Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel–Lindau protein

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von Hippel–Lindau (VHL) disease is a hereditary cancer syndrome that is characterized by the development of multiple vascular tumors and is caused by inactivation of the von Hippel–Lindau protein (pVHL). Here we show that pVHL, through its β-domain, binds directly to hypoxia-inducible factor (HIF), thereby targeting HIF for ubiquitination in an α-domain-dependent manner. This is the first function to be ascribed to the pVHL β-domain. Furthermore, we provide the first direct evidence that pVHL has a function analogous to that of an F-box protein, namely, to recruit substrates to a ubiquitination machine. These results strengthen the link between overaccumulation of HIF and development of VHL disease.

VHL disease frequently involves mutations in two pVHL subdomains, the α- and β-domains[1]. The α-domain binds to elongin C and Cul2; the resulting pVHL/elongin C/Cul2 complexes are thought to have a function in ubiquitination, as they are similar to SCF (Skp1/Cdc53/F-box) E3 ubiquitin ligases[2]. Moreover, pVHL co-immunoprecipitates with an E3-like activity[3,4]. pVHL and HIF form complexes, and cells lacking pVHL are unable to degrade HIF under normoxic conditions[5].

Results

pVHL binds to a region of HIF implicated in oxygen-dependent protoeolysis. To determine the structural requirements for binding of pVHL to HIF, we fused various fragments of HIF1α to the Gal4 DNA-binding domain, along with a haemagglutinin (HA) epiptope tag. The resulting chimaeras were mixed with ^{35}S-labelled pVHL *in vitro* translates and then immunoprecipitated with an anti-Gal4 antibody. pVHL bound to the chimaera containing HIF1α(530–652) but not to those containing HIF1α(1–529) or HIF1α(653–826) (Fig. 1a). Recovered amounts of the three chimaeras were comparable, as determined by immunoblotting against the HA tag (data not shown). In keeping with these results, wild-type HIF1α co-immunoprecipitated with pVHL, whereas a HIF1α mutant lacking residues 530–652 did not (Fig. 1b). HIF1α(530–652) contains a region that renders HIF unstable in the presence of oxygen[5–8]. HIF2α contains a similar region[5], and likewise co-immunoprecipitated with pVHL (Fig. 1b).

The pVHL β-domain is required for binding to HIF. Cells produce two pVHL isoforms as a result of alternative translation-initiation codons[9]. Both forms bound to HIF1α(530-652)(Fig. 1c, d). Subdivision of the shorter form, corresponding to residues 54–213, revealed that pVHL(63–155) bound to HIF1α(530–652), whereas pVHL(156–213) did not (Fig. 1c, d). The pVHL α-domain consists of three carboxy-terminal helices that bind to elongin C (Fig. 1d and ref. 1). pVHL(63–155) encompasses most of the pVHL β-domain. This domain has been proposed to be a protein–protein interface because many disease-associated mutations within this region affect surface residues that do not function in maintaining the overall structure of pVHL[1]. Representative β-domain pVHL missense mutants, such as pVHL(Y98H), pVHL(Y111H) and pVHL(W117R) bound to HIF1α(530–652) poorly or not at all (Fig. 1e). In contrast, the α-domain mutants pVHL(C162F), pVHL(Q164F) and pVHL(L188V) retained measurable HIF-binding activity (Fig. 1e). The observed partial decrease in the HIF-binding activity of some α-domain mutants probably reflects the fact that binding to elongin C stabilizes the conformation of pVHL by forming an intermolecular four-helix cluster[1]. Elongins B and C are abundant proteins that are presumably present in reticulocyte lysate. Elongin binding is not required, however, in the context of a C-terminally truncated version of pVHL, as shown by the behaviour of the pVHL(63–155) mutant (Fig. 1c and data not shown).

The pVHL–elongin complex binds directly to HIF. pVHL normally exists in cells as a complex with elongin B and elongin C rather than as a monomer[1,10–12]. To determine whether this complex binds directly to HIF, HA–Gal4–HIF1α(530–652) and Gal4–HA *in vitro* translates were immunoprecipitated with an anti-HA antibody and subjected to farwestern blotting with recombinant pVHL/elongin C/elongin B (VBC) complexes produced in bacteria. The VBC complex bound directly to HA–Gal4–HIF1α(530–652) but not to Gal4–HA (Fig. 2a, b). Note that ^{35}S-labelled pVHL co-immunoprecipitated with HA–Gal4–HIF1α(530–652), whereas ^{35}S-labelled elongin B and elongin C did not (Fig. 2f). These results, together with the data shown in Fig. 1, indicate that HIF may bind directly to the pVHL β-domain. As predicted by these results, VBC complexes bound directly to full-length HIF1α (Fig. 2c) and HIF2α (Fig. 2d, e), whereas binding to a HIF1α mutant lacking residues 530–652 was reproducibly diminished (Fig. 2c, compare upper and middle panels).

