Computational and Experimental Characterization of dVHL Establish a Drosophila Model of VHL Syndrome

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Introduction

The von Hippel-Lindau (VHL) cancer syndrome is a rare hereditary cancer, associated with mutations in the VHL tumor suppressor gene. It is characterized by increased susceptibility to various tumors, both benign and malignant, including central nervous system haemangioblastomas, renal cysts and renal cell carcinoma (RCC) and phaeochromocytoma [1-3]. The pVHL tumor suppressor protein is the substrate recognition subunit of a SCF-like ubiquitin-ligase that targets the Hypoxia-Inducible Factor for degradation. pVHL has a molten globule configuration with marginal thermodynamic stability. The cancer-associated mutations further destabilize it. The Drosophila homolog, dVHL, has relatively low sequence similarity to pVHL, and is also involved in regulating HIF1-α. Using in silico, in vitro and in vivo approaches we demonstrate high similarity between the structure and function of dVHL and pVHL. These proteins have a similar fold, secondary and tertiary structures, as well as thermodynamic stability. Key functional residues in dVHL are evolutionarily conserved. This structural homology underlies functional similarity of both proteins, evident by their ability to bind their reciprocal partner proteins, and by the observation that transgenic pVHL can fully maintain normal dVHL-HIF1-α downstream pathways in flies. This novel transgenic Drosophila model is thus useful for studying the VHL syndrome, and for testing drug candidates to treat it.

Abstract

The von Hippel-Lindau (VHL) cancer syndrome is associated with mutations in the VHL gene. The pVHL protein is involved in response to changes in oxygen availability as part of an E3-ligase that targets the Hypoxia-Inducible Factor for degradation. pVHL has a molten globule configuration with marginal thermodynamic stability. The cancer-associated mutations further destabilize it. The Drosophila homolog, dVHL, has relatively low sequence similarity to pVHL, and is also involved in regulating HIF1-α. Using in silico, in vitro and in vivo approaches we demonstrate high similarity between the structure and function of dVHL and pVHL. These proteins have a similar fold, secondary and tertiary structures, as well as thermodynamic stability. Key functional residues in dVHL are evolutionarily conserved. This structural homology underlies functional similarity of both proteins, evident by their ability to bind their reciprocal partner proteins, and by the observation that transgenic pVHL can fully maintain normal dVHL-HIF1-α downstream pathways in flies. This novel transgenic Drosophila model is thus useful for studying the VHL syndrome, and for testing drug candidates to treat it.

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VHL is evolutionarily conserved from worms to mammals [39]. The Drosophila VHL (dVHL) was identified by amino-acid sequence homology and lacks the N-terminal extension found in the 30 kDa isoform of the human pVHL [40]. The dVHL protein was shown to interact with the Drosophila Elongin C homolog and could also form complex with the human VCB complex [41]. The domain corresponding to ODD in the fly homolog of HIF1-α (termed SIMA) spans its amino acid residues 692-863 and contains a conserved proline residue (P850), which has been proposed to mediate the oxygen-dependent stabilization of SIMA as do P564 and/or P402 in the human HIF1-α [42,43]. Notably, dVHL was shown by pull down assay to bind a GST-tagged ODD peptide of either the human HIF1-α or its Drosophila homolog, SIMA, under normoxic conditions, providing further evidence for its functional evolutionary conservation [43].

In order to gain structural insight into the apparent conservation of the function of dVHL, and pVHL we generated in silico a model structure for dVHL which revealed overall structural similarity between the two proteins and more significantly, evolutionary conservation of the key functional amino acids in pVHL and dVHL and their orientation within the secondary structures of the proteins.

We corroborated these observations by in vitro biophysical assays under physiological conditions which showed that pVHL and dVHL are similar in their secondary and tertiary structures, thermodynamic stability and molten globule conformation. This structural homology between pVHL and dVHL may underline their reported functional homology in binding the VCB complex [41]. Indeed we determined that they similarly bind in vitro both the human and Drosophila derived ODD peptide. We further examined this homology in vivo in the context of the intact organism using transgenic Drosophila. We found that pVHL can rescue, via the HIF dependent pathway, the mutant phenotype of loss of function of dVHL.

Taken together our results indicate that the transgenic Drosophila model generated accurately represents the Mammalian pVHL-HIF1-α pathway and is thus a useful tool for studying the VHL syndrome and VHL function in a whole organism.

Results

Comparison of the human and Drosophila VHL proteins

Human pVHL and Drosophila dVHL have been shown to have similar functions through their E3 ubiquitin ligase activity [41]. To assess the structural basis for the functional similarity between the two proteins, we searched for structural similarities between the human and fly homologues. Although the structure of human pVHL has already been elucidated by crystallographic methods, this has yet to be achieved for Drosophila dVHL. We therefore built an in silico three-dimensional model for Drosophila dVHL.

