TITLE:

New lessons from an old gene: complex splicing and a novel cryptic exon in *VHL* gene cause erythrocytosis and VHL disease

RUNNING TITLE:

Complex genetics in VHL disease and erythrocytosis

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Abstract:

Chuvash polycythemia is an autosomal recessive form of erythrocytosis associated with a homozygous p.Arg200Trp mutation in the von Hippel-Lindau (*VHL*) gene. Since this discovery, additional *VHL* mutations have been identified in patients with congenital erythrocytosis, in homozygous or compound-heterozygous state. *VHL* is a major tumor suppressor gene that was first described mutated in heterozygous patients presenting with von Hippel–Lindau disease, which is characterized by the development of highly vascularized tumors. Here, we identified a new *VHL* cryptic-exon (termed E1') hidden in intron 1 that is naturally expressed in many tissues. More importantly, we identified mutations in E1' in seven families with erythrocytosis (one homozygous case and six compound-heterozygous cases with a mutation in E1' associated with mutation in *VHL* coding sequence) and in one large family, members of which develop a typical VHL disease without any alteration in the other *VHL* exons. We performed a comprehensive study and show that the mutations induced a dysregulation of the *VHL* splicing with excessive retention of E1' associated with a downregulation of VHL protein expression. In addition, we demonstrated a pathogenic role of synonymous mutations in *VHL*-Exon 2 that alter splicing through E2-skipping in five families with erythrocytosis or VHL disease. In all the studied cases, the mutations differentially impact splicing, correlating with phenotype severity. This study demonstrates that cryptic-exon-retention or exon-skipping are new *VHL* alterations and reveals a novel complex splicing regulation of the *VHL* gene. These findings open new avenues for diagnosis and research into the VHL-related-hypoxia-signaling pathway.

Introduction:

Congenital erythrocytosis represent a heterogeneous group of rare disorders. Genetic changes affecting all parts of the regulatory pathway of erythropoiesis, including oxygen sensing, erythropoietin sensitivity, or hemoglobin oxygen affinity have been described in patients with congenital erythrocytosis. The detection of underlying genetic changes in patients with presumed hematological pathology may have important implications for an adequate clinical management. However, even with the use of NGS panel diagnostics, the underlying genetic cause of presumed congenital erythrocytosis has been identified in less than one third of the patients in most published cohorts.

Molecular basis of VHL-related congenital erythrocytosis were first described in the autonomous Russian Republic of Chuvashia where this condition is an endemic disorder.[1] Chuvash polycythemia is frequently associated with rubor, vertebral hemangiomas, varicose veins and low blood pressure. Chuvash patients have reduced survival rates associated with a higher prevalence of arterial and venous thromboses and pulmonary hypertension in addition to hemorrhagic events.[2] Chuvash polycythemia arose from a homozygous c.598C>T, p.Arg200Trp (R200W) mutation in the *VHL* gene. This specific *VHL*-R200W mutation has also been identified in combination with other *VHL* mutations (compound-heterozygosity) in Chuvash polycythemia. Subsequently, other missense *VHL* mutations in both alleles have been described in patients with congenital erythrocytosis.[3,4] Interestingly, some unexplained cases of patients with erythrocytosis have been described in which only one heterozygous *VHL* mutation has been identified to date.[4-6]
VHL is located on 3p25-26 and has been reported to contain three exons (E1, E2, E3). The commonly described *VHL* transcript contains the three spliced exons that encode a 213 amino-acid (a-a) protein (pVHL213 also termed pVHL30) and a smaller isoform (pVHL160 or pVHL19) initiated from an in-frame internal translation start site.[7] A naturally occurring splice variant, expressed at low levels in some tissues, comprises E1 directly spliced to E3 and is translated into a protein product termed pVHL172 (pVHLΔE2), which functions are still under investigation.[8-12] pVHL213 and pVHL160 are involved in a variety of functions, the most studied being the regulation of the cellular oxygen-sensing pathway. The main player of this pathway is the Hypoxia Inducible Factor (HIF). Under normal oxygen supply, the α−subunits of HIF (HIF-1α, 2α and 3α) are hydroxylated by the prolyl-4 hydroxylase domain enzymes (PHD1, 2 and 3) and subsequently

targeted by pVHL, a subunit of an E3 ubiquitin-ligase complex that promotes HIF-α ubiquitination and subsequent proteasomal degradation.[13,14] Under hypoxic conditions or when *VHL* is mutated, HIF-α remains stable and heterodimerizes with HIF-β to constitute functional HIF factor. HIF transcriptionally activates a variety of genes involved in adaptation to reduced oxygen supply (e.g. erythropoiesis, angiogenesis, metabolism and cell survival). Dysregulation of the hypoxia pathway[13,14] is central to the development of erythrocytosis[1,4] (via upregulation of erythropoietin (EPO), a HIF2α target gene), but also in the development of tumors.[15] Indeed, *VHL* is a tumor suppressor gene, in which heterozygous mutations are associated with von Hippel-Lindau disease (Figure 1A).[16,17] The VHL disease, described in 1936, is an autosomal dominant disorder with high penetrance characterized by the development of highly vascularized tumors like central nervous system and retinal haemangioblastomas, pancreatic neuroendocrine tumors, pheochromocytomas and clear-cell renal cell carcinomas (ccRCC).[18-20]