Ubiquitination of HIF is pVHL-dependent. HIF is ubiquitinated under normoxic conditions, and cells lacking pVHL are unable to degrade HIF[5–7,13]. To determine directly whether pVHL ubiquitinates HIF, we carried out *in vitro* ubiquitination assays with ^{35}S-labelled versions of HIF. HIF1α(530–652) was ubiquitinated in the presence of a HeLa cell (*VHL*$^{+/+}$) S100 extract, whereas HIF1α(1–529) and HIF1α(653–826) were not (Fig. 3a–c). We confirmed the authenticity of the ubiquitin ladder formed by HIF1α(530–652) by repeating these experiments in the presence of a ubiquitin mutant (K48R) or glutathione-*S*-transferase (GST)-

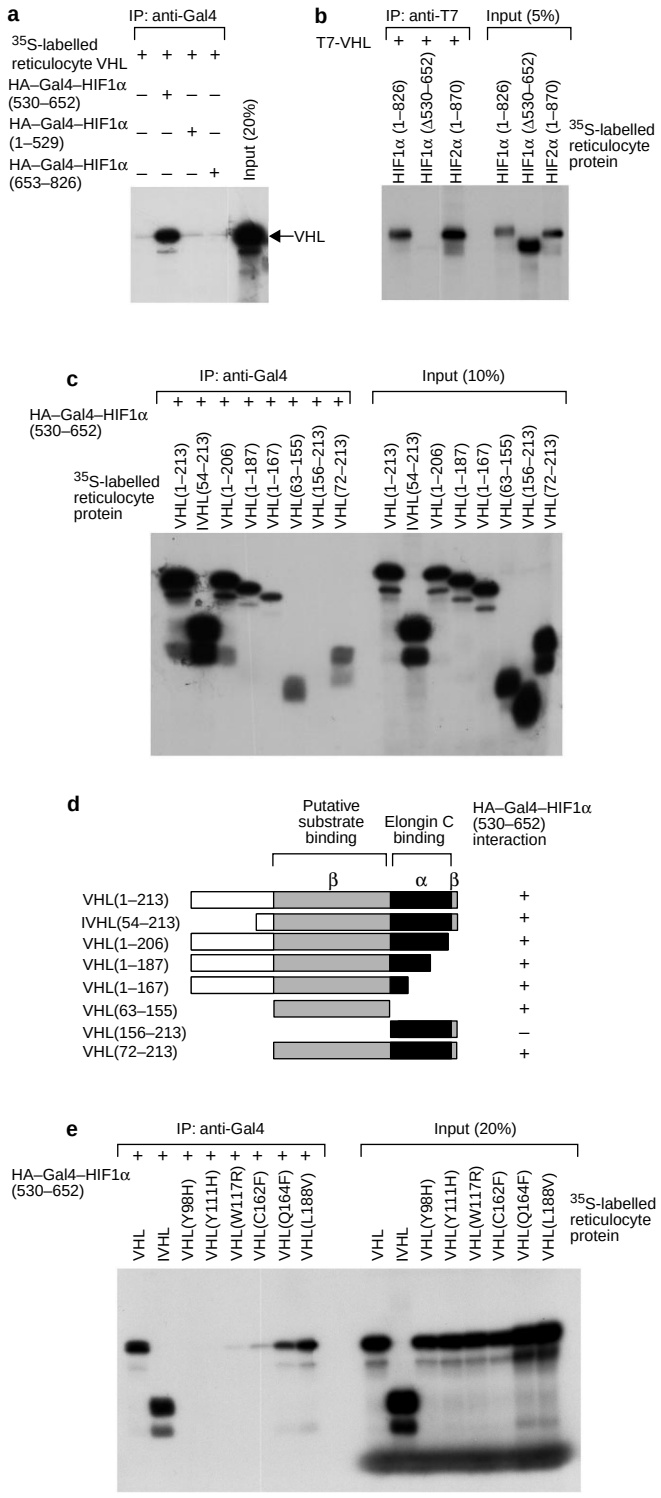

Figure 1 **The pVHL β-domain binds to a region of HIF implicated in oxygen-dependent proteolysis. a,** Immunoprecipitation (IP), with anti-Gal4 antibody, of the indicated HA–Gal4–HIF1α chimaeras in the presence of radiolabelled pVHL. **b,** Immunoprecipitation, with anti-T7 antibody, of T7-tagged pVHL in the presence of the indicated radiolabelled HIF1α and HIF2α fragments. **c,** Immunoprecipitation, with anti-Gal4 antibody, of HA–Gal4–HIF1α(530–652) in the presence of the indicated radiolabelled pVHL mutants. **d,** Schematic representation of pVHL mutants. **e,** Immunoprecipitation, with anti-Gal4 antibody, of HA–Gal4–HIF1α(530–652) in the presence of the indicated pVHL mutants.

