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The von Hippel–Lindau tumour suppressor interacts with microtubules through kinesin-2

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Abstract Synthesis and maintenance of primary cilia are regulated by the von Hippel–Lindau (VHL) tumour suppressor protein. Recent studies indicate that this regulation is linked to microtubule-dependent functions of pVHL such as orienting microtubule growth and increasing plus-end microtubule stability, however little is known how this occurs. We have identified the kinesin-2 motor complex, known to regulate cilia, as a novel and endogenous pVHL binding partner. The interaction with kinesin-2 facilitates pVHL binding to microtubules. These data suggest that microtubule-dependent functions of pVHL are influenced by kinesin-2.

Keywords: pVHL; Kinesin-2; Microtubules; Primary cilium

1. Introduction

The rare hereditary von Hippel–Lindau (VHL) disease is caused by heterozygous germline mutations in the *VHL* gene [1], which predisposes patients to a variety of cysts and tumours. Multifocal renal cysts exhibit loss of *VHL* heterozygosity [2]; moreover, kidney-specific inactivation of *vhl* in mice results in the development of kidney cysts [3]. Both isoforms, pVHL30 and pVHL19, function as an ubiquitin E3 ligase, targeting hydroxylated hypoxia inducible factor alpha (HIFα) for poly-ubiquitination and subsequent proteasomal degradation [1,4,5]. Hypoxia interferes with HIFα-hydroxylation, thereby enabling stabilised HIFα to transactivate genes necessary for adjusting to a hypoxic environment.

Recent studies support a function for pVHL in the primary cilium [6,7]. The axoneme core of cilia serves as tubulin scaffolding for the kinesin-2 complex, which transports all cargo into the cilium [8]. Accordingly, genetic disruption of any of the individual components of the kinesin-2 complex (KIF3A, KIF3B, and KAP3) invariably results in cilia dysfunction and renal cyst development [9]. Here, we report the identification of kinesin-2 as novel and endogenous pVHL binding partner, mediating binding to microtubules (MT). Since pVHL and kinesin-2 have overlapping functions regarding primary cilia regulation, these data may provide novel mechanistic insight into MT-dependent regulation of primary cilia by pVHL.

2. Materials and methods

2.1. Construction of plasmids

All expression constructs were generated using pcDNA3-derived vectors (Promega, USA) containing either N-terminal Myc- or Vsv-tag by standard cloning techniques. GFP-fusion constructs were generated by cloning into pEGFP-C1/2 (Clontech, USA). KIF3A (gift from Dr. L. Goldstein), KIF3B, ΔN-KAP3 (gift from Dr. T. Akiyama), VHL-Δ95-123 and VHL-Y112H (gift from Dr. W. Krek) constructs were cloned into the appropriate vectors. pBABE-puro was a gift from Dr. G. Nolan.

2.2. Yeast-two-hybrid screen

A human fetal brain cDNA library (Matchmaker, Clontech) was co-transfected with VHL-pMD4 into *Saccharomyces cerevisiae* strain Hf7c. Plasmids were recovered from His+/LacZ+ clones and tested for specificity with irrelevant baits and 2.5 mM or 25 mM of 3-amino-triazole (3AT).

2.3. Cell culture

HEK293T, MDCK, murine neuroblastoma N1E-115 cells, and murine C26 colorectal carcinoma cells were cultured in DMEM supplemented with antibiotics and 5–10% fetal calf serum. Primary kidney cells were isolated from 4-day-old mice by sequential trypsinisation steps and cultured in DMEM supplemented with antibiotics and 20% fetal calf serum to confluency. Cells cultured to produce cilia, received serum-free medium for an additional 3 days after reaching confluency to stimulate cilia outgrowth. Transfections were performed using Fugene-6 (Boehringer Mannheim, Germany). MG132 (5 μM; Sigma–Aldrich, USA) was used for 8 h to inhibit proteasomal degradation. Desferoxamine (DFO) (100 μM; Sigma–Aldrich) was used for 4 h to stabilise HIFα.

