

- Neurosci.* **9**, 448–459 (1997).
5. Xu, Q., Allidus, G., Hlder, N. & Wilkinson, D. G. Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the *Xenopus* and zebrafish hindbrain. *Development* **121**, 4005–4016 (1995).
  6. Xu, Q. & Wilkinson, D. G. Eph-related receptors and their ligands: mediators of contact dependent cell interactions. *J. Mol. Med.* **75**, 576–586 (1997).
  7. Flanagan, J. G. & Vanderhaeghen, P. The ephrins and Eph receptors in neural development. *Annu. Rev. Neurobiol.* **21**, 309–345 (1998).
  8. O'Leary, D. D. M. & Wilkinson, D. G. Eph receptors and ephrins in neural development. *Curr. Opin. Neurobiol.* **9**, 65–73 (1999).
  9. Holland, S. J. *et al.* Bidirectional signalling through the Eph-family receptor Nuk and its transmembrane ligands. *Nature* **383**, 722–725 (1996).
  10. Bruckner, K., Pasquale, E. B. & Klein, R. Tyrosine phosphorylation of transmembrane ligands for Eph receptors. *Science* **275**, 1640–1643 (1997).
  11. Gale, N. W. *et al.* Eph receptors and ligands comprise two major specificity subclasses, and are reciprocally compartmentalised during embryogenesis. *Neuron* **17**, 9–19 (1996).
  12. Eph Nomenclature Committee. Unified nomenclature for Eph family receptors and their ligands, the ephrins. *Cell* **90**, 403–404 (1997).
  13. Ellis, C. *et al.* A juxtamembrane autophosphorylation site in the Eph family receptor tyrosine kinase, Sek, mediates high affinity interaction with p59fyn. *Oncogene* **12**, 1727–1736 (1996).
  14. Theill, T. *et al.* Segmental expression of the EphA4 (Sek-1) gene is under direct transcriptional control of Krox-20. *Development* **125**, 443–452 (1998).
  15. Woo, K. & Fraser, S. E. Order and coherence in the fate map of the zebrafish nervous system. *Development* **121**, 2595–2609 (1995).
  16. Townes, P. L. & Holtfreter, J. Directed movements and selective adhesion of embryonic amphibian cells. *J. Exp. Zool.* **128**, 53–120 (1955).
  17. Steinberg, M. S. Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium processes and the emergence of a hierarchy among populations of embryonic cells. *J. Exp. Zool.* **173**, 395–434 (1970).
  18. Nose, A., Nagafuchi, A. & Takeichi, M. Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* **54**, 993–1001 (1988).
  19. Friedlander, D. R., Mege, R. M., Cunningham, B. A. & Edelman, G. M. Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules. *Proc. Natl Acad. Sci. USA* **86**, 7043–7047 (1989).
  20. Godt, D. & Tepass, U. *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. *Nature* **395**, 387–391 (1998).
  21. Gonzalez-Reyes, A. & St Johnston, D. The *Drosophila* A-P axis is polarised by the cadherin-mediated positioning of the oocyte. *Development* **125**, 3635–3644 (1998).
  22. Meima, L. *et al.* AL-1-induced growth cone collapse of rat cortical neurons is correlated with REK7 expression and rearrangement of the actin cytoskeleton. *Eur. J. Neurosci.* **9**, 177–188 (1997).
  23. Zisch, A. H. *et al.* Tyrosine phosphorylation of L1 family adhesion molecules: implication of the Eph kinase Cdk5. *J. Neurosci. Res.* **47**, 655–665 (1997).
  24. Lumsden, A. & Keynes, R. Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424–428 (1989).
  25. Heyman, I., Kent, A. & Lumsden, A. Cellular morphology and extracellular space at rhombomere boundaries in the chick embryo hindbrain. *Dev. Dynamics* **198**, 241–253 (1993).
  26. Xu, Q., Hlder, N., Patient, R. & Wilson, S. W. Spatially regulated expression of three receptor tyrosine kinase genes during gastrulation in the zebrafish. *Development* **120**, 287–299 (1994).
  27. Irving, C., Nieto, M. A., DasGupta, R., Charnay, P. & Wilkinson, D. G. Progressive spatial restriction of Sek-1 and Krox-20 gene expression during hindbrain segmentation. *Dev. Biol.* **173**, 26–38 (1996).