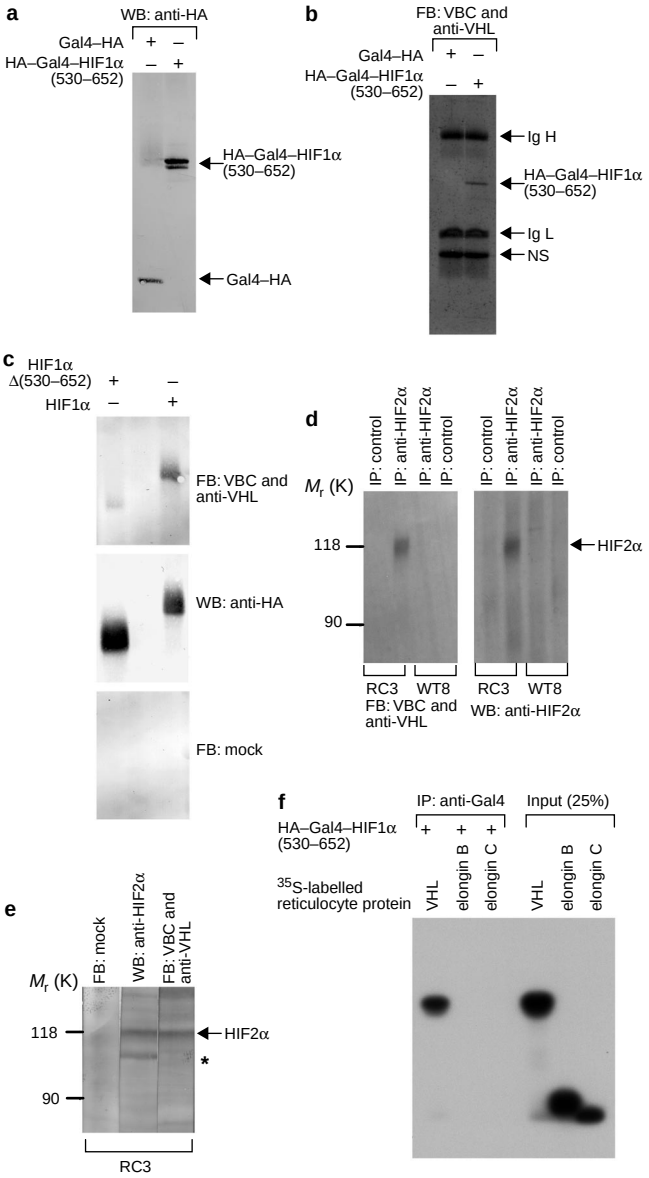

Figure 2 **pVHL binds directly to HIF. a, b,** Gal4–HA and HA–Gal4–HIF1α(530–652) *in vitro* translates were immunoprecipitated with an anti-HA antibody, resolved by SDS–PAGE, and transferred to nitrocellose filters. Filters were then probed with anti-HA antibody (**a**; WB, western blot) or with purified recombinant pVHL/elongin C/elongin B (VBC) complex and anti-VHL antibody (**b**; FB, farwestern blot). Bound pVHL complexes were detected with anti-pVHL antibody. Ig, immunoglobulin; NS, nonspecific. **c,** HA–HIF1α and HA–HIF1α(Δ530–652) *in vitro* translates were immunoprecipitated with anti-HA antibody. Bound proteins were western blotted with anti-HA antibody or farwestern blotted with recombinant VBC complex and anti-VHL antibody. A farwestern blot was also carried out in which the VBC complex was omitted (mock). **d,** Cell extracts prepared from pVHL-defective 786-O renal carcinoma cells stably transfected to produce wild-type pVHL (WT8) or transfected with an empty expression vector (RC3) were immunoprecipitated (IP) with control or anti-HIF2α antibodies. Bound proteins were western blotted with anti-HIF2α antibody or farwestern blotted with VBC complex and anti-VHL antibody. M_r (K), relative molecular mass in thousands. **e,** Cell extracts prepared from RC3 cells were resolved by SDS–PAGE, transferred to nitrocellulose, and western blotted with anti-HIF2α antibody or farwestern blotted with VBC complex and anti-VHL antibody. *, non-specific band. **f,** Immunoprecipitation, with anti-Gal4 antibody, of HA–Gal4–HIF1α(530–652) in the presence of radiolabelled pVHL, elongin B or elongin C, as indicated.

tagged ubiquitin (Fig. 3d). Thus, the region of HIF that binds to pVHL is also ubiquitinated *in vitro*. To investigate whether ubiquitination requires pVHL, we repeated these experiments with S100 extracts derived from renal carcinoma cells that lack wild-type pVHL (RC9) or that were stably transfected to produce wild-type pVHL (WT8) or pVHL(Y98H) (clone 4). Wild-type, but not mutant, pVHL S100 extract ubiquitinated HIF1α(530–652) in a dose-dependent manner (Fig. 4a–c). In contrast, the *in vitro* ubiquitination of the CDK inhibitor p27 was not affected by the presence or absence of pVHL (data not shown). Furthermore, addition of recombinant wild-type pVHL, but not of pVHL(Y98H), restored the ubiquitination of HIF1α(530–652) by the pVHL-defective S100 extract (Fig. 4c, d). Ubiquitination of HIF1α(530–652) was also restored by addition of pVHL(L188V), but not of pVHL(C162F) (Fig. 4d, e). pVHL(L188V) retained the ability to bind to HIF and to elongin C, whereas pVHL(C162F) did not (Fig. 1 and ref. 14). Comparable amounts of wild-type and mutant pVHL were used in these assays, as shown by immunoblotting against the HA tag (data not shown).

