Drosophila VHL tumor-suppressor gene regulates epithelial morphogenesis by promoting microtubule and aPKC stability

Serena Duchi¹, Luca Fagnocchi¹, Valeria Cavaliere¹, Anita Hsouna², Giuseppe Gargiulo¹,* and Tien Hsu²•,*¹

SUMMARY
Mutations in the human von Hippel-Lindau (VHL) genes are the cause of VHL disease, which displays multiple benign and malignant tumors. The VHL gene has been shown to regulate angiogenic potential and glycolic metabolism via its E3 ubiquitin ligase function against the alpha subunit of hypoxia-inducible factor (HIF). However, many other HIF-independent functions of VHL have been identified and recent evidence indicates that the canonical function cannot fully explain the VHL mutant cell phenotypes. Many of these functions have not been verified in genetically tractable systems. Using an established follicular epithelial model in Drosophila, we show that the Drosophila VHL gene is involved in epithelial morphogenesis via stabilizing microtubule bundles and aPKC. Microtubule defects in VHL mutants lead to mislocalization of aPKC and subsequent loss of epithelial integrity. Destabilizing microtubules in ex vivo culture of wild-type egg chambers can also result in aPKC mislocalization and epithelial defects. Importantly, paclitaxel-induced stabilization of microtubules can rescue the aPKC localization phenotype in Drosophila VHL mutant follicle cells. The results establish a developmental function of the VHL gene that is relevant to its tumor-suppressor activity.

KEY WORDS: Von Hippel-Lindau tumor-suppressor gene, Drosophila, Epithelial morphogenesis

INTRODUCTION
Establishing and maintaining epithelial integrity is essential for embryonic development, organogenesis and tissue remodeling. The key characteristic of epithelial cells is asymmetrical specification of membrane domains marked by domain-specific proteins (Knust and Bossinger, 2002; Nelson, 2003; Tepass, 2002). The epithelial morphogenetic mechanism, although with some variations in different epithelial tissues, is highly conserved from worm to mammal. The crucial initial step in establishing epithelial polarity is the specification of the apical domain, which is defined by the function of a complex containing atypical PKC (aPKC), Bazooka (Baz; mammalian and worm PAR-3) and PAR-6 (for a review, see Suzuki and Ohno, 2006). The PAR complex is initially recruited by activated Cdc42 to the apical domain. The three proteins were originally thought to function as a complex; however, recent evidence indicates that Baz might be required first to recruit the aPKC–PAR-6 complex to the subapical domain juxtaposed to the future adherens junction (AJ) (Harris and Peifer, 2007). The PAR complex is required for the localization of another apical complex containing Crumbs (Crb), Stardust (Std; mammalian Pals1) and Discs lost (Dlt; mammalian Patj). The PAR- and Crb-containing complexes occupy the apical-most region of the lateral membrane, just apical to the AJs.

The apical complexes in turn restrict the localization of a third complex comprising Scribble (Scrib; mammalian Scribble/Vartul), Discs large (Dlg) and Lethal(2) giant larvae (Lgl) (Betschinger et al., 2005; Bilder et al., 2003) to the basolateral domain, while Lgl also antagonizes the apical components and prevents their spreading to the basolateral side (Yamanaka et al., 2003). The antagonistic action of apical and basolateral complexes helps define the apicolateral loci eventually occupied by AJs. It is not yet completely clear how the initial localization of the apical complex is achieved.

The VHL tumor-suppressor gene mutations are the genetic cause of the familial VHL disease. Germline mutations in VHL predispose the patients to several benign and malignant tumors, including renal cell carcinoma (RCC, kidney cancer), hemangioblastoma (overgrowth of blood vessels in the retina and central nervous system) and pheochromocytoma (tumors in the adrenal glands). VHL protein has been shown to function as an E3 ubiquitin ligase. Among its best-documented targets is the alpha subunit of the hypoxia-inducible factor (HIF-α). Therefore, the canonical tumor-suppressor function of VHL is modulation of the normal oxygen-sensing mechanism that regulates angiogenic response and metabolic switch to glycolysis (Kaelin, 2008). However, how this function correlates with the origin of epithelial tumors such as RCC is unclear, although it is thought that HIF-independent mechanisms might be involved (Frew and Krek, 2007).

VHL is evolutionarily conserved. In Drosophila, the VHL gene has been implicated in tracheal tubule development and HIF-α regulation in the embryos based on biochemical and RNA interference-mediated phenotypic studies (Adryan et al., 2000; Arquier et al., 2006; Aso et al., 2000; Mortimer and Moberg, 2009). In this report, we generated the first genomic Drosophila VHL mutant and examined the function of VHL in epithelial morphogenesis using a model epithelium – the follicle cells in the egg chamber. We show that VHL regulates the proper localization and stability of aPKC in the follicle cells and that this function is, at least in part, mediated by the action of VHL on microtubule (MT) stability. Without VHL function, MTs and aPKC are destabilized, resulting in epithelial defects. These results establish a developmental function of the VHL gene that is relevant to its tumor-suppressor activity.

1Dipartimento di Biologia Evoluzionistica Sperimentale, Università di Bologna, Via Selmi 3, 40126 Bologna, Italy. 2Department of Pathology and Laboratory Medicine, and Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29425, USA.