**Acknowledgements.** We thank N. Gale and G. Yancopoulos for ephrin clones, M. Henkemeyer for the EphB2 clone and S. Fraser, J. P. Vincent, R. Krumlauf and P. Trainor for discussions. This work was supported by the MRC, an EC Biotechnology grant and an EMBO Fellowship to G.M.

Correspondence and requests for materials should be addressed to D.G.W. (e-mail: d-wilkin@nimr.mrc.ac.uk)

## The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis

Patrick H. Maxwell<sup>\*</sup>, Michael S. Wiesener<sup>\*</sup>,  
Gin-Wen Chang<sup>\*</sup>, Steven C. Clifford<sup>†</sup>, Emma C. Vaux<sup>‡</sup>,  
Matthew E. Cockman<sup>‡</sup>, Charles C. Wykoff<sup>‡</sup>,  
Christopher W. Pugh<sup>‡</sup>, Eamonn R. Maher<sup>‡</sup>  
& Peter J. Ratcliffe<sup>\*‡</sup>

<sup>\*</sup> Wellcome Trust Centre for Human Genetics, Windmill Road, Oxford OX3 7BN, UK

<sup>†</sup> Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, Birmingham B15 2TT, UK

<sup>‡</sup> Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK

**Hypoxia-inducible factor-1 (HIF-1) has a key role in cellular responses to hypoxia, including the regulation of genes involved in energy metabolism, angiogenesis and apoptosis<sup>1–4</sup>. The  $\alpha$  subunits of HIF are rapidly degraded by the proteasome under normal conditions, but are stabilized by hypoxia<sup>5</sup>. Cobaltous ions or iron chelators mimic hypoxia, indicating that the stimuli may**

**interact through effects on a ferroprotein oxygen sensor<sup>6,7</sup>. Here we demonstrate a critical role for the von Hippel-Lindau (VHL) tumour suppressor gene product pVHL in HIF-1 regulation. In VHL-defective cells, HIF  $\alpha$ -subunits are constitutively stabilized and HIF-1 is activated. Re-expression of pVHL restored oxygen-dependent instability. pVHL and HIF  $\alpha$ -subunits co-immunoprecipitate, and pVHL is present in the hypoxic HIF-1 DNA-binding complex. In cells exposed to iron chelation or cobaltous ions, HIF-1 is dissociated from pVHL. These findings indicate that the interaction between HIF-1 and pVHL is iron dependent, and that it is necessary for the oxygen-dependent degradation of HIF  $\alpha$ -subunits. Thus, constitutive HIF-1 activation may underlie the angiogenic phenotype of VHL-associated tumours. The pVHL/HIF-1 interaction provides a new focus for understanding cellular oxygen sensing.**