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Abbreviations: pVHL, von Hippel–Lindau tumour suppressor protein; HIFα, hypoxia inducible factor alpha; MT, microtubules; DFO, desferoxamine; IVTT, in vitro transcription/translation

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2.4. Retroviral transductions

After 24 h transfection with the indicated pBABE plasmids, Phoenix ecotropic packaging cells (gift from Dr. G. Nolan) were incubated at 32 °C for 24 h before harvesting culture supernatants. N1E-115 or C26 cells were transduced with sterile-filtered culture supernatants 24–36 h at 32 °C with 8 μg/ml polybrene. Fresh medium supplemented with puromycin (1 μg/ml; Sigma–Aldrich) replaced the supernatants for an additional 24 h at 37 °C.

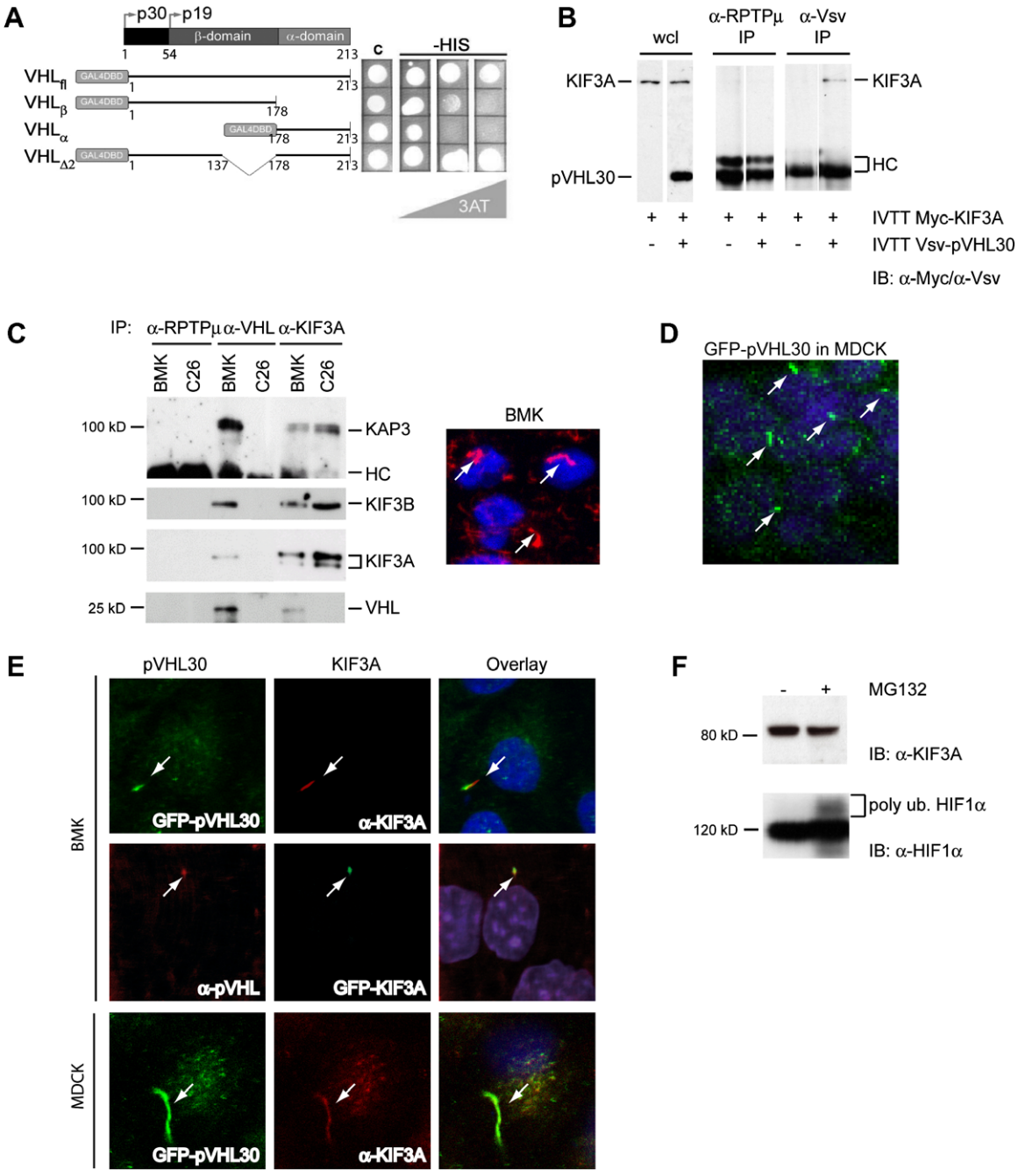

Fig. 1. pVHL interacts with kinesin-2. (A) Yeast-two-hybrid assay with Gal4 DNA binding domain fused to: full-length pVHL (VHL_{fl}), the acidic and β-domain of pVHL (VHL_{β}), the α-domain of pVHL (VHL_{α}) or pVHL lacking exon 2 ($VHL_{\Delta 2}$) used as bait and KIF3A used as prey. Right panel: colony growth of transformed yeast, using increasing 3-aminotriazole (3AT; 0, 12.5 and 25 mM) without histidine (-HIS). Control colony (c) in the presence of histidine. (B) Western blot analysis of IVTT Myc-KIF3A binding to IVTT Vsv-pVHL30. α-RPTPμ serves as unrelated antibody control. wcl, whole cell lysates; HC, immunoglobin heavy chain. IB, immunoblot. (C) Western blot analysis of endogenous KAP3, KIF3B, KIF3A and pVHL recovery after α-RPTPμ, α-VHL and α-KIF3A IPs in VHL-deficient C26 cells and ciliated baby mouse kidney (BMK). Right panel, BMK cells stained for acetylated tubulin (red), demonstrate cilia (arrows) when used for IP. DAPI (blue) stains nuclei. (D) Translocalisation of GFP-pVHL30 to multiple cilia in MDCK cells. DAPI (blue) stains nuclei. (E) pVHL30 co-localises with