*Present address: Boston University School of Medicine, Henn/Onc Section, 650 Albany Street, EBRC 440, Boston, MA 02118, USA

†Authors for correspondence (giuseppe.gargiulo@unibo.it; tienh@bu.edu)

Accepted 23 February 2010
MATERIALS AND METHODS

Drosophila strains and genetics

V 1 was used as the wild-type stock in this study. The Drosophila VHL 1 mutant was generated by replacing the wild-type copy, via homologous recombination (Rong and Golic, 2000), with a deletion that removes 80 codons encompassing the first two in-frame AUGs. The mutant stock is maintained with the balancer chromosome Cyo. Df(2R)-YEP that carries a YFP reporter gene directed from the Dfd promoter (expressed in mandibles). The Df(2R)-en-A is a chromosomal deletion that uncovers the region 47D6-48F8 on the second chromosome, including the genomic VHL locus at 47E5-47E6. Follicle cell clones mutant for VHL were generated with site-directed mitotic recombination using the Flp/FRT system (Xu and Rubin, 1993). The mutant VHL alleles were crossed onto chromosome 2R carrying the yeast recombination site FRT 13 at the base of the chromosome arm (at position 42B). The mitotic mutant clone was induced by crossing the VHL, FRT 13 fly with a transgenic strain carrying the wild-type FRT 13 chromosome (with a ubiquitin promoter-driven GFP reporter gene, ubi-GFP) and the UAS-Fip recombinase transgene under control of the T155-Gal4 driver. The genotype of the VHL mutant mosaic female is (using VHL 1 as an example): w 1 253; P(FRTw 18 )G13, VHL/P(FRTw 18 )G13, P(ubi-GFP); P[T155-Gal4], UAS-Fip+/+, T155-Gal4 directs expression in all follicle cells throughout oogenesis. FRT 182 , aPKC 98-806/Cyo is a strong loss-of-function allele and was a gift of M. W. Deng (Florida State University, USA) (Tian and Deng, 2008). The VHL full-length cDNA (Adryan et al., 2000) and the VHL 1H variant (single amino acid substitution mutation of tyrosine to histidine at position 51, equivalent to human Y98) were cloned in the UAS-based pGateway vector tagged with three HA epitopes at the 3′ end. These expression vectors were used to transform Drosophila flies. N2A71 (1:250; see above), mouse anti-tubulin (1:2000; C20, Sigma), rabbit anti-Bazooka (1:500; see above) and rabbit anti-VHL (1:10,000; see above).

RESULTS

Drosophila VHL mutant

The VHL mutation was generated using the homologous recombination strategy (Rong et al., 2002). Homozygous VHL 1 mutants are sluggish after hatching and die at the end of first instar larval stage. Wild-type, heterozygous and homozygous first instar larvae were hand-picked and subjected to genomic DNA PCR. The homozygous mutant animals show complete loss of the wild-type VHL locus at 47E5-47E6. The penetrance in potential egg chamber phenotypes, prominent epithelial defects begins soon after follicle cells diverge from the somatic stem cells and encircle the germ cells (stage 1). The epithelium reaches maturity at mid-oogenic stages (after stage 6 at ~30 hours of egg chamber development; total developmental time ~70 hours), when follicle cells cease to proliferate. To examine the phenotype in the follicular epithelium, an adult tissue, we generated mosaic mitotic mutant clones using the Fip/FRT system. Multilayering was observed with the VHL locus (at 47E), shows the same late first instar lethality, suggesting that VHL 1 is a null mutant. The lethal phenotype can be rescued by expressing a wild-type VHL cDNA under the control of the hsp70 promoter (data not shown). Therefore, VHL gene truncation is the only major genetic defect in the VHL 1 allele.

Epithelial defects in VHL mutant follicle cells

The Drosophila follicle cells exhibit the typical epithelial polarity exemplified by markers such as the apical PAR complex, AJ components and the basolateral Lgl complex (Bilder et al., 2003; Tanentzapf et al., 2000). Establishment of the epithelial polarity begins soon after follicle cells diverge from the somatic stem cells and encircle the germ cells (stage 1). The epithelium reaches maturity at mid-oogenic stages (after stage 6 at ~30 hours of egg chamber development; total developmental time ~70 hours), when follicle cells cease to proliferate. To examine the phenotype in the follicular epithelium, an adult tissue, we generated mosaic mitotic mutant clones using the Fip/FRT system. Multilayering was observed with the VHL locus (at 47E), shows the same late first instar lethality, suggesting that VHL 1 is a null mutant. The lethal phenotype can be rescued by expressing a wild-type VHL cDNA under the control of the hsp70 promoter (data not shown). Therefore, VHL gene truncation is the only major genetic defect in the VHL 1 allele.

Immunoprecipitation

Ovaries from 50-150 females were collected in 1× PBS and rinsed twice with 1× PBS. Then 180 μl protein extraction buffer (50 mM HEPPS pH 7.6, 100 mM KCl, 1 mM EGTA, 1 mM MgCl 2, 1% Triton-X 100, 10% glycerol) and 20 μl of 10× protein inhibitor cocktail (Roche) were added. The ovaries were homogenized manually on ice on a Eppendorf tissue grinder, then sonicated on ice four times with 2-second pulses and 2-second intervals and centrifuged at 6000 g for 20 minutes at 4°C. The supernatant was stored at −20°C in 50 μl aliquots and a small portion was used to perform a protein quantification assay. 500 μg of protein extracts were used for each immunoprecipitation in a total volume of 500 μl of protein extraction buffer. The extracts were pre-adsorbed with equilibrated 50 μl of 50% protein A/G agarose bead slurry (Pierce). The extracts were then incubated with ~3 μg of anti-β-tubulin or anti-GFP (either mouse or rabbit) before 50 μl of equilibrated protein A/G beads were added. Purified IgG was used as control. The precipitated protein complexes were then subjected to western blotting. Antibodies used in western blots were: mouse anti-β-tubulin (1:250; see above), mouse anti-β-actin (1:2000; Sigma), rabbit anti-Bazooka (1:5000; see above) and rabbit anti-VHL (1:10,000; see above).
determined that and the remaining 20% showed swelling (see below). We also showed flattening/stretching of epithelium (Fig. 1C,D, brackets); defects. Drosophila VHL

