Molecular and Cellular Pathobiology

Tumor Suppressor VHL Functions in the Control of Mitotic Fidelity

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Abstract

The von Hippel–Lindau (VHL) tumor suppressor protein pVHL is commonly mutated in clear cell renal cell carcinoma (ccRCC) and has been implicated in the control of multiple cellular processes that might be linked to tumor suppression, including promoting proper spindle orientation and chromosomal stability. However, it is unclear whether pVHL exerts these mitotic regulatory functions in vivo as well. Here, we applied ischemic kidney injury to stimulate cell division in otherwise quiescent mouse adult kidneys. We show that in the short term (5.5 days after surgery), Vhl-deficient kidney cells demonstrate both spindle misorientation and aneuploidy. The spindle misorientation phenotype encompassed changes in directed cell division, which may manifest in the development of cystic lesions, whereas the aneuploidy phenotype involved the occurrence of lagging chromosomes but not chromosome bridges, indicative of mitotic checkpoint impairment. Intriguingly, in the long term (4 months after the ischemic insult), Vhl-deficient kidneys displayed a heterogeneous pattern of ccRCC precursor lesions, including cysts, clear cell–type cells, and dysplasia. Together, these data provide direct evidence for a key role of pVHL in mediating oriented cell division and faithful mitotic checkpoint function in the renal epithelium, emphasizing the importance of pVHL as a controller of mitotic fidelity in vivo. Cancer Res; 74(9): 2422–31. ©2013 AACR.

Introduction

Inactivation of the VHL tumor suppressor gene plays a causal role in the development of hereditary (von Hippel–Lindau disease) and sporadic clear cell carcinoma of the kidney, establishing VHL as a critical "gatekeeper" of the renal epithelium (1). The gene product of VHL, pVHL, is a multi-functional protein implicated in the regulation of a variety of cellular processes essential for epithelial homeostasis, including cell growth and differentiation, modulation of cell-death pathways, extracellular matrix deposition, hypoxia response, and primary cilium maintenance (2, 3).

Recent cell-based studies in VHL-deficient renal carcinoma cell lines unveiled a critical role for pVHL in the regulation of two key aspects of mitosis—suppression of spindle misorientation and preservation of normal mitotic checkpoint function (4). Spindle misorientation has been linked, in this setting, to unstable astral microtubules, whereas impaired mitotic checkpoint function and resultant aneuploidy involves a mechanism causing reduced expression of Mad2, a spindle checkpoint control protein, whose changes in abundance is associated with impaired mitotic checkpoint function.

Whether these newly identified mitotic functions of pVHL are relevant in vivo in the renal epithelium is not known. However, both of these mitotic functions could potentially contribute to key characteristics of human VHL-associated pathology (1, 5, 6). In the renal epithelium, changes in spindle orientation could, in principle, alter the directionality of cell division, a phenomenon believed to contribute to cyst formation during development (7, 8). Similarly, the production of chromosome missegregation errors could culminate in the emergence of aneuploid cells, a well-known enabling feature of tumor development (9, 10). To test this directly, we resorted to an injury-regeneration model, in which kidney epithelial cells are challenged with ischemic injury and are consequently induced to reenter the cell cycle as part of a regenerative response of the tissue (11, 12). Our cell biologic and molecular analyses of primary mouse renal epithelial cells and kidney epithelia from kidney-specific Vhl-deficient and Cre-negative control mice that have been challenged with ischemic injury highlight a key role for VHL as a suppressor of spindle misorientation and chromosomal instability in vivo.

