Molecular and Cellular Pathobiology

Genetic Evidence of a Precisely Tuned Dysregulation in the Hypoxia Signaling Pathway during Oncogenesis

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models (mutated for Pten or p53) illustrate this model; however, the notion that even partial inactivation of tumor suppressors can critically contribute to tumorigenesis in humans has gained only limited acceptance in the scientific community. One puzzling case of inherited cancer syndrome is the von Hippel-Lindau (VHL) disease associated with VHL mutations. 

VHL is a TSG, and heterozygous inherited mutations are associated with multiple phenotypes. Depending on the mutation, patients are predisposed to multiple tumors, including renal cancers (that have lost the wild-type allele), and/or pheochromocytomas (that do not always lose the second allele), and in cases with particular mutations, patients are free of cancer. This wide range of patient phenotypes seemed a good example to test the hypothesis of a continuum model in humans.

The VHL gene is involved in many functions; the most studied being the regulation of the cellular oxygen-sensing pathway. The main factor of this pathway is the heterodimeric transcription factor hypoxia-inducible factor (HIF). Under normal oxygen tension, the \( \alpha \) subunits of HIF (HIF1\( \alpha \), 2\( \alpha \), and 3\( \alpha \)) are hydroxylated by the dioxygenases PHD (PHD1, 2, and 3). The hydroxylated HIF\( \alpha \)s are then targeted by the VHL protein (pVHL; the substrate recognition subunit of an E3 ubiquitin ligase complex) that promotes HIF\( \alpha \) for proteasomal degradation (2, 3). In contrast, in hypoxia, the HIF\( \alpha \) subunit is stabilized and binds to the HIF\( \beta \) subunit to form an active transcription factor that regulates expression of hundreds of gene targets notably in angiogenesis, erythropoiesis, cell survival, and tumor progression.

More than 160 distinct germline mutations in the VHL gene have been identified and are associated with distinct phenotypes (Supplementary Fig. S1). Heterozygous mutations predispose to VHL disease, the leading cause of hereditary kidney cancer. Patients with VHL disease are at high risk of developing clear cell renal cell carcinomas (ccRCC), central nervous system (CNS) and retinal hemangioblastomas, pheochromocytomas, paragangliomas, cysts and neuroendocrine tumors of the pancreas, and tumors of the endolymphatic sac. Studies of genotype–phenotype correlations originally established two types of phenotypes based on the risk of developing pheochromocytomas (4, 5): VHL type 1 (low risk) and VHL type 2 (high risk). Type-2 disease is subdivided into three categories corresponding to a low (2A) or high risk (2B) of developing ccRCC, or to a risk of pheochromocytomas and paragangliomas only (2C). VHL type-2 disease is associated with VHL missense mutations that lead to severe (types 2A and 2B) or weaker (type 2C) alterations in pVHL function (6, 7). In contrast, VHL type-1 disease is associated with mutations that strongly impair the structure of pVHL or result in the complete absence of the protein (truncated proteins or total gene deletion). The analysis of cases with gene deletions demonstrated that VHL-loss alone is sufficient to predispose to ccRCC development, and that the loss of a larger region (encompassing the HSPC300 gene) would have a protective role (8).

Whereas germline mutations in the VHL gene commonly predispose patients to the development of multiple tumors, a third category of phenotype has been described in Russian patients from the Chuvash region. These patients are homozygotes for the VHL-R200W mutation (considered less severe than classic VHL mutations; refs. 9, 10), and present with a congenital erythrocytosis (excess of red cell production) named Chuvash polycythemia. Survival in the Chuvash patients was found to be reduced compared with control groups due to higher rates of arterial and venous thromboses, and to hemorrhagic events (11). This specific VHL-R200W mutation has also been identified in combination with other VHL mutations (compound heterozygosity; Supplementary Table S1) and, since then, other missenses VHL mutations have been described (always different from the VHL mutation involved in severe VHL disease; Supplementary Table S2; ref. 12). It is known that the HIF signaling is involved in Chuvash polycythemia (9, 10, 13, 14) but the exact mechanisms that may explain the heterogeneity of the clinical phenotype [differences in serum erythropoietin level and propensity to develop thrombosis and pulmonary arterial hypertension (PAH)] are still unexplained (15, 16). However, a characteristic of these patients is the total absence of tumor development (11, 15, 16). Note worthy, the R200W mutation is always transmitted (excepted in one case; ref. 17) within a 340-kb haplotype inherited 14,000 to 62,000 years ago from a single founder event (18). The absence of tumor development in patients carrying the R200W mutation raised the possibility of the presence of a protective element within this core haplotype (11).

