Insights into the molecular features of the von Hippel–Lindau-like protein

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Abstract
We present an in silico characterization of the von Hippel–Lindau-like protein (VLP), the only known human paralog of the von Hippel–Lindau tumor suppressor protein (pVHL). Phylogenetic investigation showed VLP to be mostly conserved in upper mammals and specifically expressed in brain and testis. Structural analysis and molecular dynamics simulations show VLP to be very similar to pVHL three-dimensional organization and binding dynamics. In particular, conservation of elements at the protein interfaces suggests VLP to be a functional pVHL homolog potentially possessing multiple functions beyond HIF-1α-dependent binding activity. Our findings show that VLP may share at least seven interactors with pVHL, suggesting novel functional roles for this understudied human protein. These may occur at precise hypoxia levels where functional overlap with pVHL may permit a finer modulation of pVHL functions.

Keywords  Von Hippel–Lindau disease · Hereditary neoplastic syndrome · Cancer · Bioinformatics

Introduction
Cellular specialization arises from finely tuned interactions between different molecular components, such as proteins, DNA, RNA, transcription factors, degradative machinery, and external factors (Barabási and Oltvai 2004). This complex organization allows coordination of several metabolic networks yielding cellular metabolism. Oxygen sensing is an example of a biological network aimed at promoting metabolic adaptation to environmental variations, as is a well-characterized molecular pathway triggering complex responses to hypoxia (Bruick 2003; Bárdos and Ashcroft 2005). Oxygen variation is perceived by the cell through interactions of highly specialized proteins: the von Hippel–Lindau tumor suppressor protein (pVHL), hypoxia-inducible factor 1α (HIF-1α), and prolyl-hydroxylase protein family (PHDs), which form the so-called core hypoxia response network (Minervini et al. 2014). At physiological oxygen concentrations, PHDs catalyze the hydroxylation of two specific HIF-1α prolines (P402 and P564) (Jaakkola et al. 2001; Ivan et al. 2001). Hydroxylated HIF-1α is then targeted for proteasomal degradation by pVHL, an E3 ubiquitin ligase complex substrate recognition element (Hon et al. 2002). Hypoxia inhibits PHDs activity, impairing HIF-1α and pVHL recognition, promoting HIF-1α stabilization (McDonough et al. 2006). Once stabilized, HIF-1α is translocated to the nucleus, where it activates hypoxia response elements (HRE) and promotes hypoxia-regulated gene expression (Maxwell et al. 1999). Deregulation of this network is known to predispose to cancer onset, as in von Hippel–Lindau syndrome (Iliopoulos et al. 1995). Biological networks, beyond their complexity, are also characterized by robustness (Kitano 2004), a fundamental property which allows the cell to react to internal and external perturbations (e.g., hypoxia, nutrient variation, DNA mutations, etc.) (Kitano 2004). A bow-tie architecture is frequently used to describe biological networks (Kitano 2004; Friedlander 2005).
et al. 2015). This representation implies few highly connected hub proteins forming a conserved core, while a number of proteins and various stimuli form the network input/output. Elements in the conserved core are associated with the main tumor suppressors and oncogenes, such as p53 (Lamb and Crawford 1986; Bisio et al. 2014), pVHL (Frew and Krek 2008), and c-Myc (Zeller et al. 2006), just to name a few. Another property directly derived from a bow-tie network organization is the appearance of specialized protein hub isoforms (or alternative genes) presenting slightly different functions. The p53-like protein family is an example of this functional overlap within the members of the same family (Murray-Zmijewski et al. 2006). p53, p63, and p73 are known to form a transcription factor family (Strano et al. 2001, p. 73), acting as main regulators of cell cycle progression. Although partially redundant, each member has its own unique function, varying between coordination of cell differentiation, stress response, functional apoptosis and aging (Murray-Zmijewski et al. 2006). From a biological network perspective, functional overlap can be seen as a natural mechanism to improve network robustness, i.e., together replicating crucial nodes while allowing evolution of new functions. In 2004, the von Hippel–Lindau-like protein (VLP), a human paralog of the VHL gene, was reported in the literature (Qi et al. 2004). VLP contains 139 amino acids, corresponding to the pVHL β-domain, while the pVHL E3-component recognition domain, required for pVHL degradative function, is missing. The first characterization attempts revealed that VLP binds HIF-1α and suggested it may act as a dominant-negative VHL, protecting HIF-1α from degradation (Qi et al. 2004). Three different pVHL isoforms (Chesnel et al. 2015) are already known in the literature. Although pVHL was described to be directly involved in several different pathways interacting with various proteins (Frew and Krek 2008), the exact role of each different isoform and paralogous VLP is far from being clearly understood. Here, we present an in silico characterization of human VLP. Phylogenetic analysis was paired with in silico structural investigations. Our analysis showed that VLP is expressed in specific human tissues and shares interactors with pVHL. Collectively, our findings suggest novel functional roles for this human pVHL paralog.

