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DAB2IP modulates primary cilia formation associated with renal tumorigenesis

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Abstract

Primary cilium is a microtubule-based organelle that projects from the surfaces of most mammalian cell types and protrudes into the extracellular milieu as an antenna-like sensor to senses extracellular physical and biochemical signals, and then transmits signals into cytoplasm or nucleus to regulate numerous physical and developmental processes. Therefore, loss of primary cilia is associated to multiple cancer progression, including skin, breast, pancreas, ovarian, prostate, and kidney cancers. Our previous studies demonstrate that high prevalent loss of DAB2 Interacting Protein (DAB2IP) is associated with renal cell carcinoma, and we found a kinesin-like protein, kinesin family member 3A (KIF3a), was significantly increased in DAB2IP-interacting protein fraction. KIF3 is one of the most abundant kinesin-2 family proteins expressed in cells, and it is necessary for ciliogenesis. In this study, we observed that loss of DAB2IP in normal kidney epithelial cell significantly impair primary cilia formation. We unveiled a new mechanism of primary cilia stability via DAB2IP and KIF3a physical interaction at DAB2IP-PH domain. Furthermore, we found that KIF3a also act as a tumor suppressor in renal cell carcinoma, affect tumor development and patient survival.

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Keywords: DAB2IP, KIF3a, Primary cilia, Renal cell carcinoma, Tumor suppressor gene

Introduction

Primary cilium is a microtubule-based organelle that projects from the surfaces of most mammalian cell types and protrudes into the extracellular milieu. The primary cilium is usually immotile and acts as an antenna-like sensor in most tissues that senses extracellular physical and biochemical signals and transmits these signals into cytoplasm or nucleus to regulate numerous physical and developmental processes. Structure of primary cilia can be divided into basal body, transition zone, and axoneme; each structure is composed by multiple proteins. Studies have found that the mutation or dysfunction of these primary cilia proteins not only defect primary cilia formation, but associated with various human physiologic diseases, called "ciliopathies."

Primary cilium also plays a suppressive role in carcinogenesis, which is supported by the fact that primary cilium contains signaling pathways linked with cancer and additionally many different types of tumors, including basal cell carcinoma of skin [1], breast cancer [2], cholangiocarcinoma [3], ovarian cancer [4], pancreatic cancer [5], and prostate cancer [6] lose primary cilia. Indeed, renal cell carcinoma (RCC) also shows loss of cilia and this is related with a downregulation of the *von Hippel-Lindau* (*VHL*) tumor suppressor gene [7]. However, the underlying causes of loss of primary cilia are largely unknown.

Our previous studies demonstrate that high prevalent loss of DAB2 Interacting Protein (DAB2IP), a Ras-GTPase-activating protein, is associated with RCC. With the array analysis, we found a kinesin-like protein, kinesin family member 3A (KIF3a), was significantly increased in DAB2IP-interacting protein fraction. KIF3 is one of the most abundant kinesin-2 family protein expressed in cells, and is composed by KIF3a, KIF3b, KIF3c and a nonmotor subunit kinesin-associated protein KAP3 [8]. This kinesin-2 family protein interacts with intraflagellar transport proteins at anterograde of primary cilia, and it is necessary for primary cilia assembly and length maintenance [9]. In this study, we observed that loss of DAB2IP

Abbreviations: ac-tubulin, acetylated tubulin; AIG, anchorage independent growth assay; ATCC, American Type Culture Collection; C2, protein kinase C conserved region 2 domain; Con, vector control; DAB2IP, DAB2 Interacting Protein; DAPI, 4', 6-diamidino-2-phenylindole; FBS, fetal bovine serum; HIF, hypoxia-inducible factors; IFT, intraflagellar transport; KD, knockdown; KIF3a, kinesin family member 3A; KO, knockout; OE, overexpression; PH, Pleckstrin homology domain; qRT-PCR, Quantitative real-time PCR; RCC, renal cell carcinoma; STR, short tandem repeat; TCGA, The Cancer Genome Atlas; UTSW, University of Texas Southwestern Medical Center; VHL, von Hippel-Lindau; WT, wild type.

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in normal kidney epithelial cell significantly impair primary cilia formation. By unveiling a new mechanism of action of primary cilia stability via physical interaction between DAB2IP and KIF3a, we provide new evidence that KIF3a can act a tumor suppressor in RCC.

Materials and methods

Cell culture and cell transfection

HK2, ACHN, 769P, Madin-Darby Canine Kidney cell (MDCK), and HEK293 were purchased from American Type Culture Collection (ATCC) and maintained according to ATCC guidelines. Mice KIF3a wild type (WT) and knockout (KO) cells were kindly gifted by Dr. Igarashi and maintained as previously described [10]. All culture media were supplemented with 5% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA), and 100 U/mL penicillin, 100 μg/mL streptomycin. All human cell lines were authenticated with the short tandem repeat profiling by Genomic Core of The University of Texas Southwestern Medical Center at Dallas (UTSW) periodically and Mycoplasma testing was performed by MycoAlert kit (Lonza Walkersville, Inc. Walkersville, MD) every quarterly to ensure Mycoplasma-free.

For cell transfection, cells were seeded in 24-well plates with 70% confluency before transfection. shRNA targeting KIF3a were purchased from *RNAi-core, Academia Sinica, Taiwan* (http://rnai.genmed.sinica.edu.tw). KIF3a overexpressing plasmid was kindly provided by Dr. Igarashi. Transfection procedure was followed by manufacture instruction (Xfect Transfection Reagent, Takara Bio Europe, France), and the expression was confirmed after 48 h of transfection.

Immunofluorescence staining

Cells were seeded into chamber-slide with 90% confluency and incubated with 1% FBS culture medium overnight for primary cilia formation. The cells were fixed with 4% Paraformaldehyde and permeabilized with 0.1% Triton-X. The samples were incubated with the specific antibodies for overnight and treated with Fluorescence-conjugated secondary antibodies. Finally, they were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, NJ, USA) and observed under the BZ-x700 microscope (Keyence, Tokyo, Japan).

Mass spectrometry

Experiment was performed as described previously [11]. In brief, HEK293T cells transfected with flag tagged DAB2IP were separated into nuclear and cytosol fractions. Each fraction was immunoprecipitated with anti-flag antibody. DAB2IP interacting proteins in cytosol fraction were identified by Orbitrap Elite mass spectrometry platforms (Thermo Fisher Scientific). Proteins were identified from samples using data analysis pipeline (CPFP) of Proteomics Core at UTSW.

Immunoprecipitation (IP) and Western blot

The immunocomplexes were precipitated with Dynabeads Protein G (Life Technologies) according to the manufacture's protocol. Precipitated samples were subjected to western blot analysis.

For western blot, cells were lysed and electrophoresized on 4% to 12% gradient Bolt gels (Life Technologies). Separated proteins were electroblotted onto nitrocellulose membranes that were incubated with 5% nonfat dry milk (w/v) for 1 h and then washed in PBS containing 0.1% Tween-20. Membranes were then incubated with designed primary antibody, and designated secondary antibody. KIF3a antibody was purchased from Santa Cruz (sc-18745; Santa Cruz Biotechnology Inc. Santa Cruz, CA) and Protein Tech (13930-1-AP; Proteintech Group, Chicago, IL). DAB2IP antibody was purchased from Abcam (ab87811, Cambridge, MA). Acetylated-tubulin and Gamma-tubulin antibodies were purchased from Sigma (T7451, T5192; Sigma Chemical Co., St. Louis, Mo). ARL13B antibody was purchased from Protein Tech (17711-1-AP).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Maxwell 16 LEV SimplyRNA Purification Kit (Promega, Madison, WI) and 1 µg RNA was reversely transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR analysis was set up with the SsoAdvenced Universal SYBR Green Supermix Kit (BioRad) and carried out in MyiQ thermal cycler (Bio-Rad). The relative level of mRNA expression was determined by normalizing 18S rRNA. All experiments were repeated at least 3 times in triplicates.