In patients carrying *VHL* mutations, the precise mechanistic aspects that underpin the different phenotypes remain obscure. Although most patients carry mutations in the *VHL* gene that induce a partial or complete loss of protein function, some cases remain unsolved. Indeed, some patients with erythrocytosis have been found to be heterozygous rather than homozygous for the expected alteration[4-6] or carry homozygous synonymous mutations that leave the amino-acid sequence intact. In addition, some patients present with VHL disease in the absence of identified mutations or deletions in *VHL*, or carry heterozygous synonymous mutations. Here, we report an investigation of twelve families linked to unexplained disease, including nine families with erythrocytosis and three families with VHL disease (Figure 1A). This study has led to the discovery of a novel cryptic-exon in the *VHL* gene and a complex regulation of *VHL* splicing.

Methods:

Complete materials and methods are detailed in supplemental data.

Study approval

Informed consent for medical diagnosis and research was obtained from the patients and their relatives. This study was agreed by the CCPPRB (French Ethical Committee) Paris-Sud at Bicêtre Hospital.

Sanger sequencing

Exons and exon-intron junctions of the *VHL* gene were sequenced from DNA extracted from whole blood, as previously described.[21]

Whole Genome Sequencing

Whole genome sequencing (for Families F2, F3, F7) was performed at the clinically accredited Molecular Diagnostics Laboratory at the John Radcliffe Hospital using the Hi-Seq 4000 platform (Illumina Inc., San Diego, CA) in high-throughput mode.[22,23] Analysis of single nucleotide variants, short insertions/deletions and copy number variants was conducted and is explained in detail in the supplementary methods.

Transcript detection and quantification

After reverse-transcription reactions (ThermoScientific), exon-specific PCR was performed using primers localized to flanking E1 and E3 exons. Taqman real-time PCR were performed on 20 ng of cDNA with the qPCR Mastermix (Eurogentec). Quantification of *RPLP0* transcripts was used as internal control. The thresholds were determined using dilutions of plasmids containing coding sequences of each gene.

RNA sequencing

Library construction was performed with SureSelect Strand-Specific RNA Library Prep for the Illumina Multiplexed-kit (Agilent-Technologies). After purification (Macherey-Nagel), the fragment size of libraries was controlled using the 2200 TapeStation system (Agilent-Technologies). Ten pM of each library were pooled and prepared according to the denaturing and diluting libraries protocol for the Hiseq and GAIIx (Illumina) for cluster generation on the cBot™ system. Paired-end sequencing was carried out in a single lane on the HiSeq® 2500 system (Illumina). Processing of reads is detailed in Supplementary methods.

Reporter assays

The plasmid encoding the wild type HA-VHL was kindly provided by Prof. William G. Kaelin Jr. An expression plasmid for the hypothetical X1-protein was constructed by subcloning the coding sequence following its synthesis (LifeTechnologies). Mutations of interest were introduced by site-

directed mutagenesis (New-England Biolabs). 786-O cells were transfected (Polyplus) with constructs encoding wild-type or mutant proteins, Firefly-luciferase under the control of Hypoxia-Response-Elements (HRE-luciferase), and Renilla-luciferase for normalization[24]. After 24h, luciferase assays were performed using a Dual-Luciferase®Reporter Assay System (Promega).

Western Blotting

Cell lysates from the luciferase reporter assay were loaded into a Bis-Tris Mini Gel (4-12%) (Invitrogen). After transfert, the membrane (GE-Healthcare) was subsequently incubated with a mouse anti-HA antibody (BioLegend), and then a goat anti-mouse HRP-conjugated antibody (Jackson-Immuno-Research). Western blot using the mouse monoclonal antibody JD-1956 (Patent No. 14305925.1-1402-2014; CNRS-EFS) raised against human VHL was performed as described.[8]

Minigene experiments

Minigene constructs were prepared in pCas2 plasmid, containing two artificial exons A and B (as described[25]), between which *VHL*-Exon1' or *VHL*-Exon2 with intronic flanking sequences were cloned. Cells were transfected (Polyplus) or nucleofected (Lonza). RNA were extracted 24h after transfection and reverse transcribed. PCR amplification was performed using the PCR GoTaqQ2 kit (Promega) with primers against both artificial (A and B) exons. PCR products were resolved in a 2% agarose gel.

Results:

Identification of new VHL spliced isoforms containing a cryptic-exon.

We first focused our study on a patient with erythrocytosis where a synonymous *VHL* c.429C>T, p.Asp143Asp (D143D) mutation in heterozygous state was identified (Family 1, Table 1). No other mutations in the three *VHL* canonical exons were detected in genomic DNA of this patient. A PCR using primers in E1 and E3 was performed in cDNA samples extracted from lymphoblastoid cell lines (LCL) established from different family members. The results showed a strong decrease of the E1E2E3 isoform and an upregulation of the E1E3 isoform (Figure 1B) compared to wild-type LCL. Some minor extra fragments of larger size were observed in this patient and his mother's sample. Subcloning and sequencing of these fragments allowed us to identify new *VHL* transcript isoforms that contained intronic sequences. This intronic sequence,