Fig. 1. (more than three layers of cells; arrow in C) and stretching (brackets in C,D). The mutant epithelia show stacking of cells (arrows in B), piling-up (Fig. 1B,D). (A) VHL mutant allele contains a deletion removing the first two in-frame AUG codons. PCR primers (black line fragments) for distinguishing wild-type and VHL genomic segments are shown with expected PCR fragment sizes. Genomic DNAs from first-instar larvae of the indicated genotypes were subject to PCR amplification using the VHL-specific primers indicated on the left or rp49-specific primers as control. (B-D) VHL mutant clones were generated as described in Materials and Methods. Relevant cell types are marked in B for reference herein. FC, follicle cells; NC, nurse cells; O, oocyte. The apical surface of the follicle cells is in contact with the germ cell complex (nurse cells and the oocyte). Mutant cells are identified by a lack of GFP expression (dotted lines or brackets). Egg chambers were stained for DNA (ToPro-3 in B and DAPI in C,D). The mutant epithelia show stacking of cells (arrows in B), piling-up (more than three layers of cells; arrow in C) and stretching (brackets in C,D). (E,F) Mutant clones of Df(2R)en-A encompassing the VHL locus were generated as above. Egg chambers were stained for DNA (Propidium Iodide). Stacking of epithelial cells is seen (arrows). (G) Quantification of cell numbers in mutant clones compared with the sister wild-type cell clones. Twenty-five clones are presented with increasing sizes. There are no significant size differences between mutant and wild-type clones. Scale bars: 20 μm.

showed flattening/stretching of epithelium (Fig. 1C,D, brackets); and the remaining 20% showed swelling (see below). We also determined that VHL loss-of-function mutation did not affect cell proliferation or viability. As mitotic recombination in a heterozygous progenitor cell (VHL/ubi-GFP) generates one VHL homozygote (no GFP) and one wild type (two copies of GFP), the numbers of high GFP-expressing cells and GFP-negative cells should be equal if the mutant cells do not exhibit an altered rate of proliferation or viability. We calculated the ratio of the cell number within the VHL mutant clones versus the cell number within the sister wild-type clones (number of clones examined=25) and obtained a mean value of 0.96 (± 0.16 s.d.). Importantly, the ratio between the cell number in mutant and wild-type sister clones did not change as a function of the clone size (Fig. 1G). Furthermore, staining for mitosis and apoptosis markers phospho-histone H3 and cleaved caspase 3, respectively, also showed that VHL mutation does not affect cell proliferation or caspase-mediated apoptosis (see Fig. S1 in the supplementary material).

**Loss of VHL in follicle cell clones affects cell polarity**

Multilayering is a typical terminal phenotype for polarity defects in epithelial cells. In homozygous VHL clones, the aPKC protein was mislocalized or greatly diminished from the apical membrane domain, which abuts the germ cell complex. This defect occurred early in oogenesis in 82% (n=28) of the stage 3-4 clones (<10-24 hours of egg chamber development; stage 4 as an example in Fig. 2A). At later stages (>stage 8; >40 hours of egg chamber development) in homozygous VHL clones, mislocalization of aPKC was more pronounced, showing either cytoplasmic localization or absence in 96% (n=27) of stage 10 clones or 83% (n=29) of stage 8-9 clones (Fig. 2B,C). Interestingly, in many mutant cells (61% (n=28) of stage 8-9 clones), aPKC was detected in the perinuclear region (Fig. 2C, sharp arrows), suggesting a defect in translocation of the protein. We next analyzed the distribution of the aPKC partner Bazooka (Baz). Baz localization was largely normal in VHL mutant cells at the same stage 3-4, with only a slight decrease in the apical domain and some spreading to the lateral domain (50%, n=28; Fig. 2D). The overall levels diminished significantly later in 83% (n=29) of stage 9-10 clones (Fig. 2E). Perinuclear accumulation was also detected in 61% (n=28) of stage 10 clones (Fig. 2E). The time difference in localization defects of these two proteins indicates that Baz mislocalization at later stage is probably an indirect effect downstream of mislocalized aPKC.

The PAR complex has been shown to be a master regulator of epithelial polarity (Suzuki and Ohno, 2006). We therefore examined representative markers of different apicolateral membrane domains. The subapical region (SAR) can be visualized by Crumbs (Crb), a transmembrane protein that interacts with aPKC and is required for the establishment of this domain (Tepass et al., 1990; Wodarz et al., 1995). It has been shown that phosphorylation of Crb by aPKC in Drosophila embryonic blastodermal cells is necessary for Crb stabilization in the apical domain (Sotillos et al., 2004). In homozygous VHL clones at stage 4, apical localization of Crb was only slightly reduced (but not absent; Fig. 2F,G). Co-staining of Crb and aPKC at stage 4 showed that in the VHL clone, aPKC is undetectable while Crb is fading but still present in the apical domain (see Fig. S2A in the supplementary material). Later at stage 8, Crb was mislocalized to the basal domain and formed large aggregates in 89% (n=28) of stage 8-10 clones (Fig. 2G, arrows).