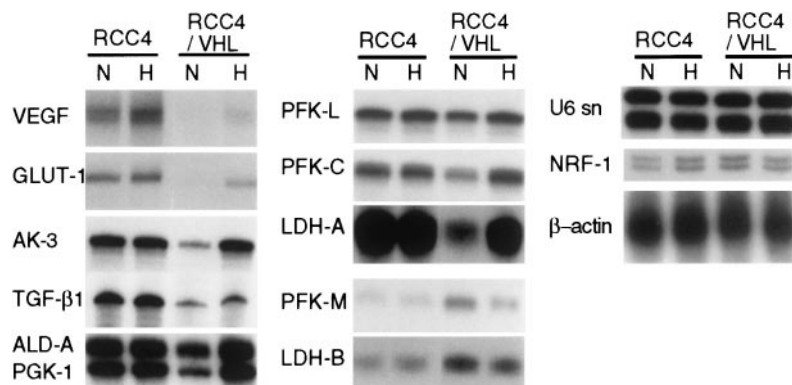
Enhanced glucose metabolism and angiogenesis are classical features of cancer<sup>8,9</sup>, involving upregulation of genes that are normally induced by hypoxia. In addition to stimulation by the hypoxic microenvironment<sup>10</sup>, genetic alterations contribute to these effects<sup>8,9</sup>. A striking example is von Hippel-Lindau (VHL) disease, a hereditary human cancer syndrome predisposing sufferers to highly angiogenic tumours<sup>11</sup>. Constitutive upregulation of hypoxically inducible messenger RNAs encoding vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT-1) in these tumour cells is correctable by re-expression of pVHL. A post-transcriptional mechanism has been proposed<sup>12,13</sup>. We studied the involvement of pVHL in oxygen-regulated gene expression using ribonuclease protection analysis of two VHL-deficient renal carcinoma lines, RCC4 and 786-O. Eleven genes encoding products involved in glucose transport, glycolysis, high-energy phosphate metabolism and angiogenesis were examined; nine are induced by hypoxia in other mammalian cells and two (LDH-B and PFK-M) are repressed by hypoxia. None of these responses was seen in the VHL-defective cell lines. Responses to hypoxia were restored by stable transfection of a wild-type VHL gene, with effects ranging from a rather modest action of hypoxia (PFK-L and LDH-B) to substantial regulation (Fig. 1 shows results for RCC4 cells; similar effects were seen in 786-O cells, data not shown). These results indicate that the previously described upregulation of hypoxia-inducible mRNAs in VHL-defective cells<sup>12,13</sup> extends to a broad range of oxygen-regulated genes and involves a constitutive 'hypoxia pattern' for both positively and negatively regulated genes.

As a number of these genes (VEGF, GLUT-1, AK-3, ALD-A, PGK-1, PFK-L and LDH-A) contain hypoxia-response elements (HREs) which bind HIF-1 and/or show altered expression in cells lacking HIF-1 (refs 2, 14 and references therein), this survey of expression in VHL-defective cells prompted us to look for effects of pVHL on HIF-1 and HRE function. RCC4 cells were co-transfected with reporter plasmids which did or did not contain HREs from the mouse phosphoglycerate-kinase-1 or erythropoietin (Epo) enhancers, and either the VHL-expression plasmid pcDNA3-VHL or an empty vector. pVHL suppressed HRE activity in normoxic cells and restored induction by hypoxia (Fig. 2a). Similar results were obtained by sequential stable transfection of RCC4 cells with an HRE reporter followed by pcDNA3-VHL (data not shown). HIF-1 itself was examined by electrophoretic mobility shift assay (EMSA), which showed a constitutive HIF-1 DNA-binding species in VHL-deficient RCC4 cells, with restoration of the normal hypoxia-inducible pattern in RCC4 cells stably transfected with pcDNA3-VHL (RCC4/VHL) (Fig. 2b). In other cells, HIF-1 activation by hypoxia involves a large increase in HIF-1 $\alpha$  abundance from low basal levels in normoxia<sup>1,15</sup>. Western blotting of whole-cell extracts showed that RCC4 cells express constitutively high levels of both HIF-1 $\alpha$  and a related molecule, HIF-2 $\alpha$  (also termed EPAS-1, HRF, HLF and MOP2) which is normally regulated in a similar way<sup>16</sup> (Fig. 2c). Constitutively high levels of these proteins were found in eight other VHL-defective cell lines, in contrast to the renal