Materials and Methods

Mouse strain information and general techniques

Kidney-specific Vhl knockout mice (Vhl−/−) were obtained as described previously (13, 14). Mice were kept under standard pathogen-free conditions in accordance with Swiss animal welfare regulations.
Ischemic kidney injury surgery

Kidney clamp surgery was performed under sterile conditions in a laminar flow hood on 6- to 9-week-old female mice. Anesthesia was initiated with 5 vol% isoflurane in pure oxygen (800 mL/minute) and maintained at approximately 2 vol% isoflurane (500 mL/minute O₂). In addition, a single dose of buprenorphine (0.2 mg/kg, s.c.) was given. To initiate the injury, a nontraumatic micro aneurysm clip (Harvard apparatus; #610186) was placed around the left renal pedicle for 30 minutes, followed by reperfusion after clamp removal. The right kidney remained unclamped and served as internal control. Animals were sacrificed 5.5 days or 4 months after injury and both kidneys were removed for further analysis. In the case of long-term experiment, one animal per surgery session was included that had been sacrificed already after 5.5 days to confirm the efficiency of the kidney clamp.

Primary kidney cell preparation and culturing

Kidneys were dissected from 6- to 7-week-old Vhl adversary male mice, and further processed under sterile conditions as described previously (15). Kidneys were cut into small pieces with a surgical razorblade and digested at 37°C for 30 minutes with collagenase 4a (200 U/ml, Worthington Biochemical Corp.). Digested tissue was passed through a 70-μm cell strainer and washed with 5% fetal calf serum (FCS) in PBS. Cells were resuspended and plated in K-1 medium [50:50 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12], supplemented with 2.5% FCS (Bioconcept), 5% horse serum (Bioconcept), 10 mmol/L Hepes (Life Technologies), 1.1 mg/ml sodium bicarbonate (Sigma), 5 μg/ml insulin (Sigma), 25 ng/ml prostaglandin E1 (Sigma), 5 × 10⁻¹² mol/L triiodothyronine (Sigma), 10 nmol/L sodium selenite (Sigma), 5 × 10⁻³ mol/L hydrocortisone, and 5 μg/ml transferrin (Sigma). Seven days after plating, cells were infected with adenovirus expressing Cre or Cre-internal ribosome entry site (IRES)-GFP and LacZ or green fluorescent protein (GFP) as control, respectively, and optionally with pLKO lentivirus overexpressing pVHL or a control cassette (R. Pawlowski, A. Ittner, data not published), and selected for 3 days on puromycin (2 μg/ml) before harvesting for subsequent assays.

Western blotting

Western blot analysis was performed as described previously (4). The antibodies used in this study were anti-mouse pVHL(m) CT antibody (16) and anti-Cdk2 (Santa Cruz Biotechnology; sc-163-G).

Transcription analyses

RNA was isolated from cultured cells of equal confluence using TRIzol (Life Technologies) according to the manufacturer's instructions. One microgram of total RNA was reversely transcribed using a premixed polymerase mix (EcoDry; ClonTech) according to the manufacturer's instructions. One microgram of total RNA was reversely transcribed using TRIzol (Life Technologies) according to the manufacturer's instructions. One microgram of total RNA was reversely transcribed using a premixed polymerase mix (EcoDry; ClonTech) and analyzed by quantitative real-time PCR (qRT-PCR) using the SYBR Green system on a LightCycler (Roche Applied Science) instrument. The following primer pairs were used (all yielding PCR products with a single melting point): glucose transporter 1 (Glut1; 5’-CAG TTC GGC TAT AAC ACT GGT G-3’ and 5’-GCC CCC GAC AGA GAA CAT GAT G-3’, phosphoglycerate kinase (PGK1); 5’-TGG AGC CAA CTC CGT TGT C-3’ and 5’-CAG GCA TTC TCG ACT TCT GGG-3’, VEGFA; 5’-CTT GTT CAG AGC GGA GAA AGC-3’ and 5’-ACA TCT GCT AGT ACG TTC GTT-3’, hypoxanthine phosphoribosyltransferase (HPRT); 5’-TCA GTC AAC GGG GGA CAT AAA-3’ and 5’-GGG GCT GTA CTG CTT AAC CAG-3’.

