Von Hippel-Lindau gene product directs cytokinesis: a new tumor suppressor function

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Summary
One of the mechanisms of tumorigenesis is that the failure of cell division results in genetically unstable, multinucleated cells. Here we show that pVHL, a tumor suppressor protein that has been implicated in the pathogenesis of renal cell carcinoma (RCC), plays an important role in regulation of cytokinesis. We found that pVHL-deficient RCC 786-O cells were multinucleated and polyploid. Reintroduction of wild-type pVHL into these cells rescued the diploid cell population, whereas the mutant pVHL-K171G failed to do so. We demonstrate that lysine 171 of pVHL is important for the final step of cytokinesis; the midbody abscission. The pVHL-K171G caused failure to localize the ESCRT-1 interacting protein Alix and the v-SNARE complex component Endobrevin to the midbody in 786-O cells, leading to defective cytokinesis. Moreover, SUMOylation of pVHL at lysine 171 might modulate its function as a cytokinesis regulator. pVHL tumor suppressor function was also disrupted by the K171G mutation, as evidenced by the xenograft tumor formation when 786-O clones expressing pVHL-K171G were injected into mice. Most RCC cell lines show a polyploid chromosome complement and consistent heterogeneity in chromosome number. Thus, this study offers a way to explain the chromosome instability in RCC and reveals a new direction for the tumor suppressor function of pVHL, which is independent of its E3 ubiquitin ligase activity.

Key words: pVHL, CIN, SUMOylation, Cytokinesis, Alix, Endobrevin

Introduction
Von Hippel Lindau (VHL) syndrome is a multisystem neoplastic disorder characterized by central nervous system and retinal hemangioblastomas, renal cell carcinoma (RCC), and pheochromocytomas. About 20–30% of VHL patients have large or partial germline deletions, 30–38% have missense mutations, and 23–27% have nonsense or frameshift mutations (Maier and Kaelin, 1997; Stolle et al., 1998). As part of the E3 ubiquitin ligase complex, the Von Hippel Lindau tumor suppressor protein (pVHL) targets the α-subunits (1, 2, and 3) of hypoxia-inducible factor α (HIF-α) for degradation (Cockman et al., 2000; Maxwell et al., 1999), a function that has been associated with the highly vascular nature of pVHL-associated neoplasms. Other functions of pVHL in preventing RCC tumorigenesis include a role in the assembly of extracellular matrix (He et al., 2004; Ohh et al., 1998) and in stabilization of both P53 and JADE-1 molecules (Roe et al., 2006; Zhou et al., 2004; Zhou et al., 2002). pVHL has also been shown to associate with and control microtubule stability and dynamics at the cell periphery (Hergovich et al., 2003; Lolkema et al., 2004). Most recently, Thoma et al. showed that pVHL inactivation leads to spindle misorientation, deregulated spindle checkpoint, and increased aneuploidy (Thoma et al., 2009). However, sufficient knowledge to establish the direct link between aneuploidy and tumorigenesis in pVHL-null patients is inadequate. In this study, we examined the novel role of pVHL in the regulation of cytokinesis and linked the chromosomal instabilities associated with defective cytokinesis to tumorigenesis.

Cytokinesis is the process by which cells physically separate after the duplication and segregation of genetic material. Constriction of the cell membrane by actin and myosin results in formation of the cleavage furrow and, finally, the midbody structure. The final stage of cell division requires cleavage of the midbody into two daughter cells. Endobrevin, a member of v-SNARE membrane fusion machinery, has been reported to localize to the midbody and be required for the terminal stage of cytokinesis (Low et al., 2003). Polo-like kinase 1 (Plk1)-dependent phosphorylation of centrosome protein 55 (Cep55) is also required for midbody localization and cytokinesis (Fabbro et al., 2005). Other investigators showed that Alix, a protein associated with the ESCRT-1 subunit Tsg101 (Strack et al., 2003; von Schwedler et al., 2003), is recruited to the midbody during cytokinesis by an interaction with Cep55 and is essential for final midbody abscission (Carlton and Martin-Serrano, 2007). Although the roles of proteins like Endobrevin, Plk1, Cep55, Alix and Tsg101 are well documented in midbody abscission, how they are regulated during midbody abscission is still under studied.

pVHL-deficient RCC 786-O cells are hypertriploid, and reintroduction of wild-type pVHL partially rescues the DNA content to a near-diploid chromosome complement (Giles and Voest, 2005). Our study shows that wild-type pVHL localizes to the cytokinetic bridge during cytokinesis and partially rescues polyploidy in these cells, whereas localization of pVHL-K171G (lysine 171 mutated to glycine) is substantially decreased at the cytokinetic bridge and fails to alter the prevalence of polyploidy.
We demonstrate that pVHL-deficient 786-O cells and 786-O-VHL-K171G cells are defective in localization of Alix and Endobrevin to the midbody. pVHL-null RCC A498 cells were also defective in localization of Alix to the midbody, whereas the cells expressing wild-type pVHL, Caki-1 (a human renal carcinoma cell line), were not. Post-translational modification or SUMOylation of pVHL at lysine 171 might be pivotal for its function as a cytokinesis regulator. Further, we report that polyploid cells expressing pVHL-K171G injected subcutaneously into mice can lead to tumor formation. Therefore, the lysine 171 residue in the α-domain of pVHL appears to be important for the regulation of cytokinesis, one of the central regulatory mechanisms of cell biology, and thus in the initiation of tumor formation.