that we termed the E1' cryptic-exon, is spliced to E1 at its 5' end, and to either E2 or E3 at its 3' terminus (Figure 1C). We showed that these isoforms are expressed in a variety of tissues and cell lines (Supplemental Fig.1). Their translation may theoretically lead to the production of a protein of 193 a-a that contains the first 114 a-a encoded by E1[26], and 79 additional a-a of unknown function encoded by E1'. During the course of our study, an automated computational analysis deposited data in NCBI for an isoform containing the E1' cryptic-exon (E1E1'E2 isoform), which was predicted to encode a protein of 193 a-a named X1 (XP_011532380.1) with the sequence described above. Computational analysis of this DNA region revealed strong conservation in primates, but a moderate to low conservation in more distant species (Supplemental Fig.2A-C); notably, the splice sites are identical to canonical sites (TTCA**AG**/tc, AG/**GT**AAG), and are highly conserved. *In silico* analysis of the donor (SD) and acceptor (SA) splice sites of E1' showed similar consensus values compared to other *VHL* exons (Figure 1C, upper panel). The capacity to translate a potential X1 protein is only conserved in higher primates (Supplemental Fig.2D). The deposited sequence has now been removed and replaced by a non-coding isoform containing E1' spliced with *VHL*-E2 and E3 (ENST00000477538.1) (Figure 1C, isoform on the bottom). This isoform may be initiated by an alternative promoter. Indeed, the sequence located at the 5' end of E1' represents a transcriptionally active region as illustrated by epigenetic marks (Supplemental Fig.3). We confirmed the expression of an additional transcript initiated from the upstream region of E1' (that we termed "Upstream E1'") in different tissues and cell lines (Supplemental Fig.1).

The new E1' cryptic-exon is mutated in patients with erythrocytosis or von Hippel-Lindau disease.

Sanger sequencing of this new cryptic-exon in the proband (F1-II.1) identified a variant not reported in databases: c.340+770T>C (Figure 2B, Table 1, Supplemental Fig. 4). Sequencing of the germline DNA of the mother revealed the same variant, indicating that the propositus F1-II.1 is compound heterozygous. This result prompted us to sequence additional patients with erythrocytosis described as heterozygous for *VHL* mutations.

We investigated Chuvash polycythemia patients for which the *VHL*-R200W had been found in heterozygous state, but no second variant had been identified. Sanger sequencing identified the same intronic variant c.340+770T>C in a singleton (F2)[5] and two affected brothers (F3) (Figure

2B). In addition, we identified a duplication c.340+694_711dup in the proband of F4 and F5[6] previously described as *VHL*-R200W/wt and *VHL*-G144R/wt respectively (Figure 2B). In F6, previously diagnosed as *VHL*-Q164H/wt, we identified a genetic variant c.340+574A>T that altered the consensus splice-acceptor (SA) site **AG**/tc of E1'. This variant is described as a rare polymorphism (rs98274567) in NCBI database (Table 1).
As biological material from the parents of F2 and F3 was not available, we cloned the intronic region containing the E1' variant and the Chuvash core-haplotype SNP (single nucleotide polymorphism) rs779808 associated with the *VHL*-R200W mutation (described[27] to be located downstream of E1', in position c.340+1150). Segregation analysis (F4, F5, F6) or Chuvash core-haplotype analysis (F2, F3) demonstrated that patients are compound heterozygous, with one mutation being inherited from each parent (Supplemental Fig.5).

In a parallel independent study, whole genome sequencing (WGS) was being used to investigate patients with Chuvash polycythemia, heterozygous for the *VHL*-R200W mutation (F2 and F3). The intronic variant c.340+770T>C was the only rare variant identified in the *VHL* gene in these patients. Other WGS filtering strategies (Supplementary Methods) did not identify any further significant mutations in biologically relevant genes (Supplemental Fig.6). This study also conducted WGS for a trio with congenital erythrocytosis for which prior whole exome sequencing (WES) had not identified any mutations (F7). No rare biologically relevant variants were identified using the filtering strategy described in Supplementary Methods. However, further inspection of the new cryptic-exon led to the identification of the c.340+816A>C variant in homozygous state in the proband, with both parents being heterozygous for this mutation.

In addition to these patients with erythrocytosis, in which no variants in *VHL* were initially detected, screening of patients with von Hippel-Lindau disease may also result in the lack of detection of canonical *VHL* exonic mutations. An example of such family, F8, with hereditary hemangioblastoma, clear-cell renal-cell carcinoma and pheochromocytoma has been studied. Microsatellite analysis demonstrated a co-segregation of markers surrounding the *VHL* region with the disease (Supplemental Fig. 7). Sequencing of the new E1' cryptic-exon in this family identified two heterozygous variants in E1': a previously unreported c.340+617C>G variant and a c.340+648T>C variant described as a rare polymorphism in databases (Table 1). These variants

segregate in six patients who developed the disease, and were absent in four healthy tested descendants, indicating their presence on a single disease-associated allele (Figure 2B). Sequencing of tumor DNAs did not display loss of heterozygosity (LOH), suggesting that a wild-type *VHL* deletion (as observed in classic VHL disease) may not be prerequisite in cells of patients with this specific *VHL* genotype (Supplemental Fig.4).

Expression study of the new VHL isoforms in patients' cells.