a subset of ciliary KIF3A in kidney cells. Confocal images of BMK and MDCK cells transfected with GFP-pVHL30 co-localising with endogenous KIF3A and conversely, GFP-KIF3A co-localises with endogenous pVHL. DAPI (blue) stains nuclei. (F) Exogenous KIF3A does not accumulate or become poly-ubiquitinated like HIF1α after MG132 treatment.

2.5. Immunoprecipitations

Approximately 1×10^6 cells were lysed in 400 μl buffer (20 mM TRIS, 1% Triton-X-100, 140 mM NaCl, 10% glycerol, pH 8.0) with protease inhibitors (Roche, Switzerland), then centrifuged 10 min at 13 000 rpm. Mouse monoclonal α-VHL (1.5 μg; Ig32; BD-Pharmingen, USA) coupled to protein A/G agarose beads (7.5 μl; Santa-Cruz Biotechnology, USA), was added to 170 μl cleared lysate. For endogenous IPs, 1×10^7 cells were lysed in 400 μl buffer of which 100 μl fresh cleared lysate was added to 25 μl protein A/G agarose beads pre-coupled to 7.5 μg mouse monoclonal α-VHL, 7.5 μg mouse monoclonal α-KIF3A (BD-Transduction Labs, USA), or 7.5 μg mouse monoclonal α-RPTPμ (clone 3D7) for specificity control. IPs were incubated 4 h at RT, washed three times and analysed by Western blot. In vitro transcription/translation (IVTT) samples were generated according to manufacturer's protocol (Promega).

2.6. Immunofluorescence staining

Fixed cells were incubated with α-KIF3A (1:500; BD-Transduction Labs), α-acetylated tubulin (1:20 000; Sigma–Aldrich) or α-VHL (1:500; Ig32, BD-Pharmingen). Goat-anti-mouse Alexa568 (1:400, Molecular probes, USA) was used as secondary antibody, as previously described [10].

2.7. Western blotting

Standard western blots [11] were incubated with α-Myc (9E10, hybridoma supernatant 1:5), α-Vsv (P4D5, hybridoma supernatant 1:5), α-VHL (1:500, Ig32, BD-Pharmingen), α-KIF3A, α-KIF3B, α-KAP3 (1:500; BD-Transduction Labs), anti-HIF1α (1:500, BD-Pharmingen) or α-MAPK (1:500, gift from Dr. O. Kranenburg).