Dot blot analysis

Nitrocellulose membranes were prepared and immersed into PBS 10 min before experiment. Total protein of 30 µg was added onto membranes at the center of grid with vacuums. The membranes were blocked by 3% BSA in PBS for 1 h. RIPA-lysed-HK cell were applied onto the membranes and incubated at 4°C for 16 h without shaking. The membranes were washed with PBS twice and incubated with KIF3a antibody at 4°C for 16 h. The membranes were washed with PBS and then incubated with anti-rabbit-HRP antibody (Santa Cruz).

Colony assay

Same amount (5×10^3) of cells were plated in 60-mm dish and incubated at 37°C, 5% CO2 for colony formation. After 14 d, colonies were fixed with 10% (v/v) methanol for 15 min and stained with 0.05% crystal violet for 30 min for colony visualization. Stained colony was washed with PBS, and air dry before taking representative image. For quantification, 10% acetic acid was used to distain, and colony forming ability was measured with spectrophotometer (OD 550 nm).

Anchorage independent growth assay (AIG)

To make the bottom layer, 1 mL of 0.6% agarose was added to 6-well plates and solidified at room temperature for at least 30 min. To prepare the top layer (0.3% agarose), 500 μL of agarose was mixed with 500 μL cell suspension containing 5000 cells. This mixture was overlaid above the bottom layer and allowed to solidify at room temperature. An additional 2 mL of culture media was added after solidification of the top layer, and cells were incubated for 2 wk at 37°C. After 14 d of growth, 1 mg/mL of MTT were added into culture medium and incubate at 37°C for 3 h. The colony numbers were counted under a phase contrast microscope. Data were presented as colony numbers per field.

Mouse xenografts

All animal work was approved by the Institutional Animal Care and Use Committee, UTSW. HK2 vector control (Con) and DAB2IP knockdown (KD) (1×10^6 cells) were subcutaneously injected into 6- to 8-wk-old male SCID mice. Seven weeks of postinjection, the tumor incidence and tumor volume were recorded. Tumor volume was determined by caliper and calculated using the ellipsoid formula ($\pi/6 \times$ length $\times$ width $\times$ depth).

Statistical analysis

All numerical data represent mean and standard deviation. Student t test was used for the determination of statistical significance between groups ($P < 0.05$). All statistical analyses were performed with GraphPad Prism software.

Results

The role of DAB2IP in primary cilia formation

According to previous studies, DAB2IP is often lost in renal cancer cell, and its expression correlated with clinical patient outcomes [12,13]. Also, we have demonstrated that DAB2IP-positive kidney tubular cells, including immortalized normal epithelial cell (HK), MDCK acquire tumorigenicity after DAB2IP knockdown (KD) compared to parental control (Con). On the other hand, DAB2IP-negative RCC cells, including ACHN, 769P and HEK293 cells, become less tumorigenic and sensitive to radiotherapy after DAB2IP overexpression [12,14]. To explore the role of DAB2IP in primary cilia formation, we compared the primary cilia formability of KD vs Con under serum starvation that is known to increase primary cilia formation [15]. As shown in **Figure 1A**, the number of ciliated cells visualized with primary cilia marker (ARL13B) was significantly reduced in KD cells compared with Con cells under normal serum (NS: 10% FBS) or serum starvation (N: 1% FBS) condition. Similarly, a normal canine kidney cell (MDCK) significantly decreased primary cilia formation after knocking down DAB2IP (**Figure 1B)** in both normal and starvation condition. On the other hand, by ectopically transfecting DAB2IP cDNA into 769P and 293 cells, the number of ciliated cells dramatically increased in DAB2IP overexpressing cell (D) compared to vector control cell (Vc) regardless of the culture condition (**Figure 1C**). Furthermore, we examined the number of ciliated cells from the kidney derived from wild type (WT) and DAB2IP knockout (KO) mice. As shown in **Figure 1D**, the percentage of primary cilia in kidney epithelia determined by ARL13B and another cilia marker acetylated tubulin (ac-tubulin) (**Supplemental Figure S1**) significantly decreased in DAB2IP KO mice. Taken together, these data indicate the presence of DAB2IP is associated with primary cilia formation and this effect is independent from serum starvation condition.

Identification of KIF3a as a potential factor in DAB2IP-regulated primary cilia formation

To elucidate the downstream factor of DAB2IP in controlling primary cilia formation, we performed Mass spectrometry analysis of immunoprecipitated DAB2IP complex from cytosolic fraction and the result indicated many ciliary related proteins as potential DAB2IP-binding partners (**Figure 2A**) based on normalized spectral index, SI_N. By mapping the protein-protein association network, these candidates were imported into STRING database [16] and the result was divided into 3 groups (**Figure 2B**). The most significant group (red) contains kinesin-like protein (KIF) family, intraflagellar transport protein (IFT), pericentrin (PCNT), and some gamma-tubulin complex components that are known as primary cilia proteins [17]. The second group (blue) contains RAB guanine nucleotide exchange factor 1 (RABGEF1), vacuolar protein sorting-associated protein 16 homolog (VPS16), and vacuolar protein sorting-associated protein 45 (VPS45) that is associated with endocytosis, ciliary membrane homeostasis, and autophagy process [18–20]. The third, the most minor, group (green) is autophagy related proteins including autophagy protein 5, and 7 (ATG5, ATG7), rapamycin-insensitive companion of mTOR (RICTOR), and regulatory-associated protein of mTOR (RPTOR) [21]. Autophagy is known to be initiated by the basal body of primary cilia, in turn, promotes primary cilia formation by degrading OFD1 protein [22–24]. Knowing DAB2IP-regulated primary cilia formation is independent from serum starvation resulted in autophagy [25], we decided to focus kinesin family proteins such as kinesin family member 3A (KIF3a) from the largest group. KIF3a is an anterograde motor protein that is known as an important factor of ciliogenesis and participated in the transport mechanism in primary cilia [26]. Indeed, with mouse kidney cells derived from KIF3a WT and knockout (KO) cells more ciliated cells were found in KIF3a KO cells compared to WT cells (**Figure 2C**). Despite of no change of DAB2IP expression among KO and WT cells (**Figure 2D**), KIF3a appears to be critical in primary cilia formation.

To elucidate the potential regulation of KIF3a by DAB2IP in kidney cells, HK Con and KD cells were employed **(Figures 2D and 4A).** Our data indicated a significant reduction of KIF3a protein expression but not mRNA level in DAB2IP KD cells compared with Con cells, suggesting that DAB2IP regulates KIF3a at translational or post-translational level. A similar reduction of KIF3a protein expression was also found in MDCK KD cell. In contrast, an elevated KIF3a protein expression was seen in 769P and 293 cells with DAB2IP overexpression (**Figure 2F**), indicating that DAB2IP worked as an upstream regulator that modulate KIF3a protein level. Taken together, these data indicate that KIF3a is a key mediator in DAB2IP-regulated primary cilia formation.