RNA-sequencing (RNA-Seq) analysis of patients' LCL and tumors demonstrated that genomic variants in E1' are associated with an up-regulation of transcripts containing the E1' cryptic-exon compared to controls (Figure 3A, Supplemental Fig.8). In addition, the mutated allele is preferentially expressed (more than 70%), suggesting a causal relationship between the genetic variants and the E1' retention (Supplemental Fig.8C).

We performed quantitative RT-PCR using TaqMan probes specific for the different splicing junctions or for the region upstream of E1' (location of probes Figure 1C), which showed a strong upregulation of a unique isoform containing E1' spliced with E1 in LCL and tumors from patients of F8 compared to control (Figure 3B). The study was therefore focused on the isoform E1-E1'. Transcripts that retain E1' spliced with E1 contain (in-frame with E1) a premature termination codon (Figure 1C) and are likely targeted for degradation according to nonsense-mediated mRNA Decay (NMD) mechanisms. We therefore investigated the expression of transcripts that contain E1 spliced with E1' in the absence or presence of puromycin, an inhibitor of NMD. Without puromycin, higher levels of expression were seen in samples with mutated E1' (Figure 3C) compared to samples with WT-E1'. Exposure to puromycin resulted in a profound induction of isoforms containing E1 spliced with E1' *versus* other isoforms (Figure 3C, Supplemental Fig.9). These results indicated that isoforms with E1-E1' junction are degraded by NMD and may fail to produce proteins. We performed RT-PCR on LCL sample of F6 carrying the mutation in the SA site, using primers in *VHL* exons E1 and E3. We observed fragments of larger size in samples from patients carrying the mutation, which were highly visible in presence of puromycin (Figure 3D). Cloning and sequencing of these fragments demonstrated a dysregulated splicing of E1' with the use of an alternative SA site located 15 nucleotides downstream (Supplemental Fig.10).

In order to measure the impact of the splicing dysregulation on pVHL expression, we performed an immunoblot with an antibody able to recognize the a-a encoded by E1 and, therefore, able to detect the different pVHL isoforms.[8] The antibody detected overexpressed exogenous X1 protein following transfection of plasmid encoding X1 (Figure 4A). However, it failed to detect the endogenous protein, even in patients' samples that overexpress the mRNA containing E1-E1'. Instead, the immunoblots showed a lower expression of all the VHL protein isoforms in patients with mutated E1' (Figures 4A and 4B).

Functional characterization of the mutated VHL-E1'.

As the hypoxia pathway represents the major pathway involved in the genesis of the secondary erythrocytosis and VHL disease[13-15,28], we performed functional studies of the hypothetical wild-type or mutated X1 proteins using Hypoxia Response Element (HRE)-dependent reporter assays. These functional tests failed to reveal any substantial effects of X1 on this pathway, either alone or in competition with pVHL (Figure 4C, Supplemental Fig.11). We next focused our study on a potential impact of the E1' variants on splicing by performing splicing reporter minigene-assays in various cell lines. The experiments showed that splicing of the wild-type E1' is barely detected (Figure 4D). Interestingly, higher molecular weight bands corresponding to the expected spliced isoforms containing E1' appeared in the presence of the mutations. Cloning and sequencing of the isoforms confirmed a retention of E1' at the expected splicing sites. The level of expression of the upper isoform, specific to E1' inclusion, was higher for mutations associated with cancers than with erythrocytosis, independently of the cell lines. Notably, the combination of both variants associated with cancer has a more pronounced effect than each variant taken independently (the SNP showing very low effect), suggesting a synergistic effect of the variants on splicing (Supplemental Fig.12). Consistently, *in silico* analysis of the c.340+617C>G predicted a severe alteration of splicing by the creation of an Exonic Splicing Enhancer (ESE) site (Supplemental Fig.13).

The minigene experiment performed with the mutated E1' at the SA site (F6) confirmed the inclusion of E1' during splicing (Figure 4D, right panel), using the same dowstream alternative SA site identified in mRNA extracted from patients' LCL (Supplemental Fig.10).

Synonymous mutations in VHL-Exon 2 induce exon-skipping.

We then focused our study on the synonymous D143D mutation in E2, identified in heterozygous state in the proband of F1. This mutation was also identified in homozygous state in two patients with erythrocytosis (F9, F10, Figure 5A, Table 1). The cDNA extracted from LCL established from the different members of these two families were sequenced. Comparison of chromatograms obtained by sequencing of DNA *versus* cDNA displayed a weaker peak of the mutated allele in cDNA, reflecting a decreased expression of the mRNA transcripts carrying the mutation.

A different synonymous mutation in E2, c.414A>G, p.Pro138Pro (P138P), has been identified in two families (F11, F12) with von Hippel-Lindau disease (Figure 5B). This heterozygous mutation segregates with the disease in three generations. Sequencing of DNA extracted from the pheochromocytoma of F11-III.1 showed a loss of the wild-type allele in the tumor, demonstrating LOH as currently described in classical VHL disease.

