7C-D). Thus, PKACA2 reduces SEPT7-SEPT7 interaction followed by disrupting septin filament formation.

PKA-mediated SEPT7 phosphorylation affected ciliogenesis

Septin-based ring filaments act as a diffusion barriers at the base of primary cilia [22]. We then investigated whether SEPT7 phosphorylation modulated primary cilia formation. Under serum starvation, cells start to grow primary cilia [29], we thus checked whether serum starvation affected SEPT7 phosphorylation. Under serum starvation, the phosphorylation of SEPT7 was reduced (Fig. 8A), suggesting that SEPT7 phosphorylation might affect ciliogenesis. To further confirm our hypothesis, different SEPT7 mutants were transfected into RPE1 cells, the model cells for ciliogenesis, and the population of cells with primary cilium was quantified. Transfection of T197A had no effect on ciliogenesis when compared with transfection of wild-type SEPT7. However, the frequency of ciliated was reduced when RPE1 cells were transfected with T197E (Fig. 8B). Thus, SEPT7 phosphorylation inhibits primary cilia formation. Then, the role of cAMP-PKA cascade was examined. Treatment of cAMP reduced the frequency of ciliated and the length of cilia (Fig. 8C-E). In addition, overexpression of PKA inhibited ciliogenesis significantly (Fig. 8F). Thus, SEPT7 phosphorylation inhibits ciliogenesis during serum starvation.

Discussion

In this study, we demonstrated that SEPT7 phosphorylation at Thr197 disrupted SEPT7-SEPT7 interaction, but did not affected SEPT2-6-7 or SEPT4-6-7 complexes, thus inhibiting septin filament polymerization. We also demonstrated SEPT7 phosphorylation was mediated by cAMP-PKA axis. Furthermore, we showed that SEPT7 phosphorylation inhibited primary cilia formation and reduced cilia length. Taken together, PKA-mediated SEPT7 phosphorylation inhibits septin filament formation, thus reducing ciliogenesis.

Phosphorylation Of Septin Affects Septin Filament Formation

Post-translational modifications play an important role in the regulation of septin – septin interactions and control the formation of high-order septin complex structure. These modifications include SUMOylation, acetylation, ubiquitination and phosphorylation. Human septins (SEPT6, SEPT7, and SEPT11) could be modified by SUMOylation and SUMO-deficient mutants interfered with septin bundling and cell division [30]. SEPT4 is a substrate of the E3 ubiquitin ligase, parkin, and accumulation of SEPT4 is found in the parkin mutant, suggesting the SEPT4 protein stability is regulated by proteasome-mediated ubiquitination pathway [31–33]. In the budding yeast, NuA4, a lysine acetyltransferase, is involved in regulating septin collar formation [34]. In addition, phosphorylation of the terminal subunit septin, Cdc11, disrupted neck filaments and influence higher-order septin architecture [35]. Phosphorylation of *Drosophila* septin Pnut (homolog of human SEPT7) in the early stages of embryogenesis disrupts the assembly of septin filament formation, thus leading to dissociation of the septin complex. Our previous work shows that phosphorylation of a germ-cell specific SEPT12 leads to a complete loss of septin ring at the sperm annulus, illustrating important roles of septin phosphorylation in regulation of septin assembly and formation of higher-order structures. The phosphorylation site of

SEPT12 locates in the GBD, and this domain is conserved among several septins, including SEPT7. We thus checked whether phosphorylation of SEPT7 affected the assembly of septin filament. Indeed, SEPT7 phosphorylation in the GBD led to defective polymerization of septin filaments. Thus, phosphorylation of septin in the GBD might affect the septin filament formation. However, it is still unclear whether other septins, in addition to SEPT7 and SEPT12, also show similar phenotype, and this hypothesis still needs to be tested in the future.

SEPT7 Phosphorylation Inhibits Primary Cilia Formation

It has been shown the septin complexes regulate cilium length and ciliogenesis. Ablation of any of these septins expression by RNA interference inhibits ciliogenesis. Depletion of SEPT7 results in the concomitant loss of SEPT2 and SEPT9 expression of the complex [22, 36]. In the *Xenopus* epidermis, SEPT7 forms a ring at the base of motile cilia. Knockdown SEPT7 cause fewer and shortened cilia [23]. Although SEPT7 is important for ciliogenesis, it is still unclear whether post-translational modifications of SEPT7 affect ciliogenesis. Here we showed that SEPT7 phosphorylation was reduced during serum deprivation and T197E mutant inhibited ciliogenesis, suggesting phosphorylation status of SEPT7 was important for the growth of primary cilia. So far it is still unclear how T197E affected ciliogenesis. Septin filaments orchestras the transition zone of primary cilia, and T197E mutant disrupted septin complex formation, thus, we speculate that T197E mutant disrupts septin filament formation followed by inhibiting ciliogenesis. However, this hypothesis still needs to be confirmed.

PKA Mediates SEPT7 Phosphorylation

In primary kidney inner medullary collecting duct (IMCD3) cells, blocking the expression of SEPT2 inhibits ciliogenesis, thus reducing the Sonic hedgehog (Hh) signaling [22]. Hedgehog signaling provides an essential role in cilia function. Knockdown of SEPT7 caused defective ciliogenesis and abrogated Hedgehog signaling in developing *Xenopus* embryos [23]. Interestingly, PKA is a conserved negative regulator of the Hh signaling transduction. Increasing cAMP concentration leads to the activation of PKA followed by inducing Gli3 repressor (Gli3R). The Gli3R translocates into the nucleus and represses gene expression. Here we showed that PKA phosphorylated SEPT7 thus inhibiting ciliogenesis; thus, PKA inhibited Hh signaling might through both activation of Gli3R or disruption of primary cilia. However, this hypothesis still needs to be confirmed.

Several studies demonstrate that septin filament might have an exclusive role in membrane diffusion barrier functions in diverse cell types, including sperm, neurons, and epithelia. In the sperm, a septin ring structure (annulus) connects the midpiece and the principal piece. The SEPT4 null mice show a disruption of diffusion barrier phenotype at the sperm tail [37]. In the epithelia, absence of septin filaments caused misalignment of microtubules, loss of apicobasal polarity [38, 39], and the cell shape [2], thus leading to defective cytokinesis [40–42]. In the nervous system, septins form diffusion barriers at dendritic spines neck to restrict the diffusion of membrane proteins across the spine [43, 44]. In this

paper, we showed PKA-mediated SEPT7 phosphorylation was important for the septin filament assembly and ciliogenesis. We speculate SEPT7 complex serves the diffusion barrier function at the ciliary base and participates in a variety of receptor-signaling pathways. However, the downstream pathways of PKA-SEPT7 await further investigation. Our findings provide an important avenue to decipher the dynamics of SEPT complex assembly as well as the physiological roles of SEPT7 complex.