In addition to their similar functions, pVHL and dVHL belong to the same superfamily according to the Pfam database, suggesting that they are evolutionarily related and may share the same folding [44]. Indeed, two fold-recognition algorithms, FFA803 and HHpred, identified pVHL19 as a structural template for dVHL [45,46]. The relatively low sequence identity between pVHL19 and dVHL (<22% sequence identity and <30% similarity as calculated using the MUSCLE algorithm [47]) Figure 1) required the use of a composite modeling approach in order to optimize the alignment between the target (dVHL) and template (pVHL19) sequences. Our modeling procedure employed fold recognition algorithms such as FFA803 and HHpred with additional sequence alignments computed by the MUSCLE algorithm. We integrated hydrophobicity and evolutionary conservation algorithms and the final model was produced using MODELLER [48–53]. Structural data regarding the pivotal amino acids for pVHL19 interactions with its binding partners were used for alignment and orientation of amino acids within the model. Additional validation was achieved by comparison of our model structure to that produced by standard modeling tools such as I-TASSER [54] and Phyre2 [55]. The model structure produced by our composite approach shares significant structural similarity to these additional models (Figures S1 and S2).

Superimposition of the resultant dVHL model structure on the crystal structure of pVHL19 (PDB ID: 1LM8) shows that the overall domain structure of pVHL19 is conserved in dVHL (Figure 2A). The β domain structure, which consists in pVHL of a seven-stranded β sandwich (resides 1–116) and an α helix (resides 154 to 163), is overall more conserved than the α domain in the dVHL model structure. The main difference between the β domain of the two structures is the absence of the smallest of the seven β strands of pVHL (S6) in the dVHL model structure, which is therefore predicted to consist of a six-stranded β sandwich and an α helix. The α domain in pVHL is comprised of three α helices (resides 122 to 159). One of these three helices in dVHL is predicted to assume a random coil conformation as the amino acid sequence which makes up this region in dVHL does not favor helix formation (Figure 2A).

The comparison between the two structures also provided hints for the functional conservation of pVHL and dVHL with regard to the binding of HIF1-α and the interactions with Elongin B, Elongin C and Cul2 (Figure 2B). HIF1-α is known to interact exclusively with the β domain of pVHL by binding alongside its β sandwich [25]. The formation of the complex between HIF1-α and pVHL is mediated primarily by the interactions between a hydroxyproline (Hyp564) of HIF1-α and the following amino acids in pVHL: W88, Y98, W117, W217, H115 and S111. These amino acids are not only conserved in the linear sequence of dVHL but in pVHL is replaced by T64 in dVHL but are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C). The orientation of these amino acids is crucial for the function of pVHL and predictably of dVHL as well. Furthermore, we found that Y98 and W117 (Y51 and W70 in pVHL) are part of the same secondary structures and assume the same orientation in the dVHL model as they are in the pVHL crystal structure (Figure 2C).
Finally, pVHL has been shown to interact with Cul2. It is proposed that this is achieved via six amino acids that make up the Cul2 box in the pVHL sequence [56]. Out of these six residues, four are conserved in dVHL and three adopt very similar orientation and are evolutionary conserved (Figure 2G, H).

To conclude the modelling analysis, despite relatively low sequence conservation between human pVHL and fly dVHL, these two proteins are predicted to adopt the same fold. Moreover, the pivotal functional amino acids in pVHL and dVHL are oriented in very similar positions, allowing the formation of the intricate network of interactions which constitute the interfaces of the VCB complex. Taking these results into consideration with the fact that the key functional amino acids are also evolutionary conserved in pVHL and dVHL, we postulate that the two orthologous proteins may be able to interact with each other’s binding partners.

We examined the similarity in folding and stability of the two proteins in vitro as well as the ability of the two proteins to regulate each other’s downstream HIF dependent pathways in vivo.

**Folding and stability of dVHL are similar to pVHL**

Several biophysical methods were employed to compare folding and stability of human pVHL and its *Drosophila* homolog dVHL.

We examined the conformational motifs, including α-helices, β-sheets and turns by Circular Dichroism (CD) spectra of soluble, bacterially expressed pVHL and dVHL, unbound to their Elongin B, C and HIF1-α partners by far UV (178–250 nm) at 25°C. This analysis indicated that pVHL and dVHL have similar secondary structure comprising of both α-helices and β-sheets, estimated by the K2D2 algorithm to represent 32% and 34% of pVHL, respectively and 14% and 32% of dVHL, respectively (Figure 4A; Table 1). These values for pVHL are in agreement with those previously reported for this protein [34].

We next evaluated the tertiary structures of the two proteins by near-UV CD spectra (205–350 nm) at 25°C. No significant signals originating from aromatic side chains were observed for either protein, suggesting a dramatic loss of the tertiary structure of the soluble and unbound dVHL and pVHL (Figure 4B). This data indicates that both proteins are in molten globule state since the signal is near zero.