The physical interaction between DAB2IP and KIF3a

Since KIF3a was identified as a potential DAB2IP-interacting protein, we further examined whether DAB2IP can physically interact with KIF3a. By immunoprecipitation with KIF3a antibody, DAB2IP was detected from HK WT cell lysate (**Figure 3A**). Vice versa, we immunoprecipitation DAB2IP-overexpressing 293 cell lysate with DAB2IP antibody, and KIF3a signal was detected (**Figure 3B**). As, expected, both KIF3a and DAB2IP proteins were clearly colocalized with primary cilia visualized by acetylated-tubulin (Ac-tubulin; primary cilia marker), and colocalized with KIF3a signal **(Figure 3C)**. Additionally, primary cilia were isolated by $Ca2^+/K^+$-shock method [27] from both HK and MDCK cell lines that express endogenous DAB2IP, and the data indicated a positive detection of DAB2IP and KIF3a proteins along with primary cilia marker Ac-tubulin (**Figure 3D**). These data suggesting that DAB2IP interacts with KIF3a at primary cilia site.

We further determined the binding domain of DAB2IP for KIF3a by expressing various flag tagged DAB2IP domain constructs in ACHN (**Supplemental Figure S2**) and 293 (DAB2IP-negative) cells and exam the KIF3a binding signal by using dot blot assay. As shown in **Figure 3E**, full length of DAB2IP showed a strong signal and slightly decreased in N terminal-DAB2IP fragment lysate then absent in C terminal-DAB2IP fragment lysate, suggesting N terminal was the major binding site of KIF3a. To validate DAB2IP N-terminal binding domain effect *in vitro*, several N-terminal domain constructs including Pleckstrin homology domain (PH), and protein kinase C conserved region 2 domain (C2) were transfected into ACHN cell. Consistently, KIF3a proteins exhibit a strong binding signal with the full length, N terminal and PH domain of DAB2IP, but very weak binding signal with C2 domain and C terminal of DAB2IP. **(Figure 3F, Supplemental Figure S2)**. To further elucidate the effect of KIF3a-DAB2IP interaction on primary cilia formation, ciliated cell percentage was analyzed in these cells by using immunofluorescence assay. Our data indicated that PH domain of DAB2IP is sufficient to maintain primary cilia similar to both full length and N-terminal DAB2IP, while C2 domain has no effect on primary cilia formation (**Figure 3G**). Based on these data, PH domain in N-terminal DAB2IP appears to be the key binding domain for KIF3a that is critical for primary cilia formation.

Post-translational regulation of KIF3a by DAB2IP

Next, we examined the functional role of this physical interaction. Previous study has indicated that DAB2IP, especially PH domain, can bind with phosphatidylinositol lipids inside of cellular membranes, these lipids including phosphatidylinositol (3,4,5)-triphosphate and phosphatidylinositol (4,5)-bisphosphate [28]. Notably, phosphatidylinositol regulates primary cilia protein trafficking, which subsequently affects primary cilia formation as well as cilia downstream signaling pathways [29]. Additionally, we found that knockdown DAB2IP reduced KIF3a protein expression but not the mRNA level **(Figure 2F and 4A)**. Thus, we believe

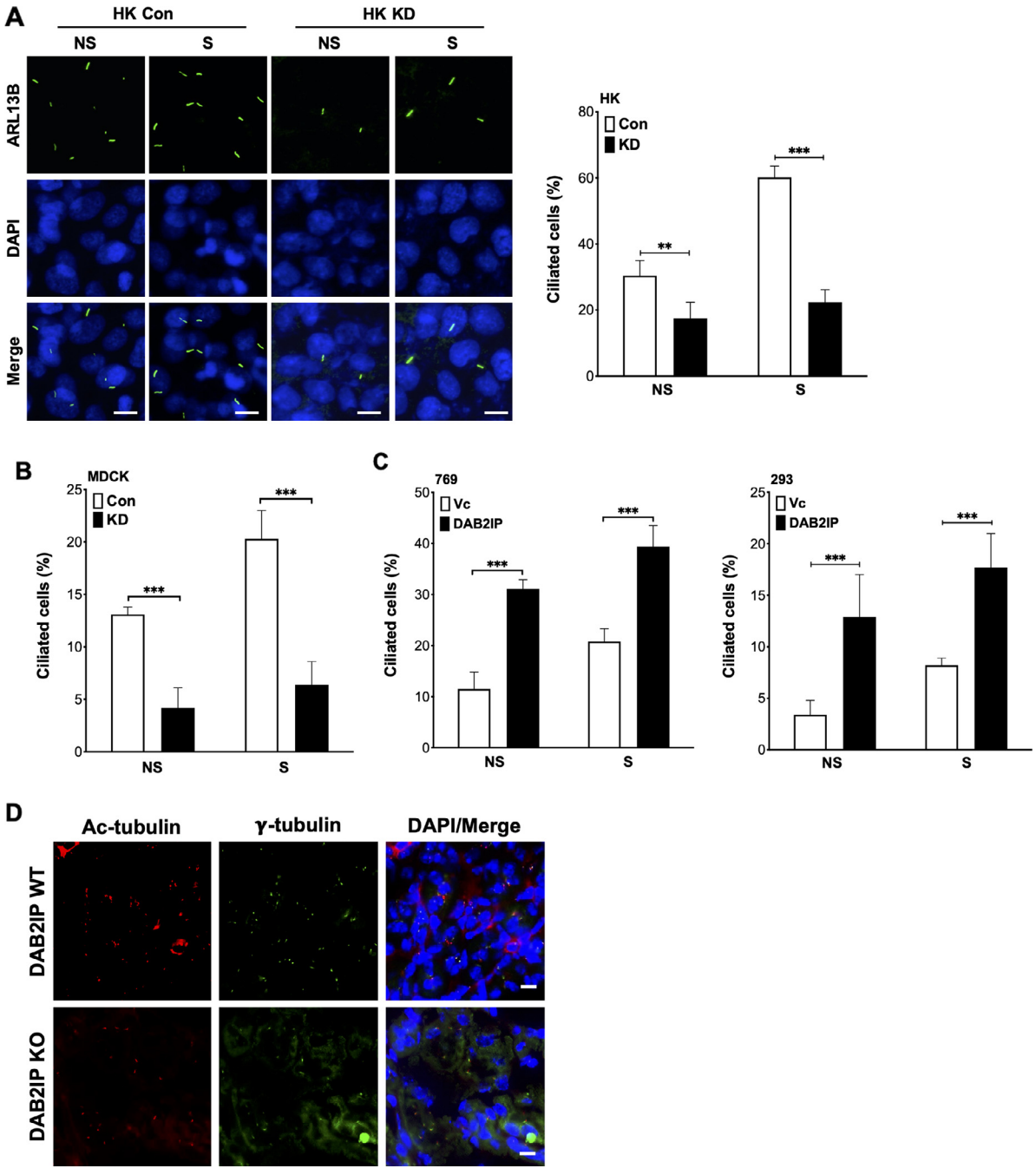


Fig. 1. The role of DAB2IP in primary cilia formation. (**A**) HK and (**B**) MDCK cells were transfected with vector control (Con) or DAB2IP knockdown vector (KD) (**C**) 769P and 293 cells were transfected with vector control (Vc) or DAB2IP expression vector (OE). All cells were cultured in either nonstarvation (NS, 5% FBS) or starvation condition (S, 1% FBS) for 16 h. All Fixed cell were stained with primary cilia marker ARL13B (Green) and nuclear counterstaining DAPI (Blue) and the number of ciliated cells was calculated (**D**) Mice kidney sections were collected in DAB2IP wild-type (WT) and knock out (KO) mice. Primary cilia were identified by primary cilia marker Ac-tubulin, and gamma-tubulin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed at least 3 times. Representative picture was shown. Statistic data represented mean ± standard deviation. DAB2IP, DAB2 Interacting Protein; FBS, fetal bovine serum; MDCK, Madin-Darby Canine Kidney cell.