IPR2016-01315

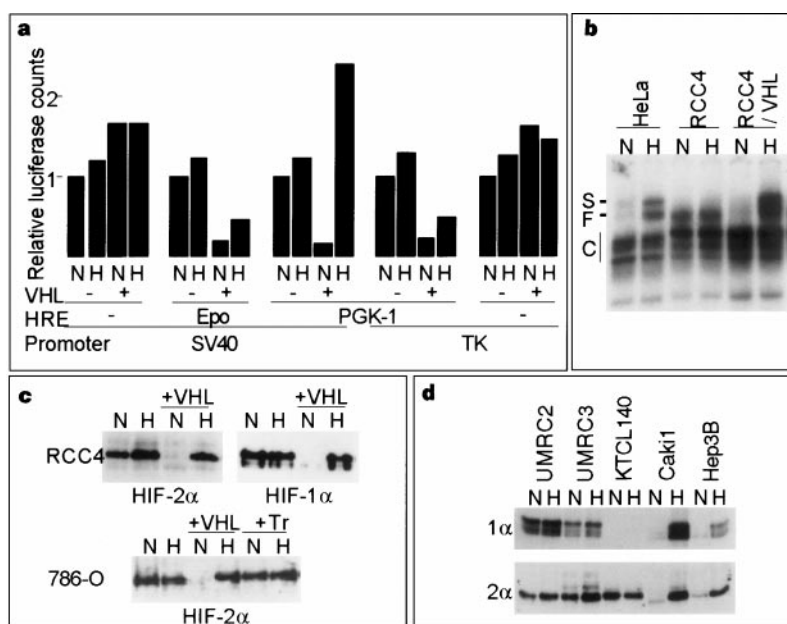
GSK Ex. 1029, pg 574

271



**Figure 1** Effect of pVHL on oxygen-regulated gene expression. mRNA analysis of RCC4 cells and stable transfectant expressing pVHL (RCC4/VHL). N, normoxia; H, hypoxia (1% O<sub>2</sub>, 16 h). VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter 1; AK-3, adenylate kinase 3; TGF-β1, transforming growth factor-β1; ALD-A, aldolase A; PGK-1, phosphoglyceratekinase 1; PFK, phos-

phofruktokinase; LDH, lactate dehydrogenase. U6 small nuclear (sn) RNA was used as an internal control. Also illustrated are two genes not influenced by VHL status or hypoxia; nuclear respiratory factor 1 (NRF-1) and β-actin. Amount of RNA analysed is detailed in Table S1 of the Supplementary Information.



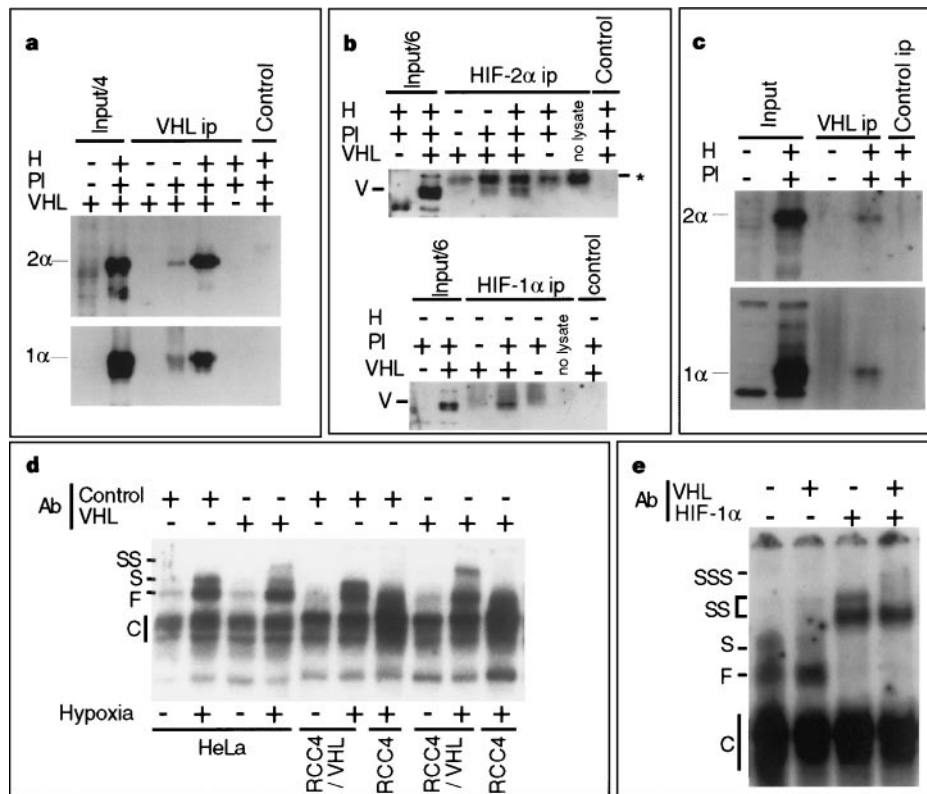
**Figure 2** Effect of pVHL on HIF-1 and HRE activity. **a**, Representative transient transfections of RCC4 cells with VHL expression vector (+) or empty vector, and luciferase reporter genes containing no HRE, or HREs from the PGK-1 or erythropoietin (Epo) genes linked to SV40 or TK promoters. N, normoxia; H, hypoxia (0.1% O<sub>2</sub>, 24 h—results were similar with 1% O<sub>2</sub>). **b**, EMSA using the Epo HRE. N, normoxia; H, hypoxia (1% O<sub>2</sub>, 4 h). In HeLa and RCC4/VHL, HIF-1 is a doublet (S, slower and F, faster mobility). RCC4 cells contain only faster mobility HIF-1, with equivalent levels in normoxia and hypoxia. Constitutive binding