Materials and methods

Expression data

Expression profile data were retrieved by combining different database searches. Bgee (Bastian et al. 2008) was used to extract and compare gene expression patterns between homologous genes from different species and CleanEx (Praz and Bucher 2009) to extract specific entries according to their biological annotation. Ensembl (Herrero et al. 2016), GeneCards (Stelzer et al. 2011), NextProt (Lane et al. 2012), GTEx Portal (2013), and Gene Expression Atlas (Kapushesky et al. 2010) were used to retrieve human gene expression data at the tissue level as well as to extract data for gene regulation and genetic variation.

Sequence feature and interactor analysis

Orthologous VLP sequences from different species were retrieved in OMA Browser (Schneider et al. 2007), OrthoDB (Zdobnov et al. 2017), EggNOG (Huerta-Cepas et al. 2016), PhylomeDB (Huerta-Cepas et al. 2014) and enriched by manual Blast (Mount 2007) search against the NR database. The extracted dataset was then manually curated to remove pVHL-specific homologous sequences and aligned with Jalview (Waterhouse et al. 2009) to derive a conservation score for each residue. The final conservation score and chemical properties were considered as a cutoff for protein–protein interaction surface prediction. Interaction surfaces were defined as regions of VLP corresponding to pVHL-binding interfaces described in VHLdb (Tabaro et al. 2016) (http://vhldb.bio.unipd.it/). A VLP interaction network was derived merging data from the BIOGRID (Chatr-Aryamontri et al. 2015), STRING (Franceschini et al. 2013), and IntAct (Hermjakob et al. 2004). Putative VPL molecular functions were predicted with INGA (Piovesan et al. 2015), while dSysMap (Mosca et al. 2015), COSMIC (Forbes et al. 2015), and PolyPhen-2 (Adzhubei et al. 2013) were used to address VLP role in human disease onset.

Phylogenetic analysis

A Bayesian analysis was performed with MrBayes 3.2.6 (Ronquist et al. 2012) using the Jones–Taylor–Thornton model of protein evolution plus invariant sites, JTT + I, identified with MEGA 7 (Kumar et al. 2016, p. 7), with 4 chains (3 heated chains and 1 cold chain) and temperature 0.1 and 1,000,000 generations. The tree layout and bootstrap values at the nodes of the tree were visually inspected with FigTree (http://tree.bio.ed.ac.uk/).

Homology modeling

The human VLP sequence (accession code: Q6RSH7) was retrieved from UniProt (UniProt Consortium 2014). The crystal structure of pVHL bound to the CODD fragment of HIF-1α at 1.85 Å (PDB code: 1LM8) was selected as template by search against the Protein Data Bank (http://www.rcsb.org/pdb). As both proteins present a long N-terminal tail that were thought to be intrinsically disordered, VLP sequence was aligned against pVHL with Jalview (Waterhouse et al. 2009) using T-Coffee (Notredame et al.
algorithm with default parameters to identify the exact boundaries of the conserved globular domain. The final alignment corresponds to VLP residues 42-139 and pVHL 60-157. Initially, five comparative models of the target sequence were built by MODELLER 9.17 (Webb and Sali 2017) using the model single module. The best model was selected by picking the model with the lowest value of discrete optimized protein energy (DOPE) calculated using the DOPE integrated method. The VLP-HIF-1α dimer was modeled through structural superimposition in Chimera (Pettersen et al. 2004). GROMACS 4.6.5 (Hess et al. 2008) was used to relax the model structures for 1000 steps of steepest descent minimization. Model quality was assessed with QMEAN (Benkert et al. 2008), TAP (Tosatto and Battistutta 2007) and PROCHECK [27]. Network of interacting residues was generated using RING 2.0 (Piovesan et al. 2016) and used to inspect the main structural feature of VLP.

