NEDD8 acts as a ‘molecular switch’ defining the functional selectivity of VHL

Ryan C. Russell & Michael Ohh⁺

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

The von Hippel–Lindau (VHL) tumour suppressor protein is important in the E3 ubiquitin ligase ECV (Elongin B/C–CUL2–VHL)-mediated destruction of hypoxia-inducible factor and the promotion of fibronectin (FN) extracellular matrix assembly. Although the precise molecular mechanism controlling the selectivity of VHL function remains unknown, a failure in either process is associated with oncogenic progression. Here, we show that VHL performs its FN-associated function independently of the ECV complex, highlighting the autonomy of these pathways. Furthermore, we show that NEDD8, a ubiquitin-like molecule, acts as a ‘molecular switch’ in which its covalent conjugation to VHL prohibits the engagement of the scaffold component CUL2 and, concomitantly, activates the association with FN. These findings provide the first mechanistic step in defining the functional selectivity of VHL and explain a previously unrecognized function of NEDD8.

Keywords: CUL2; ECV; fibronectin; HIF; NEDD8; VHL

EMBO reports (2008) 9, 486–491. doi:10.1038/embor.2008.19

INTRODUCTION

Inheritance of one faulty von Hippel–Lindau (*VHL*) allele is the cause of VHL disease, which is characterized by the development of retinal, cerebellar and spinal haemangioblastomas, pheochromocytoma and renal clear-cell carcinoma (RCC; Kaelin, 2002). Tumorigenesis begins with the loss or inactivation of the remaining wild-type *VHL* allele in a susceptible cell (Kaelin, 2002). Biallelic inactivation of the *VHL* locus is also responsible for the development of most of the sporadic RCC, establishing *VHL* as a crucial ‘gatekeeper’ gene of the renal epithelium (Kaelin, 2002).

The phenotypic variation observed in relatives that carry mutations in *VHL* (*VHL* kindred) are due to alterations in tissue-specific functions or in the severity with which certain *VHL* functions are altered. The two most well-characterized functions of *VHL* are as a negative regulator of hypoxia-inducible factor

(HIF), which is the main transcription factor that activates the expression of many hypoxia-inducible genes to counter the detrimental effects of compromised oxygen availability (Kaelin, 2002), and as a positive regulator of fibronectin (FN) extracellular matrix (ECM) assembly (Roberts & Ohh, 2008).

ECV (Elongin B/C–CUL2–VHL) is an SCF (SKP1/CDC53 or CUL1/F-box protein)-like E3 ubiquitin ligase in which VHL acts as a substrate-conferring component that recruits the α -subunits of HIF. These have been modified with hydroxyl groups on conserved prolyl residues within the oxygen-dependent degradation domain (ODD) by a class of prolyl hydroxylases in an oxygen-dependent manner (Kaelin, 2002). This mechanistic insight explains why HIF α is stabilized under hypoxia to bind to constitutively expressed HIF β (also known as aryl hydrocarbon receptor nuclear translocator) to form an active heterodimeric transcription factor. Concordantly, cells under hypoxia or tumour cells devoid of VHL irrespective of oxygen tension have enhanced expression of HIF target genes, such as *VEGF* (vascular endothelial growth factor), *GLUT1* (glucose transporter 1) and *EPO* (erythropoietin; Roberts & Ohh, 2008). The overexpression of hypoxia-inducible genes probably contributes to the hypervascular nature of VHL disease-associated tumours, and supports the idea that constitutive stabilization of HIF α is a crucial oncogenic event following the loss of VHL (Roberts & Ohh, 2008).

VHL also binds to FN and this physical interaction represents a requisite step in the promotion of correct ECM assembly (Ohh *et al*, 1998). All tumour-causing VHL mutants tested so far show a striking failure in binding to and/or in the assembly of FN (Clifford *et al*, 2001; Hoffman *et al*, 2001). Recently, VHL was also shown to interact with collagen IV (COLIV) to promote its deposition in the extracellular space (Grosfeld *et al*, 2007; Kurban *et al*, 2008). These findings underscore the significance of VHL-mediated ECM assembly in tumour suppression. Kurban *et al* (2006) showed that the loss of correct FN ECM promotes angiogenesis of RCC xenograft in an HIF-independent manner and that HIF α stabilization in the context of intact FN ECM results in tumours with low microvessel density despite the overexpression of VEGF. Tang *et al* (2006) showed, in mice with conditional knockout of *VHL* in endothelial cells, that VHL has a crucial role in the vascular FN ECM assembly, independent of its role in regulating HIF α . In addition, studies of genomic clustering in *Caenorhabditis elegans* identified a discrete HIF-independent role of VHL in ECM function