AJs are the mature epithelial cell landmark and can be visualized by the Drosophila β-catenin encoded by the armadillo (arm) locus (Peifer, 1993; Tanentzapf et al., 2000). Arm distribution was normal...
at stage 4 (Fig. 2H, H'). Co-staining of aPKC and Arm confirmed that, in VHL mutant cells at stage 4, apical aPKC is greatly diminished but Arm appears normal (see Fig. S2B in the supplementary material). Arm distribution became abnormal in VHL mutant cells after stage 6 of oogenesis. As shown in Fig. 2I', loss of VHL caused a pronounced alteration of the epithelial monolayer (swelling as an example) and Arm overaccumulated to the apical and basolateral membrane domains in 89% (n=27) of stage 8-10 clones (Fig. 2I', arrows). The pattern of Arm spreading is consistent with the notion that apical and SAR polarity proteins are crucial for the formation and restriction of AJ at the apicolateral domain of the epithelial cells.

In Drosophila follicle cells, as in other epithelial tissues, antagonistic interaction between the PAR complex and the basolateral Lgl complex is necessary for the establishment and maintenance of epithelial polarity (Bilder et al., 2003; Hutterer et al., 2004; Tanentzapf and Tepass, 2003). Lgl can compete with Baz/PAR-3 for binding to aPKC-PAR-6 and destabilize the PAR complex (Yamanaka et al., 2003). Conversely, aPKC phosphorylates Lgl, resulting in its dissociation from the membrane domain (Betschinger et al., 2005). In wild-type epithelial cells, Lgl is located in the lateral membrane domain with enrichment toward the basal side. At stage 4 in the homozygous VHL clones, Lgl was slightly reduced but present on the basolateral domain (Fig. 2J'), but greatly reduced from the membrane domain in 90% (n=29) of stage 8-10 clones (Fig. 2K', K'). Interestingly, it has been shown that a functional but mislocalized aPKC-PAR-6 complex could still phosphorylate Lgl in the cytoplasm (Hutterer et al., 2004). It is therefore probable that the mislocalized aPKC in VHL mutant cells can phosphorylate Lgl and prevent its membrane localization.

We have previously shown that RNA interference-mediated knock-down of VHL resulted in defects in tracheal development during embryogenesis (Adryan et al., 2000). Homozygous VHL 'escaper' mutant flies (~10% of adult progeny from heterozygous cross) can be recovered by re-expression of the VHL cDNA sequence from the breathless (btl) gene promoter, which is specific for the trachea and glial cells. As btl is not expressed in the follicle cells, we reasoned that the rescued adult VHL homozygous females should exhibit severe epithelial defects in the follicle cells and serve as a confirmation of the VHL clonal phenotypes. We first confirmed that overexpression of VHL from the btl promoter in wild-type females did not cause epithelial defects (Fig. 3A). Conversely, the VHL homozygous females rescued by btl > VHL were sterile, in which 70% of the ovarioles contained only degenerated egg chambers. In the rest, we observed very severe epithelial defects in their egg chambers, including prominent expansion of the epithelium into the germ cell proper (Fig. 3B, C, arrows). In other cases, the entire epithelium became multilayered as shown in the optical cross-section through the center of the egg chamber in Fig. 3D. The invading follicle cells showed a loss of apical specification as Arm was expressed evenly throughout the cell peripheries (Fig. 3B, C, arrows). In the severely multilayered epithelium (Fig. 3D),
Arm overaccumulated in the apical domain in the inner layer facing the germ line complex and spread throughout the lateral membrane domains in the stacking layers. We also observed a collapsed nurse cell complex (Fig. 3D, bracket), which, in the wild-type egg chamber, occupies the anterior half of the egg chamber at stage 10. This might be the result of germ line cytoskeletal defects or the result of structural defects in the squamous follicle cells that cover the nurse cell complex. In all egg chambers examined, aPKC was mislocalized (cytoplasmic localization or spreading throughout the lateral membrane) and greatly reduced.

MT defects

It has been shown that human VHL can promote MT stability against MT destabilizing agents colcemid and nocodazole (Hergovich et al., 2003; Lolkema et al., 2004), although the physiological role of this activity has not been elucidated. We therefore examined whether MT organization is affected in Drosophila VHL mutant follicle cells. In many organ-associated epithelial cells such as the follicle cells, MTs are not centrosome-anchored, but are instead organized in cortical bundles parallel to the apico basal axis, with the plus-end localized basally (Bartolini and Gundersen, 2006). In follicle cells, an apical meshwork of MTs also exists. As shown in Fig. 4, VHL mutant cells already displayed either disorganization or loss of MTs in pre-stage 6 clones (Fig. 4A-B’), in step with loss of apical aPKC (Fig. 4A”,B”). Loss of MTs along the lateral cortex became more obvious in later egg chambers (Fig. 4C). The MT defects were observed in all VHL clones. It has been shown that unbundled MTs are lost during fixation with standard immunostaining protocol (e.g. 4% paraformaldehyde) but can be preserved and detected using more extensive fixation/cross-linking conditions (e.g. 8% paraformaldehyde) (Theurkauf, 1994). Surprisingly, even with 8% paraformaldehyde fixation, VHL mutant cells still exhibited loss of MTs (Fig. 4D,D”), suggesting that either the stability of MTs in VHL mutant cells was severely compromised or the plus-end polymerization became defective. In all VHL homozygous egg chambers from btl-VHL-rescued mutant females, MT was barely detectable (Fig. 3E,F).