Molecular dynamics simulation

Molecular dynamics (MD) simulations were carried out in triplicates with GROMACS 4.6.7, using the CHARMM-27 force field (Mackerell et al. 2004). Hydrogen atoms were added to the system using the Gromacs pdb2gmx routine. A solvent box constituted by ~1.2 × 10^4 TIP3P water molecules and sized 69 Å was generated imposing a cutoff distance of 10 Å from the farthest point of the protein boundaries in each dimension. The system was neutralized by adding 6 Cl⁻ ions for system containing VLP alone, whereas 4 Cl⁻ for VLP complexed with HIF-1α, both wild-type and hydroxylated. The protonation state of residues was adjusted to reproduce a pH value of 7.0, i.e., all lysine residues were positively charged. Similarly, aspartate and glutamate residues were considered fully deprotonated. The simulation protocol consists of a minimization step, 100 ps of NVT (constant number of molecules, volume, and temperature) simulation, 100 ps of NPT (constant number of molecules, pressure, and temperature) simulation, and 100 ns of classical MD. Temperature for production simulations was kept at 300 K. Parrinello–Rahman was selected as the barostat of the MD, while V-rescale (modified Berendsen) was selected as the thermostat. Covalent bonds constrain was applied by using LINCS (linear constraint solver) algorithm. The electrostatic interactions were processed using the Particle Mesh Ewald (PME) grid, whereas neighbor searching was processed setting Verlet as cutoff-scheme and 20 as nstlist value to allow GPU acceleration of calculation. Periodic boundary conditions were applied to all directions. A time step of 2 fs was selected for the simulations and no constant forces were applied. The MD trajectories were recorded every 2 ps. Parameters for simulating the hydroxyproline residue were derived from Minervini et al. (2013). Mixed CPU/GPU calculations were conducted on a standard x 86 Ubuntu 16.04 Linux workstation (AMD FX-8350, Nvidia GeForce GTX 570). The Gromacs kernel was built using gcc 5.4 compiler.

![Gene expression for VHLL (ENSG00000189030.8)](image)

**Fig. 1** VLP expression in human. Box plot of human VLP gene expression data from GTEx Portal. VLP is actively expressed in testis and brain (cerebellum and basal ganglia)
Results
The human VLP is a 139 amino acid protein with 67.6% identity and 78% similarity to the pVHL region 1–157. This corresponds to the N-terminus and β-domain of pVHL (Leonardi et al. 2009). VLP was proposed to co-regulate HIF-1α stability (Qi et al. 2004); however, to date its molecular function is far from understood. The results from different in silico analyses aimed at characterizing VLP are discussed in the following.

VLP is found in cerebellum, testis, and placenta

The expression patterns associated with VLP were investigated by a virtual expression analysis searching different databases. Multiple database searches in Bgee, CleanEX, and Gene Atlas confirmed the previous observations reporting the human VLP in cerebellum and placenta (Qi et al. 2004). The Gene Cards and GTEx Portal report VLP to be also expressed in testis (Fig. 1). As these databases are derived from different sources, this data cannot be considered as a spurious observation. In particular, GTEx Portal collects novel analyses of human tissues from donors; it could be supposed that VLP expression could be caught in the near future within other tissues beyond cerebellum,

Fig. 2 VLP sequence conservation. Multiple sequence alignment of VLP showing relevant conservation for the globular domain corresponding to the pVHL β-domain (purple bar). A magenta box highlights the pVHL α-domain missing in VLP. Light blue and orange bars represent putative protein–protein interaction interfaces predicted for VLP using the previous pVHL description (Leonardi et al. 2009). VLP cancer mutations found in COSMIC are shown with red dots. Arrows highlight conserved residues stabilizing the structure. The amino acid type and number are shown in green for aromatic residues, red and blue are for negative and positive charges.
placenta, and testis. Of note, all of these tissues present a well-known susceptibility to hypoxia (Aplin 2000; Busl and Greer 2010; Reyes et al. 2012). Due to its molecular similarity with pVHL, a tissue-specific role for VLP could be hypothesized.