Heterozygous carriers for VHL-R200W either have no symptoms or rarely develop erythrocytosis, but they do not develop tumors, except for two cases of isolated CNS hemangioblastoma (19) and, intriguingly, one family with apparently typical VHL disease, suggesting an additional level of complexity to genotype-phenotype correlations (20, 21). Since it was first reported, this family has puzzled the medical and scientific VHL community (see references in Supplementary Table S1). To better understand the absence of tumors in the heterozygous carriers of the VHL-R200W mutation and to determine the cause of cancer development in this family (loss of a protective event or presence of an additional event), we have carried out an in-depth study of the medical and scientific VHL community (see references in Supplementary Table S1).
Molecular dynamics simulations
The starting models of pVHL were constructed from the crystal structure solved by Min and colleagues (PDB ID code 1lm8; ref. 22). Mutant models were generated from the wild-type model by in silico substitutions of the arginine (R) in positions 161 and 200, with a glutamine (Q) and a tryptophan (W), respectively. All the details are given in Supplementary Data.

In vitro analysis of HIF1α–pVHL binding
The HA-tagged pVHL protein was synthesized in vitro and incubated with a hydroxylated-biotinylated peptide corresponding to the HIF1α oxygen-dependant domain. HA-pVHL–HIF complexes were immunoprecipitated with streptavidin beads and analyzed by Nu-Page migration (Invitrogen). The HA-VHL bound to the hydroxylated peptides were revealed by immunoblotting using an HA-specific antibody (Santa Cruz Biotechnology). Details are described in Supplementary Data.

VHL mutants construction
To avoid cell death during reintroduction of VHL in the VHL-defective 786.O cell line (23), we used a tetracycline (or doxycycline)-inducible system to study the VHL mutants. The ViraPower T-REX Lentiviral Expression System (Invitrogen) was used to construct inducible lentivectors encoding nontagged VHL. The vectors were stably transfected into 786.O cells that overexpresses HIF2α. The 786.O cells are a gift from the laboratory of Prof. W. Kaelin (Dana-Farber Cancer Institute, Boston, MA). They have been genotyped by direct sequencing for the VHL gene after reception in our laboratory. Clones with equivalent VHL mRNA expression were selected for microarray study. All the details are indicated in Supplementary Data.

Transcriptomic study
RNA was extracted using TRizol reagent (Sigma) and purified on Qiagen columns. Five Hundred nanograms of total RNA from each RNA sample was amplified and labeled with two fluorescent dyes (Cy5 and Cy3). Labeled cRNA were hybridized to the Agilent Human Whole Genome Oligo Microarray format 4×44K (Agilent Technologies), before washing and scanning. All hybridizations were performed in duplicate with dye swap to eliminate possible dye bias. Data were extracted from scanned images using Feature Extraction software (Agilent) with default settings. Data from all hybridizations were analyzed with Rosetta Resolver software. The microarray data related to this article have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-1269. Details are described in Supplementary Data.

HIF2α target gene identification
Chromatin immunoprecipitations (ChIP) were performed on 786.O cells as previously described (24) using rabbit polyclonal antiserum to HIF2α (PM9). Preimmune serum or IgG were used as negative controls. High-throughput sequencing was performed on libraries prepared from immunoprecipitated chromatin using the Illumina ChIP-Seq kit; 36-bp single-end sequence analysis was performed on the Illumina GAII platform. The microarray analysis of 786.O cells in the presence of either control or HIF2α siRNA was performed as previously described (25). Correlation of ChIP-seq data with microarray analysis of gene expression was performed using GSEA software Version 2 (www.broadinstitute.org/gsea/).

Quantitative reverse transcription PCR
Gene expression was quantified using the TaqMan quantitative RT-PCR (Life Technologies) on the 786.O clones expressing the different VHL mutants used in the microarray study and on two additional independent clones. Probes references are available upon request.