In summary, we found SEPT7 was phosphorylated by PKA. PKA-mediated SEPT7 phosphorylation disrupted SEPT7-SEPT7 interaction, but not affected SEPT2-6-7 or SEPT4-6-7 complexes. In addition, phosphorylation of SEPT7 was reduced upon serum starvation, during which primary cilium started to grow. T197E mutant inhibited ciliogenesis upon serum starvation. Thus, phosphorylation status of SEPT7 is essential for septin filament formation and ciliogenesis.

Conclusions

Post-translational modification regulates the assembly of septin filaments. Here, we demonstrate that PKA-mediated SEPT7 phosphorylation disrupts septin filament polymerization. More importantly, phosphorylated SEPT7 also inhibits primary cilia formation. Thus, our study identified a novel phosphorylation site, which is targeted by cAMP-PKA cascade, for regulating the structure and function of septin filaments.

Abbreviations

SEPT7

septin7

PKA

protein kinase A

PKACA2

PKA catalytic subunit

GBD

GTP-binding domain

N'

N-terminus

C'

C-terminus

Declarations

Acknowledgments

This study was supported by the Core Research Laboratory, College of Medicine, National Cheng Kung University and National Cheng Kung University Hospital.

Funding

This work is supported by Ministry of Science and Technology of Taiwan, R.O.C (MOST 106-2314-B-006-056-MY3, 108-2811-B-006-509, 108-2811-B-006-5188 to Pao-Lin Kuo; MOST103-2628-B-006-001-MY3 and MOST106-2320-B-006-056-MY3 to Chia-Yih Wang).

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Author contributions

P.L. Kuo and C.Y. Wang designed the research. C.H. Lin, T.Y. Chen, and Y.R. Shen performed the research. P.L. Kuo and C.Y. Wang analyzed the data. C.Y. Wang and P.L. Kuo wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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Figures

Figure 1

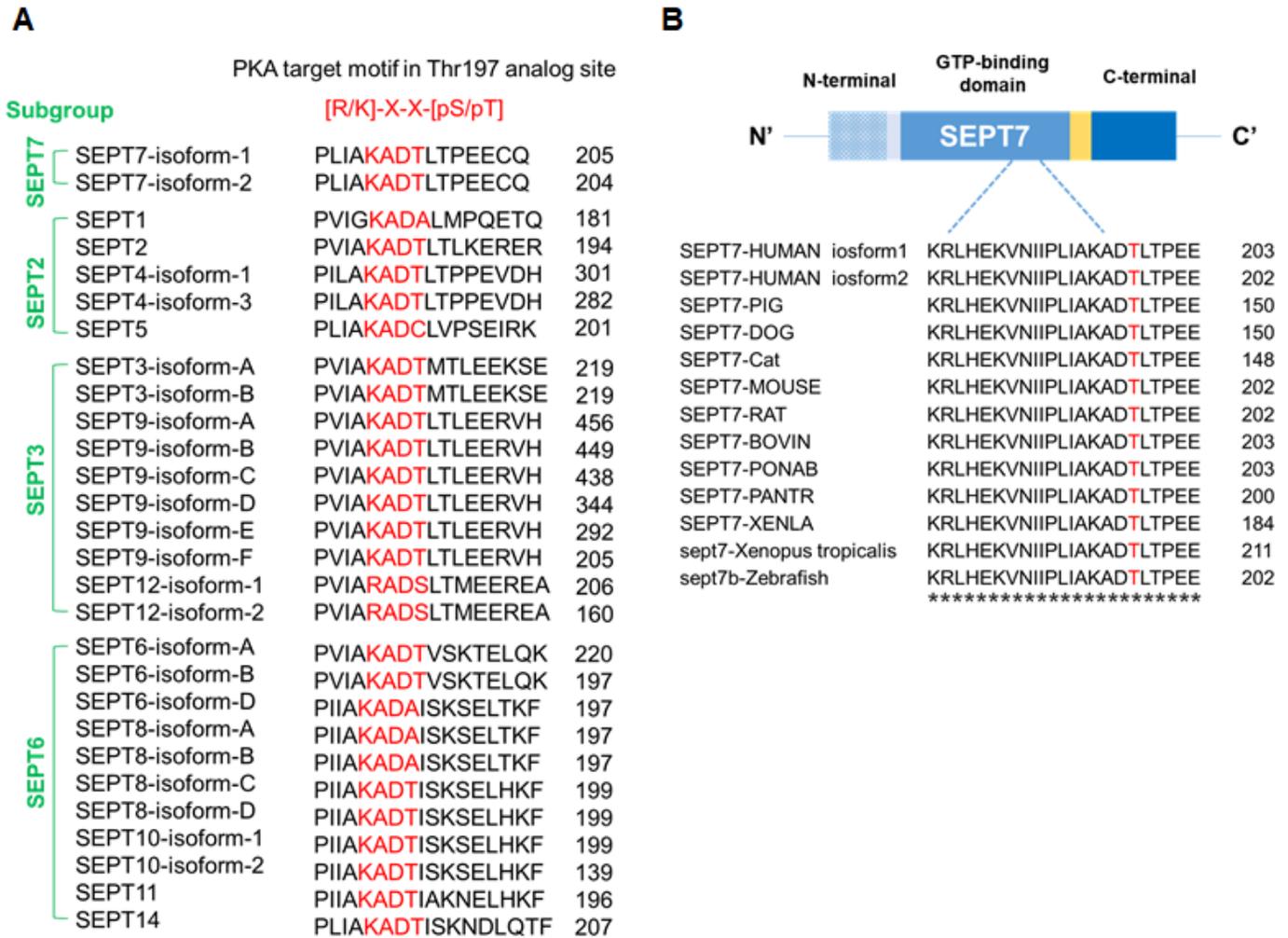


Figure 1

Conservation of PKA target SEPT7 phosphorylation site Thr197 among species. (A) Multiple sequence alignment flanking the analogous Thr197 residue of SEPT7 in 13 different human genes coding for septins. Alignment the sequences according to PKA consensus target motif [R/K]-X-X-[pS/T]. (B) Multiple sequence alignment of analogous site of Thr197 in the SEPT7 orthologues comparison of the amino acid in various species. Red indicates residues aligned are similar. At the bottom, “*” denotes identical residue in all sequences in the alignment. The amino acid sequences were analyzed by using the ClustalW2 program at EMBL-EBI.