Wild-type, but not mutant, pVHL S100 extract also ubiquitinated full-length HIF1α and HIF2α, but not a HIF1α mutant lacking residues 530–652 which, as shown above, are important for binding to pVHL (Fig. 5a–d). These results show that an intact pVHL β-domain is required for ubiquitination of HIF *in vitro*. In keeping with these results, wild-type pVHL, but not the β-domain mutant pVHL(Y98H), restored the regulation of HIF and its downstream targets such as Glut1 when re-introduced into a pVHL-defective renal carcinoma cell line (Fig. 5e and data not shown). The α-domain of pVHL binds to elongin C which, in turn, binds to Cul2 and Rbx1/Roc1 (ref. 2). A synthetic peptide that blocks the interaction of pVHL with elongin C[11,12] prevented ubiquitination of HIF1α by pVHL (Fig. 5f). In contrast, a similar peptide with a single amino-acid change that prevents it from disrupting the pVHL/elongin C interaction[12] did not. Thus, for HIF to be ubiquitinated, it must physically interact with a multiprotein complex containing pVHL and elongin C.

These results do not exclude the possibility that other proteins contribute to the regulation of HIF ubiquitination and proteolysis under well-oxygenated conditions. As has been shown[6,7], addition of proteasomal inhibitors to cells containing wild-type pVHL stabilized HIF (Fig. 5g). In contrast, addition of proteasomal inhibitors to cells lacking pVHL did not lead to a further increase in steady-state levels of HIF protein (Fig. 5g). These results indicate that pVHL may be the principal regulator of proteasome-dependent HIF proteolysis.

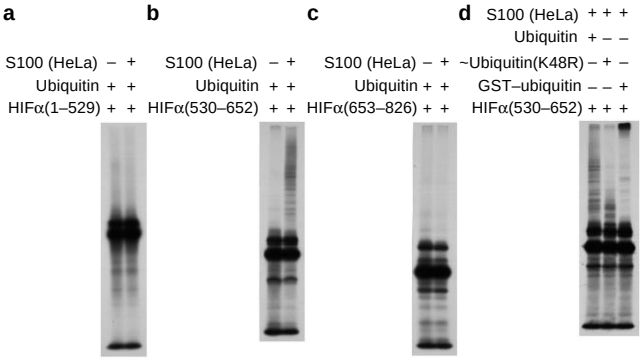

Figure 3 **Ubiquitination of the HIF pVHL-binding domain. a–c,** *In vitro* ubiquitination of the indicated radiolabelled HA–Gal4–HIF chimaeras in the presence or absence of HeLa S100 extract as indicated, and the presence of purified ubiquitin and an energy-regenerating system (see Methods). **d,** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) in the presence of wild-type ubiquitin, ubiquitin(K48R) or GST–ubiquitin.

A hypoxia-mimicking compound inhibits pVHL's ubiquitinating activity *in vitro*. To determine how HIF is stabilized in the presence of hypoxia, we carried out *in vitro* ubiquitination assays in