Suspecting an effect of the synonymous mutations on splicing, we next assessed the expression of the *VHL* transcripts in patients' samples by RT-PCR (Figure 5C). We showed a significant change in the ratio of expressed *VHL* isoforms, with a higher expression of the E1-E3 transcripts in LCL of patients homozygous for D143D and in the pheochromocytomas with P138P mutation. These results suggested an effect of the mutation on splicing regulatory elements, leading to E2 skipping. *In silico* analysis of the synonymous mutations indicated a potential effect on Exonic-Splicing Enhancer (ESE) motifs (Supplemental Fig.14).

In order to specifically quantify the different *VHL* isoforms, we performed quantitative RT-PCR on LCL using probes complementary to the *VHL* E1-E2 or E1-E3 junctions (Figure 5D). All mutated samples displayed an increase of the isoform with skipped E2 (E1-E3). The patients carrying a homozygous D143D mutation presented a severe decrease of the wild-type isoform expression level. These results demonstrated that synonymous mutations in E2 affect mRNA isoform production.

In order to evaluate a potential impact on protein expression, we performed a western blot analysis. We did not observe any overexpression of the pVHL172 isoform as expected regarding mRNA quantification studies. However, in patients homozygous for D143D, we detected a strong

downregulation of all the pVHL isoforms, which was also observed to a lesser extent in LCL samples from heterozygous patients (Figure 5E).

Proportion of VHL-E2 skipping is correlated to disease severity in minigene experiments.

In order to study the impact of the synonymous E2 mutations on splicing in different cell lines, we performed minigene-assays. We tested the implications of D143D and P138P mutations on splicing in addition to close mutations described in patients with erythrocytosis (P138L and G144R).[4,6,29] Indeed, these mutations may also impact splicing rather than VHL protein function. We first evaluated the loss-of-function of these mutants by reporter assays. We showed a conservation of their ability to downregulate HIF similar to the wild-type protein, in contrast to VHL proteins lacking E2 (pVHLΔE2) which are described to be nonfunctional in terms of regulating HIF activity[8-10,12] (Figure 6A). Minigene experiments were performed in a variety of cell lines relevant to the studied diseases, using LCL as control (Figure 6B). We demonstrated that all mutations cause E2-skipping in LCL. In other cell lines, these experiments demonstrated a major impact on the *VHL*-E2 splicing of the P138P mutation associated with cancer. The mutations P138L, D143D and G144R, all associated with erythrocytosis, displayed a weaker effect on splicing, with slight variations among cell lines but with a stronger effect in the erythroid cell line (UT7 cultured with EPO).

Splicing dysregulation of VHL is causal in the developement of disease.

RNA-seq confirmed that the synonymous mutations were not silent but instead induce potent E2-skipping (Figure 6C, Supplemental Fig.15A). Further transcriptome analyses of the pheochromocytoma carrying P138P showed an upregulation of HIF target genes (typically seen in *VHL*-related pheochromocytomas) compared to the pheochromocytoma carrying a *RET* mutation (Supplemental Fig.15B)[30]. We then re-analyzed our independent cohort of pheochromocytomas[30] using RNA-Seq data from both tumors. After unsupervised classification, we observed a segregation of the P138P pheochromocytoma with other *VHL* related-tumors (C1B cluster) whereas the control pheochromocytoma bearing the *RET* mutation was grouped with other *RET* related-tumors (Figure 6D).

Discussion:

The hypoxia pathway plays a central role in erythrocytosis or tumors developed by patients carrying *VHL* mutations. Nevertheless, the full molecular mechanisms at the origin of these different phenotypes remain to be elucidated. To date, the functional studies of *VHL* mutants have been performed on missense mutations. We describe here, for the first time, functional studies of *VHL* mutations that do not impact the coding sequence but that influence the *VHL* splicing. We discovered a complex regulation of *VHL* splicing that may help to explain the complexity of genotype/phenotype correlations observed in VHL-related disorders. Notably, we demonstrated that synonymous variants (D143D or P138P) can impact *VHL* splicing and should be considered as pathogenic mutations. Our study points to a particular region in the E2 that may be considered as a splicing regulatory domain. Therefore, it would be interesting to evaluate the impact on splicing of all the nucleotide changes described in *VHL*-E2[17] in the same way as we described for two missense mutations (P138L, G144R). We observed that, depending on the mutation in this region, the impact on splicing can be moderate (D143D, G144R, P138L) or severe (P138P), which correlates with the severity of the disease developed by individuals carrying these *VHL* mutations (erythrocytosis *versus* cancers). This observation confirms the hypothesis of a continuum-model of tumor suppression by *VHL*.[21,31]
Regarding the erythrocytosis developed in patients homozygous for D143D, both probands (F9 II.1 and F10 II.1) present mutations in the beta-globin gene (*HBB*) that induce hemoglobin instability or thalassemia (Table 1). This may compensate the strong erythropoiesis associated with a very high serum erythropoietin level associated with the D143D mutation.

More importantly, we discovered a new *VHL* cryptic-exon, E1', expressed in healthy tissues. Our study allowed the identification of E1' heterozygous mutations occurring in the second allele of six families with an erythrocytosis previously associated to a heterozygous mutation in *VHL* rather than a homozygous mutation. Our investigations further confirm that polycythemia associated with *VHL* mutation is definitely an autosomal recessive disease. In addition, we identified an E1' homozygous mutation in a patient with an erythrocytosis of unknown origin. This result demonstrates the causal role of the alteration in this new cryptic-exon to the occurrence of erythrocytosis. Importantly, we also identified E1' mutations in patients with unexplained VHL disease.