Figure 2

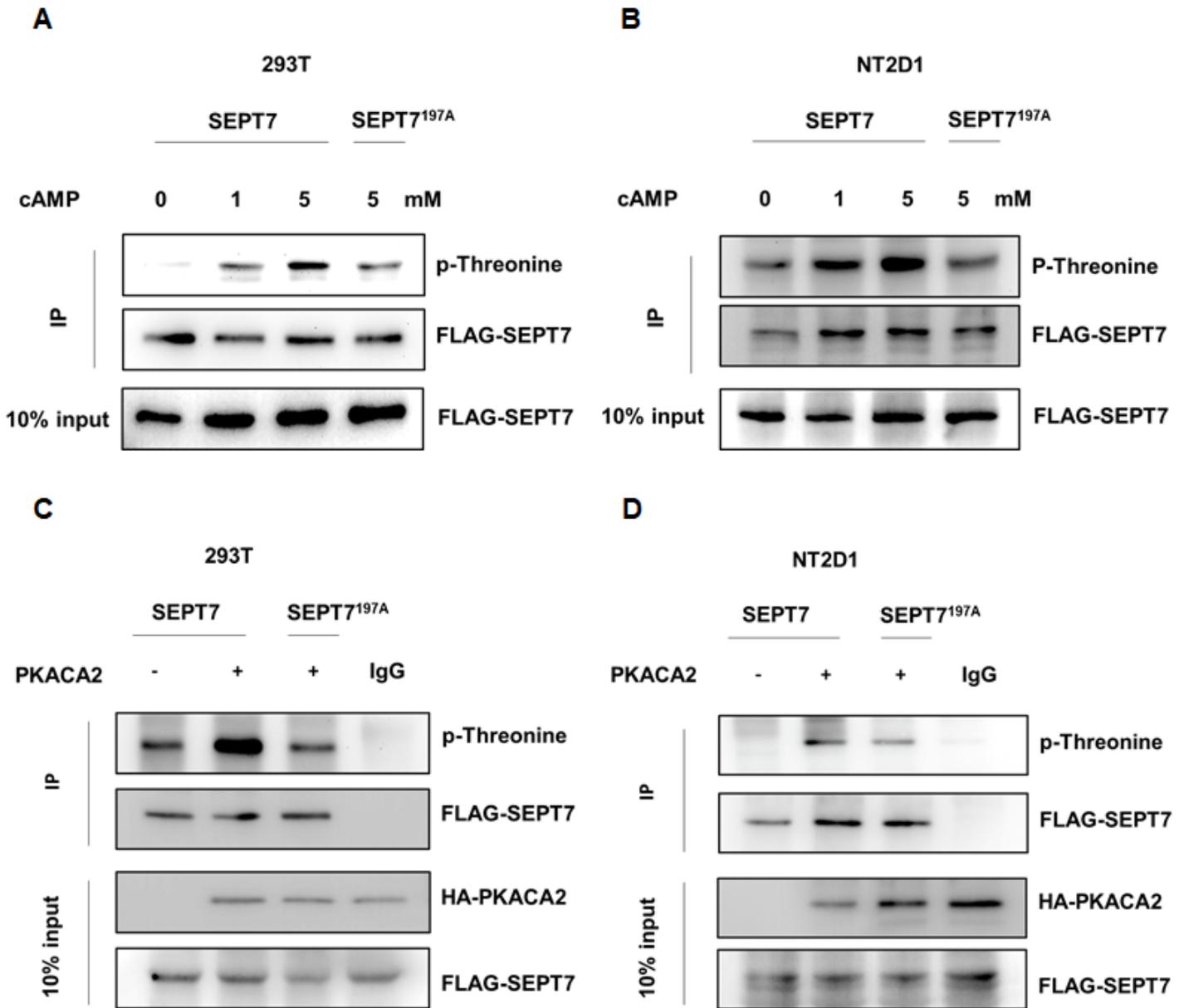


Figure 2

SEPT7 was phosphorylated at Thr197 site by PKA. Activation of PKA increased SEPT7 phosphorylation at Thr197 site following treatment of 8-Br-cAMP in a dose dependent manner, but reduced in phospho-deficient SEPT7 (T197A) mutant of 293T (A) or NTSD1 (B) cells. (A) FLAG-SEPT7 or FLAG-SEPT7 (T197A) was transfected into 293T cells following treatment of 0, 1, or 5mM 8-Br-cAMP. The levels of the Thr197 phosphorylation was increased in FLAG-SEPT7-transfected 293T cells. Extracts of FLAG-SEPT7-transfected 293T cells or FLAG-SEPT7 (T197A) mutant 293T cells following treatment of 8-Br-cAMP were analyzed by immunoprecipitation with antibodies against phospho-Threonine and FLAG. (B) FLAG-SEPT7 or FLAG-SEPT7 (T197A) mutant was transfected into NT2/D1 cells. The levels of the Thr197 phosphorylation was increased in FLAG-SEPT7-transfected NT2/D1 cells but reduced in FLAG-SEPT7 (T197A) mutant NT2/D1 cells following treatment of 8-Br-cAMP. Extracts of FLAG-SEPT7 or FLAG-SEPT7

(T197A) mutant-transfected NT2/D1 cells following treatment of 8-Br-cAMP were analyzed by immunoprecipitation with antibodies against phospho-Threonine and FLAG. PKA phosphorylates SEPT7 on Thr197. Flag-SEPT7 and Flag-SEPT7 (197A) mutant with or without HA-PKACA2, were transfected into 293T cells (C) and NT2/D cells (D) as indicated, and the expression of phosphorylated Threonine, SEPT7 and PKACA2 was detected with antibodies against phospho- Threonine, Flag and HA. IgG was served as negative control.