Staining methods

Cells grown on a coverslip were fixed for 5 minutes with cold methanol. Kidneys were cut in half and fixed overnight either with 4% paraformaldehyde (PFA) or formalin. PFA-fixed tissues were embedded in 4% low melting point agarose (W5376R; Fisher Scientific) before cutting (50 μm sections) with a vibratome (Hyrax V50; Zeiss). Formalin-fixed tissues were dehydrated on a TPC 15 Duo (Medite AG) tissue processor and subsequently treated in a paraffin-embedding station (Medite AG). Sections (4 or 10 μm) were cut on a HM 355S microtome with Cool Cut and Section-Transfer-System (Microm AG). The following primary antibodies were used: anti-α-tubulin (homemade from rat hybridoma clone YL1/2; Sigma-Aldrich), anti-phosphorylated (Ser10) histone 3 (p-H3; Cell Signaling Technology; 9706S), anti Ki-67 (Dako Cytomation; M7249), anti-thiavione-sensitive sodium chloride cotransporter (NCC; Millipore; AB3553), anti-glucose transporter 1 (Glut1; Abcam; ab14683), anti-hypoxia-inducible factor 1 α (HIF 1α; Novus Biologicals; NB100-479), anti-β-tubulin (Sigma, T3559; Abcam; ab11316), anti-E-cadherin (Cell Signaling Technology; 3195S, clone 24E10), anti-atypical protein kinase C zeta (aPKCζ; Santa Cruz Biotechnology; sc-17781). All secondary antibodies were obtained from Life Technologies. Rhodamine-labeled Dolichos Biflorus Agglutinin (DBA; Vector Laboratories Inc.; RL-1032) was used for direct labeling of distal tubules (as a replacement of NCC in case of species cross-reactivity). DAPI (4’,6-diamidino-2-phenylindole) was applied as nuclear counterstain. Hematoxylin and eosin (H&E) stainings were performed on an automatic COT 20 stainer (Medite AG). Terminal deoxynucleotidy transferase–mediated dUTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit (Roche Applied Sciences) according to the manufacturer’s protocol. Fluorescence in situ hybridization (FISH) detection of murine chromosome 7 was performed after antigen retrieval and protein digestion (45 minutes; Digest-All III; Life Technologies) with a probe provided by Heather Flynn Gilmer and Dr. Patricia T. Greipp (Mayo Clinics, Cleveland, OH; see also ref. 17). Image acquisition was performed on widefield (Zeiss Axioplan2 and Observer Z2, DeltaVision personalDV) and confocal (Zeiss LSM510 or Leica TCS SP2) fluorescence microscopes. Chromogenic stains were analyzed on a Zeiss AxioImager A1. Image analysis was performed using ImageJ (NIH), Imaris (Bitplane AG), and the ZEN software (Zeiss).

Metaphase spreads

Exponentially growing cells were arrested in metaphase using nocodazole (250 ng/mL, overnight treatment). Cells were collected and treated for 7 minutes in buffered hypotonic solution (0.05 mol/L KCl, 0.02 mol/L Tris, pH 7.5). Subsequent fixation was performed with pre-chilled methanol/acetic acid.

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(3:1) at ambient temperature for 10 minutes. Metaphase spreads were performed by dropping cell suspension at 37°C in a humidified environment and mounted in mowiol (Calbiochem) containing 1 μg/mL of DAPI.

**Spindle angle measurements**

Spindle angle measurements were performed as described elsewhere (8). Shortly, Z-stack images were acquired (0.5 μm for kidney sections and 0.3 μm for primary kidney cells) and the three-dimensional representation of the image was done using the Imaris software (Bitplane AG). Only sections with a complete mitotic spindle were included in further analysis. The three-dimensional coordinates describing the direction of the kidney tubule and the mitotic spindle were used to calculate the net angle of division. In case of cross-sections, the z-dimension was omitted as it was not relevant for the type of analysis. The following mathematical formulas were used:

Net angle:

\[
\cos \alpha = \frac{a \cdot b}{|a| \cdot |b|} = \frac{a_1b_1 + a_2b_2 + a_3b_3}{\sqrt{a_1^2 + a_2^2 + a_3^2} \cdot \sqrt{b_1^2 + b_2^2 + b_3^2}}
\]