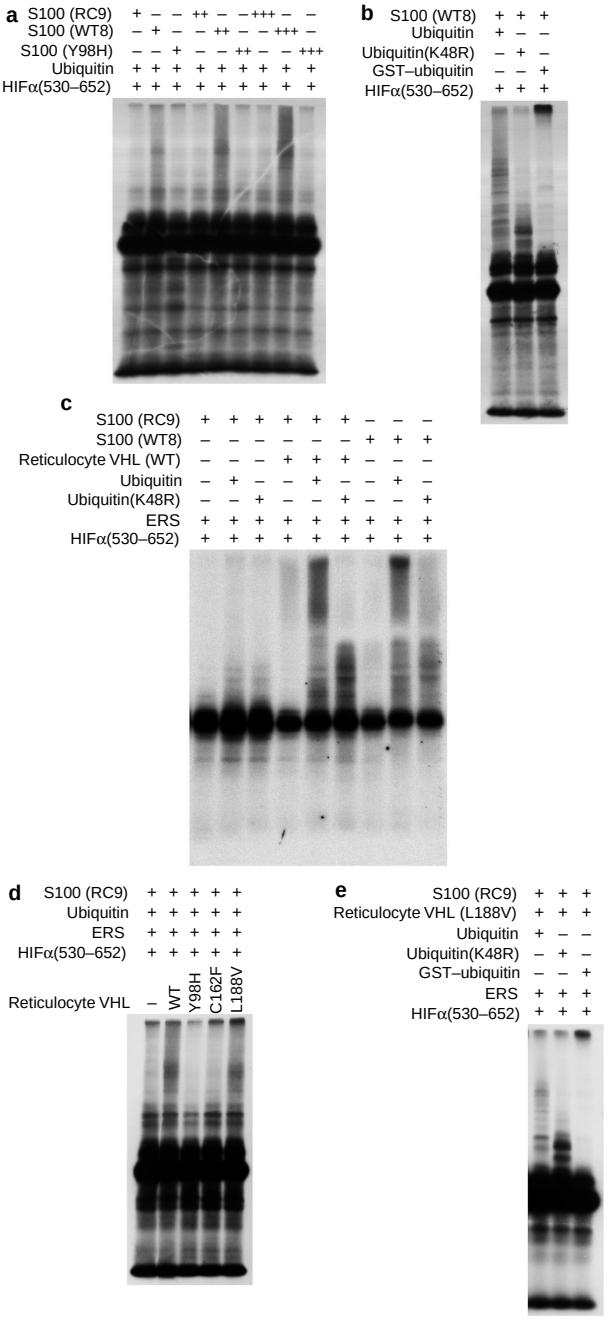

Figure 4 **pVHL-dependent ubiquitination of HIF. a,** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) in the presence of increasing amounts (as indicated by plus symbols) of S100 extracts prepared from cells lacking wild-type pVHL (RC9) or stably transfected to produce wild-type pVHL (WT8) or pVHL(Y98H). **b,** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) in the presence of wild-type ubiquitin, ubiquitin(K48R) or GST–ubiquitin. **c, d,** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) in the presence of RC9 S100 extract, to which was added wild-type (WT) or mutant pVHL *in vitro* translates. In **c,** *in vitro* ubiqutination assays were also carried out with wild-type (WT8) S100 extract for comparison. ERS, energy-regenerating system (see Methods). **e,** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) in the presence of wild-type ubiquitin, ubiquitin(K48R) or GST–ubiquitin.