2.8. Microtubule co-sedimentation assays

Transfected HEK293T cells were lysed in PTN buffer (10 mM Pipes, 30 mM TRIS, 50 mM NaCl, 1 mM EGTA, 1.25 mM EDTA, 1 mM DTT, 1% Triton-X-100, protease inhibitors, pH 6.3). IVTT samples were generated according to manufacturer's protocol (Promega). Lysates were pre-cleared by centrifugation at $100\,000 \times g$ for 45 min. Co-sedimentation assays were performed using the MAP Spin-down Biochem Kit (Cytoskeleton, USA) with the following adjustments: polymerised MT were centrifuged at $100\,000 \times g$ for 30 min before being resuspended in pre-cleared lysates containing 50 μM paclitaxel and AMP–PNP (Sigma–Aldrich; final concentration 10 mM) where indicated, and incubated for 30 min. Samples were loaded onto 100 μl of the kit's cushion buffer and centrifuged at $100\,000 \times g$ for 40 min. Pre-cleared lysates, supernatant and pellet fractions were analysed by western blot.

2.9. Reporter assays

HIF reporter assays were performed in duplicate with 0, 100, or 300 ng Myc-ΔN-KAP3 and/or 100 ng wild-type HIF1α as previously described [11].

3. Results

3.1. VHL interacts with kinesin-2

We performed a yeast-two-hybrid screen of a human fetal brain cDNA library with full-length pVHL as bait. Eight clones bound pVHL robustly in this assay, including a known pVHL interactor, chaperonin CCTη [12]. Interestingly, one of the remaining clones has a strong link to cilia regulation: KIF3A, component of the kinesin-2 microtubule motor complex. We re-transformed yeast with KIF3A and four different pVHL bait constructs and determined that the first 136 residues of pVHL largely determine this interaction (Fig. 1A). Co-immunoprecipitation (IP) assays using IVTTpVHL and KIF3A, validated the direct nature of this interaction (Fig. 1B). Suggesting physiological relevance,

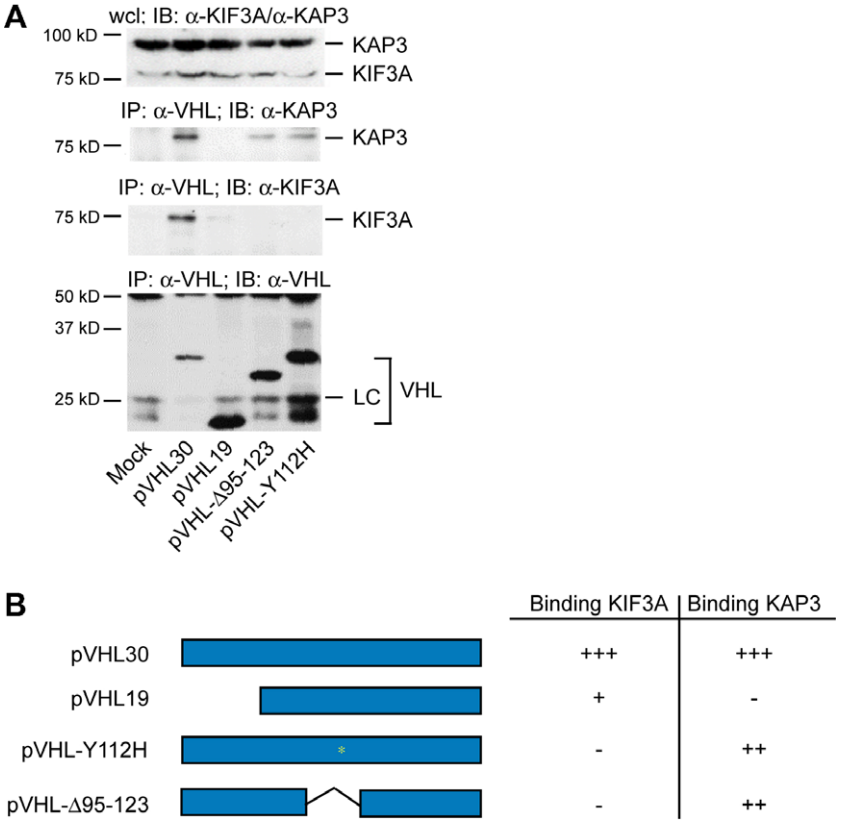

Fig. 2. Characterising pVHL-kinesin-2 interaction. (A) α-VHL IPs of retrovirally reconstituted pVHL variants in C26 cells. Upper panel, input endogenous KIF3A and KAP3. Second and third panels, co-precipitation of endogenous KAP3 and KIF3A, respectively. Lower panel, pVHL recovery. LC, immunoglobin light chains. (B) Schematic representation of pVHL allelic variants and their ability to bind KIF3A and KAP3.