The thermodynamic stability of dVHL was assessed via thermal denaturation measurements by CD. The melting temperature ($T_m$) of dVHL was found to be about 45°C [Figure 4A], identical to the $T_m$ reported for pVHL [34]. Furthermore, the unfolding of dVHL was only partially reversible (Figure S4B), and formation of amorphous aggregates could be visualized by the naked eye, as reported for pVHL [34].

Size exclusion chromatography (SEC) was next used to estimate the hydrodynamic dimensions and the compactness of the tertiary structure of pVHL and dVHL. Elution volume of partially or fully unfolded proteins is significantly smaller than that of well folded proteins due to the large increase in the Stokes radius ($R_s$). The dVHL protein eluted at a retention volume of 10.38 ml and pVHL at 10.22 ml, very close to ovalbumin, which served as a standard protein (MW = 44,000 Da) (Figure 4C). The apparent MW of these proteins, calculated from the calibration curves generated for elution of molecular weight standards, correspond to molecular mass of 38,019 Da for dVHL and 40,833 Da for pVHL. These empirical values are larger than the calculated molecular mass of both proteins (19,000 Da) and are similar to the previously published values for pVHL monomers [34,35]. The $R_s$ values of dVHL and pVHL, calculated from the calibration curve are 2.64 nm and 2.93 nm, respectively (Figure 4D). Taken together, these results indicate that soluble pVHL and dVHL, unbound to their partners (Elongin B, C and HIF1-α) are larger than their calculated molecular weight (~40 kDa vs. ~19 kDa, respectively). These results together with our near CD spectra results suggest that both proteins have a molten globule conformation.

Affinity of proteins in a molten globule conformation to the hydrophobic probe ANS (3-anilino-1-naphthalene sulfonic acid) is much stronger than in the rigidly packed or the completely unfolded state [57], as reflected by a stronger fluorescence signal. We used ANS to assess the compactness and rigidity of packing of the hydrophobic core of dVHL. Figure 4E shows that at 25°C ANS fluorescence greatly increased upon binding of dVHL. The emission spectrum indicated that dVHL, as reported for pVHL [34], has a loosely packed hydrophobic core, and could comprise a molten globule tertiary structure. We monitored ANS fluorescence intensity at different temperatures (Figure 4E). The results indicate that at 4°C the accessibility of the hydrophobic core of dVHL to the solvent is slightly reduced due to the low temperature. At 37°C and 50°C (tertiary structure thermal denaturation) the protein is unfolded and loses the packing of the hydrophobic core. It is apparent that, as found in our CD spectra studies (Figure 4A) the transition between folded and unfolded conformation of dVHL...
resulting from thermal denaturation is irreversible, as previously reported for pVHL [34].

Collectively, the results from the CD spectra measurements, SEC data and ANS packing assessment indicate that dVHL and pVHL share similar folding, structure and stability. These in vitro findings mirror our in silico results which predict high similarity between the folding and structure of these proteins.

Functional characterization of dVHL and pVHL

**In vitro.** To assess functional homology between dVHL and pVHL, we compared their ability to bind proline-hydroxylated ODD using an ELISA-based binding assay. For this purpose we synthesized two rhodamine-labeled peptides, termed Hyp402-ODD-HIF and Hyp850-ODD-SIMA, comprising the amino acid sequences surrounding P402 in HIF1-α and P850 in SIMA in which these proline residues were hydroxylated. Increasing concentrations of the two peptides were incubated, with either soluble dVHL, pVHL or with BSA as a negative control, and fluorescence intensity was measured.

The dVHL and pVHL proteins bound the Hyp402-ODD-HIF or Hyp850-ODD-SIMA peptides with similar affinities (Figure 4F). Both proteins showed higher affinity towards the HIF1-α-derived peptide than to the SIMA-derived peptide. pVHL bound both ODD peptides ~1.5 fold stronger than dVHL.

**In vivo.** In order to assess if this evolutionarily conserved binding of the target ODD takes place also in the intact organism. We next examined whether pVHL can rescue the mutant phenotype of *Drosophila* lacking endogenous dVHL. Larvae
homozygous for a null mutation in the *Drosophila* VHL exhibit characteristic sluggishness evident immediately after hatching and die at the end of first instar larval stage [70]. Ubiquitous expression of either dVHL or pVHL transgenes (encoding the 19 kDa or the 30 kDa isoforms of pVHL) by tubulin Gal4 lead to ~60% of the animals completing development and eclosing as adult flies, indicating functional complementation (Figure 5A). To further examine whether this rescue reflects restoration of the HIF1-α pathway in these flies, we examined the level of the endogenous fly HIF1-α homolog protein, SIMA. Rescue of the null dVHL phenotype would suggest that the transgene is capable of normal targeting the fly HIF1-α homolog (SIMA) for degradation (as in wild type flies). Total protein extraction was prepared from the rescued and wild type flies, as well as flies over expressing SIMA, and were immunoblotted with anti-SIMA antibodies. The SIMA protein level in the flies rescued by dVHL or by either isoform of pVHL, was found to be similar to its level in wild type flies and considerably lower than in flies over-expressing SIMA which served as a control (Figure 5B). This is in agreement with our *in vitro* study which indicated that the two proteins directly bind the ODD of the SIMA protein with similar affinities. In addition, we examined in these flies the level of expression of the SIMA target genes Glut1 and VEGF [20,58,59]. Real time qPCR indicated that the level of the transcripts of these genes in flies rescued by either dVHL or by either isoform of pVHL is similar to their level in wild type flies (Figure 5C). These results indicate that pVHL is able to directly bind SIMA in the fly milieu and to properly regulate its downstream pathways.