VHL functions via stabilizing MTs

Drosophila aPKC can control epithelial polarity during epithelialization of embryonic blastoderm by regulating MT organization (Harris and Peifer, 2007). In human cells, VHL has been shown to interact with the PAR-3–PAR-6–aPKC complex (Schermer et al., 2006), which in turn regulates MT–cortex interactions and coordinates growth of cortical MTs. Conversely, because MTs are a major transport route for epithelial markers (Musch, 2004; Rodriguez-Boulan et al., 2005), the disruption of MT might indirectly affect aPKC localization. To distinguish between these two possibilities, we first analyzed MT distribution in aPKC mutant clones. Homozygous aPKC mutant clones, genetically marked by the absence of GFP and verified by lack of aPKC antibody staining, showed disorganized MT structure but not diminished MTs (Fig. 4E,F). Even at later stages (>stage 8) when Drosophila VHL mutant clones showed consistent loss of MTs, aPKC mutant cells exhibited a disorganized MT network but no loss of MTs (Fig. 4G). Closer examination showed that instead of organized cortical bundling (Fig. 4G”), the aPKC mutant cells exhibited a dotted pattern of MT structure (Fig. 4E’,G’, empty arrowheads), indicating a truncated MT structure. Other aspects of the VHL mutant phenotypes, such as piling up (Fig. 4E, arrows) and stretching or gaps in epithelium (Fig. 4F,H, brackets), were observed. Therefore, the aPKC mutant exhibits some, but not all, of the phenotypes in the VHL mutant.

We next examined whether disruption of MTs can directly influence aPKC localization. Wild-type egg chambers were dissected and cultured ex vivo in the presence of DMSO (control), paclitaxel (MT stabilizing agent) or nocodazole (MT destabilizing agent). After a 5-hour incubation, stage 8-9 egg chambers were examined for expression of aPKC and β-tubulin. Egg chambers treated with DMSO (Fig. 5A) showed normal organization of the MT network (Fig. 5A”) and normal epithelial structure (Fig. 5A”), with apically enriched aPKC (Fig. 5A). Paclitaxel increased the levels of MT bundles throughout the egg chamber (Fig. 5B’).
there is 90% effects from epithelial destruction. Our crucial observation is that direct result of nocodazole treatment, not downstream indirect were shown to demonstrate that aPKC and MT disruption is the samples, follicle cells without disintegration of epithelial structure. In nocodazole-treated these rescued clones, the correct apical localization of aPKC was correct distribution was restored (Fig. 5G, empty arrowheads). Arrows point to mutant regions which show abnormal cell shape or stretched epithelial cells (bracket), residual MTs occupy very thin cytoplasmic space, thus appearing condensed and stained strongly. (C,C') A stage-10A egg chamber. A VHL1 clone (dotted line) shows loss of MTs along the lateral cortex. (D,D') A stage-7 egg chamber. Even with 8% formaldehyde fixation, MTs are still lost in the VHL1 clone (dotted line). (E-H') Ovaries containing aPKC clones were dissected and egg chambers stained for β-tubulin, GFP and DNA (ToPro-3 in E) or aPKC (F-H). (E,E') A stage-4 egg chamber. MTs are not diminished in the mutant cells (dotted line) but show a dotted pattern (empty arrowheads). Arrows point to mutant regions that show the piling-up phenotype. (F,F') A stage-6 egg chamber containing two aPKC clones (dotted lines), which show abnormal cell shape or stretched epithelium (brackets). MTs are not diminished in either case. (G-G') A stage-9 egg chamber containing 2 aPKC clones (dotted lines). The aPKC mutant clones show disorganized and truncated (dotted lines) MT pattern with the in vivo VHL phenotype. In 100% (n=20) of VHL1 clones, aPKC was almost completely lost from the apical domain (Fig. 5F). By contrast, in 80% of the homozgous VHL1 clones (n=20) from egg chambers treated with paclitaxel, MTs recovered and their correct distribution was restored (Fig. 5G'). Importantly, within these rescued clones, the correct apical localization of aPKC was invariably (100%) re-established (Fig. 5G, G', arrows).

Because aPKC can also regulate MT organization, we tested whether paclitaxel can also rescue the MT phenotype in aPKC mutant cells. Interestingly, only 45% of the aPKC mutant clones (n=20) could be rescued under the same treatment condition as for the VHL1 clones described above (Fig. H,I). This indicates that aPKC regulates overlapping as well as different aspects of MT organization than VHL. Taken together, the above results demonstrate that the epithelial function of VHL is mainly mediated via stabilizing MTs, although we cannot exclude the possibility that VHL can also influence the stability of aPKC as 45% of aPKC mutant clones can be similarly rescued and 20% of the VHL mutants cannot be rescued by paclitaxel treatment.