Department of Laboratory Medicine and Pathobiology, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8

⁺Corresponding author. Tel: +1 416 946 7922; Fax: +1 416 978 5959;

E-mail: michael.ohh@utoronto.ca

Received 8 October 2007; revised 10 January 2008; accepted 23 January 2008; published online 7 March 2008

(Bishop *et al*, 2004). Although these studies highlight the HIF-independent FN assembly function of VHL, the mechanism controlling the selectivity of VHL functions remained unknown.

Until recently, the ubiquitin-like molecule NEDD8 has been thought to conjugate exclusively to Cullins, in a manner analogous to ubiquitylation, to enhance the activity of SCF and ECV complexes (Pan *et al*, 2004; Sufan & Ohh, 2006). Emerging evidence has shown NEDD8 to be more versatile in substrate specificity and, importantly, several proteins involved in oncogenesis, including VHL, p53, murine double minute 2 (MDM2), p73, epidermal growth factor receptor and the breast cancer-associated protein 3, have now been shown to be targets of NEDD8 modification (Stickle *et al*, 2004; Xirodimas *et al*, 2004; Gao *et al*, 2006; Oved *et al*, 2006; Watson *et al*, 2006). In addition, the functional consequence of neddylation was found to be more diverse than previously thought, extending beyond the enhancement of the ubiquitin-proteasome pathway. Here, we provide evidence that NEDD8 acts as a 'molecular switch' in which its covalent conjugation to VHL precludes ECV formation by steric hindrance and, concomitantly, allows interaction with FN, thereby providing the first mechanistic step in the definition of the functional selectivity of VHL.

RESULTS AND DISCUSSION

ECV- and FN-functions of VHL are mutually exclusive

The ability of VHL to regulate HIF is dependent on its ability to form a functional ECV. Therefore, we investigated whether the ability of VHL to bind to FN was also dependent on ECV. VHL-associated FN was immunoprecipitated after short interfering RNA (siRNA)-mediated knockdown of CUL2, a scaffold component of ECV, in ³⁵S-radiolabelled *VHL-null* RCC4 renal carcinoma cells ectopically expressing haemagglutinin (HA)-VHL (WT) or empty plasmid (MOCK). The level of FN co-precipitating with VHL did not decrease despite a marked reduction in CUL2 expression (Fig 1; supplementary Fig S2E online). As expected, siRNA-mediated knockdown of CUL2 attenuated VHL-dependent ubiquitylation of HIF1 α ODD (supplementary Fig S2C online). Furthermore, biotinylated HIF1 α ODD-OH peptides co-precipitated ECV components without the presence of FN, as compared with FN co-precipitated from ³⁵S-radiolabelled 786-VHL RCC cells using an HA antibody directed against HA-VHL (Fig 1C, compare lanes 2 and 3). Although it is possible that HIF1 α ODD-OH peptides might have displaced FN by competition for VHL, hypoxia or hypoxia-mimetic (desferrioxamine or CoCl₂) treatment of 786-VHL cells did not increase VHL/FN interaction (supplementary Fig S3 online). These results argue against the idea that VHL binding to FN is influenced by competition with HIF α , and suggest that ECV complex is not necessarily required for VHL to bind to FN.

Disruption of NEDD8 pathway abrogates FN binding

Recently, we have shown that mutations in VHL that disrupt NEDD8 conjugation lead to a failure in binding to FN (Stickle *et al*, 2004). To investigate further whether VHL binding to FN is dependent on the NEDD8 pathway independent of ECV complex formation, ts41 Chinese hamster ovary (CHO) cells with temperature-sensitive APP-BP1 (a component of NEDD8-activating enzyme; Chen *et al*, 2000) were transiently transfected with plasmids encoding HA-VHL and green fluorescent protein (GFP)-FN. VHL binding to FN was markedly decreased in cells maintained under