This *VHL*-E1' exon remained so far unidentified because of its low expression and the fact that this deep intronic region was never explored or represented in WES data. Our data showed that these newly described E1'-containing transcripts may be polyadenylated (because captured by polydT in RNAseq) but also targeted by NMD and may therefore fail to produce a protein. However we cannot exclude the translation into a new protein (X1) not expressed in sufficient quantity to be detected by western blot. This potential X1 isoform would contain the VHL-E1 that encodes the NH2-terminal part of pVHL, including 16 residues involved in HIF binding out of the 17 described.[26] Nonetheless, the hypoxia-dependent reporter assays failed to identify a potential direct role of this isoform on the HIF pathway. Instead, our results provide compelling evidence that mutations in E1' induce a severe retention of this E1'cryptic-exon wich correlates with a defect in global VHL protein expression. Therefore, our study strongly favors a dysregulation of splicing with a consequent downregulation of pVHL expression as the underlying cause of the developed diseases. Insufficient pVHL levels but not a reduced HIF binding by the mutant pVHL (as seen in the Chuvash polycythemia mutation *VHL*-R200W[1]) finally lead to an impairement of HIF degradation. To note, the functional study of mutations identified in E1' in association with erythrocytosis demonstrated a less severe impact on splicing than mutations associated with cancer, confirming that polycythemia is associated with *VHL* hypomorphic mutations.

Our findings may have broad implications for patients with presumed congenital erythrocytosis. First, the underlying cause of congenital erythrocytosis has been identified in only about one third of the patients in most published studies. However, studies were focused on missense and nonsense changes in coding regions and known regulatory domains of candidate genes. This has also been the case in patients with suspected VHL syndrome. Our study shows that synonymous exonic changes and also changes within intronic sequences affecting exon splicing may be responsible for these disorders and have to be considered during the diagnostic process. Notably, the *VHL*-E1' exon should be added in the list of regions routinely sequenced in patients with congenital erythrocytosis. Second, the detection of underlying changes has implications on the clinical management of patients. For example, phlebotomy in patients with VHL- or HIF2α-related erythrocytosis may even worsen the clinical situation by increasing the risk and severity of pulmonary hypertension in these patients.[32] Third, the confirmation of the continuum-model of tumor suppression by VHL helps to understand the very low frequency of secondary tumors in patients with VHL-related

erythrocytosis[33]. On the other hand, this also means that later occurrence of such neoplasms cannot be definitely excluded. Therefore, regular follow-up not only concerning the risk for thromboembolic complications, pulmonary hypertension, and cardiovascular disease, but also regular examinations to exclude the presence of typical VHL-related tumors should be part of the clinical management of these patients. Patients with VHL syndrome due to E1' mutations will also benefit from regular screening for tumors. Finally, the detection of these new genetic changes will also allow appropriate genetic counselling of affected patients and their families.

In conclusion, *VHL* is a major tumor suppressor gene that plays a pivotal role in the oxygen-sensing pathway, involved in multiple physiological (e.g. angiogenesis, erythropoiesis) and pathological (e.g. cancer) processes. Our findings, related to complex splicing regulation of this gene in erythrocytosis and tumorigenesis may, therefore, open new avenues for diagnostics and research in the multiple fields related to the hypoxia-signaling pathway. Notably, we suggest further targeted exploration of the *VHL*-E1' region in unresolved cases of congenital erythrocytosis, inherited kidney cancers, hereditary paraganglioma/pheochromocytoma syndrome, hemangioblastomas, in addition to all types of sporadic tumors with altered hypoxia signaling.

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Authorship contributions:

LM, RF, SL, CA, HD, BA, GS, CS, CF, PM, BT, FS, VM, ABY, GB performed experiments; CC, SJLK, JCT, PM, KE and DE conducted genome sequening or data analysis & interpretation; LP, CM, DuS, JS, LM performed bioinformatics analyses; CH, SK, DeS, BN, MJM, AF, GC, HL, IS, ME, BK, DKM, BdPB, GF, RML, PMC, BV, VWR, GJ, KA, JN, BC, GRAP, RS conducted the medical study; GB, HD, LM, RF wrote the manuscripts; GB, CH, designed the study; GB directed the study; all authors contributed to the research and approved the final manuscript.

Disclosure of Conflicts of Interests:

The authors declare no competing financial interests.

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Table Legend:

Table 1. Description of variants identified in *VHL*-E1' and *VHL*-E2 with associated clinical manifestations. The second column indicates the position of the nucleotide variants identified in the *VHL* gene regarding the current nomenclature (sequence encoded by the *VHL* E1-E2-E3). WT: wild-type. ♦: the nucleotide change has been reported as rs982745672 with a global MAF that corresponds to T=0.00007/2 (TOPMED). ♦♦: the nucleotide change has been reported as rs73024533 with a global MAF that corresponds to C=0.0026/13 (1000 Genomes) and C=0.0050/147 (TOPMED). The third column indicates the impact either on the VHL protein (for variants located in E1, E2 or E3), or on the potential X1 protein, if encoded by E1-E1' (for variants located in E1'). Diag: diagnosis, M: Male, F: Female, N: normal values. The normal values corresponds to: Hb (Hemoglobin) (g/dL) = M: 13-18 and F: 12-15; Ht (hematocrit) (%)= M: 40-52, F: 37-47; Red cells (million/mm^3)= M: 4.2-5.7, F: 4.2-5.2; EPO (mU/ml)= 5-25.