Expression profiles of the 30 identified genes in ccRCC
The GSM14994 dataset (26) was used to analyze the expression of the 30 genes identified as direct target of the pVHL–HIF2α pathway. The Affymetrix HT Human Genome U133A Arrays [[HT_HG-U133A] was normalized with the justRNA procedure in BrB Array Tools v.4.4.0. The signature corresponding to the comparison of 52 ccRCC (VHL mutated) versus 11 normal kidney tissues was performed with the Class Comparison procedure on a restrictive list of 26 genes (probes for KCNIP3, TMEM141, ARRD3C, and ERRFI1 were absent of the dataset; ref. 26). All the details are given in Supplementary Data.

Results
The tumor occurrence in the family bearing the R200W mutation is associated with an additional event
The proband (II-2; Fig. 1A) was originally identified as a heterozygous carrier of a single c.598C>T, p.Arg200Trp (R200W) mutation in the VHL gene (20, 21). He had a history of ccRCC and of CNS and retinal hemangioblastomas, and died at the age of 62 years of CNS complications. His father (II-1) died at the age of 42 years from a cerebellar hemangioblastoma, while his paternal grandparents died at an advanced age and had no known history of VHL manifestations. Two of his daughters, of ages 28 and 36 years (IV-2, IV-3), have a personal history of CNS and retinal hemangioblastomas. Patient IV-2 recently developed a ccRCC and a pheochromocytoma, and patient IV-3 a pancreatic neuroendocrine tumor. There is no erythrocytosis in the family, the hematocrit, red blood cells count, and erythropoietin levels being normal (Supplementary Table S3). Because of the presence of pheochromocytoma and RCC, the family was classified as VHL type-2B.

We performed parallel haplotyping and genotyping of this family. To determine whether the VHL disease occurred in the genetic context of the particular Chuvash core haplotype (18), we analyzed the SNPs of the VHL region (Table 1). Despite the absence of known Chuvash ancestry, the mutated allele did localize to the Chuvash core haplotype (18). The VHL-R200W mutation was confirmed by genotyping. Remarkably, we found a second heterozygous germline mutation, c.482G>A, p.Arg161Gln (R161Q; Fig. 1B). Allele segregation showed that both mutations were present on the same allele; the mother and the healthy daughter (III-3 and IV-1)
carried two wild-type alleles, whereas the two affected daughters (IV-2 and 3) carried one copy each of the allele containing the two mutations (Fig. 1A).

Molecular dynamics simulations of pVHL mutants demonstrate a gradient in the loss of function with an additive effect of the double mutant

The impact of the single mutations (R200W and R161Q), and the double mutation (R200W+R161Q), on the structure and dynamic behavior of pVHL was studied by molecular dynamics simulations. pVHL, a 213 amino acids protein, contains two domains, \( \alpha \) and \( \beta \), connected by a highly flexible linker L8 (3).

The \( \alpha \)-domain consists of three \( \alpha \)-helices, H1, H2, and H3, forming a "box" motif that binds to adapter proteins. The substrate-binding \( \beta \)-domain consists of a seven-stranded \( \beta \)-sandwich and an \( \alpha \)-helix H4 (Supplementary Fig. S2A; ref. 22). Amino acids R200 and R161 are located in the helices H1 (\( \alpha \)-domain) and H4 (\( \beta \)-domain), respectively. Analysis of non-covalent contacts indicates that each mutation, R200W and R161Q, taken separately decreases the stabilizing interdomains interactions, while these local effects are even more pronounced in the double-mutant R200W+R161Q (Supplementary Fig. S2B and S2C and Supplementary Table S4). The interdomain mobility of pVHL, evidenced by the rotation angle