Fig 1 | ECV- and fibronectin-associated functions of VHL are mutually exclusive. (A) Human embryonic kidney 293A cells were treated with increasing amounts (10–100 nM) of CUL2 siRNA (lanes 2–4), scrambled (Scram) siRNA (lanes 5–7) or transfection reagent alone (MOCK; lane 1). Equalized whole-cell lysates were resolved on SDS-polyacrylamide gel electrophoresis and immunoblotted (IB) with CUL2 (lower panel) or vinculin (upper panel) antibodies. (B) RCC4 cells stably transfected with HA-VHL (WT) or empty plasmid (MOCK) were radiolabelled (metabolically labelled with [³⁵S]methionine). Cells were treated with (+) or without (–) CUL2 siRNA as indicated. Cell lysates were immunoprecipitated with an HA antibody and the resolved proteins were visualized by autoradiography. (C) 786-WT and 786-MOCK cells were metabolically labelled with [³⁵S]methionine, lysed and immunoprecipitated with an HA antibody (lanes 1,2) or pulled down with synthetic HIF1 α ODD-OH peptides (lanes 3,4). Bound proteins were resolved and visualized by autoradiography. The asterisk denotes nonspecific protein bands. AR, autoradiography; ECV, Elongin B/C-CUL2-VHL; FN, fibronectin; HA, haemagglutinin; IP, immunoprecipitation; RCC, renal clear cell; siRNA, short interfering RNA; VHL, von Hippel-Lindau.

a restrictive temperature as compared with cells under a permissive temperature (Fig 2A), indicating that an intact NEDD8 pathway is crucial for promoting VHL binding to FN. As expected, neddylation of VHL and CUL2 was curtailed under a non-permissive temperature (Fig 2B,C). An intact ECV able to bind to HIF1 α was observed under both restrictive and permissive temperature conditions (Fig 2C), indicating that the ability of VHL to form an ECV is not sufficient for binding to FN. Furthermore, non-neddylatable VHL(RRR) (Stickle *et al*, 2004),

Fig 2 | Restriction of a dynamic NEDD8 pathway results in the attenuation of von Hippel–Lindau binding to fibronectin. (A) CHO ts41 cells were transfected with plasmids encoding HA-VHL(WT) and GFP-FN. Cells were grown at a permissive (P; 33 °C) or non-permissive (NP; 39 °C) temperature for 15 h and radiolabelled. Cell lysates were immunoprecipitated with an HA antibody and resolved proteins were visualized by autoradiography. (B) CHO ts41 cells were transfected with plasmids encoding HA-VHL(WT) and NEDD8. Cells were grown at a permissive or non-permissive temperature for 15 h, lysed, immunoprecipitated and immunoblotted with an HA antibody. (C) CHO ts41 cells were transfected with plasmids encoding HA-VHL and HIF1 α . Cells were grown at a permissive or non-permissive temperature for 15 h, lysed and immunoprecipitated with an HIF1 α antibody. Resolved proteins were visualized by immunoblotting with HIF1 α (upper panel), CUL2 (middle panel) or HA (lower panel) antibodies. The asterisk denotes uncharacterized protein bands. AR, autoradiography; CHO, Chinese hamster ovary; FN, fibronectin; GFP, green fluorescent protein; HA, haemagglutinin; HIF1 α , hypoxia-inducible factor 1 α ; IB, immunoblot; IP, immunoprecipitation; VHL, von Hippel–Lindau; WT, wild type.

although with a similar subcellular distribution pattern as VHL(WT) (supplementary Fig S4 online), has a compromised ability to bind to FN (Stickle *et al*, 2004; supplementary Fig S2 online). These results indicate that neddylation of VHL does not promote FN binding by altering the subcellular localization of VHL, which binds to the cytosol-exposed region of FN in the endoplasmic reticulum/Golgi (supplementary Fig S4 online).