immunoprecipitating endogenous pVHL readily pulled down KIF3A, KIF3B and KAP3 in ciliated primary mouse kidney cells (Fig. 1C); likewise, α-KIF3A pulled down pVHL, while an irrelevant antibody (α-RPTPμ) did not retrieve either pVHL or KIF3A (Fig. 1C). Confocal microscopy showed endogenous pVHL or GFP-pVHL30 translocation to multiple cilia in kidney cells partially overlapping with KIF3A localisation (Fig. 1D and E). Because pVHL functions as an E3 ubiquitin ligase, we blocked proteasomal degradation with MG132 and observed no increased cellular levels or poly-ubiquitination of KIF3A (Fig. 1F). Taken together, these data suggest endogenous interaction between pVHL and kinesin-2 in primary kidney cells, not affecting KIF3A turnover.

3.2. Mutant VHL alleles do not bind kinesin-2

We characterised the binding of pVHL to kinesin-2 by testing pVHL30, pVHL19, pVHL-Δ95-123, implicated in defective regulation of MT stability [13] and pVHL-Y112H, a patient missense mutation. Retroviral reconstitution of these VHL allelic variants in VHL-deficient C26 cells was confirmed by α-VHL IP and analysed for binding to endogenous KIF3A and KAP3 (Fig. 2A). While transduced pVHL30 precipitated endogenous KIF3A and KAP3, pVHL19 was less effective at co-precipitating KIF3A and failed to co-precipitate KAP3. The opposite was true for the deletion variant pVHL-Δ95-123 and the Y112H patient mutation, which failed to co-precipitate KIF3A, but recovered some KAP3. These data indicate that the N-terminal acidic domain mediates binding of pVHL30 to KAP3 and that the MT-binding domain is required for KIF3A binding to pVHL (Fig. 2B).

3.3. pVHL-KIF3A binding does not affect HIFα function

To experiment with the pVHL-KIF3A interaction, we checked whether pVHL could bind a dominant-negative vari-