Cross-section angle:

\[
\cos \alpha = \frac{a \cdot b}{|a| \cdot |b|} = \frac{a_1b_1 + a_2b_2}{\sqrt{a_1^2 + a_2^2} \cdot \sqrt{b_1^2 + b_2^2}}
\]

**Statistical and error analyses**

Data are plotted as mean ± SD, mean ± SEM, or median (indicated in the figure legend). If applicable, Gaussian error propagation was used. In cases in which normal distribution could be assumed, a two-sided unpaired Student t test was applied; otherwise the Mann–Whitney U test was used. P-values were *, P < 0.05; **, P < 0.01; and ***, P < 0.001. For the sake of clarity, nonsignificant associations were only mentioned when instructive. Significant changes in chromosome number distribution were tested for by using the Mood test (18) in the statistical package R (19). Differences in the frequency of lagging chromosome (single or multiple combined vs. normal and chromosome bridges) as well as chromosome FISH counts were assessed by the \( \chi^2 \) test.

**Results**

**Loss of Vhl in primary mouse renal epithelial cells in vitro induces spindle misorientation and aneuploidy**

To assess the influence of Vhl loss-of-function on spindle misorientation and aneuploidy generation in the context of mouse primary renal epithelial cells (MREC), we isolated renal epithelial tubule cells from mice homozygously carrying loxP-flanked Vhl alleles (Vhl\(^{fl/fl}\)) and genetically inactivated Vhl by adenoviral transduction with Cre-recombinase (AdCre). As a control, the same cells were infected with an adenovirus expressing LacZ (AdLacZ). Immunoblotting confirmed the successful excision of Vhl (Fig. 1A). Using immunofluorescence microscopy, we analyzed spindle assembly checkpoint-dependent and -independent chromosome segregation errors that include in particular lagging chromosomes and chromosome bridges, respectively (Fig. 1B). Quantification of these images revealed an increased occurrence of single and multiple lagging chromosomes, while the rate of chromosome bridges remained unchanged (Fig. 1C), indicative of a weakened spindle checkpoint. To further corroborate this finding, we counted the number of chromosomes in metaphase spreads prepared from wild-type and Vhl-deficient MRECs. As illustrated in Fig. 1D, the distribution of chromosome numbers in Vhl-deleted cells is significantly broader than in the corresponding Vhl-positive MRECs, suggestive of increased levels of aneuploidy.

In parallel, we investigated whether ablation of Vhl also leads to spindle misorientation in MRECs. As a measure of misorientation, we determined the spindle angle, defined by the direction of the spindle relative to the glass support (Fig. 1E). Although the mitotic spindles in AdLacZ-treated cells preferentially aligned parallel to the support, inactivation of pVHL caused the spindle to be more frequently tilted (Fig. 1F). Quantification of the spindle angle showed a significant increase of the median spindle angle in Vhl\(^{-/-}\) cells compared with control cells (Fig. 1G). At the same time, the spindle diameter remained unaffected (Fig. 1H).

To independently confirm the pVHL specificity, we performed a rescue experiment in which we reexpressed full-length pVHL in Vhl\(^{fl/fl}\) MRECs transduced with AdGFP or AdCre (coexpressing GFP). GFP positivity combined with immunofluorescence stainings for HIF1α, which is stabilized upon pVHL depletion, provided us with a single cell–based readout for the absence or presence of pVHL (Supplementary Fig. S1A and S1B). Assessment of mRNA levels of HIF1α target genes also confirmed that Vhl was successfully deleted or reexpressed (Supplementary Fig. S1C). As shown in Supplementary Fig. S1D and S1E, we could recapitulate that the loss of Vhl introduces chromosome missegregation and spindle misorientation, whereas reexpression of full-length pVHL rescued these phenotypes, consistent with the view that suppression of these phenomena is part of the proposed "gate-keeper" function of pVHL in kidney epithelial cells.