**VLP is mainly conserved among primates**

Next, we asked whether VLP is conserved among other species. To answer this question, a comparative sequences analysis was done. Retrieving orthologous sequences is particularly challenging due to high sequence identity with the pVHL protein. Although an initial search using an automatic procedure generated a list of 47 putative orthologs, manual inspection showed that only a limited number of sequences can be correctly classified as VLP. In particular, VLP is mainly present in primates (*Homo sapiens, Pan paniscus, Nomascus leucogenys, Aotus nancymaeae, Rhinopithecus roxellana, Mandrillus leucophaeus*), with *Vicugna pacos* and *Monodelphis domestica* (the gray short-tailed opossum, a marsupial) representing the only two other mammals whose sequences are not ambiguous and clearly orthologous to human VLP. A multiple sequence alignment comparing a total of six VHL orthologs from distinct upper mammals shows the VLP globular region, corresponding to the β-domain of pVHL, to be highly conserved (> 85% similarity). A longer primate-specific region formed by three repetitions of the xEEx motif was found at the N-terminus (Fig. 2). This sequence organization is comparable to pVHL30, the longest human pVHL isoform (Minervini et al. 2015) which presents a 54 residue tail formed by 5 GxEEx repetitions. Although debated, this tail was proposed to mediate isoform-specific functions presumed to have emerged during primate evolution, i.e., a further protein–protein interaction interface (Minervini et al. 2015).

**VLP phylogenetic analysis**

Despite great efforts in retrieving orthologous sequences of human VLP from various sources, multiple sequence alignment suggested that not all “VHL-like proteins” are truly orthologous to human VLP. Indeed, most of them share a sequence and domain composition resembling VHL rather than human VLP. Due to this ambiguity, we discarded these putative sequences from the following analysis. We next performed a Bayesian inference phylogenetic analysis to better investigate the evolutionary relationships between VLP, its paralog VHL, and their different functions. Our phylogeny suggests a complex evolutionary relationship behind VLP and its paralog VHL. A group of primate VHL sequences can be identified, including *Homo sapiens*, while the VLP and VHL proteins from *Vicugna pacos* seem to form a separated group, sharing higher sequence similarity with the VHL group (Fig. 3). As the Alpaca (*Vicugna pacos*) is a camelid evolved to live at higher altitudes of South American mountains, the observed differences may reflect an adaptation to constitutive low oxygen concentrations. The analysis also highlights a well-defined group including all VLP sequences from Primates, suborder Haplorhini, with a clear distinction for each family: Hominidae, including *Homo sapiens, Gorilla gorilla, Pan paniscus* and *Pongo abelii*, and Hylobatidae represented by *Nomascus leucogenys*. On the other hand, we found no trivial localization for *Monodelphis domestica* as its putative VLP shares features with both VHL and VLP groups. These findings, paired with the high sequence similarity shared by the VHL β-domain and VLP sequences, suggest that one (or more) remote gene duplication during mammalian evolution could easily explain the differences observed between VLP sequences from distant groups. Further genetic characterization will help to clarify this point.

**VLP presents a structured pVHL-like β-domain**

A VLP homology model, generated starting from pVHL structure (PDB code 1LM8) (Min et al. 2002), shows a clear pVHL β-domain fold, completely lacking the elongin-binding α-domain (Fig. 4). Superimposition of the model accounting for VLP residues 42–139 and pVHL (AA 60–157) 3D structure (0.2 Å RMSD for the backbone heavy atoms) highlights full conservation (100% identity) for the β2- and β6-strands and a short loop in pVHL. The model quality calculated with QMEAN is 0.649, TAP estimates a 0.757 confidence value, whereas a Ramachandran plot
calculated with PROCHECK (Laskowski et al. 1993) estimated that 88.2% of the total amino acids reside in most favored regions, with remaining 11.9% in additional allowed regions (Figure S1). No residue was identified in disallowed regions. All together these pieces of evidence, paired with the high sequence similarity between VLP and pVHL, suggest that our VLP model is reliable. The network of interacting residues generated with RING (Piovesan et al. 2016) showed an extended cluster of conserved aromatic residues engaging multiple cation π interactions and forming the VLP hydrophobic core (Fig. 5). Similarly, three conserved pairs of ionic interactions seem to further stabilize the structure. We then decided to perform molecular dynamics simulations to verify VLP fold stability. The RMSD traces for the backbone heavy atoms for three trajectories of 100 ns MD simulations show that the VLP globular domain is almost stable, with an average 1.8 Å RMSD value, whereas the analysis of backbone fluctuations shows the only flexible regions to correspond to both the N- and C-terminal tails (Figure S2). At the end of simulations, we observe an increased number of electrostatic interactions due to rearrangement of Ser47 and His97. However, comparison with RING of different MD frames extracted every 2 ns shows that the major contribution to the β-domain stability is given by Van Der Waals interactions among hydrophobic residues Ile56, Ile58, Leu83, Phe89, Ile91, Ala 131, Ile133 mostly shared with pVHL.