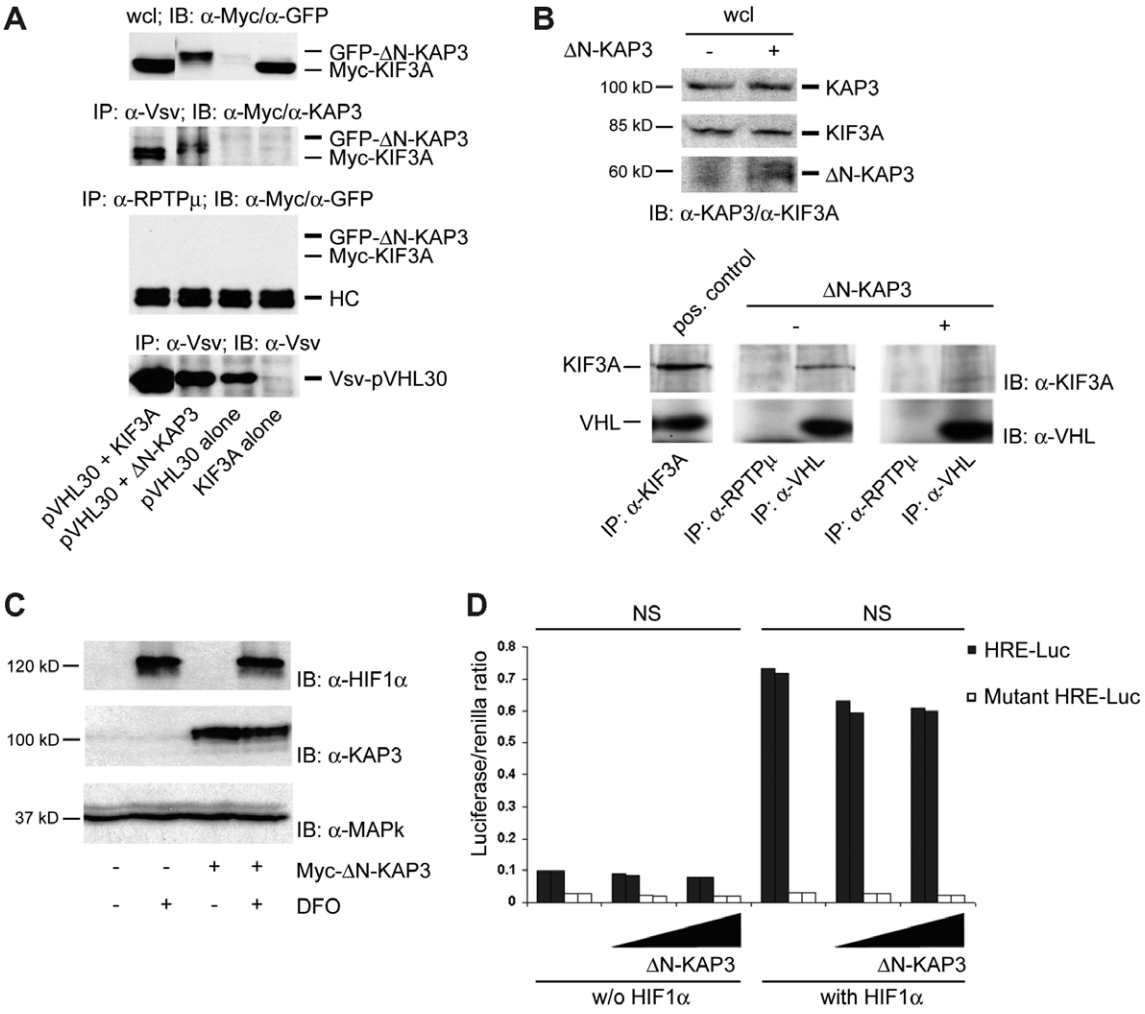

Fig. 3. ΔN-KAP3 disrupts pVHL-KIF3A binding. (A) α-Vsv IPs of transfected Vsv-pVHL30 with Myc-KIF3A or GFP-ΔN-KAP3. Upper panel, transfection efficiency for Myc-KIF3A and GFP-ΔN-KAP3. Second panel, Myc-KIF3A and GFP-ΔN-KAP3 recovery in α-Vsv IP fractions. Third panel, aspecific protein recovery in α-RPTPμ IPs. Bottom panel, Vsv-pVHL30 recovery. (B) ΔN-KAP3 disrupts endogenous pVHL-KIF3A interaction. Upper panel, endogenous KAP3 and KIF3A. KIF3A IP shows specific recovery of KIF3A (positive control). Retrovirally transduced pBABE-puro with either no insert (−) or ΔN-KAP3 (+) specifically affects KIF3A recovery in α-VHL IPs. α-RPTPμ, unrelated antibody control. All IPs are from the same blot, same exposure, but lanes have been rearranged to improve presentation. (C) HIF1α response to DFO not affected by ΔN-KAP3. Upper panel shows endogenous HIF1α expression in HEK293T; lanes treated with DFO or transfected with empty vector/Myc-ΔN-KAP3, as indicated. Middle panel, expression Myc-ΔN-KAP3. Lower panel, MAPK loading control. (D) Hypoxia Response Element reporters (HRE-Luc; filled bars) or mutated HRE (Mutant HRE-Luc; empty bars) with 0, 100 or 300 ng Myc-ΔN-KAP3 with or without 100 ng HIF1α. Response to 100 ng HIF1α was statistically significant ($P = 0.0004$). NS, not significant.

ant of the non-motor subunit KAP3 (missing residues 1–184; ΔN-KAP3), which disrupts binding between tumour suppressor APC and kinesin-2 [14]. Indeed, exogenously expressed Vsv-pVHL30 could co-precipitate GFP-tagged ΔN-KAP3 (Fig. 3A). To validate an effect of ΔN-KAP3 on pVHL30-KIF3A binding, α-VHL IPs were performed with N1E-115 cells either mock or ΔN-KAP3-transduced, and loss of endogenous binding between pVHL30 and KIF3A was observed (Fig. 3B). ΔN-KAP3 however, does not affect HIF1α stabilisation in response to DFO (Fig. 3C); nor are HIF responsive reporter assays affected by increasing amounts of ΔN-KAP3 (Fig. 3D). Therefore, we concluded that the interaction between pVHL and KIF3A does not affect HIF1α regulation or function.