**VHL regulates MTs and aPKC stability**

The functional relationship among VHL, aPKC and MTs was further tested in cultured Drosophila S2 cells and in ovaries expressing the VHL full-length sequence or VHL RNA interference construct (VHLi) under heat-shock promoter control. Protein extracts were especially in the nurse cells (yellow arrow). Follicle cells were somewhat thickened but maintained the monolayer characteristics (Fig. 5B'). aPKC localization was also normal (Fig. 5B). By contrast, nocodazole treatment greatly diminished MTs throughout the epithelium (Fig. 5C', D', E') and caused stacking (Fig. 5C', E', sharp arrows). Concomitantly, aPKC was either diminished or mislocalized, not only in cells that showed epithelial defects (Fig. 5C, sharp arrows), but also in morphologically normal epithelium (Fig. 5C, arrows; Fig. 5D). Actin filaments showed some disorganization but were largely intact (Fig. 5E), indicating that nocodazole treatment for 5 hours does not cause general disintegration of the follicular epithelium. In nocodazole-treated samples, follicle cells without disintegration of epithelial structure were shown to demonstrate that aPKC and MT disruption is the direct result of nocodazole treatment, not downstream indirect effects from epithelial destruction. Our crucial observation is that there is 90% (n=20) of aPKC mislocalization similar to that observed in the VHL mutant in vivo. It is interesting to note that these defects occurred in stage 9-10 egg chambers within 5 hours of nocodazole treatment without cell division (follicle cell division ceased after stage 6). This suggests that maintenance of epithelial integrity is a dynamic and continuous process, for which MTs are crucially important.

To further demonstrate that VHL function in follicle cells is mediated by MT organization, we examined whether stabilizing MTs can rescue VHL phenotypes. As shown in Fig. 5F,G, ex vivo cultured egg chambers containing VHL1 mutant clones (Fig. 5F', G', dashed lines) were treated with either solvent (DMSO) or paclitaxel. Homozygous VHL1 clones from stage 9 egg chambers without drug treatment showed that MTs are compromised in the mutant cells, with significant loss along the lateral cortex (Fig. 5F'), consistent with the in vivo VHL phenotype. In 100% (n=20) of VHL1 clones, aPKC was almost completely lost from the apical domain (Fig. 5F). By contrast, in 80% of the homozgous VHL1 clones (n=20) from egg chambers treated with paclitaxel, MTs recovered and their correct distribution was restored (Fig. 5G'). Importantly, within these rescued clones, the correct apical localization of aPKC was invariably (100%) re-established (Fig. 5G, G', arrows).
subjected to western blotting. Overexpression of full-length VHL increased the VHL protein level modestly and expression of VHLi significantly reduced VHL protein levels in both S2 cells (Fig. 6A) and in ovaries (Fig. 6B). In S2 cells, overexpression of VHL increased the levels of aPKC and PAR-6, whereas knockdown of VHL greatly reduced their levels (Fig. 6A). However, the effects of altered VHL levels on the levels of aPKC and PAR-6 were present but less pronounced in vivo (Fig. 6B). Also interestingly, although VHL knockdown did not alter the total cellular tubulin level in S2 cells, in ovaries, VHL knockdown greatly reduced tubulin levels, consistent with the immunostaining results (Fig. 4). Perhaps in cell culture, the MTs are less prone to dynamic instability and therefore more stable. This is particularly true when compared with follicular epithelium, in which the epithelial sheet movement at stage 9, accompanied by rapid growth into a columnar shape, requires extensive remodeling and turnover of cytoskeletons.

We next examined whether VHL can interact with tubulin and aPKC in vivo. For this purpose, we used transgenic flies expressing aPKC-GFP fusion protein, which has been shown to be fully functional (Tian and Deng, 2008). Ovarian extracts expressing aPKC-GFP fusion proteins were processed for immunoprecipitation. As shown in Fig. 6C, aPKC-GFP pulled down its known partner...
Fig. 6. VHL interacts with MTs and aPKC. Head-to-head VHL cDNA duplex (dVHL) or full-length VHL (dVHL) were cloned into the pCaspe-hs vector and transfected into Schneider (S2) cells or used to generate transgenic flies. (A) Lysates from S2 cells transfected with control plasmid or vectors expressing full-length VHL or VHL duplex were western-blotted for indicated proteins. (B) Ovaries from females carrying hs-VHL (two lines) or VHLi were dissected and protein lysates processed for western blotting with the indicated antibodies. (C) Protein lysates from ovaries carrying Cy2-Gal4/UAS-aPKC-GFP were processed for immunoprecipitation (IP) and probed with the antibodies indicated on the left. Input denotes western blots of total protein lysates without IP.

Baz, but not tubulin (left panels), while VHL was co-immunoprecipitated by either β-tubulin or aPKC-GFP (right panels). Interestingly, VHL interactions with MTs and aPKC are probably not within the same complex as the majority of aPKC does not associate with microtubules (aPKC-GFP does not bring down tubulin). These results are consistent with a direct regulatory function of VHL on MTs and suggest an additional VHL function in associating with microtubules (Gwathmey et al., 1996). Importantly, the expression levels of the wild-type and Y-to-H mutant VHL proteins were the same in the follicle cells, measured by ImageJ software analysis of confocal images of two-cell clusters from five different same-stage egg chambers of each strain. VHL showed localization along the MT bundles in cultured S2 cells (see Fig. S5B in the supplementary material). The effects of the wild-type and YH mutant VHL on MT stability were further tested ex vivo. As shown before, nocodazole treatment on a normal egg chamber disrupted the MT organization and reduced the overall levels of MT bundles (compare Fig. 7A,B). Overexpression of VHL1-HA counteracted the destructive effects of nocodazole (compare Fig. 7B-D). By contrast, the VHL1-HA mutant had only limited effect (compare Fig. 7E,F). The MTs in these follicle cells contained only a mesh in the apical region. These observations are quantified in Fig. 7G. Interestingly, without intact MT bundles, the VHL1 protein itself became mislocalized. It did not colocalize with the disorganized MT and became more nuclear (Fig. 7F, insets). This indicates that cytoplasmic distribution of the VHL protein is itself MT-dependent.