**VLP interacts with HIF-1α through interface B**

Although interaction between VLP and HIF-1α is not completely new (Qi et al. 2004), driving molecular details remain unknown. Due to its similarity with pVHL, the interaction was proposed to rely on similar residues (Qi

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**Fig. 4** Overview of VLP structural features. Structural comparison of domain organization and electrostatic surface between VLP and pVHL. VLP homology model in ribbon representation with semi-transparent surface colored by conservation (purple, high; turquoise, low). VLP corresponds to the β-domain of pVHL (blue box). Helices are colored in green, beta strands in orange. Residues stabilizing the VLP structure are shown as sticks and labeled as in Fig. 2
et al. 2004). The corresponding VLP complex was modeled by superimposition with the pVHL/HIF-1α complex crystal structure. Our structural model shows amino acid substitutions at the putative HIF-1α interaction interface which may interfere with VLP/HIF-1α interaction. In particular, Ser111 of pVHL, which is known to engage interaction with hydrolyzed proline residues of HIF-1α, is substituted with Asn93, a polar residue larger than serine. Similarly, pVHL Arg107 which forms an ionic interaction with Asp571 of HIF-1α is changed in Phe89,
a hydrophobic amino acid unable to support electrostatic interaction. Multiple independent MD runs were performed to test complex stability with and without HIF-1α hydroxylation (Fig. 6). These showed VLP interacting with HIF-1α through the β3- and β4-strands. The binding is maintained by Van Der Waals interactions between VLP residues Leu53 Trp70, Tyr80, His92, Phe94, which form a hydrophobic niche within the binding pocket, and the HIF-1α region comprising residues 561–565. The remaining residues of the HIF-1α fragment appear to follow a different fate depending on presence of hydroxylated proline 564. The hydroxylated complex appears more stable during the simulation, in particular, a hydrogen bound between hydroxyproline 564 and histidine 97 of VLP lower the average distance between the two proteins, yielding further hydrophobic interactions with interface B residues (Fig. 6). As a direct consequence of a lower average distance, residues 571–574 of hydroxylated HIF-1α form 3 further hydrogen bonds with VLP backbone, resembling the association between pVHL and HIF-1α. Contrarily, the C-end of wild-type HIF-1α does not form these hydrogen bonds, yielding less thigh interaction with VLP. Interestingly, the Asn93 which is in VLP in place of pVHL Ser111 appears to be able to sustain the interaction with the hydroxyproline 564. Similarly, the Arg87 of VLP is predicted to rescue the interaction with Asp571 of HIF-1α. Collectively, our simulations showed that hydroxylation of proline 564 may play a regulative role in the interaction with VLP.

**VLP presents conserved structural motifs suggesting overlapping pVHL functions**

In a previous research, we proposed three different pVHL protein–protein interaction interfaces (A, B, and C, respectively) (Leonardi et al. 2009). For each interface, conserved residues are known to engage in specific interactions with a subset of pVHL binding partners (Tabaro et al. 2016). We asked whether a similar surface organization is also conserved in VLP. Considering the high sequence identity between the two proteins, we mapped putative binding regions on the VLP interface. Residues forming interfaces B and C are mostly conserved in VLP, i.e., 63.6% and 81.5%, respectively. Considering the similarity between the two proteins, at least seven pVHL interactors can be inferred for VLP (Fig. 7). Putative pathways in which VLP plays a role were assigned based on these findings. VLP may be involved in regulating HIF-1α degradation, cell migration, and cell cycle regulation interacting with USP33 and USP20 through interface B. Both are well-known deubiquitinating enzymes involved in various
cellular processes (Curcio-Morelli et al. 2003; Shenoy et al. 2009, p. 2; Berthouze et al. 2009). For interface C, interactions with EP300, EEF1A1, AR (androgen receptor), and PSMC3 were predicted, linking VLP with proteins involved in gene transcription and tissue differentiation. These putative interactors can be found expressed in cerebellum and/or testis (see Fig. 8), suggesting they can co-exist with VLP in these tissues. Taken together, these data suggest VLP to be involved in various pathways, such as cell cycle regulation, nuclear export, growth and development of male reproductive organs, and ATP-dependent degradation of ubiquitinated proteins.