Figure3

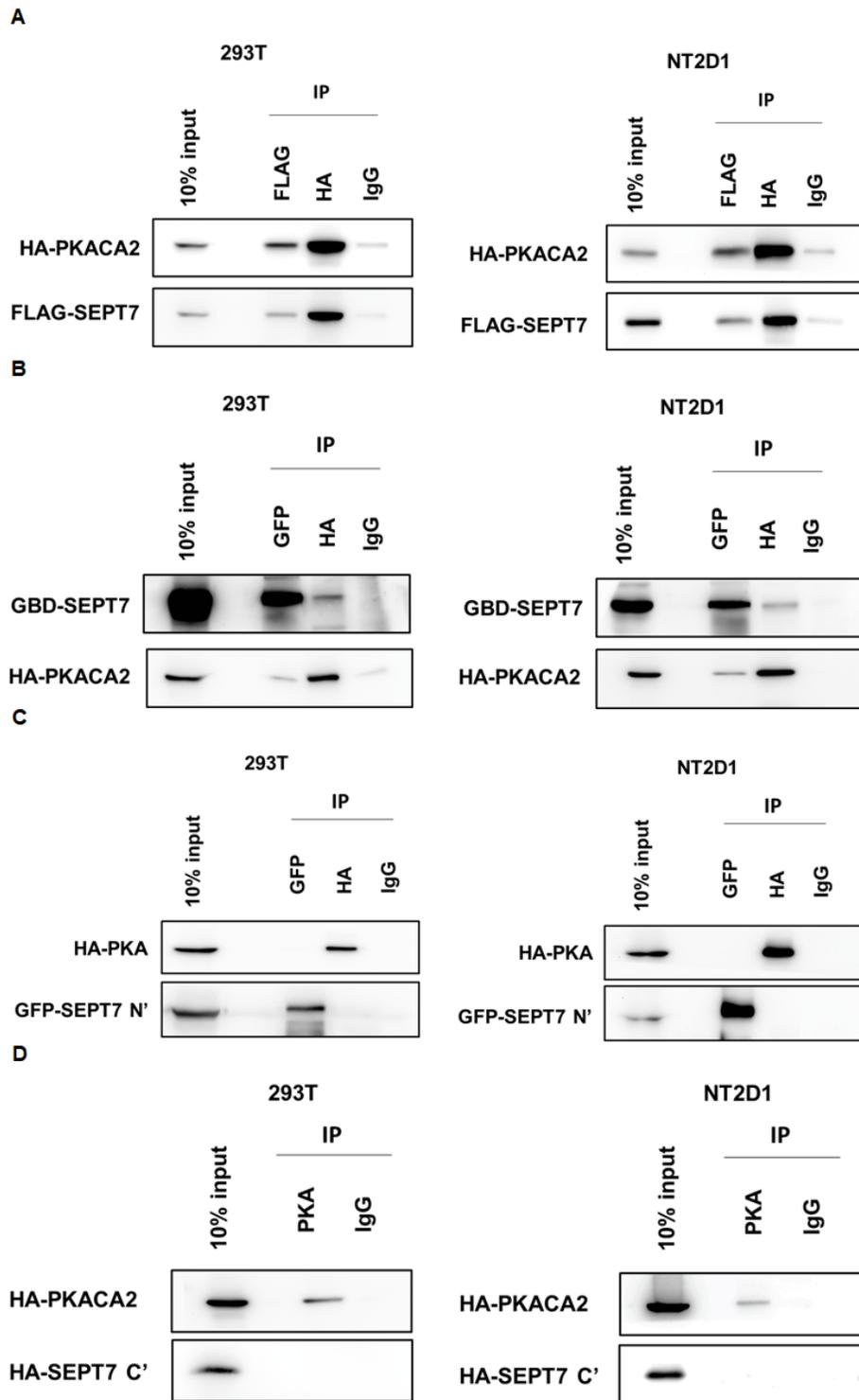
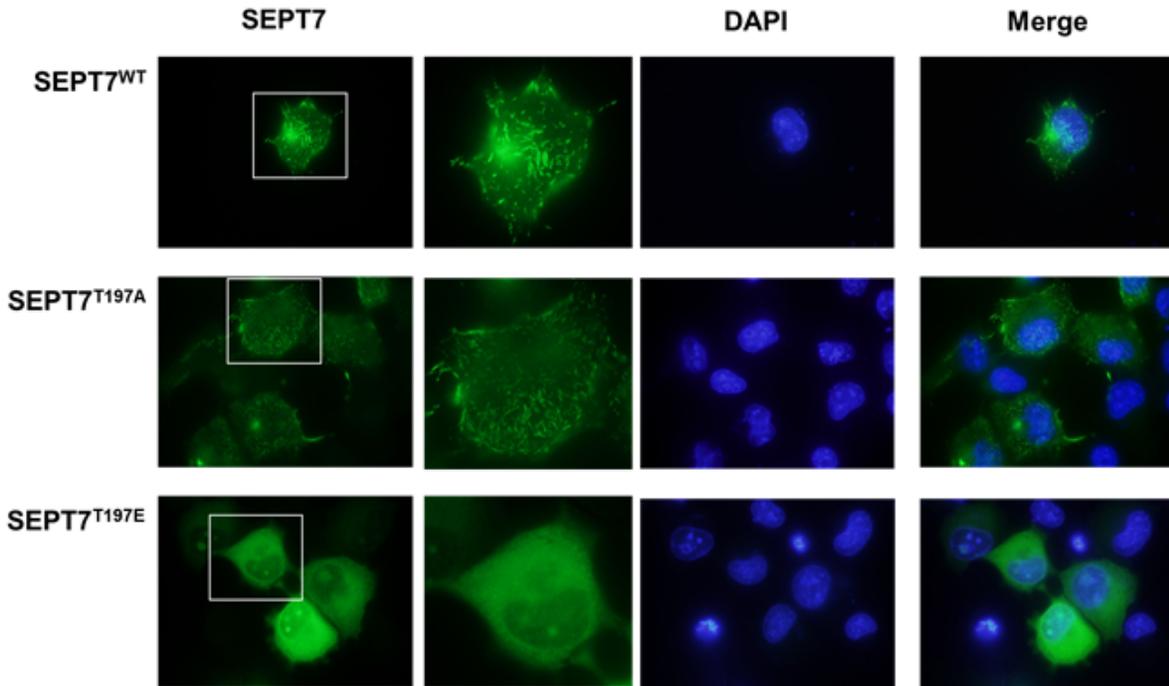