the interaction of DAB2IP and KIF3a at the cilia axoneme, is critical for maintaining primary cilia. First, we examined whether DAB2IP can regulate KIF3a protein expression at post-translational level. We treated 769P WT cells with proteasome inhibitor MG132 to analyze the stability of KIF3a protein and data indicated that this treatment led to the accumulation of KIF3a as well as DAB2IP proteins, suggesting that DAB2IP can protect KIF3a from proteasome degradation process (**Figure 4B**). Hence, HK Con and KD, ACHN -Vc and -DAB2IP cells were treated with cycloheximide to block protein synthesis, and protein lysates were collected at 0, 24, 36, and 48 h post-treatment. We found that in HK Con and ACHN-DAB2IP cells loses KIF3a expression at 48 h post-treatment. On the other hand, DAB2IP low cells (HK KD and ACHN-Vc) decreased KIF3a signal at 24 h post-treatment (**Figure 4C**), suggesting that DAB2IP indeed prolongs the half-life of KIF3a. We then transfected DAB2IP or KIF3a vector

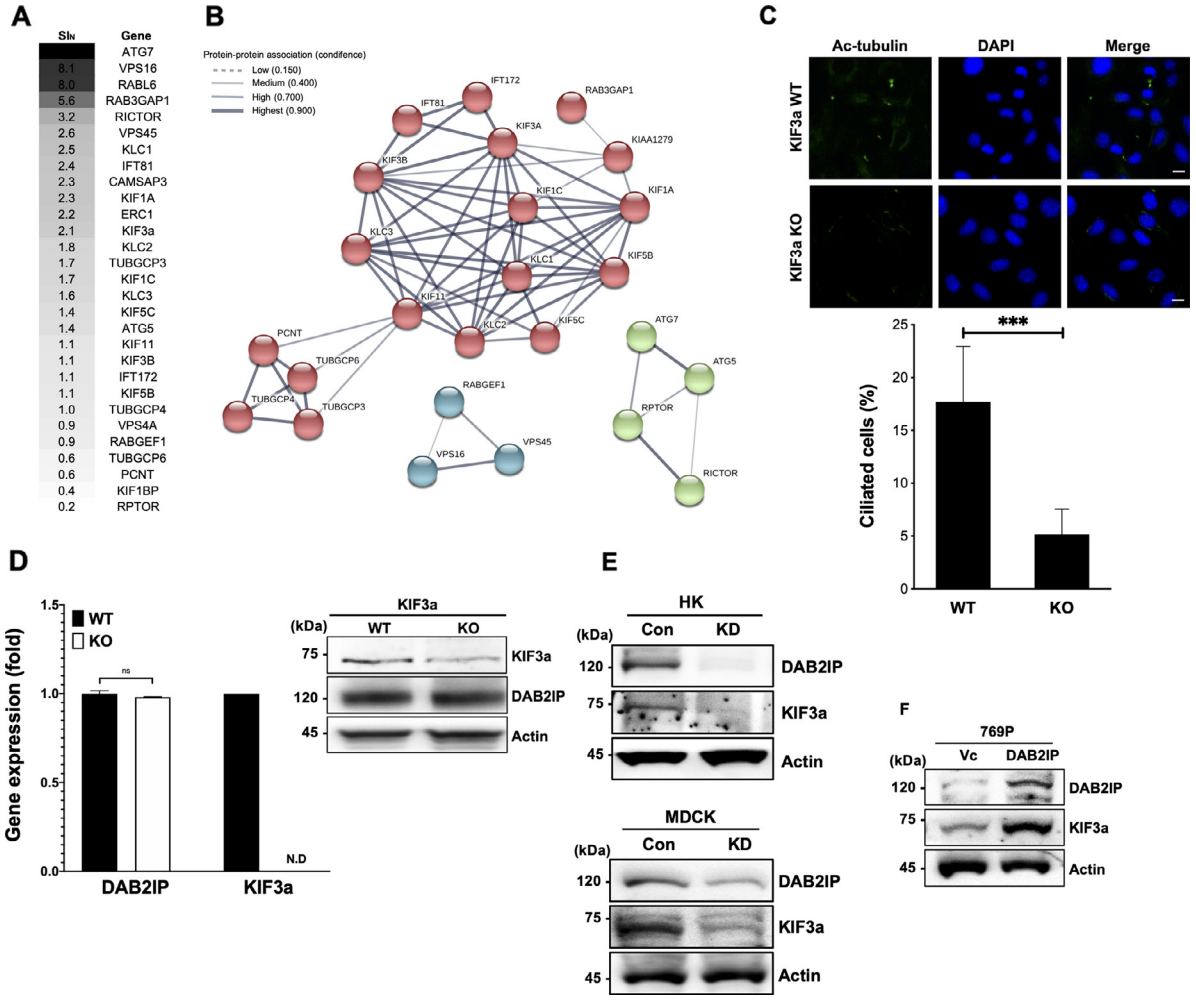


Fig. 2. KIF3a as an interactive partner of DAB2IP in primary cilia regulation. (**A**) Primary cilia related protein enriched in DAB2IP binding fraction were listed according to normalized spectral index (SI_N) and listed as heatmap format. (**B**) The protein-protein association was schemed in STING database according. Unconnected proteins were removed. Colored nodes represent query proteins. Edges represent protein-protein associations, and the density of edges represents the confidence of association. (**C**) Cells purified from KIF3a WT and KIF3a KO mice were used to analyze primary cilia forming rate. Ac-tubulin (Green) was used to visualize primary cilia, DAPI (Blue) was used to indicate nucleus. (**D**) The expression of DAB2IP and KIF3a in mouse cells was confirmed by qRT-PCR and western blot. (**E and F**) DAB2IP was either knocked down (KD) in HK and MDCK cells or overexpressed in (OE) 769P and 293 cells. The protein expression of DAB2IP and KIF3a were examined by western blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed at least 3 times. Representative picture was shown. Statistic data represented mean ± standard deviation. DAB2IP, DAB2 Interacting Protein; DAPI, 6-diamidino-2-phenylindole; KIF3a, kinesin family member 3A; MDCK, Madin-Darby Canine Kidney cell; qRT-PCR, Quantitative real-time PCR.

into 769P cells with low endogenous expression of both proteins. After confirming the expression status of both genes (Figure 4**D**), these cells were subjected to immunostaining for primary cilia detection. As shown in Figure 4**E**, consistent with our previous result, the transfection of DAB2IP into cells significantly increased the primary cilia formation. In contrast, the transfection of KIF3a alone only exhibited slightly increased primary cilia formation. As expected, the transfection of both DAB2IP and KIF3a, primary cilia formation was similar to DAB2IP transfection group. These data indicate that DAB2IP plays an important role in primary cilia formation by stabilizing KIF3a in primary cilia axoneme.