**VLP mutations**

COSMIC contains 36 mutated VLP samples, including 25 missense, 9 synonymous, 1 nonsense substitution, and 1 non-stop gain substitutions. No other complex mutations were found. Both confirmed somatic mutations and variants of unknown origin are described. A schematic view of the distribution of missense mutations on the VLP sequence and their pathogenicity predictions is presented in Table 1. Several residues that our investigation predicts to be relevant in determining VLP fold stability are mutated in cancer (Fig. 9). Interestingly, tissues and organs in which VLP is found mutated are mainly different from those affected in VHL syndrome (Fig. 10). Considering only missense mutations, 33.6% were found in adenocarcinoma of the large intestine, 15.4% in gastric adenocarcinoma, and 11.5% in endometrioid carcinoma. Other organs include biliary tract, breast, small intestine, kidney, liver, lung, ovary, esophagus, and skin.

**Discussion**

Oxygen sensing in mammal cell occurs at many levels, yielding both acute and chronic adaptation. The pVHL/HIF-1α axis is deputed to this role through fine regulation of several hypoxia responsive elements. From a biological network point of view, pVHL is a conserved core element, or a master protein, driving important cell functions. Robustness is a property of biological systems. A relevant aspect of robustness is a clear distinction between proteins acting as signals (modulators) and members of the central control (conserved core) (Kitano 2004). To guarantee system
stability, defined as resistance to external aggressions such as mutations and metabolic imbalance, the central core elements frequently evolve specialized isoforms with partially overlapping functions. This concept is well known for the combined functions of p53 protein family (Murray-Zmijewski et al. 2006). A similar assumption of functional family can be hypothesized for pVHL. Currently, three isoforms and a paralog protein are described in human for this important tumor suppressor (Hon et al. 2002, p. 19; Frew and Krek 2008, p. 19; Chesnel et al. 2015, p. 19). The two major isoforms, i.e., pVHL19 and pVHL30, show different cellular localization and the exact nature of their functional segregation remains far from understood. Fewer molecular details are available for VLP. It is thought not to possess ubiquitin ligase activity, missing the pVHL VCB-forming α-domain (Leonardi et al. 2009). This clearly excludes VLP from being an ubiquitin E3 ligase. Our analysis showed that this protein is mainly found in primates, where it apparently evolved structural features posing it halfway between pVHL19 and pVHL30. Human VLP is found to be expressed in three specific tissues characterized by relevant susceptibility to hypoxic injury. The brain, in particular, is well known for its sensibility to variation of oxygen concentration. VLP was proposed as a dominant-negative VHL, exerting a HIF-1α protective function (Qi et al. 2004). It is known that neuron-specific inactivation of HIF-1α correlates with increased brain injury during transient focal cerebral ischemia in mice models (Baranova et al. 2007). In the same study, it was