Results
Depletion of pVHL leads to chromosome instability
Missense and truncating mutations of the VHL gene are often reported in human cancer, indicating the tumor suppressor role of pVHL (Crossey et al., 1994; Kim and Kaelin, 2004; Richards et al., 1993; Richards et al., 1994; Zbar et al., 1996). To delineate a functional link between chromosome instability (CIN) and pVHL function, we used isogenic RCC lines that are either deficient in pVHL (786-O-empty) or have stable expression of wild-type pVHL [786-O-VHL(wt)] or mutated pVHL (e.g. 786-O-VHL-K171G). Immunofluorescent staining of 786-O-empty cells with anti-α-tubulin antibody revealed a significantly increased frequency of multinucleated cells ($P=0.003$) in the absence of pVHL expression (Fig. 1A,B). Cells expressing pVHL-K171G showed a similarly
increased occurrence of multinucleation following anti-α-tubulin staining (Fig. 1A,B) but not pVHL-N90G cells (asparagine 90 to glycine mutants) (Fig. 1B). pVHL mutation at the asparagine 90 site, one of the reported pVHL missence mutation sites (Stebbins et al., 1999), and N90I (asparagine 90 to isoleucine) mutation have been shown to be defective in HIF-α ubiquitylation (Cockman et al., 2000). Here, N90G mutation of pVHL was used as a negative control for cytokinetic function. Notably, unresolved cytokinetic bridge structures were more frequently observed (Fig. 1B) in pVHL-K171G-expressing and pVHL-deficient 786-O cells than in 786-O cells expressing wild-type pVHL or pVHL-N90G during the terminal stage of mitosis. Moreover, depletion of pVHL with siRNA in the cell lines expressing wild-type pVHL, HeLa and Caki-1, resulted in disruption of cytokinesis and increased frequencies of multinucleated cells (P=0.03 and 0.02, respectively) (Fig. 1C). Consistent with that, overexpression of the pVHL-K171G mutant, but not wild-type pVHL, in HeLa cells also led to similar defects (Fig. 1C). We assume that overexpression of pVHL-K171G in HeLa cells expressing wild-type pVHL acted as a dominant-negative and over-road the wild-type pVHL function.

A twofold increase in aneuploidy due to reduced spindle assembly checkpoint activity and an absence of tetraploid cells has been reported in pVHL-deficient primary mouse embryonic fibroblasts (MEFs) (Thoma et al., 2009). However, enumeration of chromosomes in metaphase spreads of pVHL-deficient 786-O cells revealed substantial numerical chromosome aberrations (>80%) consisting mostly of polyploidy (Fig. 1D,E). Furthermore, reintroduction of wild-type pVHL, but not pVHL-K171G, rescued the population of diploid cells (P=0.002) (Fig. 1D,E). Thus, cytogenetic analysis revealed that, unlike the absence of polyploid cells in pVHL-deficient MEFs reported by Thoma and associates (Thoma et al., 2009), the RCC lines contain hypertriploid and tetraploid cells. When considered together, these data suggest that massive disruption of the mitotic function of these cells and potential disruption of cytokinesis leads to unresolved cytokinetic bridges and multinucleated cells in the absence of normal pVHL function.