Neddylation of VHL precludes ECV complex formation

VHL contains two important functional domains: α and β (19). The β -domain is required for binding to substrates (Stebbins *et al*, 1999; Ohh *et al*, 2000) and the α -domain is required for binding to Elongin C (Stebbins *et al*, 1999), which acts as a bridge connecting VHL to the rest of the ECV components. Residues spanning 158–172 (Elongin B/C-box) within the α -domain have been shown to be necessary and sufficient for binding to Elongin C (Ohh *et al*, 1999),

and Lys 159 has been shown to be the main acceptor site of NEDD8 (Stickle *et al*, 2004). ECV is analogous to SCF both structurally and functionally. Although ECV has not been crystallized, SCF (Zheng *et al*, 2002) and the VHL–Elongin B/C (VBC) complex (Stebbins *et al*, 1999; Min *et al*, 2002) have been solved. To determine the possible effects of NEDD8 conjugation to VHL, we superimposed the VBC complex against SCF. In particular, SKP1 and its orthologue Elongin C polypeptide backbones were aligned within 1.3 Å, giving confidence that CUL1 would be positioned similarly to CUL2 in the context of ECV. On the basis of this prediction, a VHL mutant unable to bind to Elongin C owing to a mutation within the Elongin B/C-box (excluding Lys 159 and Lys 171) would be more accessible for NEDD8 modification. Human embryonic kidney (HEK)293A cells were transfected with plasmids encoding HA-VHL(WT), non-neddylatable HA-VHL(RRR) and HA-VHL(C162F), a well-established α -domain mutant unable to bind to Elongin C (Ohh *et al*, 2000, 1999). As expected, HA-VHL(WT) generated a slower migrating T7-NEDD8-conjugated HA-VHL, whereas HA-VHL(RRR) failed to generate a NEDD8-modified isoform (Fig 3C). Consistent with the steric hindrance model, HA-VHL(C162F) was neddylated to a greater extent in comparison with VHL(WT) (Fig 3C). Furthermore, although the neddylated VHL comprises a minor fraction of total VHL, significantly less neddylated VHL was found in complex with CUL2 (Fig 3D, compare lanes 1 and 2), indicating an exclusion of neddylated VHL in the ECV complex. These results indicate that neddylation of VHL generates a steric clash preventing its association with the ECV complex.

CUL2 is excluded from the VHL–FN complex

The NEDD8-induced steric hindrance model predicts the exclusion of one or more ECV components, which might be necessary for the promotion of FN-mediated function. To determine directly whether ECV components are excluded from the VHL–FN complex, we performed affinity purification of intracellular FN from 786-MOCK, 786-WT and 786-C162F cells. FN-containing complexes were then competitively eluted from the Sepharose beads and immunoprecipitated with an HA antibody selecting for the FN complexes associated with HA-VHL. As expected, HA-VHL(WT) was present in the affinity-purified FN complex and co-precipitated FN, whereas a disease-causing HA-VHL(C162F) mutant, which has an intrinsic defect in FN binding (Stickle *et al*, 2004), was absent in the affinity-purified FN complex (Fig 4A, lanes 4,5). Equal amounts of whole-cell extracts were separated by SDS–polyacrylamide gel electrophoresis, and immunoblotted for total FN and HA-VHL, which indicated the presence of FN in all the indicated cell types (Fig 4A, lanes 1–3). Notably, FN is known to bind to COLIV, which has been shown recently to interact with VHL. However, the affinity-purified FN co-precipitated by means of HA-VHL did not contain COLIV (supplementary Fig S1B online), indicating that VHL binds to FN independently of COLIV. Next, anti-HA immunoprecipitations were performed on the whole-cell extracts or affinity-purified intracellular FN complexes generated from 786-MOCK, 786-WT

Fig 3 | NEDD8 modification of von Hippel-Lindau generates steric hindrance blocking the formation of ECV. (A) VBC (VHL-Elongin B/C) crystallized with HIF1 α ODD peptide was visualized using DeepView/Swiss-PdbViewer v3.7. The complex was viewed with side chains and showing van der Waals forces. Lys 159, the primary site of neddylation, is shown in orange. (B) The backbone of Elongin C in the VBC (1LM8.pdb) was overlaid with the backbone of Elongin C orthologue SKP1 in the SCF (SKP1/CDC53 or CUL1/F-box protein) complex (1LDK.pdb). By using the iterative Magic Fit function of DeepView/Swiss-PdbViewer, a fit was generated with an overlap consisting of 99 residues between Elongin C and SKP1 with an r.m.s.d. of 1.26 Å. (C) U2OS cells were transfected with plasmids encoding HA-VHL(WT), HA-VHL(RRR), HA-VHL(C162F), T7-NEDD8, or empty vector (MOCK). Cells were then lysed, immunoprecipitated and immunoblotted with an HA antibody. The asterisk denotes uncharacterized protein bands. (D) Human embryonic kidney 293A cells were transfected with the indicated combination of plasmids encoding HA-CUL2, T7-VHL and NEDD8. Immunoprecipitation with an HA (lanes 2,3) or T7 (lanes 1,4) antibody was performed on pooled lysates. Resolved proteins were immunoblotted with a CUL2 (top panel), T7 (middle panel) or VHL (bottom panel) antibody. A long exposure of anti-T7 immunoblot was performed to visualize better neddylation VHL. ECV, Elongin B/C-CUL2-VHL; HA, haemagglutinin; HIF1 α , hypoxia-inducible factor 1 α ; IB, immunoblot; IP, immunoprecipitation; VHL, von Hippel-Lindau; WT, wild type.