The role of KIF3a in RCC development

Since loss of primary cilia is associated with renal tumorigenesis and KIF3a is critical for primary cilia formation, we hypothesize a tumor suppressive role of KIF3a in RCC development. Data from murine KIF3a WT and KO cells or human HK WT and KIF3a KD cells demonstrated that loss of KIF3a expression can significantly increase colony formation (Figure 5**A**). On the other hand, 769P cells with KIF3a overexpression (OE) exhibit less colony formation compared with the control (Figure 5**B**). Nevertheless, it appeared that KIF3a is not engaged in the growth regulation of renal cells (**Supplemental Figure S3**). Also, we found knockdown KIF3a in HK cells decreased anchorage independent growth (AIG) compared to the control cells, vice versa, significant decreased AIG in KIF3a 769P cells with KIF3a OE compared with the control cells (Figure 5**C**), and both cell exhibit similar growth rate under similar culture condition (**Supplemental Figure S3**). Furthermore, HK Con and KIF3a KD cells were subcutaneously injected into mice to examine their tumor incidence and growth. As expected, KIF3a KD cells dramatically increase the tumor take compared to the control cells (from 20% to 100%), and the average tumor volume was also increased significantly (187.6 ± 85.7 mm^3 vs 37.3 ± 4.3 mm^3) (Figure 5**D**). By examining those tumors, we found weak or no expression of KIF3a protein regardless from Con or KD cells (Figure 5**E**). Taken together, these data indicate that loss of KIF3a can facilitate RCC development.

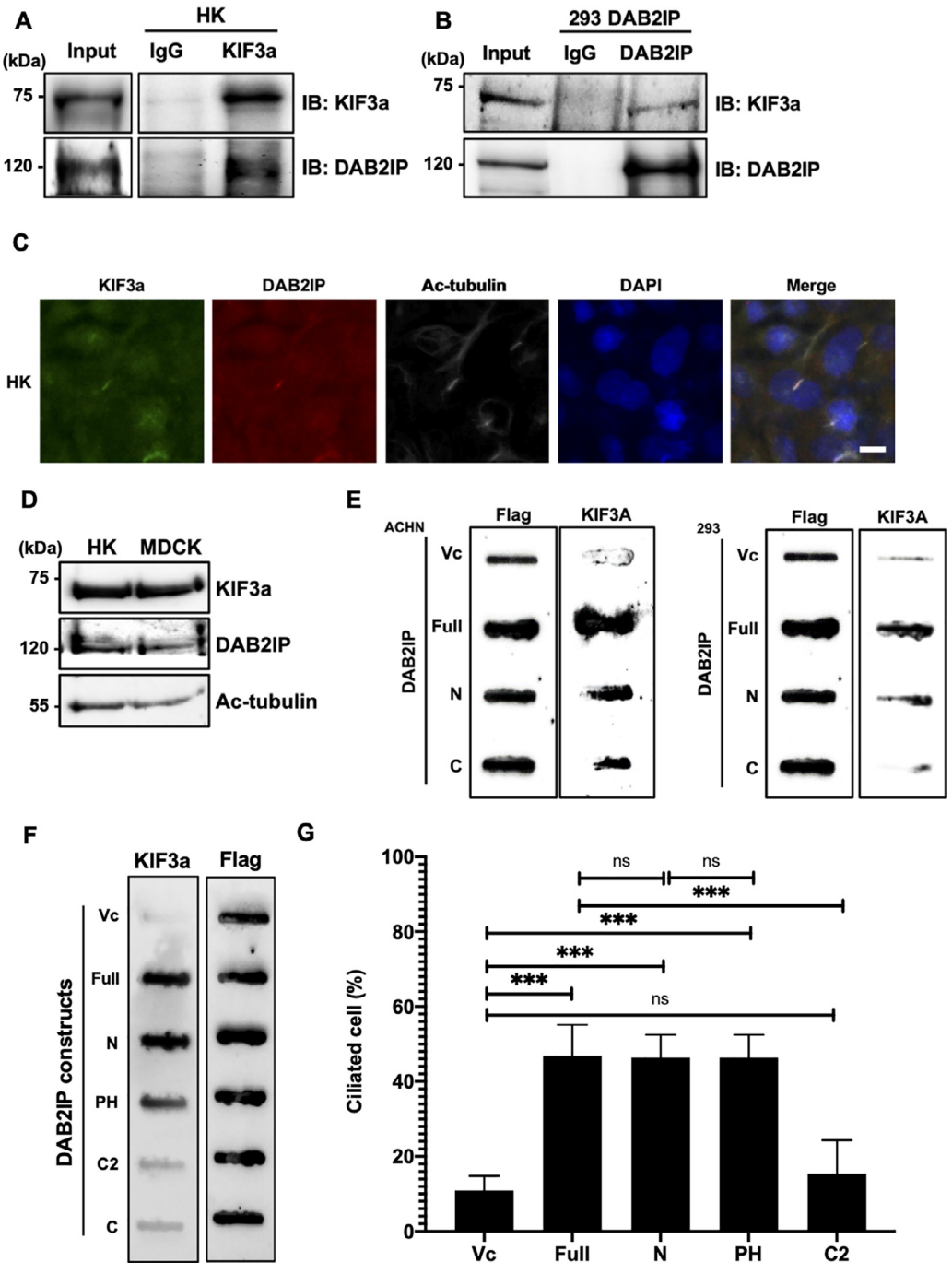


Fig. 3. Interaction of DAB2IP with KIF3a at its N-terminal PH domain. (**A**) HK cell was immunoprecipitated with KIF3a antibody, and the expression of DAB2IP and KIF3a were then immunoblotted. (**B**) 293 cells-DAB2IP OE was immunoprecipitated with DAB2IP antibody, and the expression of DAB2IP and KIF3a were then immunoblotted. (**C**) HK cell was used in immunofluorescence staining with KIF3a (Green), DAB2IP (Red), and Ac-tubulin (White) antibodies. DAPI (Blue) staining represented nucleus. (**D**) Primary cilia were cut off and isolated from HK and MDCK cells. KIF3a, DAB2IP and Ac-tubulin protein expression were analyzed. (**E**) ACHN and 293 cells were transfected with various DAB2IP constructs including vector control (Vc), Full length (Full), N terminal (N), and C terminal (C) of DAB2IP, and dot blot assay was used to analyze KIF3a binding efficacy. (**F**) DAB2IP constructs including Vc, Full, N, PH, C2, and C terminal were overexpressed in 293 cells, and the expression was determined by Flag. Dot blot assay was used to analyze KIF3a binding efficacy. (**G**) ACHN cells transfected with various DAB2IP domain constructs were used to analyze the degree of primary cilia formation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed for at least at least 3 times. Representative picture was shown. Statistic data represented mean ± standard deviation. DAB2IP, DAB2 Interacting Protein; KIF3a, kinesin family member 3A; MDCK, Madin-Darby Canine Kidney cell; PH, Pleckstrin homology.