**pVHL localizes to the cytokinetic bridge and regulates cytokinesis**

The involvement of pVHL in the regulation of microtubule stability and mitotic spindle orientation has been reported previously (Hergovich et al., 2003; Thoma et al., 2009). To further investigate the potential link between pVHL function and mitotic progression, we first studied the cellular localization of pVHL throughout mitosis. A reconstituted RCC line, 786-O, that stably expresses wild-type pVHL was subjected to immunofluorescence staining with anti-pVHL antibody followed by confocal microscopy. pVHL co-localized with α-tubulin to the spindle midzone during late anaphase (Fig. 2A) and to the cytokinetic bridge during cytokinesis (Fig. 2A,C). Similar staining of endogenous pVHL using anti-pVHL antibody in RCC Caki-1 cells indicated normal localization of pVHL at the cytokinetic bridge during cytokinesis (Fig. 2B). These observations corroborate the findings of Thoma and colleagues, who have also shown the co-localization of pVHL with α -tubulin at the spindle midzone and to the cytokinetic bridge during mitosis (Thoma et al., 2009). Immunofluorescence staining of the isogenic pVHL-null 786-O cell line did not detect any such signal either in interphase or telophase (Fig. 2A), confirming the specificity of the immunofluorescence staining. Consistent with that, treatment of HeLa cells with pVHL-specific siRNA abolished the anti-pVHL antibody signal as compared with cells treated with control siRNA (supplementary material Fig. S1A), and further verified the specificity of the pVHL antibody. To evaluate the exact location of pVHL at the cytokinetic bridge, wild-type pVHL-expressing 786-O or HeLa cells were co-stained for pVHL and either the midbody component mitotic kinesin-like protein (MKLP-1) or septin-6. The results showed that pVHL co-localized with MKLP-1 (Fig. 2D) at the central spindle region during anaphase in both wild-type pVHL-expressing 786-O and HeLa cells. Three-dimensional analysis (Fig. 2E) of the confocal images for the pVHL and MKLP-1 co-staining in wild-type pVHL-expressing 786-O cells further confirmed the co-localization of pVHL with MKLP-1 during anaphase. However, pVHL was not detected at the tip of the midbody or at the Fleming body with MKLP-1 or septin-6 during the terminal step of cytokinesis (Fig. 2D,F). Moreover, we observed that MKLP-1 was properly localized at the midbody of pVHL-null 786-O cells (data not shown), ruling out any role of pVHL for the midbody localization of MKLP-1.

Finally, we checked various pVHL point mutants for their localization ability using immunofluorescence staining. Interestingly, the same K171G mutant of pVHL that showed elevated multinucleation also showed significantly reduced localization of pVHL to the spindle midzone during late anaphase and at the cytokinetic bridge during cytokinesis (Fig. 2A,C). By contrast, other reported pVHL mutants (N90G, Y112H and L188V) did not show a significant reduction of their localization at the cytokinetic bridge (Fig. 2A,C and data not shown). We also observed reduced nuclear localization of pVHL with K171G mutation (Fig. 2A) during interphase.

**pVHL is required for localization of microtubule-associated proteins at the terminal midbody**

The final stage of cytokinesis requires cleavage of the midbody, which consists of dense spindle microtubules, by membrane fusion. Normal localization of wild-type pVHL to the cytokinetic bridge and the increased frequency of multinucleation in cells lacking pVHL at their cytokinetic bridge suggested an involvement of pVHL in cytokinesis. To explore this potential role, we analyzed the localization of a number of known cytokinesis regulatory proteins in pVHL-deficient 786-O and isogenic wild-type pVHL reconstituted cells. These cells were stained with antibodies against cytokinesis regulatory kinases Plk1 and Aurora B, proteins associated with the midbody components Cep55, Alix, Tsg101 and the v-SNARE complex component Endobrevin. Depletion of pVHL did not affect the localization of either of the mitotic kinases (Plk1 or Aurora B) (Fig. 3A,B) or the microtubule-associated midbody components Cep55 (Fig. 3G) and Tsg101 (Fig. 3H). By contrast, the Cep55-interacting protein Alix, which is recruited by Cep55 at the terminal stage of cytokinesis, was absent from the midbody (Fig. 3C,D). Similar to Alix, Endobrevin was also absent from the midbody (Fig. 3E,F). We also verified the localization of the above markers in the RCC cells A498 (pVHL-null) and Caki-1 (wild-type pVHL). Absence of pVHL did not affect the localization of Plk1, AuroraB, Cep55 and Tsg101 (Fig. 3I) in A498 cells. However, as expected, Alix was absent from the midbody (Fig. 3I) in these cells. Furthermore, we found that all cytokinesis regulatory markers tested in wild-type pVHL-expressing Caki-1 cells were properly localized to the midbody (Fig. 3I).

Next, to verify the specificity of these cytokinesis-associated defects in pVHL-deficient cells, we expressed various point mutants of pVHL in 786-O cells and performed immunofluorescence analysis of the midbody localization of all the midbody markers.
described above. Supplementary material Table S1 summarizes the effect of pVHL mutation on the localization of all the markers tested. Interestingly, only the K171G mutation of pVHL, which leads to displacement of pVHL from the cytokinetic bridge, also excluded Alix and Endobrevin from the midbody (Fig. 3C–F and supplementary material Table S1). However, western blot analysis showed no significant difference in the expression of any of these proteins in pVHL-deficient cells compared with wild-type pVHL-expressing cells (supplementary material Fig. S1B). We, therefore, suggest that the multinucleated phenotype and CIN in pVHL-deficient or pVHL-K171G mutated cells resulted from the impairment of midbody cleavage.
Fig. 3. Localization of the midbody markers. (A–C,E,G) RCC cell lines were co-stained with antibodies against α-tubulin (red) and Plk1, AuroraB, Alix, Endobrevin and Cep55 (green). Images of midbodies are shown. Cells expressing pVHL-K171G show a defect in localization of Alix and Endobrevin but not with Plk1, Cep55 and AuroraB at the midbody. (D,F) Frequency of Alix (D) and Endobrevin (F) localization at the midbody. Bars represent the percentage of cells with prominent, weak or absent Alix and Endobrevin localization at the midbody. (H) 786-O-empty and 786-O cells expressing wild-type pVHL were co-stained with antibodies against α-tubulin (green) and Tsg101 (red). (I) RCC A498 and Caki-1 cells were co-stained with antibodies against α-tubulin (green) and Plk1, Cep55, AuroraB, Alix and Tsg101 (red). Images of midbodies are shown, arrows indicate midbody localization at different midbody markers. Figures represent three separate experiments with similar results.
pVHL associates with cytokinesis regulatory midbody components