Table 1. SNP sequencing

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>rs1056286 T/C</th>
<th>rs722509 A/T</th>
<th>rs779805 A/G</th>
<th>rs779808 T/G</th>
<th>rs1678607 T/G</th>
<th>rs1642742 A/G</th>
<th>rs696356 A/C</th>
<th>rs378630 A/C</th>
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<tbody>
<tr>
<td>III-3</td>
<td>wt/wt</td>
<td>C/C</td>
<td>T/A</td>
<td>A/G</td>
<td>C/T</td>
<td>G/G</td>
<td>G/A</td>
<td>C/A</td>
<td>C/A</td>
</tr>
<tr>
<td>IV-1</td>
<td>wt/wt</td>
<td>C/T</td>
<td>T/T</td>
<td>A/A</td>
<td>T/T</td>
<td>G/G</td>
<td>A/A</td>
<td>A/A</td>
<td>C/C</td>
</tr>
<tr>
<td>IV-2</td>
<td>R200W+R161Q/wt</td>
<td>C/T</td>
<td>T/A</td>
<td>A/G</td>
<td>C/T</td>
<td>G/T</td>
<td>G/A</td>
<td>C/A</td>
<td>C/A</td>
</tr>
<tr>
<td>IV-3</td>
<td>R200W+R161Q/wt</td>
<td>C/T</td>
<td>T/A</td>
<td>G/G</td>
<td>C/C</td>
<td>G/T</td>
<td>G/G</td>
<td>C/C</td>
<td>A/A</td>
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Haplotype associated with the R200W+R161Q allele

Chuvash haplotype

<table>
<thead>
<tr>
<th>Frequency in control population*</th>
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<tbody>
<tr>
<td>0.36</td>
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<td>0.8</td>
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<tr>
<td>0.39</td>
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<tr>
<td>0.4</td>
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<td>0.13</td>
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<tr>
<td>0.39</td>
</tr>
<tr>
<td>0.67</td>
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<tr>
<td>0.34</td>
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</table>

NOTE: The eight SNPs described by Liu and colleagues (18) were sequenced in four family members. The haplotype associated with the R200W+R161Q allele was deduced and compared with the Chuvash core haplotype.

*From ref. 18, the combined probabilities of harboring the entire haplotype by chance is 0.05%.
was observed with the molecular dynamics simulations: wild-different mutants in the same order of severity (Fig. 3B) as that the HIF2 expressing the wild-type or various mutated pVHL has shown expression (Supplementary Fig. S3). Immunoblotting of clones Clones were selected on the basis of equivalent mRNA level protein products that fully mimicked the endogenous protein. The pVHL proteins were not tagged to provide the system. The pVHL proteins were not tagged to provide the

between the α- and β-domains, was previously shown to be of crucial importance for the correct positioning of the HIF substrate (27). Present molecular dynamics simulations show that the values of this angle are almost similar for the wild-type protein (57°) and the R200W mutant (63°; Fig. 2 and Supplementary Table S5). In contrast, the angle values for the mutants R161Q (77°) and R200W+R161Q (49°) differ strongly and represent two extreme conformations, open and closed, respectively.

Functional studies of the pVHL mutants show a gradual loss of function correlated to the phenotype severity To evaluate the impact of these different mutations on HIF regulation, we performed a functional in vitro analysis by measuring the ability of the mutants to bind to a proline-hydroxylated HIF1α peptide (HIF-OH). The R200W mutation had no effect on the ability of pVHL to bind to HIF-OH compared with the wild-type protein (Fig. 3A). In contrast, R161Q moderately affected this binding and R200W+R161Q completely prevented it. Therefore, the addition of the two mutations appeared to have an additional impact on the pVHL loss of function. We then performed a comprehensive in cellulo study. Each VHL-mutant construct harboring missense mutation was then reintroduced into the VHL-defective renal cancer cell line 786.O, which overexpresses HIF2α. Many attempts were unsuccessful to reintroduce classical VHL constructions in the cells, stably or transiently, toxicity (23) and nonreproducible results being observed, especially for the wild-type and the VHL-R200W vectors. We therefore used a lentiviral-inducible system. The pVHL proteins were not tagged to provide protein products that fully mimicked the endogenous protein. Clones were selected on the basis of equivalent mRNA level expression (Supplementary Fig. S3). Immunoblotting of clones expressing the wild-type or various mutated pVHL has shown that the HIF2α protein was differentially stabilized by the different mutants in the same order of severity (Fig. 3B) as was observed with the molecular dynamics simulations: wild-type $\geq$ R200W > R161Q > R200W+R161Q. As the inducible system showed weak variable leaks (Fig 3B, overexpressed pVHL blot), we therefore compared mutants only after full induction for the following experiments. To pursue this further, transcriptomic profiles from cells expressing the main representative mutants of each type of VHL disease [C162F (type 1), R167Q (type 2B), Y98H (type 2A), L188V (type 2C)] were obtained by microarray analysis. A hierarchical unsupervised clustering analysis showed a close correlation between the importance of the pVHL loss of function and the severity of the developed phenotype (Fig. 3C).