3.4. KIF3A increases pVHL binding to microtubules

To address the possibility that kinesin-2 mediates the previously reported binding of pVHL30 to MT [13], we performed MT co-sedimentation assays with lysates of HEK293T cells transfected with Vsv-pVHL30 (Fig. 4A). To determine whether this association of pVHL with MT is direct, we repeated this assay using highly purified tubulin and in vitro translated full-length pVHL; yet pVHL30 never bound MT in this assay. These data suggest that endogenous cellular factors, e.g. kinesin-2, enhance binding of pVHL to MT. Two groups have previously performed microtubule co-sedimentation assays with pVHL. Most recently, Schermer et al. [15] and Thoma et al. [7] show that cell lysates overexpressing pVHL copelleted with polymerised tubulin; however, endogenous factors such as kinesin-2 are also present in these cell lysates. Schermer et al. [15] also show that VHL IPs tubulin, again in a lysate setting. The discrepancy between our data and Hergovich's published data using IVTTpVHL30 [13] might be explained by different protocols used in the two studies; we used a sucrose cushion in the co-sedimentation assay. This sucrose cushion serves as a density gradient separating the lysate from the pellet and increasing the specificity of the spun down fraction. To address the different outcomes, we directly compared the two methods with in vitro translated pVHL30 in the presence or absence of a sucrose cushion separation. In the absence of the sucrose cushion we found more pVHL in the pellet with MT than in the pellet without MT (Fig. 4A). However, using the modified assay (with sucrose cushion) we found no direct interaction between MT and