species are indicated (C). **c**, Immunoblots of whole cell extracts for HIF α-subunits. Upper panels; RCC4 and RCC4/VHL cells (+VHL). Lower panel; 786-O cells stably transfected with vector alone, a full length VHL gene (+VHL), or a truncated VHL gene (1-115; +Tr). HIF-1α was not detected in 786-O cells. **d**, Immunoblot of UMRC2, UMRC3 and KTCL140 (renal carcinoma lines with VHL mutations<sup>39</sup>), Caki-1 (renal carcinoma line expressing wild-type pVHL) and the hepatoma line Hep3B.

carcinoma line Caki-1 (which expresses pVHL normally<sup>17</sup>) and a wide range of previously reported cell lines (Fig. 2d and Supplementary Information S2). Certain VHL-defective cells (for example, 786-O, KTCL140) expressed HIF-2α at a high constitutive level but did not express detectable HIF-1α (Fig. 2c, d and Supplementary Information S2). Examination of stable transfectants of RCC4 and 786-O cells demonstrated that expression of the wild-type, but not truncated, VHL gene restored regulation of HIF α-subunits by oxygen without affecting the levels of mRNA encoding either subunit (data not shown).

To investigate the role of pVHL in HIF-1 regulation we tested for interactions between HIF α-subunits and pVHL using a combination of hypoxia and/or proteasomal blockade to induce HIF α-subunits. Anti-pVHL immunoprecipitates of extracts from protea-

somally blocked RCC4/VHL cells, but not RCC4 cells, contained both HIF-1α and HIF-2α (Fig. 3a). Similar results were obtained with hypoxia in the absence of proteasomal blockade. In the inverse reactions, immunoprecipitating antibodies to HIF-2α or HIF-1α co-precipitated pVHL, although a smaller proportion of the total was captured (Fig. 3b). Anti-pVHL immunoprecipitations also demonstrated the interaction in HeLa cells, which express pVHL normally (Fig. 3c). We next tested whether pVHL is incorporated in the HIF-1 DNA-binding complex. Addition of antibodies raised against pVHL (but not control antibodies) to nuclear extract from RCC4/VHL cells and HeLa cells produced a change in mobility, which was not observed with VHL-defective RCC4 cells (Fig. 3d). HIF-1 migrated as two species. Only the slower-mobility HIF-1 species was shifted by anti-pVHL, whereas both species were shifted



**Figure 3** Association of pVHL with HIF-1. **a**, Immunoblots for HIF  $\alpha$ -subunits (2 $\alpha$ , 1 $\alpha$ ) of IG32 (VHL ip) and control immunoprecipitates (using VG-7be) of RCC4/VHL (VHL+) and RCC4 (VHL-) cells exposed (4 h) to normoxia or hypoxia (1% O<sub>2</sub>; H+) with or without proteasomal inhibition (PI+). Aliquots of selected input lysates were also immunoblotted. **b**, Immunoprecipitation of RCC4/VHL (VHL+) and RCC4 (VHL-) extracts with polyclonal antibodies to HIF  $\alpha$ -subunits or normal rabbit immunoglobulin (control ip) followed by immunoblotting for pVHL (V). A cross-reacting species arose from the HIF-2 $\alpha$  antibody (asterisk). **c**, Immunoprecipitation of HeLa extracts with IG32 (VHL<sub>ip</sub>) or pAb419 (control) followed by