To further demonstrate that the human protein, pVHL is capable of normal regulation of the fly HIF1-α homolog SIMA we over-expressed SIMA in the *Drosophila* eyes using the GMR-Gal4. Over-expression of SIMA resulted in a rough-eye phenotype in adult flies, evident by loss of regularity of the lattice-like organization of the ommatidia (Figure 6A). When SIMA was co-expressed in the eyes together with dVHL, as a control, or with either pVHL19 or pVHL30 the rough-eye phenotype was rescued (Figure 6A). At 29°C over-expressing SIMA in the eye resulted in lethality at the pupal stage, while when co-expression with either dVHL of pVHL the lethality was rescued (movie S1). This rescue was accompanied by reduction in the level of SIMA to the level typical of normal flies (Figure 6B). These results indicate that pVHL recognizes SIMA in the fly and targets it for degradation as does dVHL.

In a reciprocal experiment, we tested whether dVHL or pVHL can recognize, in the fly, ODD - the hallmark fragment of HIF1-α (see Introduction). When co-expressed in the fly eye imaginal discs together with either isoform of pVHL, the GFP signal of the ODD-GFP fusion protein was markedly reduced in comparison to control flies (Figure 7A). This reduction was similar to that caused by co-expression of ODD-GFP and dVHL (Figure 7A, B), as previously reported [43]. Western blot analysis of extracts from heads of the corresponding adults verified that the reduced GFP signal reflected lower levels of the ODD-GFP protein in the

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**Table 1.** Analysis from k2d2 program of pVHL and dVHL suggest similar secondary structure content of α-helix, β-sheet and random structure.

|         | pVHL | dVHL |
|---------|------|------|
| α-helix (%) | 14   | 29   |
| β-sheet (%)  | 32   | 34   |
| Random (%)   | 54   | 37   |

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*Figure 3. Comparison of the hydrophobicity of pVHL and dVHL.* The interface between pVHL (surface) and Elongin C (cartoon, orange) is greatly hydrophobic, with only a few key hydrophilic residues in the periphery. This pattern is conserved in dVHL. (A) Hydrophobicity of pVHL (PDB ID: 1LM8) shown with its binding partner human Elongin C. (B) Hydrophobicity of the dVHL model structure superimposed with human Elongin C (from PDB ID: 1LM8).

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The presence of dVHL or either isoform of pVHL (Figure 7B). These results indicate that under normoxic conditions both dVHL and pVHL recognize the ODD domain of HIF1-α and target it for degradation.

To further verify that pVHL functions normally in the fly milieu, we monitored its subcellular localization. In cells of the eye imaginal discs the transgenic pVHL was found both in the cytoplasm and in the nucleus as reported for this protein in mammalian cells [60] (Figure 8).

Overall our results demonstrate structural and conformational similarity between dVHL and pVHL, underlining their functional homology. Not only are they able to directly bind the reciprocal binding partners, but pVHL is able to replace dVHL in Drosophila, while keeping the HIF1-α pathway intact.

**Discussion**

Lower model organisms such as Drosophila melanogaster and Caenorhabditis elegans have often provided the first glimpse into the mechanism of action of human cancer-related proteins, thus making a substantial contribution to the elucidation of the molecular basis of the disease. More than 50% of the proteins that are associated with human diseases, including cancer, have orthologues in D. melanogaster [61–63]. In many cases, the function of a given protein is also conserved evidenced by the fact that the corresponding human protein can rescue the loss of function of its Drosophila orthologue [64]. Thus, studies of the fly orthologue as well as investigation of the transgenic human proteins in flies, which are highly amenable for genetic manipulations, provide important information on the human disease-associated protein and the pathways it is involved in. This is
exemplified especially regarding to the contribution of Drosophila to basic and applied aspects of cancer research (for recent reviews see [65,66]). The highly ordered, lattice-like, arrangement of the ommatidia in the fly eyes is especially suitable for large scale screens for genetic modifiers and for drug candidates [64–66]. Flies have also greatly facilitated drug screening [64]. Such studies have shown that the Drosophila VHL (dVHL) can interact with Drosophila Elongin C in vitro [40,41] and form a complex with the human Elongin CB complex and mouse Rbx-1, but not with the human Elongin A. Thus, the ubiquitin ligase function of pVHL is conserved in Drosophila. Subsequently, Irisarri et al. (2009) and Arquier et al. (2006) demonstrated in vivo that dVHL is involved in degradation of hydroxylated oxygen-dependent degradation domain (ODD) of both the human HIF1-α and of the Drosophila homolog, SIMA. Studies of dVHL have suggested the involvement of the VHL protein in regulation of endocytosis and epithelial cell motility which was subsequently demonstrated for pVHL as well [67–69]. Research on dVHL has also indicated a role for the VHL protein in microtubule stabilization during epithelial morphogenesis [70]. Yet, this remains to be demonstrated for pVHL as well.