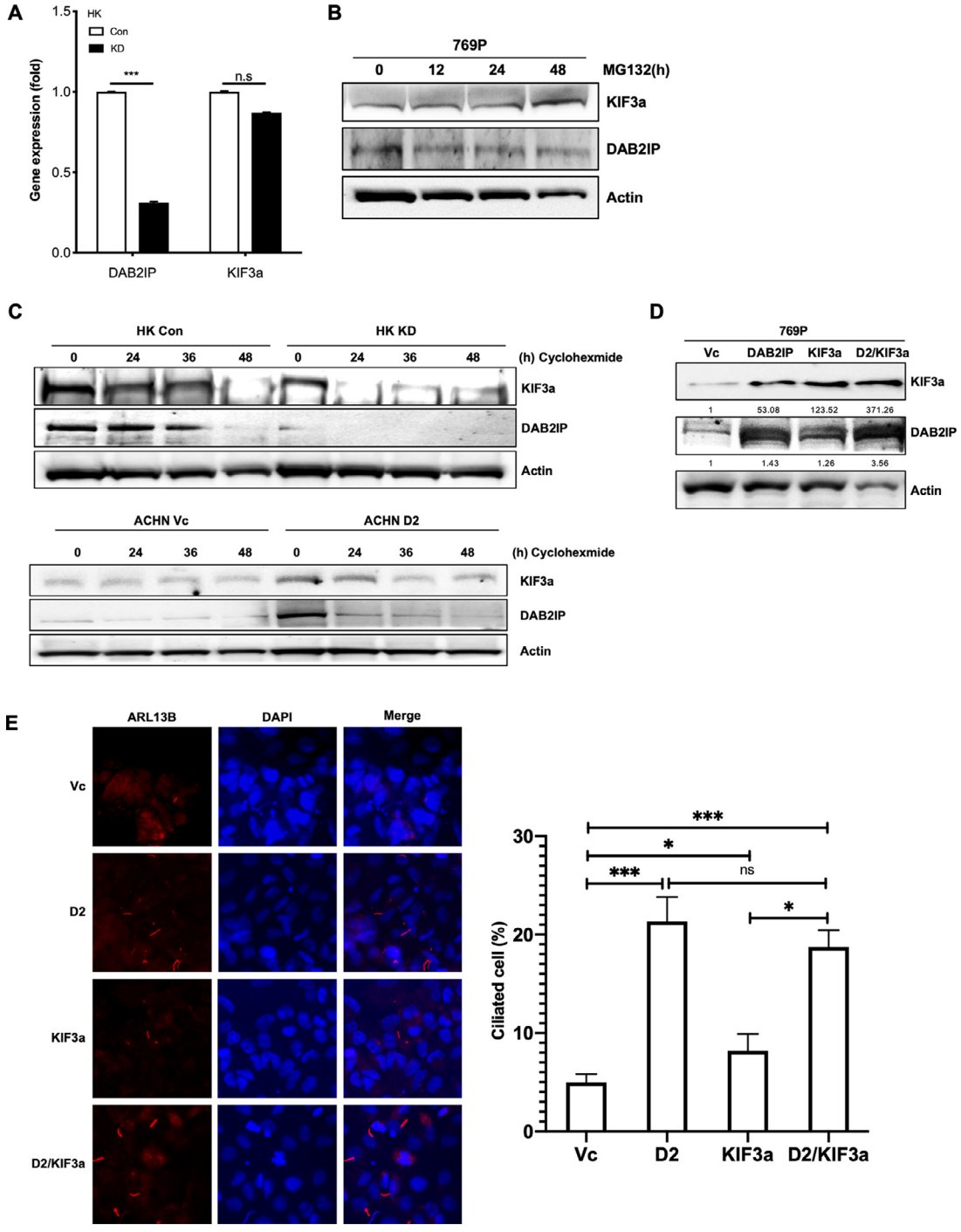
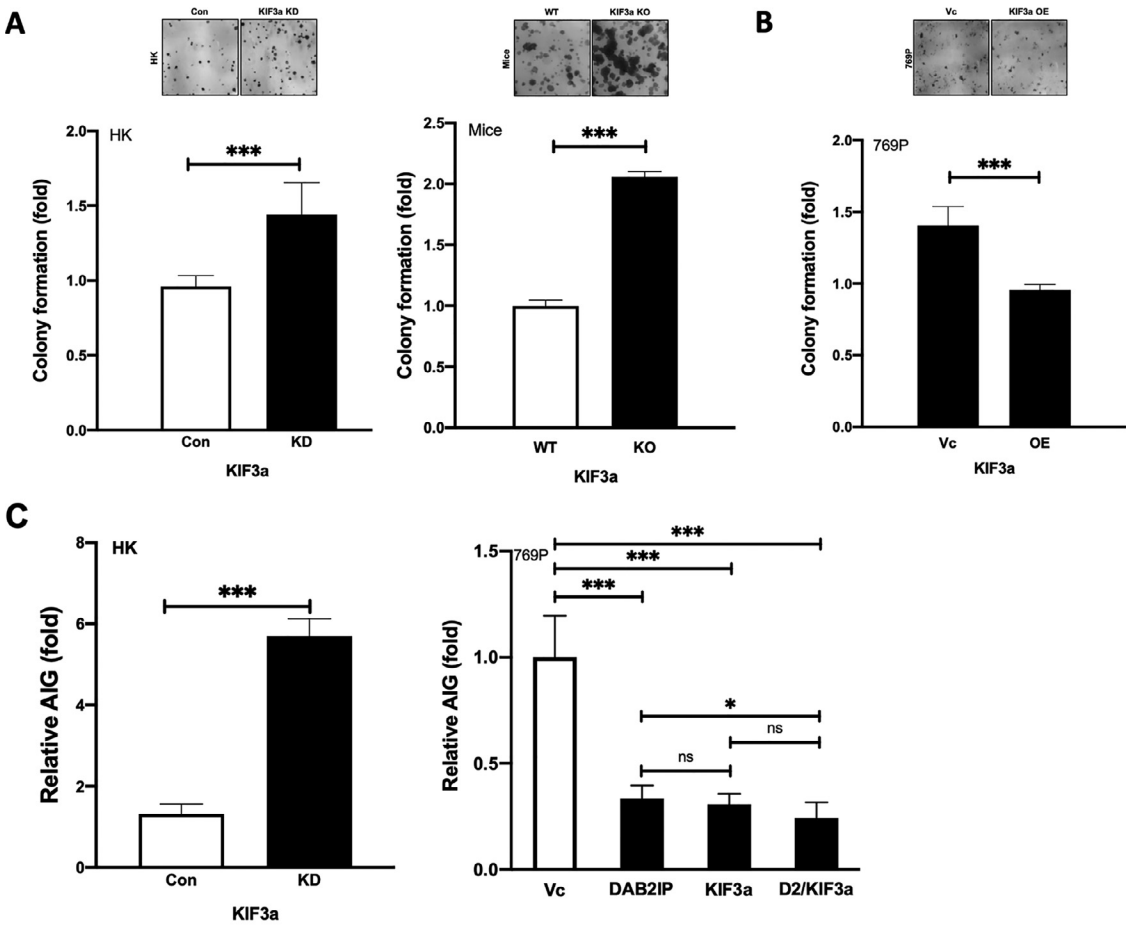


Fig. 4. The effect of DAB2IP on the protein stability of KIF3a leading to primary cilia formation. (**A**) DAB2IP and KIF3a mRNA expression were detected in HK control (Con) and DAB2IP knock down (KD) cells. (**B**) 769P wild-type (WT) cells were treated with 2.5 μM MG132 for different time points (0, 12, 24, 48 h) and the expression of KIF3a and DAB2IP protein was analyzed by western blot. (**C**) HK-Con or -DAB2IP KD cells and ACHN-Vc or -DAB2IP OE cells were treated with 10 nM at different time points (0, 12, 24, 48 h) and the expression of KIF3a and DAB2IP were analyzed by western blot. (**D**) 769P cells transfected with DAB2IP or/and KIF3a expression vectors and the expression of KIF3a and DAB2IP protein was determined by western blot. (**E**) Various 769P sublines-DAB2IP or KIF3a OE were used to examine the primary cilia formation. ARL13B (Red) was used to indicate primary cilia. DAPI was used to indicate nucleus. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed at least 3 times. Representative picture was shown. Statistic data represented mean ± standard deviation. DAB2IP, DAB2 Interacting Protein; DAPI, 6-diamidino-2-phenylindole; KIF3a, kinesin family member 3A.