To investigate how pVHL directs the localization of Alix or Endobrevin to the midbody of dividing cells, the presence of pVHL in Alix and Endobrevin immunoprecipitates from mitotic 293T cells expressing 3×FLAG-HA–VHL(wt) and 3×FLAG-HA–VHL-K171G (see Materials and Methods) were evaluated by western blot. 293T cells were synchronized at the G1–S phase with a double thymidine block and followed by release for 8.5 hours to enrich mitotic populations of cells. We observed that wild-type...
pVHL is associated with both Alix and Endobrevin (Fig. 4A,B), and that the K171G mutation decreased the association of both Alix and Endobrevin with pVHL (Fig. 4A,B). Immunoprecipitation data also revealed that Alix and Endobrevin were in the same complex (Fig. 4B), and that the K171G mutation of pVHL impaired the interaction of Alix with Endobrevin in 293T cells. We made a similar observation when lysates from synchronized 786-O cells expressing wild-type pVHL or pVHL-K171G were immunoprecipitated with Endobrevin antibody and immunoblotted with antibody against Alix (supplementary material Fig. S1C).

Because interactions between Alix, Cep55 and Tsg101 are important for efficient cytokinesis (Morita et al., 2007), we also assessed the influence of the pVHL mutant on these complexes. As expected, we observed decreased interaction of Alix with Cep55 in 293T or RCC 786-O cells expressing pVHL-K171G compared with cells expressing wild-type pVHL (Fig. 4C and supplementary material Fig. S1D). Additionally, we found that the association between Alix and Tsg101 increased in the presence of wild-type pVHL compared with the pVHL-K171G mutant in 293T cells (Fig. 4D). Although we have observed the reduced staining of pVHL-K171G to the cytokinetic bridge during cytokinesis (Fig. 2A), we could not detect any change in association of pVHL with Cep55 or Tsg101 (Fig. 4C,D, respectively) in 293T whole cell lysates. Interestingly, we found decreased interaction of Tsg101 with Cep55 (Fig. 4E).

Thus, K171G mutation of pVHL appears to alter the interactions of pVHL with Alix and thereby inhibits the association of Alix with proteins involved in midbody abscission, i.e. Cep55, Tsg101, and Endobrevin by exerting a dominant-negative effect. The data also suggest that the lysine 171 site of pVHL is important for the interaction of pVHL with Endobrevin.

**SUMOylation of pVHL enables cytokinetic regulatory function**

Existence of lysine 171 within a common SUMOylation motif ΨKxE (Rodriguez et al., 2001; Sampson et al., 2001) (Fig. 5A) indicated a possible modification of pVHL by SUMOylation. Interestingly, co-transfection of pVHL together with SUMO-1 or SUMO-2 and Ubc9 constructs in HeLa cells yielded clear, slowly migrating bands corresponding to the estimated size specific to SUMOylated pVHL (Fig. 5B). Both SUMO-1 and SUMO-2 were conjugated to pVHL to a similar extent in the in vivo SUMOylation assays (Fig. 5C). Slow-migrating bands detected by anti-FLAG immunoblotting disappeared when the SUMOylation assay was performed with a SUMOylation-defective form of SUMOs (SUMO-1ΔGG or SUMO-2ΔGG), suggesting that the bands are SUMOylated pVHL (supplementary material Fig. S2A). The identity of SUMOylated pVHL was further confirmed by Ni-NTA-agarose pull-down assay against His-tagged SUMO-1 or SUMO-2, followed by immunoblotting with anti-FLAG antibody.
pVHL was clearly detected in pull-down samples as a SUMO-conjugated protein (supplementary material Fig. S2B).