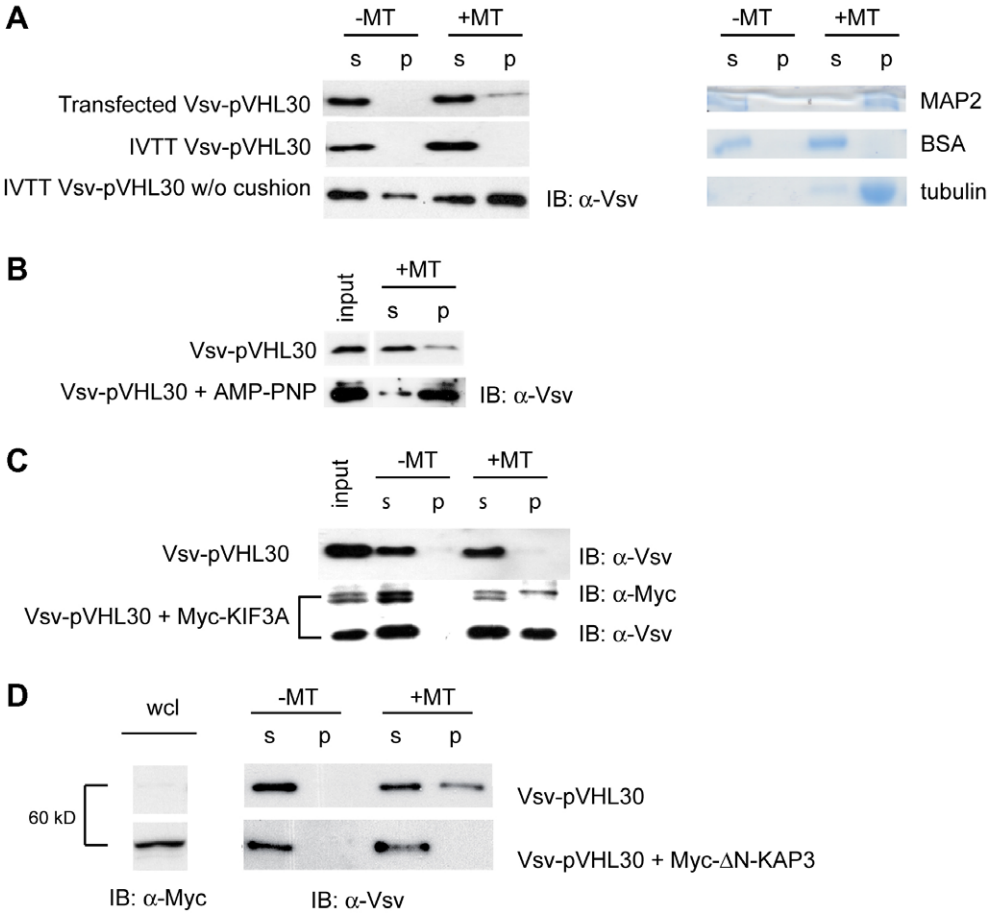

Fig. 4. KIF3A mediates binding of pVHL to microtubules. (A) Left panels, transfected vs. IVTT Vsv-pVHL30 recovered from supernatant (s) or pellet (p) in a microtubule co-sedimentation assay. MT, microtubules. Lower panel, same assay repeated without the recommended cushion buffer using IVTT Vsv-pVHL30. Right panels, Coomassie Blue stainings of MT co-sedimentation controls tubulin, Microtubule-Associated Protein 2 (MAP2; serves as a positive control) and Bovine Serum albumin (BSA; serves as a negative control). (B) Recovery of transfected Vsv-pVHL30 in supernatant and pellet of microtubule spin down fractions without (upper panel) or with AMP–PNP (lower panel). (C) Upper panel, recovery of transfected Vsv-pVHL30 from supernatant and pellet with or without MT. Middle and lower panel, recovery of pVHL and KIF3A using HEK293T transfected with Vsv-pVHL30 and Myc-KIF3A. (D) Upper panel MT co-sedimentation of Vsv-pVHL30 transfected with empty vector or with Myc-ΔN-KAP3 (60 kD). Expression Myc-ΔN-KAP3 shown on the left.

pVHL (Fig. 4A). Because MT motor proteins such as KIF3A/3B require nucleotides for MT-binding, we repeated these assays in the presence of AMP–PNP, a non-hydrolysable form of ATP. Treatment with AMP–PNP shifts more pVHL into the pellet fraction (Fig. 4B) consistent with the involvement of a motor protein in MT-binding by pVHL.

To study the effect of KIF3A on MT-binding of pVHL, we performed MT co-sedimentation assays using lysates of HEK293T cells transfected with Vsv-pVHL30 and Myc-KIF3A or empty vector. Myc-KIF3A enhanced binding of Vsv-pVHL30 to MTs (Fig. 4C). Interestingly, co-transfecting Vsv-pVHL30 with Myc-ΔN-KAP3 greatly reduced pVHL30 association with MTs (Fig. 4D).

4. Discussion

Here we describe KIF3A as a novel pVHL binding partner and confirm direct binding between these proteins. Endogenous binding of pVHL to KIF3A and other subunits of the anterograde kinesin-2 microtubule motor is also observed, which can be compromised either by pVHL mutations or by overexpression of a dominant-negative form of KAP3, ΔN-KAP3. We identified two domains within pVHL to be necessary for binding kinesin-2: the N-terminal acidic domain (residues 1–54) facilitates KAP3 binding, and residues 95–123 bind KIF3A. How pVHL-ΔN-KAP3 binding prevents the pVHL-moiety from simultaneous interaction with intact kinesin-2 could be explained by steric hindrance. Because pVHL residues 95–123 have already been implicated in microtubule binding [13], we performed microtubule co-sedimentation assays and observed that a significant portion – if not all – of pVHL binding to MT is mediated through interaction with kinesin-2. These findings correlate well with the regions and mutations of pVHL described as being deficient in stabilising MT [13] and deficient in maintaining primary cilia in kidney epithelial cells [7], suggesting that kinesin-2 binding mediates microtubule-related functions of pVHL.

Notably, KAP3 is the first protein reported to bind the acidic domain of pVHL. Phosphorylation of the acidic domain has shown to be important for the full tumour suppressor function of pVHL in mouse xenografts [16]. KAP3 has also been shown to exert tumour suppressor function, as illustrated by the malignant transformation of neural progenitor cells by targeted deletion of KAP3 [17]. Whether these two tumour suppressors influence each others' function has yet to be determined.

Recent studies describe pVHL interaction with the cell polarity complex Par3-Par6-aPKCζ, [6]. Like pVHL, this complex also regulates ciliogenesis, however Par3-Par6-aPKCζ-mediated ciliogenesis has been established to operate through kinesin-2 [18]. A similar mechanism for pVHL-driven ciliogenesis has been hypothesised [6], however kinesin-2 binding to pVHL could not be detected in their cell system. Our data adds an unique element to ongoing studies concerning the role of pVHL in cilia regulation.

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