Gradual pVHL loss of function induces a gradual dysregulation of direct HIF2α target genes To determine the genes that may be responsible for tumor development in patients with VHL disease and the absence of tumors associated with the VHL-R200W mutation, we tried to identify relevant differentially regulated genes, but could not find any. Transcriptomic data are always difficult to analyze because the deregulation of one gene can induce a cascade of signalization and induction of many indirect genes that drowns crucial information on initiating events. Therefore, to focus on the first step of regulation by pVHL, and because HIF2α, the main VHL target, is the most tumorigenic factor associated with cancer development (28), we focused on genes directly regulated by HIF2α. We then correlated the list of genes regulated by the reintroduction of the wild-type VHL into 786.O cells (283 genes were obtained from the above microarray data) with a list of direct target genes of HIF2α in 786.O cells [defined as the genes most regulated by HIF2α siRNA within 250 kb of each HIF2α binding site identified by ChiP sequencing (ChiP-seq); Fig. 4A; ref. 29]. This yielded a set of 30 genes that were stringently defined as being directly regulated by the pVHL–HIF2α axis. We tested the expression level of 10 genes randomly selected from this set in different 786.O clones, in which VHL mutants had been reintroduced (R200W and R161Q mutations, individually or in association, in which three independent clones with equivalent expression of VHL were obtained; Fig. 4B). Overall, we observed a highly significant expression gradient for these genes (overall $P < 1 \times 10^{-10}$ that matched precisely the order established above, with a function of the VHL-R200W mutation always very close to the wild-type protein. Nine individual genes displayed a gradient response with a $P < 0.02$ (Supplementary Table S6).

The target genes of the pVHL–HIF2α pathway are deregulated in RCC To validate our identified genes on naturally occurring tumors, our set of 30 candidate genes was analyzed via the Webtool SEEK (Search-Based Exploration of Expression Compendium; http://seek.princeton.edu/) that prioritizes public human mRNA microarray datasets from many tumor types according to the genes of interest. When we analyzed our 30 genes with SEEK, they turned out to be significantly deregulated in microarrays from many series of RCC (data not shown). We selected one dataset of ccRCC with detailed VHL status (GSM14994 dataset; ref. 26). The expression profiles of our 30 candidate genes were studied in VHL-mutated ccRCC compared with normal kidney tissues. Among 26 genes with
validated probes, 17 were differentially expressed with a threshold of 0.001 as nominal P value (15 with a P value around $10^{-7}$ and less). They are shown on the heatmap (Fig. 5 and Supplementary Table S7).

**Discussion**

We carried out a comprehensive study of the only family in which the VHL-R200W mutation was wrongly assumed to cause VHL disease (20, 21). We show that the R200W mutation alone is definitely not associated with the disease because the family actually carries another pathogenic mutation, R161Q, on the same VHL allele, and the double-mutated allele segregates with disease. Our SNP analysis showed that the doubled mutations lie on the Chuvash core haplotype transmitted with the R200W mutation. We can thus conclude that this haplotype does not contain any protective elements against cancer development and that the VHL mutations are, by themselves, predominantly responsible for manifestations of the VHL disease.

The family under study is VHL type 2B (pheochromocytoma and high risk of kidney cancer), and the disease is caused by the combination of two germline mutations that, when considered separately, are associated with different phenotypes (Supplementary Table S1): R200W (either no symptoms or erythrocytosis) and R161Q (VHL type 2A, pheochromocytomas and low risk of kidney cancer). Although it is not excluded that this family carry other genetic alterations (linked or not to VHL), we conducted a comprehensive functional and comparative...
analysis of these VHL mutations to clarify the complexity of genotype–phenotype correlations, and to understand the basis of either tumor development in patients with VHL disease or the absence of cancer in carriers of the VHL-R200W mutation.

The molecular dynamics simulations of the pVHL mutants revealed a highly constrained and reduced interdomain mobility of the double mutant compared with the single one. We suppose that the anticooperative allosteric effects of the two mutations impede optimal positioning of the substrate HIF.