The results presented here regarding the structural similarity between the dVHL and pVHL proteins provide insights into their functional homology. We have shown that pVHL is a molten globule protein [34], and have subsequently demonstrated that it is an intrinsically disordered protein (IDP), and is functional as such [35]. Here we show that dVHL, like pVHL, has a secondary structure but lacks well-defined tertiary structure and attains a molten globule conformation. Our results indicate that dVHL, like pVHL, is functional without being uniquely folded into a rigid 3D structure. Thus dVHL like pVHL is an IDP.

The demonstration of homology between IDPs is a major endeavor since they do not display sequence conservation. In addition, due to the lack of well-defined 3D structure, IDP regions fail to scatter X-rays coherently, hampering attempts to solve their structure by X-ray crystallography [71–73].

The in silico predicted homology in the conformation of the key functional amino acids of dVHL and pVHL, as well as their evolutionary conservation, supported by our in vitro results, provide a structural basis for understanding the homology in their function as well as their ability to interact with each other’s binding partners. dVHL can bind in vivo the hydroxylated ODD domain of the human HIF1-α and target it for degradation [43] and here we showed that transgenic pVHL can do the same in flies. Moreover, we demonstrated that transgenic pVHL can rescue the lethal phenotype of dVHL null mutants of Drosophila. These results suggest that pVHL is capable of interacting, in flies, with the Drosophila members of the VCB complex, thus regulating the fly HIF1-α homolog, SIMA. Indeed, we have shown that this rescue flies involves regulation of the HIF1-α/SIMA dependent pathway, evidenced by the level of its target genes.

The Drosophila model described in the present work should facilitate further studies on the function of pVHL and the pathways it regulates. The rough eye phenotype caused by over-
expression of SIMA, which we have shown to be suppressed by transgenic expression of normal pVHL, should be highly conducive for conducting screens for drug candidates for the VHL syndrome in Drosophila whole model organism. These, can be subsequently verified molecularly by examining the downstream HIF1-α dependent pathway which are conserved between flies and humans.

**Materials and Methods**

*In Silico Analysis*

The amino acid sequences of human and *Drosophila melanogaster* VHL proteins were obtained from Uniprot [74]. Pairwise alignment of these two sequences was constructed using the MUSCLE algorithm [47]. As these two orthologs show only < 22% sequence identity and <30% similarity, we utilized fold recognition and homology detection servers in order to find structural templates for *Drosophila* VHL. The FAS03 fold recognition server identified the structure of human pVHL19 (PDB ID 1LM8) as the best template with a significance score of –77.3 [43]. The HHpred remote protein homology detection and structure prediction server also identified human pVHL19 as a suitable template [46]. Sequence alignments were constructed using the MUSCLE algorithm and manually corrected based on data from FAS03 and HHpred as well as PSIPRED (for secondary structure predictions) and ConSurf (for evolutionary conservation considerations) [49–52,75]. The models were constructed by MODELLER with the best one selected based on structural data regarding key structural and functional amino acids as well as hydrophobicity [53]. Additionally, the quality parameters of each model were estimated with ProSA and Procheck [76,77]. The coordinate file (PDB format) of our model structure can be found at doi:10.5061/dryad.14pp7.

**Gene Subcloning**

The open reading frame of pVHL, kindly provided by Dr. Nikola Pavletich (Memorial Sloan-Kettering Cancer Center, New York), and the dVHL ORF following reengineering to adapt it to the *E. coli* codon usage for obtaining better yield, were cloned into the pET14b vector (Novagen) using NdeI and XhoI (TaKaRa) restriction enzymes.

**Proteins Expression and Purification**

The vectors were transformed to Rosetta strain of *E. coli*. Transformed cells were grown at 37°C, 200 rpm, in 2XYT medium (Difco) under antibiotic selection (100 μg/ml ampicillin). The cells were grown to an OD$_{600}$ = 2.5 and induced by adding...
1 mM of Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) for 3 hours at 37°C. The pVHL and dVHL proteins were found to be insoluble in the bacterial inclusion bodies. The cells were broken using a high-pressure homogenizer and adding 40 mg of lysozyme (Sigma-Aldrich) in TE 50:20 buffer (50 mM Tris-HCl pH 8, 20 mM EDTA). Following disruption, the inclusion bodies were recovered and washed in TE 50:20 buffer with 1%v/v Triton using centrifugation. Inclusion bodies pellet was dissolved in the presence of 6 M guanidinium hydrochloride and diluted to 10 mg/ml protein concentration. The protein was refolded from fully reduced and unfolded samples by dialysis in step-wise decreasing concentrations of the denaturant guanidine hydrochloride to working buffer (10 mM Tris-HCl pH 8.5, 200 mM NaCl). pVHL and dVHL were purified to >97% purity, and migrated on SDS-PAGE as a 19 kDa proteins. Protein concentration was determined from absorbance at 280 nm using ε280 nm = 18450 cm⁻¹ M⁻¹ for pVHL and ε280 nm = 33920 cm⁻¹ M⁻¹ for dVHL.