and 786-C162F cells (Fig 4B). HA-VHL(WT) co-precipitated CUL2 from the whole-cell extracts as expected, whereas HA-VHL(WT) in the FN complex did not (Fig 4B, compare lanes 2 and 5). In parallel, an anti-HA immunoprecipitation from the affinity-purified FN complex from 35 S-radiolabelled 786-VHL cells showed an absence of CUL2, but the presence of Elongins B and C in the HA-VHL-FN complex (Fig 4C). These results show that CUL2 is excluded from the VHL-FN complex. Notably, the exclusive presence of unneddylation VHL in complex with FN (Fig 4; supplementary Figs S1,S5B online) also indicates that the neddylation of VHL represents an intermediary step that prohibits CUL2 engagement, which is proceeded by deneddylation of VHL allowing unhindered association with FN.

NEDD8 acts as a 'molecular switch'

In keeping with the prediction based on the composite VBC-CUL1 structure (Fig 3), NEDD8 modification of VHL prevents CUL2 engagement and thus is excluded from the ECV complex. This 'freed', although minor, pool of VHL binds to FN,

representing a requisite step in the eventual assembly of the extracellular FN matrix. VHL in complex with FN is unmodified, which indicates that VHL is transiently modified by a dynamic neddylation and deneddylation process. In agreement with this, inhibition of the NEDD8 pathway or ablation of the NEDD8 conjugation sites on VHL markedly attenuated the ability of VHL to interact with FN while preserving ECV integrity. The requirement of this dynamic process also explains why the non-neddylation VHL(RRR) mutant is defective in FN binding and assembly.

The preclusion of CUL2 in the VHL-FN complex also indicates that the physical presence of CUL2 might be inhibitory in the engagement of FN to VHL. In support of this idea, a near-complete knockdown of CUL2 increased the amount of FN bound to VHL (supplementary Fig S2E online). In a complementary experiment, HA-VHL from 786-WT and 786-RRR cells was immunoprecipitated and washed under high stringency salt and detergent conditions to strip away VHL-associated proteins. The 'stripped' VHL was then mixed with radiolabelled VHL-null 786-O cell

Fig 4 | The von Hippel–Lindau–fibronectin complex excludes the ECV component CUL2. (A) Equal amounts of whole-cell extracts (WCEs) generated from 786-MOCK, 786-WT and 786-C162F cells were resolved and immunoblotted with an FN (top panel) or HA (bottom panel) antibodies (lanes 1–3). FN complexes were affinity purified from the indicated WCEs, and bound proteins were competitively eluted and immunoprecipitated with an HA antibody (lanes 4–6). Resolved proteins were immunoblotted with an FN (top panel) or HA (bottom panel) antibodies. (B) HA-VHL was immunoprecipitated with an HA antibody from either purified FN complexes (lanes 1–3) or WCEs (lanes 4–6) generated from pooled cell lysate from the indicated cell lines. Bound proteins were resolved and immunoblotted with a CUL2 (top panel) or HA (bottom panel) antibody. (C) Cells were radiolabelled and prepared as in (B). Resolved proteins were visualized by autoradiography. AR, autoradiography; ECV, Elongin B/C–CUL2–VHL; FN, fibronectin; HA, haemagglutinin; IB, immunoblot; IP, immunoprecipitation; VHL, von Hippel–Lindau; WT, wild type.