Figure 3

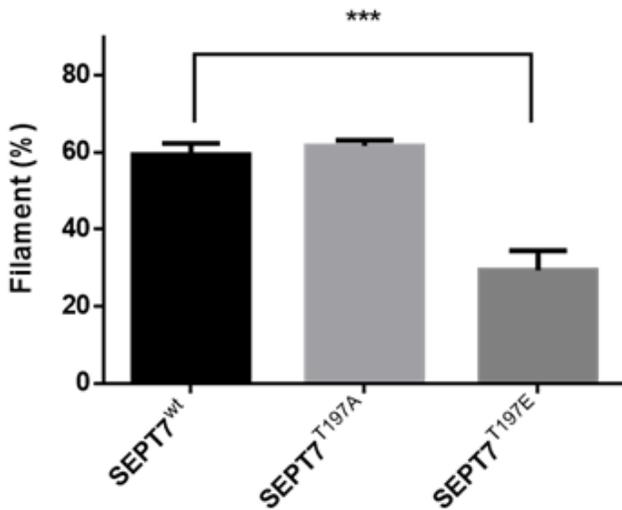
SEPT7 interacts with PKA via the GTP-binding domain. SEPT7 interacted with PKA via its GTP-binding domain (A-B) not its N-terminus (N') (C) or C-terminus (C') (D) domains. FLAG-SEPT7 and HA-PKACA2 were co-transfected to 293T Cells or NT2/D1 cells followed by performing immune-precipitation assay. (A) PKACA2 was detected in the precipitant of FLAG-SEPT7 and vice versa. Extracts of FLAG-SEPT7 and HA-PKACA2 co-transfected 293T or NT2/D1 cells were immunoprecipitated by FLAG or HA with antibodies against HA and FLAG. Transfected IgG served as a negative control. Lanes showing 10% of the input are also present. (B) GBD-SEPT7 and HA-PKACA2 were co-transfected to 293T Cells or NT2/D1 cells followed by performing immune-precipitation assay. Extracts of GFP-GBD-SEPT7 and HA-PKACA2 co-transfected 293T or NT2/D1 cells were immunoprecipitated by GFP or HA with antibodies against HA and GFP. (C) Co-transfected GFP-SEPT7-N terminal and HA-PKACA2 in 293T Cells or NT2/D1 cells followed by performing immune-precipitation assay. Extracts of GFP-N'-SEPT7 and HA-PKACA2 co-transfected 293T or NT2/D1 cells were immunoprecipitated by GFP or HA with antibodies against HA and GFP. (D) Co-transfected HA-SEPT7-C terminal and HA-PKACA2 in 293T Cells or NT2/D1 cells followed by performing immune-precipitation assay. Extracts of HA-C'-SEPT7 and HA-PKACA2 co-transfected 293T or NT2/D1 cells were immunoprecipitated by PKA with antibody against PKA. Transfected IgG served as a negative control. Lanes showing 10% of the input are also present.

Figure 4

A



B



C

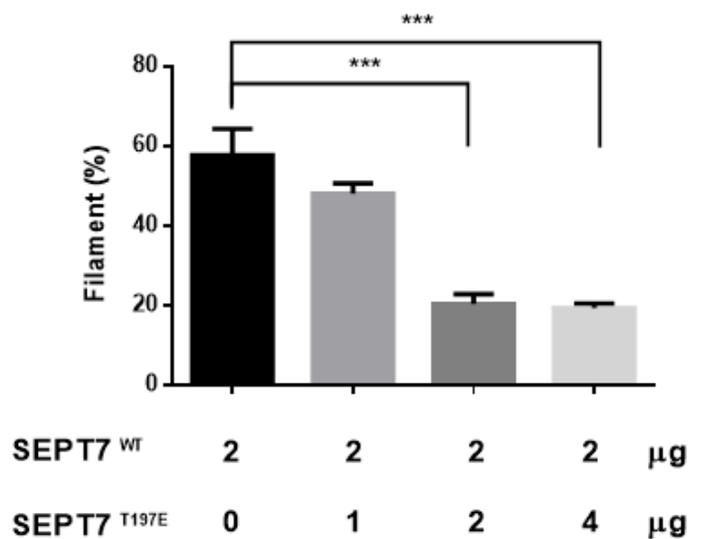


Figure 4

SEPT7 phosphorylation disrupts septin filament formation. (A-B) The plasmids of FLAG-SEPT7, FLAG-SEPT7 T197A (de-phosphorylated status), or FLAG-SEPT7 T197E (constitutive phosphorylated status) was transfected into NT2/D1 cells. (A) Filament disruption was shown in FLAG-SEPT7 T197E-transfected NT2/D1 cells. SEPT7 filament of NT2/D1 cells in the transfection of FLAG-SEPT7, FLAG-SEPT7 T197A, or FLAG-SEPT7 T197E were analyzed with antibodies against FLAG (green) and DAPI

(blue). (B) Quantitation of SEPT7 filament in FLAG-SEPT7, FLAG- SEPT7 T197A, or FLAG- SEPT7 T197E-transfected NT2/D1 cells. SEPT7 phosphorylation decreased SEPT7 filament formation. (C) FLAG-SEPT7 and FLAG- SEPT7 T197E were co-transfected into NT2/D1. Decreased SEPT filament were shown in co-transfection of FLAG-SEPT7 and 0, 1, 2 or 4 μ g FLAG- SEPT7 T197E NT2/D1 cells. Quantitation of SEPT7 filament in FLAG-SEPT7and FLAG- SEPT7 T197E co-transfected NT2/D1 cells. The quantification bar was based on the observation of more than 100 cells for each experiment in C-D. Data are represented as means \pm SEM (n=3). From three independent experiments* ** P < 0.001.