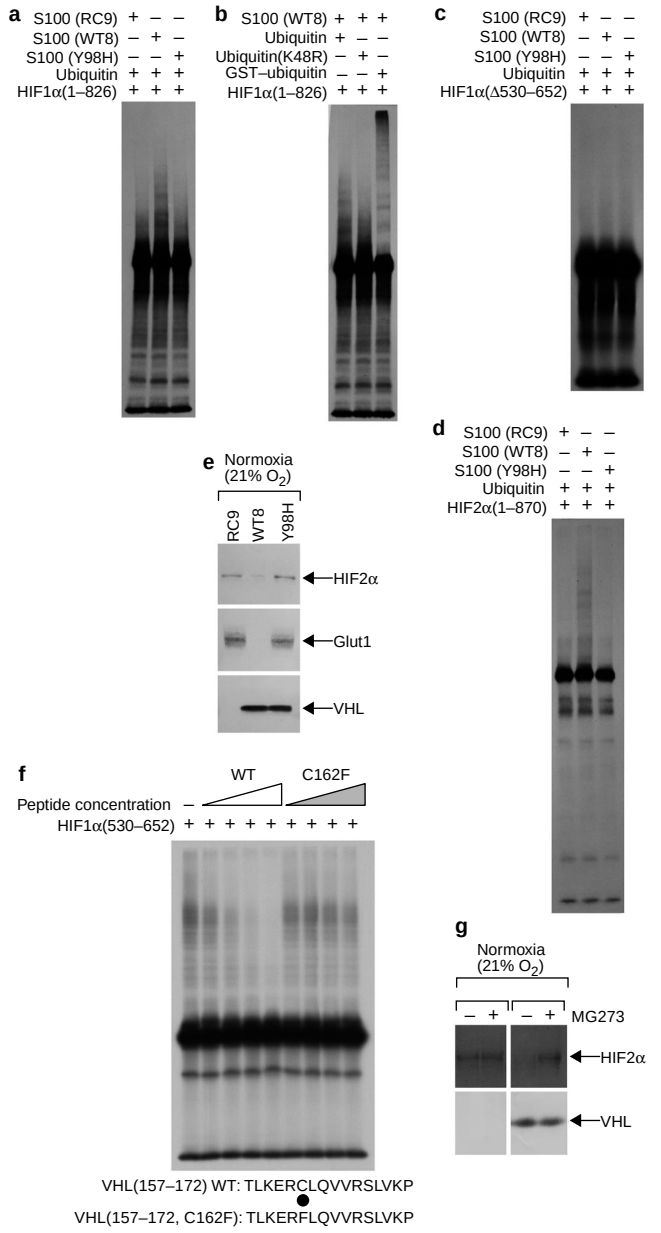

Figure 5 **Ubiquitination of HIF requires the pVHL α- and β-domains. a,** *In vitro* ubiquitination of radiolabelled full-length HIF1α in the presence of S100 extracts prepared from cells lacking wild-type pVHL (RC9) or stably transfected to produce wild-type pVHL (WT8) or pVHL(Y98H). **b,** *In vitro* ubiquitination of radiolabelled HIF1α in the presence of wild-type ubiquitin, ubiquitin(K48R) or GST–ubiquitin. **c,** *In vitro* ubiquitination of radiolabelled full-length HIF1α lacking residues 530–652 (HIF1α(Δ530–652)). **d,** *In vitro* ubiquitination of radiolabelled full-length HIF2α in the presence of S100 extracts prepared from cells lacking wild-type pVHL (RC9) or stably transfected to produce wild-type pVHL (WT8) or pVHL(Y98H). **e,** Immunoblotting against HIF2α, Glut1 and VHL in RC9, WT8, and Y98H cells under normoxic conditions. **f,** *In vitro* ubiquitination of radiolabelled HIF1α(530–652) in the presence of a WT8 S100 extract and increasing amounts (as indicated by plus symbols) of wild-type or C162F pVHL(157–172) peptides. Amino-acid sequences of the peptides used are shown at the bottom. **g,** Immunoblotting against HIF2α and pVHL in RC9 and WT8 cells under normoxic conditions, in the presence or absence of the proteasome inhibitor MG273.

the presence or absence of hypoxia mimetics such as desferrioxamine, which stabilizes HIF[8,15]. Treatment with desferrioxamine

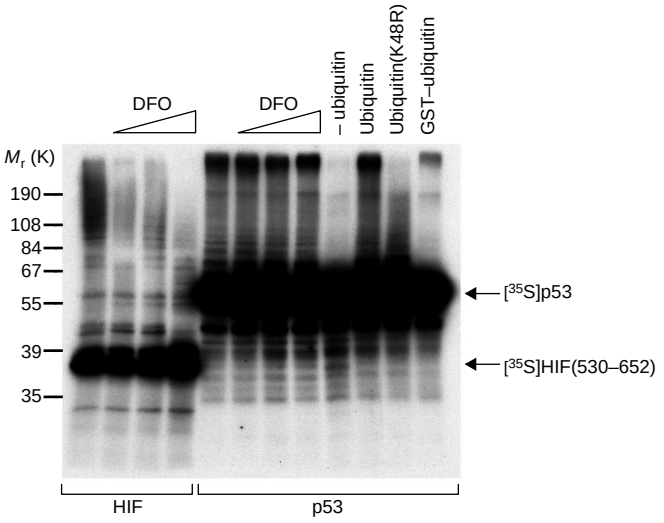

Figure 6 **Ubiquitination of HIF inhibited by a hypoxia mimetic.** *In vitro* ubiquitination of radiolabelled HA–Gal4–HIF1α(530–652) and p53 in the presence of a wild-type (WT8) S100 extract and increasing amounts of desferroxamine (100 μM, 1 mM, 10 mM), as indicated. Where indicated, the p53 ubiquitination reaction was carried out without exogenous ubiquitin (– ubiquitin) or in the presence of ubiquitin(K48R) or GST–ubiquitin. M_r (K), relative molecular mass in thousands.

blocked ubiquitination of HIF by pVHL *in vitro* (Fig. 6). This effect was specific, as the ubiquitination of p53, which was tested in parallel, was not affected (Fig. 6). Although treatment of cells with desferrioxamine leads to p53 stabilization, this is probably due to the ability of these compounds to stabilize HIF, which in turn leads to p53 stabilization[15]. Whether hypoxia and desferroxamine utilize the same molecular mechanism(s) to stabilize HIF *in vivo* remains to be determined[5,16].

Discussion

These results provide the first demonstration that pVHL is directly involved in targeting proteins for ubiquitination, and establish HIF as a substrate for this pVHL activity. This function requires both an intact β-domain and an intact α-domain. The β-domain binds directly to HIF1α and HIF2α, whereas the α-domain binds to elongin C, which in turn nucleates a complex that contains elongin B, Cul2 and Rbx1/Roc1 (ref. 2), and may contain further proteins besides these. Cul2 is highly similar to Cdc53. In yeast, Cdc53 and Rbx1/Roc1 are involved in recruitment of an E2 ubiquitin-conjugating enzyme, such as Cdc34 (ref. 2). The relevant E2(s) for the pVHL complex are currently unknown.

These observations, together with the knowledge that the β- and α-domains are hotspots for mutations in VHL disease, indicate that the vascular tumours that characterize VHL disease may be caused by inappropriate accumulation of HIF under normoxic conditions, leading to overproduction of angiogenic peptides such as vascular endothelial growth factor (VEGF). In keeping with this view, several independent VHL kindreds that carry the pVHL (L188V) mutation have been described[17,18]. These families do not develop the vascular tumours that are typical of VHL disease; instead they exclusively develop pheochromocytoma. pVHL (L188V) retains the ability to degrade HIF, as shown here, and suppresses the accumulation of HIF-inducible messenger RNAs[14]. It will be important to determine whether pVHL ubiquitinates proteins other than HIF, and whether pVHL has further functions that are unrelated to its role in ubiquitination. □

Methods

Cells.