Additionally, we found that among three lysine residues of pVHL, only K171R abrogated both SUMO-1 and SUMO-2 modifications of pVHL but not K159R or K196R (Fig. 5D). 786-O cells stably expressing wild-type pVHL or the pVHL-K171G mutant were transfected with SUMO-1 or SUMO-2 and UbC9-expressing plasmids. SUMOylated pVHL bands were abolished by pVHL-K171G mutation in 786-O cells (Fig. 5E). Moreover, the K171G mutation also abrogated the SUMO modification of pVHL in HeLa cells (supplementary material Fig. S2C). We did not find any change in ubiquitylation or neddylation of pVHL-K171G compared with wild-type pVHL (data not shown).

**pVHL-K171G mutation fails to suppress tumor formation in nude mouse xenograft model**

The pVHL-K171G mutation leads to a cytokinesis defect that ultimately develops a phenotype of multinucleation and polyploidy. Reintroduction of wild-type pVHL into RCC has been reported to block the ability of these cells to form tumors in nude mice (Iliopoulos et al., 1995; Schoenfeld et al., 1998). Therefore, we studied whether the K171G mutation compromised the tumor suppressor function of pVHL.

pVHL is a component of the E3 ubiquitin ligase complex that targets the α-subunit of HIF for degradation (Maxwell et al., 1999). Tumor cells devoid of functional pVHL produce inordinate amounts of HIF-regulated hypoxia-inducible genes such as VEGF (Gnarra et al., 1996; Levy et al., 1996; Siemeister et al., 1996). The expression of VEGF mainly accounts for the vascular phenotype of pVHL-associated tumors. Glucose transporter-1 (Glut-1) expression is also increased in pVHL-defective RCC (Iliopoulos et al., 1996; Ozcan et al., 2007). Using western blot analysis, we found a significant reduction of HIF-2α expression in mutant stable cell lines compared with 786-O-empty cells, with a magnitude of reduction similar to that observed in 786-O-VHL(wt) cells (Fig. 6A). However, pVHL-null, wild-type and mutant 786-O cells showed similar levels of HIF-2α mRNA expression (Fig. 6B). Thus, it seems that the K171G mutation does not impair the HIF-2α degradation function of pVHL. However, we detected more significant upregulation of both VEGF-A and Glut-1 expression in 786-O-VHL-K171G stable clones than in 786-O-VHL(wt) cells using real-time polymerase chain reaction (PCR) (Fig. 6C), suggesting that non-HIF-2α pathways account for upregulation of these proteins.

Next, we subcutaneously injected the following cells into nude mice: 786-O-empty, 786-O clones expressing wild-type pVHL, and two 786-O clones (C3 and C3.1) expressing the pVHL-K171G mutant. Tumor take and size were monitored and measured weekly (Fig. 6D and supplementary material Table S2). As expected, 786-O-empty cells formed tumors (9 of 10), whereas mice injected with 786-O-VHL(wt) were tumor-free (0 of 10) (Fig. 6D and supplementary material Table S2). Notably, mice injected with C3 and C3.1 clones of 786-O-VHL-K171G also developed tumors (Fig. 6D and supplementary material Table S2), but the average tumor weight and tumor-take time were lower for 786-O-VHL-K171G.

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**Fig. 6. Effect of pVHL mutation on tumor suppressor function.** (A) Effect of pVHL-K171G on HIF-2α expression. HIF-2α expression was determined in 786-O-empty cells, 786-O-VHL(wt) and two clones of 786-O-VHL-K171G using western blot. K171G mutation did not show any effect on the pVHL-mediated HIF-2α degradation. (B) HIF-2α mRNA expression. Relative HIF-2α mRNA expressions are shown for 786-O-empty, 786-O-VHL(wt) and 786-O-VHL-K171G (C3) cells. (C) Relative expressions of VEGF-A and glucose transferase-1 (Glut-1). Increased expression of VEGF-A and Glut-1 mRNAs were found in both clones C3 and C3.1 of the 786-O-VHL-K171G stable cell line. Figures represent three separate experiments with similar results. (D) pVHL-K171G fails to suppress tumor formation in a nude mice xenograft model. The graph shows tumor growth over time in nude mice injected with the stable clones 786-O-empty, 786-O-VHL(wt) and 786-O-VHL-K171G (C3 and C3.1).
K171G than for 786-O-empty tumors. The results demonstrate that the K171G mutation of pVHL affects the proper cytokinesis of the dividing cells, leads to polyploidy, and impairs pVHL tumor suppressor activity.

Furthermore, we performed western blot analysis for pVHL and HIF-2α on lysates from 786-O-empty and 786-O-VHL-K171G tumors and assessed HIF-2α expression in tumor sections by immunohistochemistry with anti-HIF-2α antibody. Interestingly, we observed a significant induction of HIF-2α expression by both western blot and immunostaining (supplementary material Fig. S3A,B). Given the cell line data, this HIF-2α upregulation in tumors of 786-O-VHL-K171G-injected mice is probably a secondary effect, possibly due to the development of a hypoxic environment in 786-O-VHL-K171G tumors.