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7 weeks	Tumor take (%)	Tumor Volume (mean ± SD)
HK Con	20%	37.3 ± 4.3
HK KIF3a KD	100%	187.6 ± 85.7

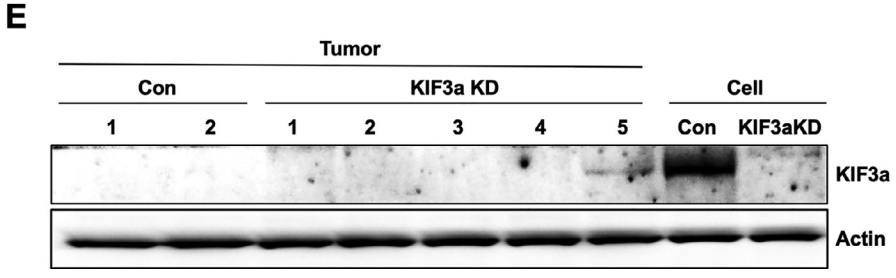

Fig. 5. The effect of KIF3a on renal tumorigenesis. (**A**) HK Con and KD cells (*right panel*), mice derived KIF3a WT and KO (*middle panel*) and (**B**) 769P Vc and KIF3a OE cells were subjected to colony forming assay with 100 cells plated onto 6-well plate for 1 wk. All colonies were stained with crystal violet and determined fold change compared to their independent control. (**C**) HK (Con, KD) and 769P (Vc, KIF3a OE) cells were subjected to anchorage independent growth (AIG) assay with 5,000 cells mixed in upper 0.6% agarose soft agar and covered on top of the 1.2 % agarose bottom agar. All cells were cultured for 2 wk and colonies were counted and determined fold change compared to their control. (**D**) HK Con and KIF3a KD (1×10^6 cells) were subcutaneously injected in SCID mice. Tumor incidence and tumor volumes were measured at Wk 7. (**E**) KIF3a expression in harvested tumors was analyzed by western blot. Cell lysates from HK Con and KIF3a KD were used as the control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed at least 3 times. Representative picture was shown. Statistic data represented mean ± standard deviation. KIF3a, kinesin family member 3A.

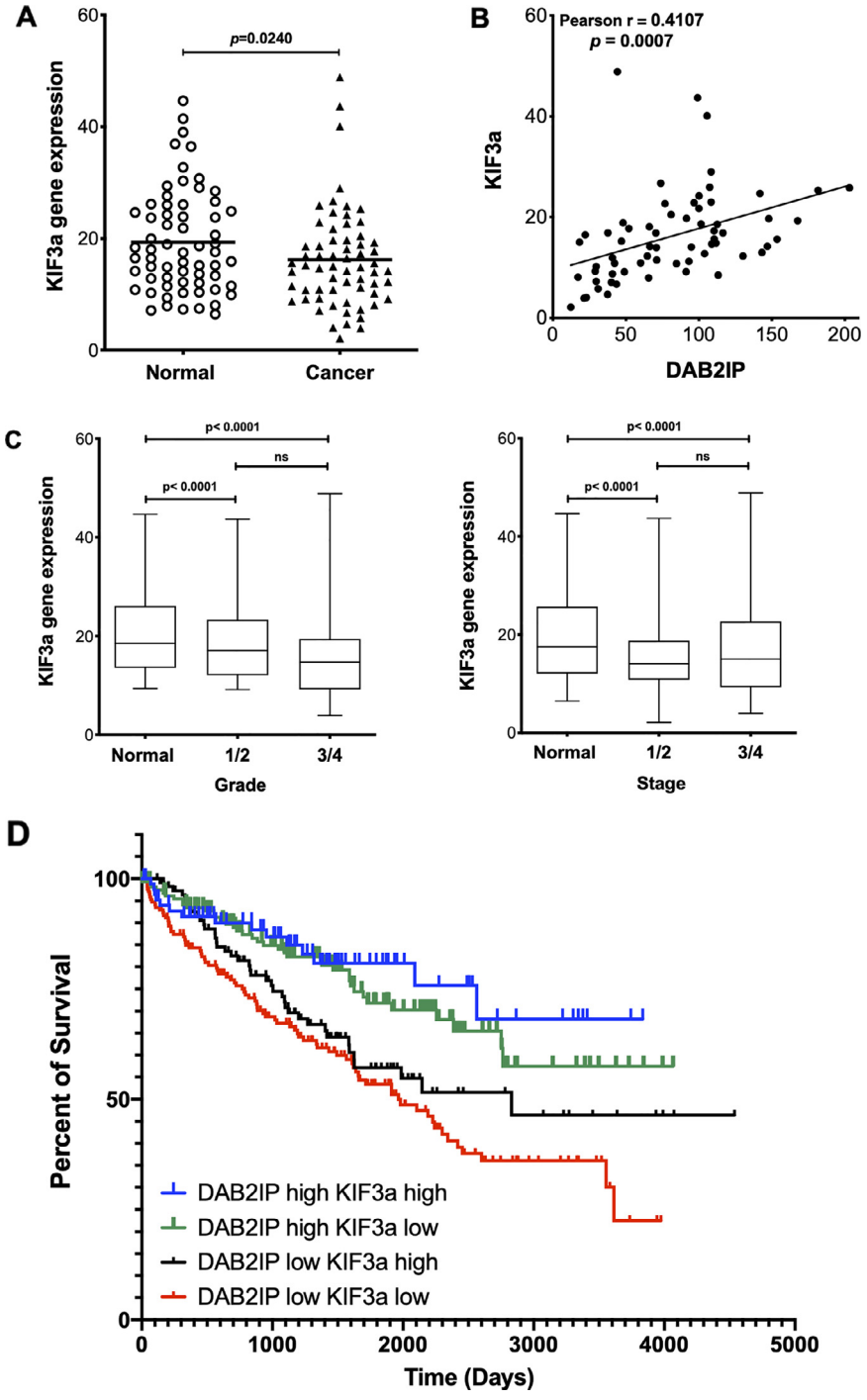

Fig. 6. Clinical relevance of KIF3a in RCC development. (**A**) KIF3a gene expression level was obtained from TCGA database. Paired sample (adjacent normal vs. tumor) were selected. (**B**) KIF3a and DAB2IP gene expression levels were obtained from TCGA database, and Pearson correlation were performed. (**C**) KIF3a gene expression levels were categorized according to individual tumor grade (normal, grade 1 and 2, grade 3 and 4) or stage (normal, stage 1 and 2, stage 3 and 4). (**D**) Patients were separated by the mid-value of KIF3a and DAB2IP expression in tumor, groups including DAB2IP^{low}/ KIF3a^{low} (Red), DAB2IP^{low}/ KIF3a^{high} (Black), DAB2IP^{high}/ KIF3a^{low} (Green), and DAB2IP^{high}/ KIF3a^{high} (Blue). The overall survival of patients was plotted accordingly. DAB2IP, DAB2 Interacting Protein; KIF3a, kinesin family member 3A; RCC, renal cell carcinoma; TCGA, The Cancer Genome Atlas.