lysates and re-immunoprecipitated. Under such conditions, the ability of VHL(RRR) to bind to *de novo* FN was restored to a level comparable with that of VHL(WT). VHL(C162F) was still unable to bind to FN (supplementary Figs S2B,S5C online), which is consistent with the idea that Elongins B and C are required for VHL–FN interaction. Furthermore, a direct interaction between FN and VHL was shown to not require CUL2, as a VBC complex lacking CUL2 was sufficient to bind to FN (Hoffman *et al*, 2001). These results indicate that the deficiency of non-neddylatable VHL is due to its inability to disengage CUL2 or CUL2-associated inhibitory factors in the absence of dynamic NEDD8 processing. Elongins B and C are likely to provide stability to the unstable tertiary structure of VHL (Stebbins *et al*, 1999; Feldman *et al*, 2003) in the VHL–FN complex. The structural requirement provided by Elongins B and C perhaps explains why α -domain VHL mutants, including C162F, fail to bind to FN. In this regard, and analogous to HIF α , FN binding by VHL requires direct physical interaction and the association of Elongins.

VHL is important in ECV-mediated destruction of HIF α and the assembly of FN ECM. Neddylation of VHL prohibits the engagement of CUL2 and concomitantly activates the association with FN. Thus, NEDD8 acts as a molecular switch that defines the functional selectivity of VHL, and provides the first mechanistic demarcation of HIF-dependent and HIF-independent pathways.

METHODS

Cells. 786-O RCC, U2OS osteosarcoma and HEK293A cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal

bovine serum (Sigma, Milwaukee, WI, USA) at 37 °C in a humidified 5% CO₂ atmosphere. 786-O subclones ectopically expressing wild-type VHL (786-WT) or empty plasmid (786-MOCK), RCC4 cells ectopically expressing wild-type VHL (RCC4-WT) or empty plasmid (RCC4-MOCK), and ts41 CHO cells were as described previously (Ohh *et al*, 1998; Chen *et al*, 2000; Clifford *et al*, 2001).

Antibodies. Monoclonal HA (12CA5) and HIF1 α antibodies were obtained from Boehringer Ingelheim (Laval, QC, Canada) and Novus Biological (Littleton, CO, USA), respectively. Monoclonal T7 antibody was obtained from Novagen (Madison, WI, USA). Monoclonal vinculin, tubulin and heteronuclear ribonuclear protein antibodies were obtained from Abcam (Cambridge, MA, USA). Polyclonal GLUT1 and CUL2 antibodies were obtained from Alpha Diagnostics (San Antonio, TX, USA) and Zymed (San Francisco, CA, USA), respectively. Monoclonal VHL antibody (IG32) was as described previously (Ohh *et al*, 1998).

Plasmids. See the supplementary information online.

Immunoprecipitation and immunoblotting. Immunoprecipitation and western blotting were performed as described previously (Ohh *et al*, 1998).

Affinity purification. Gelatin-Sepharose beads (Amersham Pharmaceuticals, Piscataway, NJ, USA) were used to affinity purify FN from whole-cell extracts by rocking at 4 °C for 3 h. FN complexes were eluted in 250 mM arginine in PBS, rocking for 10 min at 22 °C, as previously described (Vuento & Vaheri, 1978).

Metabolic labelling. Metabolic labelling was performed as described previously (Ohh *et al*, 1998).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