**Analysis of proliferative and apoptotic response of Vhl-deficient kidney cells in vivo following renal injury**

Next, we applied an ischemic injury model to kidney-specific Vhl knockout mice (Vhl\(^{fl/fl}\)) and Cre-negative littermates. In this model, kidney epithelial cells are encouraged to reenter the cell cycle as part of a regenerative response of the tissue. To this end, we subjected mice to unilateral clamping of the left kidney pedicle before kidney reperfusion. The right kidney remained unstressed and served as internal control. Both kidneys were harvested at 5.5 days after surgery (Fig. 2A), as, at this time point, we observed maximal injury-induced kidney cell proliferation. Kidney weight did not significantly change between either clamped and the corresponding contralateral control kidney or Cre-negative and Vhl\(^{fl/fl}\) littermates (Fig. 2B). Macroscopically, the clamped kidney seemed slightly paler (Supplementary Fig. S2A). We did not observe any differences in the extent of clamping-induced injury after 5.5 days between clamped Cre-negative and Vhl\(^{fl/fl}\) kidneys (Supplementary Fig. S2B). In addition, in each case, as demonstrated in Supplementary Fig. S3A and S3B, we reconfirmed the pVHL status by
specific immunostainings for HIF1α and Glut1, which are well-established readouts for Vhl negativity (20, 21).

To provide evidence that induction of proliferation had occurred, we immunofluorescently stained kidney tissues with antibodies recognizing the proliferation marker Ki-67, and phosphorylated histone 3 (p-H3) to label mitotic cells. Upon injury, we observed a dramatic increase in staining for both Ki-67 and p-H3 in clamped kidneys compared with unclamped control kidneys (Supplementary Fig. S3C). In addition, the observed high rates of proliferation were accompanied by enhanced apoptosis, as evidenced by increased TUNEL positivity in injured compared with control kidneys (Supplementary Fig. S3D). Kidney-specific Vhl deletion did not measurably affect neither the proliferative nor the apoptotic response compared with Cre-negative kidneys (Fig. 2C–G). Thus, Vhl deficiency does not affect, to any noticeable degree, the proliferative and apoptotic responses of kidneys to ischemic injury.
pVHL suppresses spindle misorientation in kidney tubule cells in vivo

To assess whether mitotic spindle orientation is perturbed in Vhl<sup>−/−</sup> kidneys that underwent ischemic injury, we costained kidney sections with anti–p-H3 and anti–α-tubulin to visualize the mitotic spindle. As an additional spindle marker, we included γ-tubulin that localizes predominantly to the centrosomal region in our analysis. Depending on whether individual kidney tubules are cut along their longitudinal axis or cross-sectional plane, two different types of angles can be distinguished. The net angle is defined by the relative directions of the mitotic spindle and the kidney tubule (Fig. 3A). Immunofluorescence microscopy revealed that deletion of Vhl significantly affected the net angle (Fig. 3B). The cross-section angle that describes the deviation of the mitotic spindle from the plane perpendicular to the apical–basal axis (Fig. 3C) was, likewise, affected by Vhl deletion (Fig. 3D). Quantification of these immunofluorescence images corroborated that pVHL is a major suppressor of both net and cross-section spindle angle changes in vivo (Fig. 3E and F, respectively). The outcome of this analysis was not affected by using either α- or γ-tubulin as markers for marking the entire spindle and its poles, respectively (Supplementary Fig. S4A). Because in our conditional knockout model, Cre expression is confined to distal but not proximal tubule cells, we compared the effect of Vhl deletion on spindle angles in both tubule types. As marker proteins for these cell types, we used Glut1 positivity as a readout for successful Vhl knockout in Vhl<sup>−/−</sup> kidneys and the distal tubule marker thiazide-sensitive NCC in Cre-negative kidneys (Supplementary Fig. S4B and S4C). Importantly, changes in both the net and cross-section spindle angles occurred preferentially in distal kidney tubule cells of Vhl<sup>−/−</sup> mice (Fig. 3G and H). These data suggest that the loss of pVHL influences the orientation of cell division within the kidney tissue and argue for a critical function for pVHL in the suppression of mitotic spindle misorientation in vivo.