The results show a gradient in the loss of function of the different mutants with an additive effect of the double mutant: R200W > R161Q > R200W + R161Q > C162F.

In cellulo study was performed to compare these mutations with classical mutants involved in the different VHL phenotypes. As it is very difficult to reintroduce a TSG in a cell line, which has undergone multiple selective pressure to delete it, without affecting its genetic status, we constructed a very sensitive cell line model with inducible untagged pVHL constructs. We chose the most studied ccRCC cell line (786.O, often used in therapeutic drug development). We did not identify any relevant candidate genes differentially regulated by the different mutants that may explain the occurrence of tumors or not. Although VHL plays roles in the HIF-independent pathway, we then focused our functional studies on the HIF2α pathway, HIF2α being the isoform that plays a key role in the regulation of the expression of erythropoietin in erythrocytosis (13) and in the development of ccRCC (28) and pheochromocytoma (30, 31). We identified a set of genes directly regulated by the

Figure 4. A, identification of direct target genes regulated by pVHL–HIF2α. The Venn diagram illustrates the intersection between genes regulated by VHL (left) and direct HIF2α target genes (right). Genes regulated by VHL were identified by microarrays analysis of 786.O cells transduced with wild-type VHL (283 genes from 318 signatures). HIF2α binding loci were identified by ChiP followed by high-throughput sequencing (Chip-seq; ref. 29) using both HIF2α and HIF1α antibodies. HIF2α direct targets were then defined as the most regulated transcript (in a microarray analysis of 786.O cells treated with either control siRNA or HIF2α siRNA) that lies within 250 kb of each of the HIF2α binding sites. The list of the 30 genes in the intersection of the Venn diagram is detailed below. B, the TaqMan real-time quantitative RT-PCR analysis. Transcript quantification was performed on three independent clones of 786.O cells transduced with wild-type (wt), mutants VHL, or empty vectors. Relative expression was normalized to endogenous 18S rRNA and expressed as fold change compared with 786.O cells transduced with empty vector. Statistical calculations are detailed in Supplementary Table S6.
pVHL–HIF2α pathway, and observed a distinct and graded ability of the different VHL mutants to regulate these genes: the more important the pVHL loss of function is, the more dysregulated the HIF2α target genes are. To validate our results in naturally occurring tumors, we studied the expression profiles of the 30 genes identified above in massive human expression compendium that contains thousands of public expression datasets from various cancer types (SEEK). A large amount of kidney cancers datasets was significantly selected. The datasets from Beroukhim and colleagues (26) with known VHL status, were carefully analyzed, and 17 genes were highly overexpressed in ccRCC that have lost VHL. These genes have a role in oncogenesis (Supplementary Table S7), and some of them are overexpressed in tumors and associated with poor prognosis [STC2 and ADM in RCC (32–34); PFKFB3 and NDRG1 in other tumors (see references in Supplementary Table S7)]. Being direct target genes of the VHL–HIF2α pathway, these genes may in fact be driver oncogenes and may be involved in early oncogenic events. They should be explored as potential markers or targets for future drugs.