Figure 7. The GFP fluorescence signal caused by over-expression of ODD-GFP is lowered by co-expression of either dVHL, pVHL19, or pVHL30. (A) GFP levels were analyzed by confocal microscopy. Genotypes: GMR-Gal4; UAS-ODD-GFP/UAS-RFP (left), GMR-Gal4; UAS-ODD-GFP/UAS-dVHL (second from left) GMR-Gal4; UAS-ODD-GFP/UAS-pVHL19 (third from the left) and GMR-Gal4; UAS-ODD-GFP; UAS-pVHL30 (right). Scale bar, 20 μm (B) Western blot analysis showing the levels of ODD-GFP protein extract from flies heads. β-actin was used as loading control. (1) GMR-Gal4; UAS-ODD-GFP/UAS-RFP. (2) GMR-Gal4. (3) GMR-Gal4; UAS-ODD-GFP/UAS-dVHL. (4) GMR-Gal4; UAS-ODD-GFP/UAS-pVHL19. (5) GMR-Gal4; UAS-ODD-GFP/UAS-pVHL30. doi:10.1371/journal.pone.0109864.g007

Figure 8. Subcellular localization of pVHL in transgenic Drosophila. Immunofluorescence staining of the Drosophila eye imaginal discs over expressing pVHL30 by GMR Gal4. (A) Bright field (B) Nuclei stained using DAPI (blue) (C) Actin stained using phalloidin 568 (red) (D) Anti-pVHL antibody was used for identification of pVHL protein (purple). (E) Merge. Upper panel scale bar: 20 μm; lower panel scale bar: 10 μm (zoom in). doi:10.1371/journal.pone.0109864.g008
Size Exclusion Chromatography (SEC)

Gel filtration experiments were performed using Superdex75 HR 10/30 size exclusion column (GE Healthcare) with a separation range of 3–70 kDa connected to an FPLC prime automated liquid chromatography system (Amersham Biosciences). The running buffer used was 10 mM Tris-HCl pH 8, 200 mM NaCl. The column was calibrated using gel filtration molecular weight standard (Bio-Rad). The following proteins were used for calibration: Thyroglobulin (670 kDa, Rs = 8.6 nm), γ-globulin (158 kDa, Rs = 5.1 nm), Ovalbumin (44 kDa, Rs = 2.8 nm), Myoglobin (17 kDa, Rs = 1.9 nm), Vitamin B12 (1.35 kDa), and Dextran blue (2 MDa).

A 0.5 ml protein sample at a final concentration of 5 μM was filtered and chromatographically analyzed using a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm, elution volumes were determined from UV chromatogram. The partition volume, Ve, and total bed volume, Vt, using the expression: 

\[ V_0 = V_t - V_e \]

were determined from UV chromatogram. The partition coefficient, Kav, was calculated from the elution volume of the sample, Ve, and total bed volume, Vt, using the expression: 

\[ Kav = (V_e - F_0)/(F_t - F_0) \]

Stokes radius (Rs) for all proteins was calculated by plotting \( \sqrt{-\log Kav} \) versus known Rs. Calibration curves and equations were established.

CD Spectra Measurements

CD spectra at far- (200–250 nm) and near-UV (250–320 nm) were recorded with a Chirascan CD Spectrometer (Applied Photophysics) equipped with a temperature-controlled cell using a cell of path length 0.5 cm; bandwidth was 1 nm, and averaging time was 30 s for each measurement. Protein concentration was 5 μM in a buffer containing 10 mM Tris-HCl [pH 8] and 200 mM NaCl.

8-Anilino-1-naphthalene Sulfonic Acid (ANS)

Fluorescence Studies

Fluorescence emission spectra of two solutions were generated with excitation wavelength of 350 nm. The first solution contained 5 μM ANS in buffer (10 mM Tris-HCl, pH 8, and 200 mM NaCl). The second solution was similar and contained protein at a final concentration of 5 μM. Samples were equilibrated at room temperature. The fluorescence emission spectra were recorded (Horiba Jobin Yvon FL3-11 Spectrofluorometer). The cuvette length was 1 cm. Measurements were conducted at 4, 25, 37, and 50°C. Baseline corrections were made with buffer lacking protein and ANS.