786-O renal carcinoma cell subclones transfected to produce wild-type pVHL (WT8), pVHL(Y98H)(clone 4), or transfected with the empty expression vector (pRC9, pRc3) were as described[14,19]. HeLa cells were obtained from H. Franklin Bunn (Boston, Massachusetts). MG273 was from Leukosite (Cambridge, Massachusetts) and was used at a final concentration of 5μM.

Antibodies.

Monoclonal anti-Gal4(DBD; RK5C1) and polyclonal anti-HA (Y-11) antibodies were from Santa Cruz. Monoclonal anti-HA (12CA5) antibody was from Boehringer; monoclonal anti-T7 antibody was from Novagen (Madison, Wisconsin). Monoclonal anti-HIF1α antibody was a gift from D. Livingston (Bostin, Massachusetts); monoclonal anti-HIF2α antibody was a gift from P. Maxwell and P. Ratcliffe (Oxford, UK). Monoclonal anti-VHL (IG32) antibody was as described[12]. Polyclonal anti-HIF2α and anti-fibronectin antibodies were from Novus Biologicals (Littleton, Colorado) and Sigma, respectively. Polyclonal anti-GLUT-1 antibody was from Alpha Diagnostics (San Antonio, Texas).

Plasmids.

Human HIF1α and HIF2α complementary DNAs were gifts from D. Livingston and P. Ratcliffe, respectively. To make pGal4–HA, the region of pAS2 (Clontech) encoding the Gal4 DNA-binding domain and the HA tag was amplified by the polymerase chain reaction (PCR), using primers introducing 5′ *Kpn*I and 3′ *Bam*HI sites, and subcloned into pcDNA3 (Invitrogen) cut with these two restriction enzymes. To make pHA–Gal4–HIF1α(1–529), pHA–Gal4–HIF1α(530–652), and pHA–Gal4–HIF1α(653–826), the corresponding regions of the HIF1α cDNA were PCR-amplified with primers introducing 5′ *Bam*HI and 3′ XbaI sites, and ligated into pGal4–HA cut with these two restriction enyzmes. To make pcDNA3–HA–HIF1α and pcDNA3–HA–HIF2α, the HIF1α and HIF2α open reading frames were likewise amplified, digested with *Bam*HI and *Xba*I, and ligated into pcDNA3–HA cut with these two restriction enzymes. To make pcDNA3–HA–HIF1α(Δ530-652), HIF1α cDNA encoding residues 1–529 was PCR-amplified with a sense primer introducing a 5′ *Bam*HI site, and the region encoding residues 653–826 was PCR-amplified with an antisense primer introducing a 3′ *Xba*I site. The PCR products were phosphorylated *in vitro* with polynucleotide kinase, digested with *Bam*HI and *Xba*I, respectively, and inserted into pcDNA3–HA cut with *Bam*HI and *Xba*I in a three-way ligation. All plasmids made by PCR were confirmed by DNA sequencing.

pRc–CMV plasmids encoding HA-tagged, wild-type pVHL, and mutant derivatives thereof, were as described[14,20], except for pRc–CMV–HAVHL(Y111H), which was made in a similar fashion. To make pcDNA3–HAVHL(63–155) and pcDNA3–HAVHL(156–213), the corresponding VHL cDNAs were amplified by PCR with primers introducing 5′ *Bam*HI and 3′ *Xho*I sites, and ligated into pcDNA3–HA cut with these two restriction enzymes. To make pcDNA3–T7–VHL, the *Bam*HI–*Xba*I VHL cDNA fragment from pRc–HA–VHL was ligated into pcDNA3–T7 cut with these two restriction enzymes. pcDNA3/T7/elongin C and pcDNA3/HA/elongin B were as described[14,20].

VHL–HIF binding assays.

TNT reticulocyte (Promega) translates (5μl) made in the presence or absence of [^{35}S]methionine were incubated in 200μl EBC buffer (50mM Tris pH8, 120mM NaCl and 0.5% NP-40) with the indicated antibodies and protein A/sepharose for 2h at 4°C. The sepharose was then washed four times with NETN (20mM Tris pH8, 100mM NaCl, 0.5% NP-40 and 1mM EDTA). Bound proteins were resolved by SDS–PAGE and detected by fluorography. For farwestern analysis, nitrocellulose filters were blocked overnight at 4°C in Hyb75 (HEPES pH7.7, 75mM KCl, 0.1mM EDTA, 2.5mM $MgCl_2$ and 0.05% NP-40) with 2% (*w/v*) powdered milk, and then incubated overnight at 4°C with recombinant pVHL/elongin B/elongin C complexes[1] (2μgml^{-1} in Hyb75 with 1% milk). After three washes with Hyb75, filters were incubated for 3h with anti-VHL antibody (IG32; 1μgml^{-1} in Hyb75 with 1% milk). After a further three washes with Hyb75, bound IG32 was detected using an alkaline-phosphatase-conjugated goat anti-mouse antibody.

Preparation of S100 cell extracts.