Figure 2. Kidney injury induces proliferative and apoptotic response independently of pVHL status. A, timeline of the renal injury experiment. B, quantification of the postinjury kidney weight for Cre-negative and Vhl<sup>−/−</sup> mice; both clamped and control kidneys included (n = 4 mice per genotype, mean ± SD). C, representative images showing proliferative response by p-H3 (green) and Ki-67 (red) in kidneys of Cre-negative and Vhl<sup>−/−</sup> mice following renal injury (counterstained with DAPI; scale bar, 50 μm). D, quantification of Ki-67 staining in Cre-negative and Vhl<sup>−/−</sup> mice 5.5 days postinjury (n = 14 independent slides per genotype, mean ± SEM, Student t test). E, quantification of p-H3 staining in Cre-negative and Vhl<sup>−/−</sup> mice postinjury (n = 7 independent slides per genotype, mean ± SEM, Student t test). F, assessment of apoptotic nuclei (red) by TUNEL assay in clamped kidneys; scale bar, 20 μm. G, quantification of TUNEL staining in Cre-negative and Vhl<sup>−/−</sup> mice postinjury (n = 7 independent slides per genotype, mean ± SEM, Student t test).
Vhl deletion prompts chromosome missegregation and aneuploidy in injury-exposed kidney epithelial cells

We next asked whether kidney tubule cells lacking Vhl and challenged by ischemia would, unlike their Cre-negative littermates, display increased chromosome segregation errors and aneuploidy. To address this, we quantified the frequency of chromosome missegregations in clamped Cre-negative and Vhl<sup>−/−</sup> kidneys 5.5 days after ischemic injury (scale bar, 20 μm). Dotted lines represent the direction of the kidney tubule, whereas thin lines indicate the spindle orientation. Insets, enlargement of mitotic spindle. Top/bottom, relative z-stack position. C, schematic representation of the cross-section spindle angle (α) calculation: a, plane perpendicular to apical–basal polarity axis; b, position of mitotic spindle. D, example pictures showing spindle orientation in cross-sections of kidney tubules treated as in B. E, distribution of the net spindle angle (n = 64 for Cre-negative and n = 74 for Vhl<sup>−/−</sup>). F, same as E but for the cross-section angle (n = 56 for Cre-negative and n = 42 for Vhl<sup>−/−</sup>). G, quantification of the net spindle angle with respect to proximal and distal tubules (bar indicates median, n = 4 mice per genotype, Mann–Whitney U test). H, same as G but for the cross-section angle.

VHL Suppresses Spindle Misorientation and Aneuploidy In Vivo

Vhl deletion prompts chromosome missegregation and aneuploidy in injury-exposed kidney epithelial cells

We next asked whether kidney tubule cells lacking Vhl and challenged by ischemia would, unlike their Cre-negative littermates, display increased chromosome segregation errors and aneuploidy. To address this, we quantified the frequency of chromosome missegregations in clamped Cre-negative and Vhl<sup>−/−</sup> kidneys using p-H3 as a marker for kidney tubule cells in late anaphase or early telophase. In analogy to the <i>in vitro</i> experiments in primary kidney cells, we analyzed the occurrence of lagging chromosomes (single or multiple) and chromosome bridges (Supplementary Fig. S5A). The former allowed estimation of the rate of newly generated aneuploidy, whereas the latter served as internal control. Quantification of these errors in tubule cells revealed that genetic deletion of Vhl increased the frequency of lagging chromosomes without affecting the rate of chromosome bridges analyzed in the same samples (Fig. 4A). Analysis of this phenomenon separately in distal and proximal tubules, revealed, like before for spindle misorientation, a selective increase in the percentage of lagging chromosomes in Vhl<sup>−/−</sup> distal (but not proximal) tubule cells (Fig. 4B), suggesting that this effect on chromosome missegregation is pVHL specific. Together, these results support the interpretation that Vhl loss impairs the mitotic checkpoint, thereby increasing the chance that cells with unattached chromosomes might undergo cell division.
To examine whether kidney-specific \( Vhl \) loss results in an aneuploid phenotype, as the increased percentage of lagging chromosomes would suggest, we performed FISH with a probe against chromosome 7 on kidney sections from clamped mice. Indeed, as shown in Fig. 4C, \( Vhl \) deletion resulted in a considerable number of cells with abnormal chromosome 7 copy numbers. Quantitative analysis of chromosome copy numbers per nucleus revealed that the relative frequency of nuclei differing from chromosome 7 disomy was in fact significantly increased in \( Vhl^{D/D} \) mice (Fig. 4D). Discrimination between proximal and distal tubule cells further supported the \( pVHL \) specificity of this phenomenon, because the relative frequency of nuclei with a copy number deviating from two was increased specifically in distal tubules of kidney-specific \( Vhl \) knockout mice (Fig. 4E). These data suggest that distal tubule cells of the kidney lacking \( Vhl \) are prone to chromosome segregation errors and aneuploidy when challenged to commit to mitotic cell divisions by short-term ischemic injury.