Altogether, our results show a gradual loss of function of the different VHL mutants that could explain the different associated phenotypes: (i) no tumor for R200W mutant with a function similarly close to wild-type pVHL, (ii) an intermediate dysfunction leading to low risk of developing ccRCC for the R161Q mutant, (iii) and VHL type 2B disease with high risk of ccRCC, as described in the present family where R200W+R161Q is associated with a severe loss of function. Remarkably, these findings do not fit the classical Knudson “two-hit” model of TSG, which implies that malignant transformation is the result of full inactivation of the TSG. Instead, and in addition to previously published VHL functional studies (6, 10, 35, 36), they support the new “TSGs continuum model,” demonstrated in mice, in which more flexible mechanisms including tissue specificity and gene dosage-sensitivity underlie tumor predisposition (1, 37). Our description of combined mutations in VHL, each proportionally affecting the patients phenotype, validates this model for the first time in humans and indicates that precisely defined quantitative aspects of structural and functional dysregulation are key to tumor predisposition. Therefore, on the basis of our results and other studies, we can depict a model demonstrating a gradual tumor susceptibility correlated with a gradual pVHL loss of function (Fig. 6). (i) heterozygous carriers of germline VHL mutations with a weak effect (such as R200W) never develop tumors (except for two isolated cases of CNS hemangioblastomas; ref. 19). The function of the VHL-R200W mutant is close but not totally equal to the wild-type protein (Fig. 3C), and further studies targeting the genes dysregulated in this context are needed to understand the phenotype observed in homozygotes. In these patients, the exact mechanisms of the occurrence of erythrocytosis (achievement of a threshold that induces HIF and erythropoietin production and/or direct effect on erythroid progenitors proliferation due to
particular properties of the VHL mutations) and occurrence of thrombotic events or PAH still need to be determined (9, 13, 14, 16, 38). Nevertheless, this mutation never induces sufficient selective pressure to allow tumorigenesis and as we evidenced, it may be due to an insufficient dysregulation of the tumorigenic HIF pathway. (ii) A moderate impact on pVHL function, although not enough to initiate renal cancer, appears sufficient for initiation of pheochromocytoma development from neural cells (notion of tissues specificity and quasi-haploinsufficiency). No difference has been observed in pheochromocytoma occurrence between type 2A and 2B (64% and 63%, respectively; ref. 5). However, it is noteworthy that germline VHL deletions are rarely associated with pheochromocytomas and a second hit (loss of the VHL wild-type allele) is often absent in the tumor (39). This observation suggests an obligate haploinsufficiency (when antitumorigenic mechanisms are induced by a complete loss of TSG usually due to the activation of fail-safe mechanisms; ref. 1). Indeed, in neural cells, overexpression of EGLN3 (a target gene of HIF) induces apoptosis and is therefore incompatible with the development of pheochromocytoma (40). We can therefore conclude that, in pheochromocytoma, pVHL mutations function as hypomorphs, not as dominant-negative alleles. (iii) A severe VHL loss of function is needed for ccRCC development. A gradient of predisposition depending on the VHL mutation has already been suggested in ccRCC (6). The threshold of pVHL loss of activity could be reached with VHL type 2A mutants (the typical Y98H mutation predispose to ccRCC in 3% of cases; ref. 41) and a maximal risk of ccRCC development occurs with type 2B mutants (severe pVHL loss of function) and type 1 mutations (total VHL loss of function; ref. 42).

It is remarkable that VHL mutations in erythrocytosis patients are different to those identified in patients with VHL (Supplementary Table S2; refs. 5, 12). There are a few exceptions (43–47), for example, Y175C and L188V have been associated with both erythrocytosis and pheochromocytoma. These mutations may be considered as "borderline," with a loss of function insufficient to induce renal cancer but still capable to cause pheochromocytoma. The L188V mutant perfectly illustrates this threshold: it is a typical VHL type 2C mutation (48), its loss of function is difficult to assess (7, 35),
and it has been described in rare patients with no symptoms or with erythrocytosis (43, 45–47).

Finally, our results suggest that the dysregulation of HIF may play a key role in the continuum model of tumor suppression by VHL. This model may be of major importance in our understanding of tumor risk in patients carrying mutations in other genes belonging the HIF pathway. Indeed, patients with germline mutations in PHD2/EGLN1 and HIF2A/EPAS1 can develop pheochromocytomas or paragangliomas (equivalent to adrenocortical pheochromocytomas; refs. 49–51). A comparative study of PHD2 mutations showed a differential regulation of HIF that perfectly fits with the model described here (52). The absence of renal cancer in patients with PHD2 and HIF24 mutations could be explained by an only partial inactivation of PHD2 (possibly compensated by PHD1 and 3; ref. 53), and by the persistence of some HIF2α degradation (the germline activating mutations described in HIF24 gene being always outside of the major hydroxylated prolines).

In conclusion, the unique concurrence of two mutations that interact positively to perturb a specific structural feature of pVHL has allowed functional–clinical correlates, improving as a result our understanding of the genotype–phenotype associations in VHL disease. The additive effect of two pathogenic germline mutations supports the quantitative continuum model of tumor suppression.

Disclosure of Potential Conflicts of Interest

W. Kaelin is a consultant/advisory board for Fibrogen. No potential conflicts of interest were disclosed by the other authors.

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