Discussion

Loss of pVHL can activate cellular pathways that are strongly associated with both tumor initiation and progression. Because upregulation of HIFs is thought to be an important pathway that mediates tumor progression in pVHL-deficient states, the pathways mediating tumor initiation are not clearly defined. Although aneuploidy, a form of CIN in pVHL-deficient MEFs, has been linked to a deregulated spindle assembly checkpoint (Thoma et al., 2009), the source of increased multinucleation and polyploidy in pVHL-deficient RCC remains unexplained. In this study, we observed that pVHL-deficient 786-O cells show increased multinucleation and the frequent occurrence of unresolved cytokinetic bridge structures. Moreover, cytogenetic analysis revealed that pVHL-deficient 786-O cells are polyploid in nature. Consistent with that, an increase in multinucleation by knocking down pVHL or overexpressing pVHL-K171G in HeLa or Caki-1 cells further supports our hypothesis.

We also report that pVHL co-localizes with MKLP-1 at the spindle midzone during late anaphase but becomes separated at the terminal midbody. More specifically, MKLP-1 localizes to the Flemming body, whereas pVHL localizes to the stem region of cytokinetic bridge during cell cleavage. Because polyploidy can be attributed to failed cytokinesis (Chi and Jeang, 2007), a number of key cytokinetic regulatory proteins, which are required for the final abscission step, have been analyzed. We observed that pVHL-null 786-O cells and 786-O cells expressing pVHL-K171G mutant are defective in localization to the midbody of Alix and Endobrevin, but not of upstream effectors Aurora B, Plk1 and Cep55 or downstream effector Tsg101. The lysine 171 residue in pVHL is unique because we did not find any effect on the localization of Alix or Endobrevin by other reported mutants of pVHL, such as Y98H and Y112H. We also found that pVHL-deficient-A498 cells were also defective in Alix localization at the midbody but not wild-type pVHL Caki-1 cells. Recent studies have established that the Cep55, Alix and Tsg101 axis functions at the terminal midbody and regulates the final cleavage (Morita et al., 2007). Alix and Tsg101 concentrate at the centrosome and are then recruited to the midbody through direct interaction with the centrosome-associated protein Cep55 (Morita et al., 2007). When we checked the association of pVHL with Alix, Cep55 and Tsg101, we found a decreased association of pVHL-K171G compared with wild-type pVHL with Alix but not with Cep55 or Tsg101. We also observed decreased interaction of Alix with both Cep55 and Tsg101 in cells overexpressing pVHL-K171G. pVHL-K171G also affected the interaction between Cep55 and Tsg101. Therefore, we propose that pVHL is a part of the Cep55–Alix–Tsg101 complex, and that pVHL interaction with Alix is important for the recruitment of Alix to the Cep55–Tsg101 complex at the midbody. Endobrevin, a member of SNARE membrane fusion machinery, localizes to the midbody and is required for the final abscission step (Low et al., 2003) during cytokinesis. Our data also suggest that Endobrevin is a component of the pVHL–Alix–Cep55–Tsg101 complex, and that Endobrevin interaction with pVHL is crucial for the localization of Endobrevin at the midbody.

The involvement of lysine 171 of pVHL in the regulation of midbody abscission intrigued us to explore the functional importance of the site. We identified the tumor suppressor pVHL as a target for SUMOylation. We observed reduced interaction of pVHL-K171G with Alix and Endobrevin in absence of SUMOylation. Thus, we suggest that SUMO modification of pVHL is necessary for the complex formation with Alix and Endobrevin. However, the complex formation of pVHL with Cep55 or Tsg101 is independent of pVHL SUMOylation. We also observed significantly reduced nuclear localization of pVHL-K171G in the absence of SUMOylation. In-depth studies are underway to delineate the mechanism of how SUMOylation alters the function of pVHL as a cytokinesis regulator. This study reveals a previously unexplored role of the pVHL.

pVHL95-123 and, more specifically, type 2A pVHL mutants Y98H and Y112H have been shown to disrupt the microtubule-destabilizing function of pVHL (Hergovich et al., 2003). Recently, Thoma and associates showed that pVHL Y98H and Y112H also failed to correct the spindle misorientation phenotype of 786-O cells but were not associated with the level of the mitotic arrest deficient protein 2 (Mad-2) (Thoma et al., 2009). However, we did not find a change in pVHL-directed localization of Alix or Endobrevin with reported pVHL mutants such as Y112H, N90G, or L188V (type 2C). Therefore, spindle assembly checkpoint failure and defective cytokinesis are different pVHL-regulated events.

Inactivation of a number of tumor suppressor genes has been implicated in cytokinesis failure and CIN (Caldwell et al., 2007; Daniels et al., 2004; Fujiwara et al., 2005). We report that pVHL-null and pVHL-K171G mutant cells are polyploid because of cytokinesis failure and are able to form tumors in mice. Although K171G is not a reported mutation, pVHL truncating mutations that affect pVHL are defective in localization to the midbody of Alix and Endobrevin with reported pVHL mutants such as Y112H, N90G, or L188V (type 2C). Therefore, spindle assembly checkpoint failure and defective cytokinesis are different pVHL-regulated events.