**Development of ccRCC precursor lesions following recovery of \( Vhl^{A/A} \) kidneys from injury**

To assess possible long-term effects of kidney injury as a function of \( pVHL \) status, we kept \( Vhl^{A/A} \) and corresponding Cre-negative littermates for 4 months after the injury and then analyzed the potential morphologic changes in the kidney. Importantly, analysis of H&E staining of sections derived from clamped and contralateral kidneys of Cre-negative animals did not reveal any histologic differences, demonstrating that the ischemia-induced damage is reversible in control animals (Fig. 5A). In contrast to Cre-negative animals, clamped \( Vhl^{A/A} \) kidneys displayed three major categories of morphologic alterations. These included the appearance of dysplastic spots with multilayered tubules and nuclear crowding, clear cells, and simple tubular cysts (Fig. 5B–E). Quantification of the number of spots of each aberration type per kidney section revealed that clamping significantly increased the relative frequency and extent of the aberrations in \( Vhl^{A/A} \) kidneys, whereas Cre-negative mice hardly showed any defects both in clamped and unclamped kidneys (Fig. 5F–H). Analysis of proliferation by Ki-67 staining revealed a moderate increase of basal proliferation in clamped and, to a lesser extent, in nonclamped \( Vhl^{A/A} \) kidneys compared with control conditions, while the apoptotic rate remained unchanged (Supplementary Fig. S6A and S6B). In parallel, also a loss of distal tubule polarity, as evidenced by E-cadherin and atypical protein kinase C zeta (aPKCz) staining, was detectable, in particular in the dysplastic spots of clamped \( Vhl^{A/A} \) kidneys (Fig. 5I and J). These results indicate that the stimuli induced by kidney clamp surgery are able to accelerate the occurrence of lesions widely believed to constitute precursors of human ccRCC.
Figure 5. *Vhl*−/− mice develop ccRCC-precursor lesions 4 months postinjury. A, representative H&E staining of clamped and nonclamped kidneys from Cre-negative animals 4 months post surgery; scale bar, 50 μm. B, same as A, but for *Vhl*−/− mice. C, H&E staining of dysplastic spot (arrow) in clamped *Vhl*−/− kidney; scale bar, 20 μm. D, H&E staining of clear cells (arrow) in clamped *Vhl*−/− kidney; scale bar, 20 μm. E, H&E staining of cyst (arrow) in clamped *Vhl*−/− kidney; scale bar, 20 μm. F, quantification of the dysplastic spots frequency per kidney section (normalized to relative area; localized, < 50 μm²; extended, > 50 μm²; n = 4 mice per genotype, Student t test with both categories combined). G, quantification of clear cells frequency per kidney section (normalized to relative area, n = 4 mice per genotype, Student t test with both categories combined). H, quantification of tubular cysts frequency per kidney section (normalized to relative area; small, ~ 1 tubule diameter; large, > 1 tubule diameter; n = 4 mice per genotype, Student t test). I, aberrant E-cadherin (green) staining in *Vhl*−/− mice that underwent ischemic injury compared with operated Cre-negative mice; DBA (yellow), distal tubule marker; DAPI in blue. J, aberrant aPKCz staining (red) in *Vhl*−/− mice that underwent ischemic injury compared with operated Cre-negative mice; NCC (yellow), distal tubule marker; Glut1 (yellow), *Vhl* knockout marker; DAPI in blue; scale bar, 20 μm.
Discussion

In this study, we have used an ischemic kidney regeneration model to unveil potential functions of pVHL in orderly mitotic progression in vivo. Our data show that Vhl nullizygosity in renal epithelial cells results in spindle misorientation and chromosome missegregation.