![Fig. 8 Expression profiles of seven predicted VLP interactors. Box plots of human USP20, USP33, EP300, EEF1A1, AR, PRKCZ, and PSMC3 genes expression data from GTEx Portal. Arrows highlight human tissues in which VLP is expressed](image-url)
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also observed that following an initial hypoxic stimulus, HIF-1α levels remained elevated after 8 days from a normal oxygenated blood perfusion. Our MD simulations suggest that VLP forms stronger interactions with hydroxylated than unmodified HIF-1α. We believe this data in particular may suggest a precise role for VLP in brain tissue remodeling after a pathological hypoxia insult. In this context, a putative interaction with EP300 (E1A binding protein p300) is worth mentioning. In severe anoxic conditions, p53 outcompetes HIF-1α for EP300 (Van Roey et al. 2014), promoting pVHL-independent HIF-1α degradation and leading to a demodulation of hypoxia response. VLP may play a role regulating the competitive p53/HIF-1α interaction with EP300. Our analysis also sheds light on HIF-1α-independent functions, in particular the putative interaction with EEF1A1 (elongation factor 1-alpha 1). This protein is functionally connected with the testis-specific protein Y-encoded (TSPY1) (Kido and Lau 2008), a male specific protein encoded on the Y chromosome expressed in testicular tissue and correlated with spermatogenesis and gonadoblastoma insurgence. As EEF1A1 mediates pVHL nuclear exporting (Khacho et al. 2008), our investigations suggest the same mechanism to be present in VLP. Combining these findings with VLP expression in testis, we suggest this protein to have a novel regulatory role modulating EEF1A1/TSPY1 interaction.

Conclusion

Our analysis shows that VLP shares structural features with the pVHL oncosuppressor in terms of structure conservation and protein–protein interaction partners. It also suggests that VLP could be a member of a larger pVHL family sharing regulative functions. pVHL is a key component of proteasome-mediated degradation system, while VLP clearly misses this function. Nevertheless, it may play a relevant role in HIF-1α stabilization. In particular, its propensity to form a more stable complex with hydroxylated HIF-1α suggests an active role in the modulation of hypoxia response. VLP appears to be structurally very similar to pVHL. Conservation of functional elements at interfaces B and C, in particular, extends the VLP range of activity to HIF-1α-independent functions. These may

Table 1 VLP missense mutations and pathogenicity prediction

| Position | Wild-type | Mutant | Conserved | PolyPhen 2          |
|----------|-----------|--------|-----------|---------------------|
| 15       | Ala       | Val    | Yes       | Possibly damaging  |
| 17       | Thr       | Ala    | Yes       | Benign              |
| 26       | Cys       | Tyr    | Yes       | Possibly damaging  |
| 32       | Ala       | Val    | Yes       | Benign              |
| 39       | Arg       | Gly    | No        | Benign              |
| 49       | Asn       | Lys    | Yes       | Probably damaging  |
| 51       | Arg       | Cys    | No        | Benign              |
| 52       | Glu       | Gly    | Yes       | Probably damaging  |
| 55       | Arg       | Trp    | No        | Probably damaging  |
| 60       | Asn       | Lys    | Yes       | Probably damaging  |
| 62       | Ser       | Asn    | Yes       | Probably damaging  |
| 66       | Val       | Met    | Yes       | Probably damaging  |
| 69       | Val       | Leu    | Yes       | Benign              |
| 78       | Leu       | Met    | No        | Probably damaging  |
| 80       | Tyr       | Asp    | Yes       | Probably damaging  |
| 86       | Gly       | Ser    | Yes       | Probably damaging  |
| 90       | Arg       | His    | No        | Possibly damaging  |
| 91       | Ile       | Thr    | Yes       | Benign              |
| 94       | Phe       | Leu    | Yes       | Possibly damaging  |
| 98       | Pro       | His    | Yes       | Probably damaging  |
| 99       | Trp       | Arg    | Yes       | Probably damaging  |
| 105      | Arg       | Ser    | Yes       | Benign              |
| 114      | Glu       | Lys    | Yes       | Possibly damaging  |
| 120      | Pro       | Leu    | Yes       | Probably damaging  |
| 121      | Ser       | Tyr    | Yes       | Probably damaging  |

For each mutation found in COSMIC, we report the amino acid position and type for both wild-type and mutated residues. Conservation in sequence and PolyPhen predictions (benign, possibly damaging or probably damaging) are also shown

with spermatogenesis and gonadoblastoma insurgence. As EEF1A1 mediates pVHL nuclear exporting (Khacho et al. 2008), our investigations suggest the same mechanism to be present in VLP. Combining these findings with VLP expression in testis, we suggest this protein to have a novel regulatory role modulating EEF1A1/TSPY1 interaction.
occur within a precise hypoxia gradient where a finer modulation of pVHL functions would be desirable as in the case of brain remodeling after ischemia injury. The degree to which these in silico results apply to living organisms is unclear. Experimental studies will have to be carried out to clarify VLP functions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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