Materials and Methods

Cell culture and transfection

Human RCC 786-O cells were cultured as described by Sinha et al. (Sinha et al., 2008). 786-O cells were stably transfected with pcDNA3.1hygro (786-O-empty), pRC-HA–VHL [786-O-VHL(ori)], pcDNA3.1hygro–HA–VHL–K171G (786-O-VHL–K171G), or pcDNA3.1hygro–HA–VHL–N90G (786-O-VHL–N90G) and maintained in DMEM medium with 10% FBS supplemented with G418 (1 mg/ml) or hygromycin (150 μg/ml). 293T cells were grown in DMEM medium and transfected with 2 μg of pBabe-puro-HA–VHL-L188V or pBabe-puro-HA–VHL-Y112H DNA using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the
SUMOylated pVHL potentiates cytokinesis

Mutant SUMO constructs with deletion of C-terminal di-glycine residues were generated by adding a stop codon on the site for the first glycine using PCR and subcloned in p3×FLAG-CMV10 vector (Sigma-Aldrich). SUMOylation assay was carried out as described above.

HeLa cells were also transfected with pRChA–VHL(wt) and pCDNA3.1Hygro–HA–VHL-K171G using PolyFect reagent. SUMOylation assay was carried out as described above.

RNA preparation and quantitative real time PCR
RNA preparation and real-time PCR (RT-PCR) was carried out as described previously (Sinha et al., 2009a). VEGF-A, Glut-1, HIF-2α and ACTB primers were purchased from SA Biosciences (Frederick, MD). Relative expression was calculated using the comparative C_t method (Pichiorri et al., 2008).

Tumor model and in vivo anti-tumor activity
Details of the RCC subcutaneous tumor model are described in supplementary material Table S2. Female, 6-week-old, nude mice were obtained from the National Institutes of Health and housed in the institutional animal facilities. All animal work was performed under protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. To establish tumor growth in mice, we injected 5×10^6 786-O-empty, 786-O-VHL(wt), and two clones (C3 and C3.1) of 786-O-VHL-K171G cells, resuspended in 100 μl of PBS, subcutaneously into the right flank. Tumors were allowed to grow for 4 weeks. Beginning at week 4 after tumor cell injection, tumors were measured every week, and primary tumor volumes were calculated by the formula: volume = 1/2 × x × y × z, where x is the longest tumor axis, y is the second longest axis, and z is the shortest tumor axis. At week 9, all mice injected with 786-O-empty cells were sacrificed by asphyxiation with CO_2. At week 13, 786-O-VHL-K171G tumor-bearing mice were sacrificed; tumors were removed, measured and prepared for immunohistochemistry and western blot.

Histological study
Tumors were removed and fixed in neutral buffered 10% formalin at room temperature for 24 hours prior to embedding in paraffin and sectioning. Sections were deparaffinized and then subjected to hematoxylin-eosin and HIF-2α immunohistochemistry staining according to the manufacturer’s instructions. Stable dianimobenzidine was used as a chromogen substrate, and the HIF-2α sections were counterstained with a hematoxylin solution. Photographs of the entire cross-section were digitized using an Olympus camera (DP70).

Statistical analysis
Statistical analysis was performed with statistical SPSS software (version 11.5; Chicago, IL). The independent-samples t-test was used to test the probability of significant differences between groups. Statistical significance was defined as P<0.05; statistically high significance was defined as P<0.01. Error bars were given on the basis of standard deviation values calculated.

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References
Baker, D. J., Jeganathan, K. B., Malureau, L., Perez-Terzic, C., Terzic, A. and Van Deursen, J. M. (2006). Early aging-associated phenotypes in Bub3/Rael hypolipidsufficient mice. J. Cell Biol. 172, 529-540.
Calderwood, C. M., Green, R. A. and Kaplan, K. B. (2007). APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. J. Cell Biol. 178, 1109-1120.
Carlston, J. G. and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroglobulin: a role for the ESCRT machinery. Science 316, 1908-1912.
Chi, Y. H. and Jeang, K. T. (2007). Aneuploidy and cancer. J. Cell. Biochem. 102, 531-538.
Cockman, M. E., Masson, N., Mole, D. R., Jaakkola, P., Chung, G. W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratcliffe, P. J. and Maxwell, P. H. (2000). Hypoxia-inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J. Biol. Chem. 275, 25733-25741.