Figure5

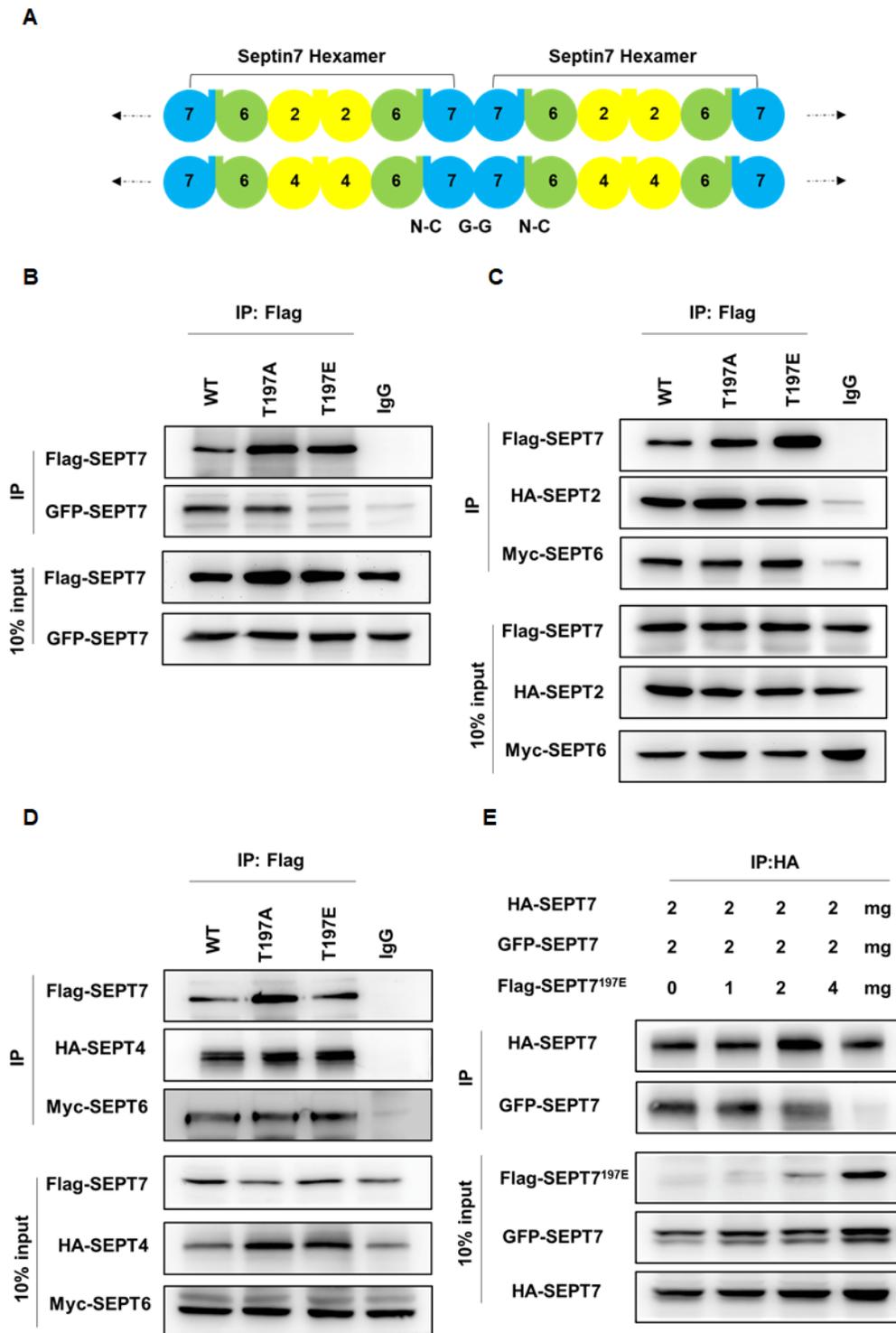


Figure 5

Mimetic phosphorylated Thr197 of SEPT7 disrupts SEPT 7-7 complex but not affected SEPT 2-SEPT 4 and SEPT 6 interaction. (A) Schematic representation of a heteromeric SEPT7-SEPT6-SEPT2 or SEPT7-SEPT6-SEPT4 complex. SEPT7 has been included at the terminal positions (shown in Blue). The SEPT 7- SEPT 7 pairwise interactions occur at the G-interface, whilst the SEPT 7- SEPT 6 dimer is stabilized by NC interface. SEPT7 phosphorylation affected SEPT7-SEPT7 interaction (B) but not SEPT 2-SEPT 4 and SEPT 6 interaction (C-E). (B) FLAG-SEPT7 and GFP-SEPT7 were co-transfected into NT2/D1 cells. Lysates from transiently transfected cells were immunoprecipitated by FLAG or GFP with antibodies against GFP and FLAG. (C-D) Myc-SEPT6, HA-SEPT4 and HA-SEPT2 with various Flag-SEPT7 plasmids into NT2/D1 cell and lysates were immunoprecipitated with an anti-Flag antibody. The expression of SEPT2, 4, 6 and SEPT7 were detected by anti-HA, anti-Myc and anti-FLAG antibodies, respectively. IgG served as negative control. (E) Co-transfection of HA-SEPT7 and GFP-SEPT7 with various Flag-SEPT7 T197E plasmids into NT2/D1 cell. A total of 2 µg of the HA-SEPT7 and GFP-SEPT7 plasmid was mixed with 0, 1, 2 or 4 µg of the Flag-SEPT7 T197E plasmid and transfected into NT2/D1 cells. Immunoblotting was performed using anti- Flag, anti- GFP and anti-HA antibodies.