Protein function

An ELISA-based binding assay was used. The peptide substrates were: SIMA (SFEAFAGRAPHYIPIDDD), HIF1-α P402 (DALTLAPAGDTISLDF). These target peptides were synthesized by Hy Laboratory Ltd (Israel). They correspond to residues 841–857 of SIMA, and residues 395–413 of HIF1-α, respectively. These peptides comprised the amino acid Proline and had this residue hydroxylated to be recognized by the VHL proteins. The peptides were tagged with the fluorophore tetramethylrhodamine (TAMRA). pVHL or dVHL were diluted in salt buffer (10 mM Tris-HCL, pH 8, 200 mM NaCl and 200 mM GnHCl) to 10 μg/ml. 100 μl of the tested protein was added to each well of an ELISA plate (Costar EIA). Experiments were performed in triplicates and error bars are presented. Plates were incubated overnight at 4°C. Wells were washed four times with high salt buffer. Blocking buffer (high salt buffer containing 5% w/v BSA) was added to all wells to block protein binding sites left open in the wells. Labeled Hsp102-ODD-HIF or Hsp850-ODD-SIMA target peptide solutions, at increasing concentrations, were added to the wells. Plates were incubated for 1 h at 25°C and were washed thereafter four times with buffer. Fluorescence intensity at λ580 nm was measured following excitation at λ380 nm. BSA protein (Amresco) served as a negative control.

Flies and crosses

Wild-type [wt] (Oregon R) and mutant strains were reared on standard cornmeal-molasses medium at 25°C. Experiments were carried out at 25°C or 29°C as indicated. Adult offspring (F1) from the crosses were collected up to 9 days after the beginning of their eclosion at 25°C in order to avoid offspring from the next generation (F2).

Drosophila strains and genetics

Oregon-R was used as the wild-type stock in this study. The Drosophila dvHL1 mutant was generated by replacing the wild-type copy with a deletion that removes 81 codons encompassing the first two in-frame AUU (a generous gift from T. Hsu, Boston University, USA). The cDNA of dvHL, pVHL19 and the full length pVHL30 variant (carrying the extra 51 amino acids at the N terminal of the protein) were cloned in the UAS-based pUAST vector using EcoRI and XhoI restriction enzymes. These expression vectors were used to transform the w118 flies (BestGene, CA). We used transgenic strains carrying stable insertions on chromosome 3 that showed the highest protein expression. Tubulin-Gal4 was kindly provided by O. Gerlitz (Hebrew University of Jerusalem, Israel), and used for inducing UAS-dependent ubiquitous expression. GMR-Gal4 was used to drive expression of VHL in the eye imaginal disc cells. UAS-ODD-GFP transgenic strains carrying stable insertion on chromosome 3 were used [43]. Unless specified otherwise, all fly stocks are from the Bloomington Stock Center.

Antibodies

Rabbit anti-dvHL antibodies were obtained from Adar Biotech (Israel), using dVHL immunogenic selected peptide (IRVRVPKSSQSPDQL). The polyclonal antibodies were characterized using a western blot analysis. Rabbit polyclonal antibodies were obtained from the following sources: anti-GFP antibody (Abcam), anti-pVHL (Abcam) and anti-SIMA antibody (generous gift from P. Wappler, Instituto Leloir, Buenos Aires, Argentina). Mouse monoclonal anti-β-actin (Abcam). Secondary antibodies used were from Santa Cruz Biotechnology (horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit antibodies). For immunofluorescence experiments, pVHL was detected with a CY5 conjugate anti-rabbit antibody (Santa Cruz Biotechnology).

Protein extraction

Collected flies or fly heads were homogenized in 1:1 volume of either extraction buffer (5 mM MgCl2, 5 mM DTT, 1 mM PMSF, 1 x Complete protease inhibitor cocktail (Roche), 1 x PBS, pH 7.5). The samples were centrifuged for 30 min at 14,000 rpm at 4°C. Protein concentration was measured using Bradford reagent (Sigma). Laemmli sample buffer (BioRad) was added to 40–100 μg of protein from each sample, followed by boiling for 10 min, centrifugation for 5 min at 14,000 rpm and analysis by SDS-PAGE and Western blot.

Immunoblotting

Protein extracts were separated by 4–20% gradient SDS-PAGE (GeBA, Israel) and transferred on to PVDF using iBlot 7-Minute Blotting System (Life Technologies). The membrane was blocked.
for 1 h in blocking solution (5% milk powder, 0.02% sodium-azide in 1×TBS), and then incubated in the primary antibody diluted in blocking solution. The membrane was then washed 3 times for 15 min each in TTBS (0.1% Tween-20 in 1×TBS), incubated for 40 min in the secondary antibody and washed 3 times for 10 min in TTBS. The membrane was developed using EZ-ECL (Biological Industries), according to the manufacturer’s instructions, and exposed to Fuji Medical X-Ray Film for up to 5 min. Films were developed using Kodak X-OMAT 2000.