Crossley, P. A., Richards, F. M., Foster, K., Green, J. S., Prowse, A., Latif, F., Lerman, M. I., Zbar, B., Affara, N. A., Ferguson-Smith, M. A. et al. (1994). Identification of intragenic mutations in the von Hippel-Lindau disease tumour suppressor gene and correlation with disease phenotype. Hum. Mol. Genet. 3, 1303-1308.

Daines, M. J., Wang, Y., Lee, M. and Venkitaraman, A. R. (2004). Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science 306, 876-879.

Fabbro, M., Zhou, B. B., Takahashi, M., Sarveev, B., Lal, P., Graham, M. E., Gabrielli, B. G., Robinson, P. J., Nigg, E. A., Ono, Y. et al. (2005). Cdk1/Erk-2 and PI3K-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. Dev. Cell 9, 477-488.

Fujiiwara, T., Bandi, M., Nitta, M., Ivanova, E. V., Bronson, R. T. and Pellman, D. (2005). Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature 437, 1043-1047.

Giles, R. H. and Voest, E. E. (2005). Tumor suppressors APC and VHL: gatekeepers of the intestine and kidney. In Developmental Biology of Neoplastic Growth, Vol. 40 (ed. A. M. Coelho), pp. 151-181. Utrecht: Springer Berlin Heidelberg.

Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Ozcan, A., Shen, S. S., Zhai, Q. J. and Truong, L. D. (2008). Role of VHL gene mutation in human cancer. Hum. Mol. Genet. 3, 595-598.

Gonzalez, R. M., Dargemont, C. and Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. J. Biol. Chem. 276, 12654-12659.

Roe, J. S., Kim, H., Lee, S. M., Kim, S. T., Cho, E. J. and Youn, H. D. (2006). p53 stabilization and transactivation by a von Hippel-Lindau protein. Mol. Cell 22, 395-405.

Sampson, D. A., Wang, M. and Matunis, M. J. (2001). The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. J. Biol. Chem. 276, 21664-21669.

Schoenfeld, A., Davidowitz, E. J. and Burk, R. D. (1998). A second major native von Hippel-Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. Proc. Natl. Acad. Sci. USA 95, 8817-8822.

Siemer, G., Weinid, K., Mohrs, K., Barleon, B., Martin-Baron, G. and Marne, D. (1996). Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. Cancer Res. 56, 2299-2301.

Sinha, S., Cao, Y., Dutta, S., Wang, E. and Mukhopadhyay, D. (2008). VEGF neutralizing antibody increases the therapeutic efficacy of vinorelbine for renal cell carcinoma. J. Cell. Mol. Med. 12, 647-658.

Sinha, S., Dutta, S., Dutta, K., Ghosh, A. K. and Mukhopadhyay, D. (2009a). Von Hippel-Lindau gene product modulates TS11B expression in renal cell carcinoma: impact on vascular endothelial growth factor expression in hypoxia. J. Biol. Chem. 284, 32610-32618.

Sinha, S., Vohra, P. K., Bhattacharya, R., Dutta, S., Sinha, S. and Mukhopadhyay, D. (2009b). Dopamine regulates phosphorylation of VEGF receptor 2 by engaging Src-homology-2-domain-containing protein tyrosine phosphatase 2. J. Cell. Sci. 122, 3385-3392.

Stebbins, C. E., Kaelin, W. G., Jr and Pavletich, N. P. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. Science 284, 455-461.

Stolle, C., Glenn, G., Zbar, B., Humphrey, S. J., Choyke, P., Walther, M., Pack, S., Hurley, K., Andrey, C., Klaussner, R. et al. (1998). Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. Hum. Mutat. 12, 417-423.

Strack, B., Calistra, A., Craig, S., Popova, E. and Gottlinger, H. G. (2003). APL1/ALIX is a binding partner for HIV-1 p6 and ELAV p9 functioning in virus budding. Cell 114, 689-699.

Thoma, C. R., Too, A., Gubbott, K. L., Reggi, S. P., Frew, I. J., Schraml, P., Hergovich, A., Mohc, H., Meraldi, P. and Krek, W. (2009). VHL loss causes spindle misorientation and chromosome instability. Nat. Cell Biol. 11, 139-146.

Mohr, S., Auffert, A., Ebert, G., Zschiedrich, F., Richter, H., Ikeda, N., Kamada, K., Stetser, E., Cremer, M. et al. (2009). The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. J. Biol. Chem. 277, 39887-39898.

Zbar, B., Kishida, T., Chen, F., Schmidt, L., Maher, E. R., Richards, F. M., Crosse, P. A., Webster, A. R., Affara, N. A., Ferguson-Smith, M. A. et al. (1996). Germline mutations in the von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. Hum. Mutat. 8, 348-357.

Zhou, M. I., Wang, H., Ross, J. J., Kuzmin, I., Xu, C. and Cohen, H. T. (2002). The von Hippel-Lindau tumor suppressor stabilizes novel plant homoeodomain protein Jade-1. J. Biol. Chem. 277, 39887-39898.