Figure6

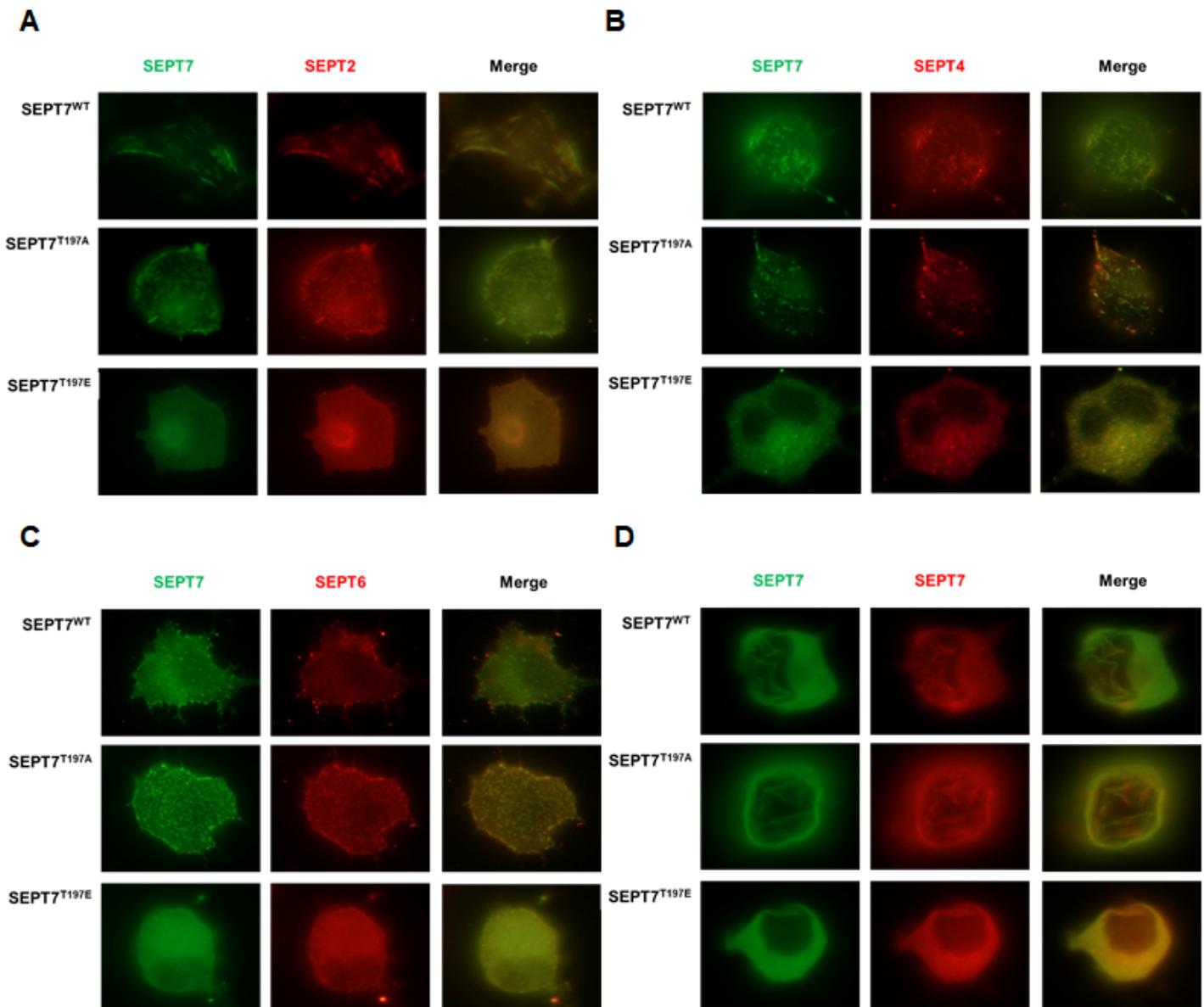


Figure 6

SEPT7 phosphorylation disrupts SEPT 7-6-2 and 7-6-4 complex. SEPT7 filament in wild type or dephosphorylation co-localized to other SEPT filament. SEPT7 phosphorylation disrupted SEPT2, SEPT4, SEPT6 or SEPT7 filament formation. Co-transfection of the plasmids FLAG-SEPT7 WT, FLAG-SEPT7T197A, or FLAG - SEPT7T197E and SEPT2 (A), SEPT4 (B), SEPT6 (C) or SEPT7 (D). Immunostaining of SEPT filament and DAPI in co-transfected NT2/D1 cells were analyzed with antibodies against SEPT7 (green) and co-stained with SEPT2 (A), SEPT4 (B), SEPT6 (C), or SEPT7 (D) (green).

Figure 7

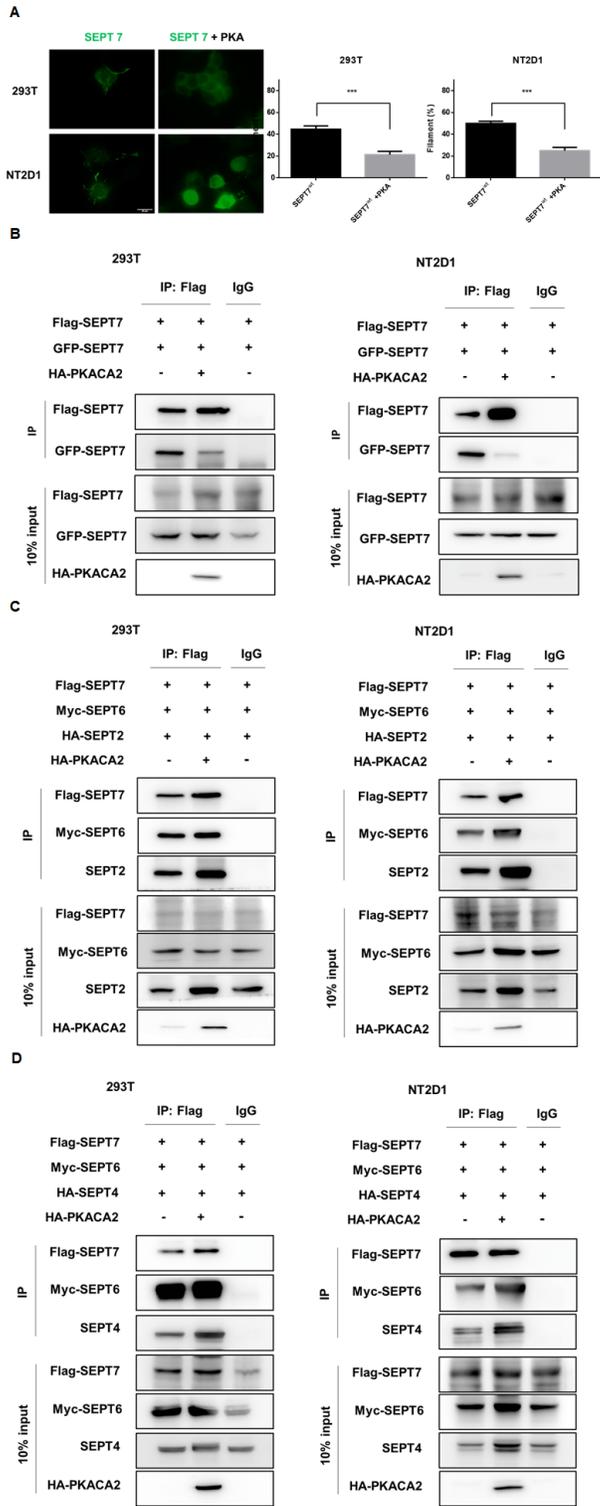


Figure 7

Overexpression of PKA disrupts SEPT7 filament formation. (A) FLAG-SEPT7 and HA-PKACA2 were transfected into 293T or NT2/D1 cells. SEPT7 filament were disrupted in 293T or NT2/D1 cells in presence of PKACA2. Immunostaining of SEPT7 filament and DAPI in the presence or absence of PKACA2 co-transfected SEPT7 293T or NT2/D1 cells were analyzed with antibodies against SEPT7 (green) and DAPI (blue). Quantitation of SEPT7 filament in the presence or absence of PKACA2 in 293T

or NT2/D1 cell lines. The quantification bar was based on the observation of more than 100 cells for each experiment. Data are represented as means \pm SEM (n=3). *** P < 0.001. PKACA2 reduces SEPT7-SEPT7 interaction followed by disrupting septin filament formation. (B) 293T or NT2/D1 cell lines were co-transfected with FLAG-SEPT7 or GFP-SEPT7 (B), Myc-SEPT6 (C and D), HA-SEPT2 (C), or HA-SEPT4 (D) in the presence or absence of PKACA2. Cell lysates were immunoprecipitated with antibody against FLAG, and IgG served as a negative control. Following precipitation, samples were analyzed by immunoblotting assay with antibodies against GFP, Myc, or SEPT2.