Clinical relevance of KIF3a expression in RCC patients

We further determined any clinical correlation of KIF3a expression using The Cancer Genome Atlas (TCGA) database. The apparently decreased KIF3a expression is associated with RCC tumors compared with adjacent benign tissues from a paired sample cohort (**Figure 6A**) and the KIF3a gene expression was strongly correlated with DAB2IP gene expression (Pearson score = 0.4107, $P = 0.0007$) (**Figure 6B**). However, the onset of loss of KIF3a expression appears to be an early event during renal carcinogenesis since its decline is independent with tumor stage or grade

(**Figure 6C**). Knowing an inverse clinical correlation of DAB2IP expression with overall survival of RCC patients and mutual interaction between of these 2 genes [14], we therefore combine DAB2IP and KIF3a expression to determine whether both combinations have better prognostic value. Indeed, a combination of both DAB2IP and KIF3a markers appeared to have better predictor power (**Figure 6D**).

Discussion

Primary cilium is a microtubule-based structure that protrudes from the centrosomal mother centriole (also known as basal body) into the extracellular milieu [30]. Since cilium axoneme is anchored at basal body originated from mitosis, primary cilia are oscillated with the cell cycle by protruding out during postmitotic (G1 or G0) phase then shortening or diminishing prior to mitosis. The length of primary cilia is depended on cilium axoneme structure. The axoneme is closely controlled by IFT proteins and motors. IFT protein form 2 kinds of complex, IFT complex A and complex B. IFT complex A binds to dynein-2 motor and is responsible for retrograde transport of the cilium proteins. IFT complex B binds to kinesin-2 motor and is responsible for anterograde transport. The axoneme is covered by the extension of cell membrane but has its unique lipids and receptors, thus given cilium the ability to detect changes in the extracellular environment and transduce signal to the cell to regulate developmental and physiological processes [31]. Therefore, dysfunction of primary cilia caused by either the gene mutation or protein dysfunction is associated with multiple diseases known as ciliopathies [32]. It is known that primary cilia regulated signaling pathways including Hedgehog, Wnt, G protein-coupled receptors, transforming growth factor-β/bone morphogenetic protein receptors, receptor tyrosine kinases and platelet-derived growth factor signals. Notably, these signaling pathways play active role in renal tumorigenesis. Studies have found Hedgehog signal was reactivated and promotes tumor growth in RCC [33,34]. Wnt signal was correlated with tumor growth, stage, and grade [35]. Abnormal G protein-coupled receptor signal and receptor tyrosine kinases account for tumor vascularization phenotypes during renal tumorigenesis [36]. Transforming growth factor-β and bone morphogenetic protein receptors have been reported to correlate with RCC progression [37,38]. Finally, several tyrosine kinase inhibitors mainly targeting the vascular endothelial growth factor receptor and platelet-derived growth factor receptor, such as Sunitinib, Pazopanib, and Tivozanib, have been used to treat metastatic RCC [39].

DAB2IP known as AIP1 or DIP1/2, has been found to be a potent tumor suppressor in many types of cancer including prostate, lung, gastrointestinal tumors as well as RCC [40]. In RCC, hypoxia-inducible factors (HIF) are highly activated due to frequent loss of von Hippel-Lindau (*VHL*) gene, while *VHL* have been shown to regulate ciliogenesis in RCC [41]. We have shown that loss of DAB2IP is correlated with the elevated expression of HIF-2α in RCC [14], implying a link between DAB2IP and primary cilia formation. By profiling DAB2IP-binding proteins in cytosol fraction, we identified primary cilia regulatory factor KIF3a as a potential candidate. KIF3a is, a reserved subunit of kinesin-2 complex, critical for primary cilia formation [42,43]. There are 2 kinesin-2 complexes including KIF3a/3b/KAP3 or KIF3a/3c/KAP3. The KIF3a/3b complex is known to function as transport motor on axoneme, while KIF3A/3c complex was mainly found in neuron cell [44]. Loss of KIF3a has been found to cause ciliopathies like cystic kidney disease [10]. Also, loss of KIF3a in combination with *VHL* and *p53* gene deletions is found in neoplastic renal lesions [45]. Yet, despite loss of primary cilia phenotype is frequently associated with RCC tissues, the role of KIF3a in primary cilia formation underlying RCC tumorigenesis still remains undetermined.

Herein, we have demonstrated that DAB2IP is able to prevent KIF3a from proteasome degradation and interact with KIF3a through its N-terminal PH domain that is related with the plasma membrane localization, supporting that DAB2IP is able to protect KIF3a located at cilia axoneme [46]. In addition, KIF3a is a potent tumor suppressor in renal tumorigenesis, thus, we believe DAB2IP-KIF3a interaction also play a critical role in maintaining normal cell homeostasis mediated through cilia signaling. Studies have indicated the loss of DAB2IP gene is correlated with poor outcome of RCC patients. We searched TCGA database to document that DAB2IP gene copy number was decreased and correlated with tumor grade and stage. However, KIF3a copy number was higher in RCC specimens compared with normal specimens but no significant different among tumor grades or stages **(Supplemental Figure S4).** Nevertheless, other clinical transcriptomic data indicate reduced KIF3a expression in RCC specimens compared with normal specimens (Figure 6A), which is correlated with tumor grade and stage leading to poor overall survival of RCC patients (Figure 6C and D) as well as increased KIF3a turnover in DAB2IP-negative RCC cells (Figure 4), suggesting that KIF3a status is likely regulated post-transcriptionally and/or post-translationally.

Primary cilia can be regulated by starvation [22], while starvation could also induce autophagy signaling through mTOR [47]. Autophagy plays as a double-edged sword in primary cilia formation that degrades OFD1 protein to promote ciliogenesis or degrade primary cilia intraflagellar transport protein to retract cilia formation [22,23]. However, it appears that DAB2IP-regulated primary cilia are independent from autophagy, which implies a new regulatory mechanism of primary cilia formation. In addition, our previous study demonstrated that loss of DAB2IP in RCC enhance the resistance of mTOR-targeted therapy [14]. Furthermore, studies have shown that mTOR functioned through primary cilia [48,49], suggesting that mTOR signaling was sabotaged during the dysfunction of primary cilia. Taken together, these data shade a light that loss primary cilia which contributed from decreased DAB2IP, might participated in the resistance of mTOR-targeted therapy.

Conclusion

In this present study, we are the first to report the interaction of DAB2IP with KIF3a is critical for primary cilia; DAB2IP is shown to regulate KIF3a protein turnover, indicating that DAB2IP could stabilize KIF3a in the axoneme for the maintenance of integrity of primary cilia. Additionally, we have also shown that loss of KIF3a could promotes RCC tumorigenesis, suggesting that DAB2IP-KIF3a complex associated with primary cilia is one of critical homeostatic machinery in normal kidney epithelia. In conclusion, these appear to be a new function of DAB2IP in normal kidney cells by regulating primary cilia formation, which is independent from starvation-induced primary cilia pathway. Mechanistically, KIF3a is identified to physically interact with N-terminal PH domain of DAB2IP resulting in increasing its half-life and primary cilia formation. Furthermore, loss of KIF3a leads to the destruction of primary cilia further promotes renal tumorigenesis.

Credit author statement

Chun-Jung Lin: Conceptualization, methodology, writing- original draft preparation, investigation.

Andrew Dang: Methodology, investigation.

Elizabeth Hernandez: Methodology, investigation.

Jer-Tsong Hsieh: Conceptualization, supervision, writing- reviewing and editing.

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Declaration of competing interest

All authors declare that they have no competing financial or nonfinancial interests that might have influenced the performance or presentation of the work described in this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2020.12.002.

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