DISCUSSION

The role of VHL and MTs in epithelial morphogenesis

In this report, we show that Drosophila VHL is important for establishing and maintaining epithelial integrity via its regulation of MT and aPKC stability. We observed MT disruption and epithelial

This set of early eggs serves as a convenient internal control. Conversely, 60% of stage 9 egg chambers analyzed (n=36) from hs-VHLi females displayed a multilayered phenotype, whereas 43% of stage 9 egg chambers from UAS-aPKC-GFP-expressing flies (n=36) displayed the same phenotype. The extent of rescue, although notable, is not statistically significant (based on Pearson’s Chi-squared test, P=0.2357; see Fig. S3 in the supplementary material). The partial rescue might be because of an insufficient expression level of aPKC-GFP. This is not probable, however, as the same Cy2-Gal4-driven aPKC-GFP can rescue an aPKC mutant (data not shown). More plausible is that the direct stabilizing (versus proper localization) action of VHL on aPKC plays an appreciable but minor role in epithelial morphogenesis.

A disease-related VHL mutant defective in MT stabilization cannot rescue the aPKC localization phenotype

A few disease-causing mutations in human VHL have been shown to ameliorate the ability of VHL to promote MT stability in cultured cells (Hergovich et al., 2003). One of these amino acid substitutions affects a highly conserved tyrosine residue (Y98 to histidine). We generated the equivalent mutation in Drosophila VHL (Y51 to H in the Drosophila counterpart) as a hemagglutinin fusion protein. The wild-type VHL-HA transgene (VHLwt-HA) is functional as it can rescue VHL1 phenotypes in 71% of the VHL mutant clones (n=24), whereas the Y-to-H mutant (VHLYH-HA) cannot in 100% of the clones examined (n=26; see Fig. S4 in the supplementary material). Anti-HA staining in VHLwt-HA-expressing egg chambers showed punctate cytoplasmic localization of the wild-type VHLwt-HA fusion protein (see Fig. S5A in the supplementary material). There was substantial co-localization of VHLwt-HA and the cortical MT bundles along the lateral membrane, in agreement with the immunoprecipitation result, although not all VHL punctae were associated with MTs (see Fig. S5A in the supplementary material). Consistent with the property of the human Y98H mutant protein, Drosophila VHLYH-HA retained, at least partly, its ability to colocalize with the MT (see Fig. S5B in the supplementary material). Importantly, the expression levels of the wild-type and YH mutant VHL proteins were the same in the follicle cells, measured by ImageJ software analysis of confocal images of two-cell clusters from five different same-stage egg chambers of each strain. VHL showed localization along the MT bundles in cultured S2 cells (see Fig. S5B in the supplementary material). The effects of the wild-type and YH mutant VHL on MT stability were further tested ex vivo. As shown before, nocodazole treatment on a normal egg chamber disrupted the MT organization and reduced the overall levels of MT bundles (compare Fig. 7A,B). Overexpression of VHLwt-HA counteracted the destructive effects of nocodazole (compare Fig. 7B-D). By contrast, the VHLYH-HA mutant had only limited effect (compare Fig. 7E,F). The MTs in these follicle cells contained only a mesh in the apical region. These observations are quantified in Fig. 7G. Interestingly, without intact MT bundles, the VHLYH protein itself became mislocalized. It did not colocalize with the disorganized MT and became more nuclear (Fig. 7F, insets). This indicates that cytoplasmic distribution of the VHL protein is itself MT-dependent.

DISCUSSION

The role of VHL and MTs in epithelial morphogenesis

In this report, we show that Drosophila VHL is important for establishing and maintaining epithelial integrity via its regulation of MT and aPKC stability. We observed MT disruption and epithelial
phenotypes early in oogenesis. This indicates that MT bundles in developing epithelial cells are crucial for epithelial development and are under pressure from dynamic instability. Without stabilizing activity provided by VHL, MTs are disorganized and ultimately disintegrate, resulting in loss of epithelial integrity. We show that disrupted MTs interfere with proper localization of aPKC, which in turn leads to mislocalization of downstream epithelial markers and epithelial defects. Our ex vivo experiment also demonstrates that epithelial defects can occur within a short time (relative to the entire oogenesis time frame) after destabilizing MTs in non-proliferating epithelial cells. This indicates that the maintenance of epithelial integrity is a dynamic and continuous process even in a stable epithelium, for which MTs are crucially important. Previous studies using RNA interference-mediated knockdown demonstrated a morphogenic role of VHL in trachea development (Adryan et al., 2000; Mortimer and Moberg, 2009). The tracheal phenotypes appear to be the result of elevated cell motility and ectopic chemotactic signaling. Therefore, the tracheal function of VHL might be mediated via different VHL targets in a tissue-specific context. Alternatively, regulation of MT stabilization might also be the underlying mechanism. We favor a separate, tissue-specific function for VHL as the tracheal defects in VHL knockdown can be relieved by decreased expression of breathless, which encodes the chemotactic signaling receptor in the trachea. The two VHL functions, however, are not necessarily mutually exclusive. These different organ systems might in the future serve as a model for testing whether the various functions assigned to VHL are tissue-specific and context-dependent.