Figure 8

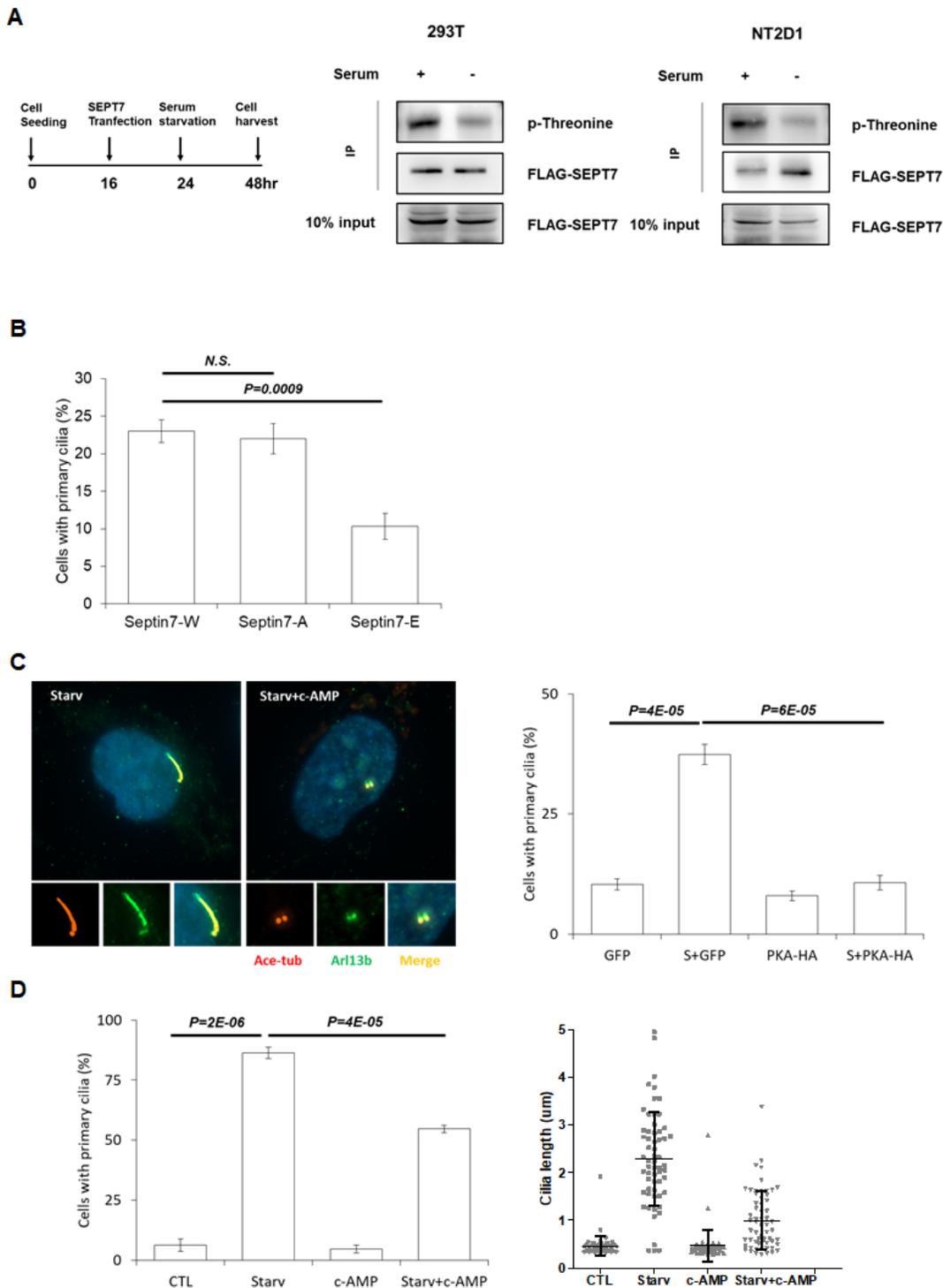


Figure 8

PKA-mediated SEPT7 phosphorylation affected ciliogenesis. (A) The levels of the Thr197 phosphorylation in 293T or NT2D1 cells was reduced after overexpression FLAG-SEPT7 in the medium with or without serum for 24 h. Extracts of SEPT7-transfected cells following with or without serum were analyzed by immunoblotting with antibodies against Flag and phospho- Threonine. Lanes showing 10% of the input are also present. SEPT7 phosphorylation inhibited primary cilia formation. (B) Quantitation of primary cilia in FLAG-SEPT7, FLAG- SEPT7 T197A, or FLAG- SEPT7 T197E-transfected RPE1 cells in the presence or absence of serum. Activated of PKA followed by 8-Br-cAMP inhibited primary cilia formation (C-D) and ciliary length (E) under serum starvation. (C) Immunostaining of primary cilia and DAPI in scramble control (CTL) or 8-Br-cAMP treated RPE1 cells. RPE1 cells were analyzed with antibodies against acetylated tubulin (red) and co-stained with acetylated tubulin (ace-tub, red) and Arl13b (green). (D) Quantitation of primary cilia of 8-Br-cAMP -treated RPE1 cells in the presence or absence of serum. (E) Quantitation of ciliary length of 8-Br-cAMP -treated RPE1 cells in the presence or absence of serum. Overexpression PKA inhibited primary cilia formation under serum starvation. (F) RPE1 cells were transfected GFP or HA-PKA in the presence or absence of serum. Quantitation of primary cilia of GFP or HA-PKA –transfected RPE1 cells under serum starvation. These results are mean +/- SD from three independent experiments; more than 100 cells were counted in each individual group.