This work was supported by funds from the Canadian Cancer Society of the National Cancer Institute of Canada. R.C.R. is a recipient of the Natural Sciences and Engineering Research Council of Canada scholarship; M.O. is a Canada Research Chair in Molecular Oncology.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Bishop T *et al* (2004) Genetic analysis of pathways regulated by the von Hippel–Lindau tumor suppressor in *Caenorhabditis elegans*. *PLoS Biol* **2**: E289
- Chen Y, McPhie D, Hirschberg J, Neve R (2000) The amyloid precursor protein-binding protein APP-BP1 drives the cell cycle through the S–M checkpoint and causes apoptosis in neurons. *J Biol Chem* **275**: 8929–8935
- Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, Ratcliffe PJ, Maher ER (2001) Contrasting effects on HIF-1 α regulation by disease-causing pVHL mutations correlate with patterns of tumorigenesis in von Hippel–Lindau disease. *Hum Mol Genet* **10**: 1029–1038
- Feldman DE, Spiess C, Howard DE, Frydman J (2003) Tumorigenic mutations in VHL disrupt folding *in vivo* by interfering with chaperonin binding. *Mol Cell* **12**: 1213–1224
- Gao F, Cheng J, Shi T, Yeh ET (2006) Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NF κ B-dependent transcription. *Nat Cell Biol* **8**: 1171–1177
- Grosfeld A, Stolze IP, Cockman ME, Pugh CW, Edelmann M, Kessler B, Bullock AN, Ratcliffe PJ, Masson N (2007) Interaction of hydroxylated collagen IV with the von Hippel–Lindau tumor suppressor. *J Biol Chem* **282**: 13264–13269
- Hoffman MA, Ohh M, Yang H, Klco JM, Ivan M, Kaelin WG Jr (2001) von Hippel–Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* **10**: 1019–1027
- Kaelin WG Jr (2002) Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer* **2**: 673–682
- Kurban G, Hudon V, Duplan E, Ohh M, Pause A (2006) Characterization of a von Hippel–Lindau pathway involved in extracellular matrix remodeling, cell invasion, and angiogenesis. *Cancer Res* **66**: 1313–1319
- Kurban G, Duplan E, Ramlal N, Hudon V, Sado Y, Ninomiya Y, Pause A (2008) Collagen matrix assembly is driven by the interaction of von Hippel–Lindau tumor suppressor protein with hydroxylated collagen IV α 2. *Oncogene* **27**: 1004–1012
- Min JH, Yang H, Ivan M, Gertler F, Kaelin WG Jr, Pavletich NP (2002) Structure of an HIF-1 α –pVHL complex: hydroxyproline recognition in signaling. *Science* **296**: 1886–1889
- Ohh M *et al* (1998) The von Hippel–Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* **1**: 959–968
- Ohh M, Takagi Y, Aso T, Stebbins C, Pavletich N, Zbar B, Conaway R, Conaway J, Kaelin WJ (1999) Synthetic peptides define critical contacts between elongin C, elongin B, and the von Hippel–Lindau protein. *J Clin Invest* **104**: 1583–1591
- Ohh M, Park CW, Ivan M, Hoffman MA, Kim T-Y, Huang LE, Chau V, Kaelin WG (2000) Ubiquitination of HIF requires direct binding to the von Hippel–Lindau protein β domain. *Nat Cell Biol* **2**: 423–427
- Oved S *et al* (2006) Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. *J Biol Chem* **281**: 21640–21651
- Pan ZQ, Kentsis A, Dias DC, Yamoah K, Wu K (2004) Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* **23**: 1985–1997
- Roberts AM, Ohh M (2008) Beyond the hypoxia-inducible factor-centric tumour suppressor model of von Hippel–Lindau. *Curr Opin Oncol* **20**: 83–89
- Stebbins CE, Kaelin WG, Pavletich NP (1999) Structure of the VHL–ElonginC–ElonginB complex: implications for VHL tumor suppressor function. *Science* **284**: 455–461
- Stickle NH, Chung J, Klco JM, Hill RP, Kaelin WG Jr, Ohh M (2004) pVHL modification by NEDD8 is required for fibronectin matrix assembly and suppression of tumor development. *Mol Cell Biol* **24**: 3251–3261
- Sufan RI, Ohh M (2006) Role of the NEDD8 modification of Cul2 in the sequential activation of ECV complex. *Neoplasia* **8**: 956–963
- Tang N, Mack F, Haase VH, Simon MC, Johnson RS (2006) pVHL function is essential for endothelial extracellular matrix deposition. *Mol Cell Biol* **26**: 2519–2530
- Vuento M, Vaheri A (1978) Dissociation of fibronectin from gelatin–agarose by amino compounds. *Biochem J* **175**: 333–336
- Watson IR, Blanch A, Lin DC, Ohh M, Irwin MS (2006) Mdm2-mediated NEDD8 modification of Tap73 regulates its transactivation function. *J Biol Chem* **281**: 34096–34103
- Xirodimas DP, Saville MK, Bourdon JC, Hay RT, Lane DP (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118**: 83–97
- Zheng N *et al* (2002) Structure of the Cul1–Rbx1–Skp1–F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**: 703–709