Figures Legend:

Figure 1: Clinical manifestations of patients carrying *VHL* mutations and identification of a new *VHL* spliced isoform containing a cryptic-exon.

(A) Mutations in the *VHL* gene predispose to different phenotypes. Von Hippel–Lindau disease is characterized by the development of central nervous system (CNS) and retinal hemangioblastomas, pheochromocytomas, clear-cell renal cell carcinomas (RCC), pancreatic cysts, neuroendocrine tumors, and endolymphatic sac tumors. Chuvash polycythemia is characterized by an elevated red blood cell number and can be associated with vertebral hemangiomas, varicose veins, thromboembolic events, and pulmonary hypertension, but never with tumors. This study describes families with typical VHL-associated phenotypes associated with an unexpected VHL status (i.e. either synonymous mutations or no alterations in *VHL*). Identification of novel *VHL* spliced isoforms containing a cryptic-exon that was found to be mutated in patients. (B) RT-PCR using primers specific for E1 and E3 was performed on mRNA extracted from lymphoblastoid cell line (LCL) established from Controls and patients of the Family 1. WT: wild-type. *: denotes larger fragments that were subsequently cloned and sequenced. (C) Schematic representation of the *VHL* gene and its products. The different *VHL* exons are represented on a scale: E1: 340bp from the ATG initiation codon, E1': 259bp, E2: 123 bp, E3: 179bp to the Stop termination codon. The full-

length *VHL* mRNA isoform encodes pVHL213 (also named pVHL30). *VHL*-E1 contains an internal translation initiation codon that initiates the production of pVHL160 protein (pVHL19). *: the isoforms containing E1' spliced with *VHL* exons have been identified by cloning and sequencing in the laboratory but have been described later on by NCBI as transcripts able to produce a protein termed X1. Consensus values of donor (SD) and acceptor (SA) splice sites sequences are indicated above the *VHL* gene, as calculated by the Human Splicing Finder *in silico* tool. Horizontal blue lines indicate the location of probes used in Taqman assays.

Figure 2: Identification of mutations in the new *VHL* cryptic-exon in six patients with erythrocytosis and a large family with the VHL disease.
(A) Schematic representation of the *VHL* gene and location of the identified mutations in the new *VHL* cryptic-exon E1'. (B) Pedigree of families with erythrocytosis or von Hippel Lindau disease. The genotypes have been elucidated by sequencing both parents and proband (F1, F5, F6), deduced by sequencing of one parent and proband (F4, the mutation deduced being under brackets), or deduced from allele cloning of proband carrying the conserved Chuvash mutation and core haplotype (F2, F3) confirming the transmission of the mutations by one of each parents (for F2 and F3, the identity of the transmitting parent being unknown, the mutation is represented by a white circle under brackets). The genotype of parents from F7 has been elucidated from WGS data. The numbers in italics (F8) indicate the age of the patient at tumor diagnosis.

Figure 3: Expression study of the new *VHL* transcripts isoforms in patient cells.
(A) Sashimi plots for RNA-Seq data obtained from samples (LCL or pheochromocytoma, PHEO), of three patients from F8. The positions of the different *VHL* exons are indicated, with the maximal number of reads indicated at the right. Splice junctions are denoted by the horizontal links, with details provided in Supplemental Fig. 8. (B) TaqMan quantification of the different *VHL* isoforms from samples of F8 performed using probes specific to the *VHL* E1-E2, the translated sequence upstream E1' or E1-E1' junctions. Relative gene expression has been normalized to LCL control (C1) fixed at 1 (mean results of technical duplicates). C: healthy control. (C) TaqMan quantification of the different *VHL* isoforms in LCLs (established from two independent controls and from patients of F1, F2 and F8) cultured in the absence or presence of puromycin, an inhibitor of Nonsense-Mediated mRNA Decay. TaqMan probes are specific to the *VHL* E1-E1' or E1-E2

junctions. The graph resumes experiments performed on LCL cultured independently 3 times and quantified in duplicates. Data are represented as the mean +/- SEM. Statistical p value: * p<0.05, ** p<0.005 based on a *t*-test. (D) RT-PCR using primers specific for E1 and E3 was performed on mRNA extracted from lymphoblastoid cell line (LCL) established from controls and patients of the Family 6 (carrying the mutation c.340+574A>T that targets the Splice Acceptor (SA) site of E1'). On the right, the spliced isoforms are schematically represented. *: denotes larger fragments that contains E1' spliced with E1, but with 15 nucleotides deleted (represented in red) by the use of an alternative SA site (sequences of the cloned bands are presented in Supplemental Fig.10).

Figure 4: Functional studies of mutations in *VHL*-E1'.