Human VHL has been shown to translocate aPKC to MTs, thereby influencing MT reorganization (Schermer et al., 2006). In this study, we show that the aPKC mutant can affect MT organization but not stability, whereas VHL can influence both. Conversely, we show that disruption of MTs alone can result in aPKC mislocalization resembling that observed in VHL mutant cells. Importantly, paclitaxel-induced MT stabilization can rescue aPKC localization in VHL mutant follicle cells. We therefore conclude that a major function of VHL in the follicular epithelium is regulation of MT stability. Loss of MTs leads to aPKC mislocalization resembling that observed in VHL mutant phenotype. Indeed, we also demonstrate that VHL can co-immunoprecipitate with tubulin or aPKC, and that, at least in S2 cells, aPKC levels can be affected by VHL levels without affecting tubulin. Taken together, it appears that the epithelial function of VHL is mediated through stabilization of MT, with an auxiliary role in directly stabilizing aPKC.

It has been suggested that VHL interacts with MTs via the kinesin 2 family of motors (Lolkema et al., 2007). Future studies using our epithelial system should also address this issue in vivo. Also interestingly, we show that the YH mutant can associate with MT but has little MT-stabilizing activity. This suggests that the YH mutant might be defective in recruiting other proteins, possibly including aPKC, that are important for regulating MT functions. In light of the role of Drosophila VHL in regulating MT stability, a function presumably important for all cells, it is curious that the tissue-specific btl-driven VHL expression can rescue the homozygous lethality of VHL. We have shown that tracheal defects are the major embryonic phenotype observed in VHL mutant (Adryan et al., 2000) (data not shown). In the course of attempting to rescue the tracheal phenotype with btl-driven VHL...
(A.H. and T.H., unpublished), we noted the appearance of rescued homozygous adults. This indicates that the MT stabilizing function of VHL is not required in all tissues. It is possible that although VHL can enhance MT stability, by itself it is not an essential factor for MT polymerization. As such, some tissues might be less dependent on VHL levels. In the follicular environment, MT rearrangement, including depolymerization and repolymerization, is crucial when the entire epithelial sheet moves over the germ cell complex while the cells grow increasingly columnar. MT stabilization facilitated by VHL might be of particular importance during this process.

### Tumor-suppressor function of VHL

The best-documented function of VHL is its E3 ubiquitin ligase activity that targets the alpha subunit of the HIF transcription factor. This activity provides an elegant mechanistic explanation for the hypervascularity of many of the VHL tumors and for a potential contributor to the metabolic switch to glycolysis, as HIF can upregulate pro-angiogenic factors such as vascular-endothelial growth factor and components in the glucose metabolic pathway. However, recent evidence has suggested that VHL is a multifunctional protein. It can function as a regulator of matrix deposition (Ohh et al., 1998), integrin assembly (Esteban-Barragan et al., 2002), endocytosis (Champion et al., 2008; Hsu et al., 2006), kinase activity (Yang et al., 2007), senescence (Young et al., 2008), protein stabilities (Chitalia et al., 2008; Roe and Youn, 2006) and tight junction formation (Calzada et al., 2006; Harten et al., 2009), among many others (Frew and Krek, 2007). Whether tight junction disassembly in VHL mutant cells is HIF-dependent is still unresolved; however, other – HIF-independent – functions appear to facilitate protein stability or activity instead of destabilizing them as a ubiquitin ligase. Such chaperon/adaptor function has also been implicated in promoting stability of MTs (Hergovich et al., 2003; Lolkema et al., 2004). The MT-stabilizing function, although potentially highly significant, has so far only been linked to ciliogenesis and mitotic spindle orientation in cultured RCC and renal tubule cells (Esteban et al., 2006; Lolkema et al., 2008; Lutz and Burke, 2006; Schermer et al., 2006; Thoma et al., 2009). The physiological and developmental significance of this function has not been elucidated in vivo. Indeed, it is unclear how loss of many of these HIF-independent functions contributes to VHL tumor formation because of a lack of tractable genetic models.

One crucial element in tumorigenesis is the breakdown of epithelial integrity that ultimately leads to epithelial-to-mesenchymal transition. This report provides the first demonstration of a potential tumor-suppressor function for VHL in regulating epithelial morphogenesis via its role in promoting MT stability. Future studies should exploit further this genetic system for elucidating how a myriad of disease-related VHL point mutations might differentially influence such function.

### Acknowledgements

We thank W. M. Deng (Florida State University), D. Grifoni (University of Bologna), T. Schüpbach (Princeton University) and A. Wodarz (Georg-August-University Göttingen) for generous gifts of antibodies and fly stocks. K. Lavenburg contributed to the initial cloning of the Drosophila VHL genomic sequence for subsequent mutagenesis. The work was supported by grants from the National Institutes of Health (PO1CA78582 and R01CA109860) to T.H., a grant from the University of Bologna (RFO 2007) to G.G. and V.C. and a Marco Polo Fellowship from the University of Bologna to S.D. Deposited in PMC for release after 12 months.

### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042804/-/DC1

### References


VHL regulates epithelial morphogenesis