We show that 4 months after kidney injury, Vhl loss caused changes in the mouse renal epithelium, leading to localized dysplasia with multilayered tubules and nuclear crowding, nodules with cytoplasmic clearing, simple tubular cysts, and regions of aberrant polarity. Small nodules with clear cells and cysts are likely contributors to key aspects of VHL-associated pathology (5, 22–24). The very small spots with clear cells are similar to changes observed in humans with VHL disease (25). In addition, such patients also develop multiple small cysts, similar to those seen in our in VhlΔΔ mice. Recently, such lesions have been shown to have lost the VHL locus in the cyst-lining cells (26). In line with this, a serial computed tomography (CT) study of patients with VHL disease manifested that cystic lesion precede solid tumor forms during disease progression (24). Intriguingly, we also observed dysplastic spots with multilayered tubules and nuclear crowding in close proximity to the nodules with cytoplasmic clearing and tubular cysts. Such dysplastic spots may represent very early forms of complex renal cystic epithelium described in humans with VHL disease (27).

Although a direct relationship between the observed mitotic alterations and the development of dysplasia, tubular cysts, and clear cells cannot be assumed, multiple observations suggest that spindle misorientation and chromosome instability constitute tumor-initiating events that may manifest themselves in these phenotypes. An immediate consequence of the observed increase in spindle angles in Vhl-deleted cells is likely to be misoriented cell division. During kidney development, the cell division axis of the proliferating tubular epithelial cells is oriented along the tubular axis allowing for an orderly lengthening of the tube (8). Consequently, changes in the direction of cell division might well contribute to loss of regular tissue organization and a kidney tubule widening, ultimately facilitating dysplasia and cyst formation. Previous work has linked the suppression of spindle misorientation by pVHL to its microtubule stabilization function (4, 28). This function is also related to the acknowledged role of pVHL in maintaining the primary cilium (16) and has been proposed as one mechanism for cyst formation when disrupted (13). Given the results presented here, the cystic and dysplastic phenotype, that is a characteristic feature of VHL-associated renal pathology, might be the result of both unscheduled cilia resorption and misoriented cell division. The suppression of both of these processes represents core HIF-independent functions of pVHL through microtubule stabilization.

Chromosome missegregation errors were abundantly detected in Vhl-deficient renal epithelial cells 5.5 days after ischemic injury. That they were specifically emerging in the distal tubular cells is consistent with the fact that in this model Cre-recombinase is active in this subset of tubular cells, implying that suppression of chromosomal instability is a relevant tumor suppression function of Vhl in vivo. In cultured mammalian cells, pVHL inactivation has been associated with a weakened mitotic checkpoint due to pVHL loss-of-function-mediated downregulation of the Mad2 mitotic checkpoint protein. Lowering levels of Mad2 without eliminating it entirely provides a potential means of perturbing the mitotic checkpoint in a manner that is compatible with cell survival, but results in a multigorgan tumor phenotype (29). Given that loss of VHL is considered an early event in the development of ccRCC, the initiation of a program of chromosomal instability would enforce the occurrence of further aberrations in the face of increased cell proliferation. In this regard, mutations in genes whose products promote aberrant cell proliferation are frequently observed in ccRCC and include components of the PI3K pathway (6). Irrespective of this, aneuploidy contributes to tumor development in a stochastic manner. This phenomenon could explain the patch-like occurrence of aberrant spots that we observed in clamped VhlΔΔ kidneys after 4 months. This stochastic behavior could also be reflected in the intratumor heterogeneity observed in ccRCC (6, 30).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M.P. Hell, M. Duda, H. Moch, W. Krek
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