Immunofluorescence staining

Females or males larvae at the 3rd instar wandering stage were dissected in PBS for separation of the eyes imaginal discs. The separated tissue was fixed for 20 min in fixation solution (4% paraformaldehyde in PBS) and washed 3 times in PBT (0.3% Triton in PBS). The tissues were then blocked for 1 h in blocking solution (5% BSA in PBT) and incubated overnight at 4°C in 200 µl of the primary antibody diluted 1:50 in blocking solution and containing 1:100 red phalloidin (Life Technologies). The samples were washed 3 times for 10 min each in PBT, incubated for 2 h in the secondary antibody 1:200 and washed 3 times for 10 min in PBT. After being thoroughly washed with PBS, cells were mounted using ProLong Antifade containing DAPI (Invitrogen). Images were taken with LSM510 confocal microscope (Zeiss).

Reverse Transcription and Real-time PCR

Total RNA from 10 adult flies was isolated with RNeasy mini kit with on-column DNase digestion (Qiagen). First-strand cDNA was generated by using the Verso CDNA Kit (Thermo). Real-time PCR was performed in triplicate with KAPA Fast SYBR master mix (KAPA Biosystems) (Qiagen) and the StepOnePlus Real-Time PCR Systems (Life Technologies). Primers for rp49, dVHL, pVHL, SIMA, Glut1 and VEGF (pfx in flies) were purchased from Hy labs (Israel). All values were normalized to the level of rp49 mRNA abundance and to the wild type (Oregon R) flies. Each primer pair was calibrated using the Absolute Quantification program with increasing concentrations of cDNA, from 1:32 to 1:1 dilutions of the original cDNA. Primers used: rp49 (forward 5′-ACC GAT GTT GGG CAT CAG ATA-3′; revers 5′- TAA GCT GTG GTT CAG ATG GC-3′). SIMA (forward 5′- CTG CCC GAG AGC AAT CCG TA-3′; revers 5′- CGG TGG TGT TAG GGG TGG AG-3′). dVHL (forward 5′- CAG AGT CCG GAT CAG CTG GTT GAC G-3′; revers 5′- GAC TCG ATG CAG GTG AGG CTT AGT-3′). pVHL (forward 5′ CTC ACG GGG TTC TCT TCT GCA AT-3′; revers 5′- GTT AAC CAG AAG CCG ATG TGT GC-3′). Glut1 (forward 5′- ACC TAC TCG ATT TTC TCG GGG GTG-3′; revers 5′- GCC CAG CAT GCC GCC GAT-3′). VEGF pfx (forward 5′- ATG CAT CCA TTT CTC ATC CTG C-3′; revers 5′- TCC TTT GCA GCA GCT GCC CTC-3′).

Scanning electron microscopy (SEM)

SEM was performed as described [78]. All SEM images from at least three female adult eyes of each genotype were analyzed.

Supporting Information

Figure S1 Superposition of the novel model structure produced by our composite approach with models produced by standard modeling tools. The model structure produced by our composite approach (blue) shares significant structural similarity to the models produced by I-TASSER (yellow) and Phyre2 (green).

Figure S2 Superposition of the amino acids involved in dVHL interactions with its binding partners. (A) Superposition of the key amino acids involved in HIF1-α binding. These are residues W70, H68, T64, and Y51and W41 in dVHL. (B) Superposition of the key amino acids involved in Elongin C binding. These are residues L154, L134, V130, C126 and L122 in dVHL. (C) Superposition of the key amino acids involved in Cul2 binding. These are residues L158, L154 and L150 in dVHL. The orientation of these key amino acids as predicted by our composite approach (blue) is similar to that of the corresponding amino acids of the models produced by I-TASSER (yellow) and Phyre2 (green).

Figure S3 Pairwise sequence alignment of the Drosophila and Human Elongin C orthologs. Pairwise sequence alignment between human Elongin C and Drosophila Elongin C, as calculated by the MUSCLE algorithm, showing conservation of the hydrophobicity between the two proteins. Hydrophobic residues (red), neutral residues (purple) and hydrophilic residues (blue).

Figure S4 Thermal denaturation assay. Thermal denaturation of wild type dVHL. (A) Denaturation. Changes in ellipticity monitored at 200–250 nm. Dashed line marks the Tm of dVHL. (B) Renaturation. Changes in ellipticity monitored at 200–250 nm.

Movie S1 The eye defect caused by over-expression of SIMA is suppressed by co-expression of either dVHL, pVHL19, or pVHL30 at 29°C. 1. GMR-Gal4; UAS-SIMA. UAS-RFP (no hatching). 2. GMR-Gal4; UAS-SIMA; UAS-dVHL. 3. GMR-Gal4; UAS-SIMA; UAS-pVHL19. 4. GMR-Gal4; UAS-SIMA; UAS-pVHL30. At 29°C over expression of SIMA by GMR-Gal4 caused pupal lethality (1) that could be rescued by co-expression of either dVHL, pVHL19 or pVHL30 (evidenced as flies motility 2–4).

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Author Contributions

Conceived and designed the experiments: MDS LS. Performed the experiments: MDS LS. Analyzed the data: MDS LS. Contributed reagents/materials/analysis tools: MDS LS GH. Wrote the paper: MDS LS EG DS

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