To prepare S100 cell extracts, HeLa and 786-O subclones were resuspended and incubated in ice-cold hypotonic buffer (20mM Tris pH7.4, 5mM $MgCl_2$, 8mM KCl, 1mM dithiothreitol, 0.5mM phenylmethyl sulphonyl fluoride, 10μgml^{-1} leupeptin, 1μgml^{-1} pepstatin, 0.1mM PABA and 10μgml^{-1} aprotinin) for 15min. Cells were subjected to three freeze/thaw cycles and pelleted by centrifugation at 14,000*g* for 5min. The resulting supernatant was ultracentrifuged at 100,000*g* for 4h. The supernatant (S100 fraction) was aliquoted and stored at –80°C.

In vitro ubiquitination assay.

[^{35}S]-labelled translates (2μl) were incubated in the presence of S100 extracts (100–200μg) supplemented with 8μgul^{-1} ubiquitin (Sigma), 100ngul^{-1} ubiquitin aldehyde (BostonBiochem, Cambridge, Massachusetts) and energy-regenerating system (20mM Tris pH7.4, 2mM ATP, 5mM $MgCl_2$, 40mM creatine phosphate and 0.5μgul^{-1} creatine kinase) in a reaction volume of 20–30μl for 1–2h at 30°C. Products were immunoprecipitated with anti-Gal4 or anti-HA antibody and resolved by SDS–PAGE. For peptide-inhibition experiments, S100 extracts were incubated with 1, 2, 3 or 4μg of the indicated peptides for 30min at 37°C before addition to the ubiquitination reaction. Ubiquitin(K48R) and GST–Ubiquitin (both from BostonBiochem) were used at 1.3μgul^{-1}.

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1. Stebbins, C. E., Kaelin, W. G. J. & Pavletich, N. P. Structure of the VHL–elonginC–elonginB complex: implications for VHL tumor suppressor function. *Science* **284,** 455–461 (1999).
2. Kaelin, W. G. Cancer. Many vessels, faulty gene. *Nature* **399,** 203–204 (1999).
3. Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M. & Krek, W. The von Hippel–Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes Dev.* **13,** 1822–1833 (1999).
4. Iwai, K. *et al.* Identification of the von Hippel–Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc. Natl Acad. Sci. USA* **96,** 12436–12441 (1999).
5. Maxwell, P. *et al.* The von Hippel–Lindau gene product is necessary for oxgyen-dependent proteolysis of hypoxia-inducible factor α subunits. *Nature* **399,** 271–275 (1999).
6. Huang, L. E., Gu, J., Schau, M. & Bunn, H. F. Regulation of hypoxia-inducible factor 1 alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl Acad. Sci. USA* **95,** 7987–7992 (1998).
7. Kallio, P., Wilson, W., O'Brien, S., Makino, Y. & Poellinger, L. Regulation of the hypoxia-inducible transcription factor 1 alpha by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **274,** 6519–6525 (1999).
8. Pugh, C., O'Rourke, J., Nagao, M., Gleadle, J. & Ratcliffe, P. Activation of hypoxia-inducible factor-1: definition of regulatory domains within the alpha subunit. *J. Biol. Chem.* **272,** 11205–11214 (1997).
9. Ohh, M. & Kaelin, W. G. J. The von Hippel–Lindau tumour suppressor protein: new perspectives. *Mol. Med. Today* **5,** 257–263 (1999).
10. Kamura, T. *et al.* The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev.* **12,** 3872–3881 (1998).
11. Duan, D. R. *et al.* Inhibition of transcriptional elongation by the VHL tumor suppressor protein. *Science* **269,** 1402–1406 (1995).
12. Kibel, A., Iliopoulos, O., DeCaprio, J. D. & Kaelin, W. G. Binding of the von Hippel–Lindau tumor suppressor protein to elongin B and C. *Science* **269,** 1444–1446 (1995).
13. Salceda, S. & Caro, J. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.* **272,** 22642–2267 (1997).
14. Ohh, M. *et al.* Synthetic peptides define critical contacts between elongin C, elongin B, and the von Hippel–Lindau protein. *J. Clin. Invest.* **104,** 1583–1591 (1999).
15. An, W. *et al.* Stabilization of wild-type p53 by hypoxia-inducible factor 1 alpha. *Nature* **392,** 405–408 (1998).
16. Chandel, N. *et al.* Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc. Natl Acad. Sci. USA* **95,** 11715–11720 (1998).
17. Ritter, M. *et al.* Isolated familial pheochromocytoma as a variant of von Hippel–Lindau Disease. *J. Clin. Endocrinol. Met.* **81,** 1035–1037 (1996).
18. Crossey, P. A. *et al.* Identification of intragenic mutations in the von Hippel–Lindau disease tumor suppressor gene and correlation with disease phenotype. *Hum. Mol. Genet.* **3,** 1303–1308 (1994).
19. Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W. G. Tumor suppression by the human von Hippel–Lindau gene product. *Nature Med.* **1,** 822–826 (1995).
20. Lonergan, K. M. *et al.* Regulation of hypoxia-inducible mRNAs by the von Hippel–Lindau protein requires binding to complexes containing elongins B/C and Cul2. *Mol. Cell Biol.* **18,** 732–741 (1998).

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