(A, B) Immunoblot analysis of patient LCLs. A representative immunoblot (A) and quantification of 3 different immunoblots are presented (B). Relative gene expression has been normalized to GAPDH expression and results obtained with LCL control (C1) have been fixed at 100%. Data are represented as the mean +/- SEM. ** p<0.005, *** p<0.0005, **** p<0.0005 based on a *t*-test. A VHL-antibody which recognized VHL-E1 was able to detect the hypothetical X1 protein. In the most left lane, control LCLs were transfected with an expression vector containing the coding sequence for X1. Five µg of protein were blotted *vs.* 45µg for other samples to avoid signal saturation with overexpressed X1. (C) Functional HRE-dependent reporter assays were performed in 786-O cells (i.e. *VHL* negative cells that constitutively express HIF-2**α**). The results are expressed as relative Firefly luciferase activity with *Renilla* luciferase as an internal control. 1.0 unit denotes the basal activity of endogenous HIF-2α using the HRE-luciferase reporter plasmid. The ability of wild type and mutated X1 to downregulate Firefly luciferase activity (related to HIF activity) was compared to pVHL and in competition with pVHL. An immunoblot using an antibody specific to the hemagglutinin tag was used to detect HA-VHL and HA-X1. The X1-L128V+L138P corresponds to a potential impact of the c.340+648T>C and c.340+617C>G variations on the hypothetical X1 protein. Three independent experiments were performed. ** p<0.005, based on *t*-test. (D) Characterization of *VHL*-E1' retention by the Minigene experiment (representative picture of agarose gel from n=3). RT-PCR was performed on mRNA obtained from cell lines transfected with a minigene construct containing *VHL*-E1’ (wild-type or mutated) flanked by large intronic sequences cloned between the *SERPING1* exons (exons A and B, targeted by the RT-PCR primers). The plasmids were transfected and the expression of the spliced chimeric transcripts (containing

EA and EB with or without E1') was analyzed. Two wild type constructs containing E1' were used; one contains SNP rs779808, 340+1150T>C. Bands corresponding to EA and EB spliced together or with *VHL*-E1' are indicated on the right. * corresponds to unspecific bands verified by sequencing. The minigene experiment performed with the construction carrying the mutation c.340+574A>T (that targets the Splice Acceptor (SA) site of E1' in F6) confirmed the use of an alternative SA site (right panel) with the deletion of 15 nucleotides (represented in red).

Figure 5: Genetic and expression study of synonymous mutations in *VHL*-E2.

(A, B) Pedigree and sequence chromatograms of germline DNA, tumor (pheochromocytoma) DNA or cDNA prepared from two families (F9, F10) with erythrocytosis (A), and two families (F11, F12) with von Hippel Lindau disease. (C) Results of RT-PCR using mRNA extracted from LCLs (F9, F10) and leukocytes and tumor material (pheochromocytoma) (F11, F12). (D) TaqMan quantification of the different *VHL* isoforms in LCLs (established from patients of F9, F10 and F11) cultured in the absence or presence of puromycin. TaqMan probes are specific to the *VHL* E1-E2 or E1-E3 junctions. Relative gene expression has been normalized to LCL control (C1). C: healthy control, F: Families. *** $P < 0.0005$, ** $P < 0.005$ (E) Immunoblot analysis of patient LCLs. A representative immunoblot (upper panel) and quantification of 4 different immunoblots are displayed (lower panel). Relative gene expression has been normalized to GAPDH expression and results obtained with LCL control (C1) have been fixed at 100%. Data are represented as the mean +/- SEM. ** $p<0.005$, *** $p<0.0005$, **** $p<0.0005$ based on a *t*-test.

Figure 6: Functional study of synonymous mutations in *VHL*-E2.

(A) Functional HRE-dependent reporter assays were performed in 786-O cells to evaluate the impact of *VHL* mutations in E2 (P138L and G144R) on VHL protein activity. The VHL protein lacking E2 (pVHL172/VHLΔE2) is used as a negative controle. The results are expressed as Firefly luciferase activity relative to Renilla luciferase as an internal control. 1.0 unit denotes the basal activity of endogenous HIF-2α using the HRE-luciferase reporter plasmid. Immunoblots using an antibody specific for the hemagglutinin tag were used to detect HA-VHL. (B) Characterization of *VHL* E2-skipping by minigene analyses. Minigene experiments were performed in a variety of cell lines relevant to the studied diseases: renal (293T, 786-O, HK2), pheochromocytoma (PC12), the erythroid cell line (UT7 cultured with EPO); and LCL. RT-PCR was performed on mRNA obtained

from cell lines transfected with a minigene construct containing *VHL*-E2 flanked by intronic sequences cloned between the *SERPING1* exons (exons A and B, targeted by the RT-PCR primers). Bands corresponding to EA and EB spliced together or with *VHL*-E2 are indicated on the right (C) Sashimi plots for RNA-Seq data. The positions of the different *VHL* exons are indicated, with the maximum number of reads for each exon indicated at the right. Splice junctions are denoted by the horizontal links, with details provided in Supplementary Fig. 15A. (D) Heatmap of pheochromocytoma transcriptome data. A comparison of transcriptome data for the pheochromocytoma from patient F11 III.1 (with P138P mutation) vs. Affymetrix data from the largest available cohort of paraganglioma/pheochromocytomas (recruited by the French COMETE network) that identified homogeneous molecular subgroups associated with susceptibility genes (Burnichon et al., 2011). exp: relative expression compared to the median.