immunoblotting for HIF  $\alpha$ -subunits. **d**, Anti-pVHL supershifts. IG32 (VHL Ab+) or VG-7be (Control Ab+) was added to binding reactions of nuclear extracts from normoxic or hypoxic (1% O<sub>2</sub>, 4 h; H+) cells. Anti-pVHL supershifted (SS) the slower HIF-1 species (S) in HeLa and hypoxic RCC4/VHL cells. No supershift was seen with RCC4 cells, which lack pVHL. **e**, Anti-pVHL, anti-HIF-1 $\alpha$  and combination supershifts in HeLa cells. Anti-pVHL (IG32, +VHL Ab) supershifted slower mobility HIF-1. Anti-HIF-1 $\alpha$  (clone 54) supershifted both components (SS). Addition of both antibodies 'super-super-shifted' (SSS) slower mobility HIF-1.

by anti-HIF-1 $\alpha$  (Fig. 3e). Similar results were obtained in other cell lines (Hep3B, Caki-1, MRC5-V2 and 293; data not shown). Furthermore, whereas RCC4/VHL, HeLa and other cells contained both HIF-1 species, RCC4 extracts contained only the faster-mobility species (Figs 3d, Fig. 2b). Thus, VHL-defective cells lack the slower-mobility species which is restored by re-expression of pVHL, and shifted by anti-pVHL. This indicates that the DNA-binding HIF-1 doublet arises from two species—containing or not containing pVHL. Combination supershift analysis confirmed that the slower-mobility species contained both HIF-1 $\alpha$  and pVHL (Fig. 3e).

HIF-1 activation by hypoxia is mimicked by cobaltous ions and iron chelation<sup>6,7</sup>. We therefore tested whether the pVHL/HIF-1 interaction was regulated by these stimuli. Proteasomal blockade induces an HIF-1 DNA-binding complex in normoxic cells<sup>18</sup>; comparison of this normoxic complex with EMSA of hypoxic cells with or without proteasomal inhibitors showed a similar shift and anti-pVHL supershift (Fig. 4a and data not shown). Together with immunoprecipitation data, this indicates that the interaction with pVHL occurs in both normoxic and hypoxic cells. In contrast, EMSA analysis of RCC4/VHL cells treated with cobalt and the iron chelator desferrioxamine (DFO) demonstrated only the faster mobility HIF-1. This did not supershift with anti-pVHL, indicating that the pVHL/HIF-1 complex could not form in cells exposed to these stimuli. Similar results were obtained in other cell types and are consistent with hitherto unexplained mobility differences between previous analyses of HIF-1 from cobalt- or DFO-versus hypoxia-stimulated cells<sup>7</sup>, indicating that this is a general

effect. Treatment with DFO 4 h before hypoxia prevented the formation of the pVHL/HIF-1 complex (Fig. 4b). Addition of iron chelators could not break the pVHL/HIF-1 complex *in vitro*, whereas addition of *in vitro*-translated wild-type pVHL (but not a truncated pVHL) could restore the slower-mobility species to nuclear extracts of proteasomally blocked, normoxic and hypoxic RCC4 cells, but not to cells treated with DFO or cobalt (Fig. 4c, and Fig. S1a of Supplementary Information). Immunoprecipitation studies also indicated that the interaction between HIF-1 and pVHL is iron-dependent. Whereas both HIF-1 $\alpha$  and HIF-2 $\alpha$  were contained in anti-pVHL immunoprecipitates from hypoxic RCC4/VHL cells, neither was contained in precipitates from DFO- or cobalt-treated cells (Fig. 4d). The iron-dependent interaction between HIF  $\alpha$ -subunits and pVHL may be direct or indirect. However, *in vitro*-translated wild-type pVHL did not bind to an *in vitro*-translated HIF-1 DNA-binding complex (Fig. S1b in Supplementary Information), in contrast to the interaction with RCC4 extracts (Fig. 4c), indicating that an additional factor or modification of HIF-1 not represented in rabbit reticulocyte lysates is necessary for the association.

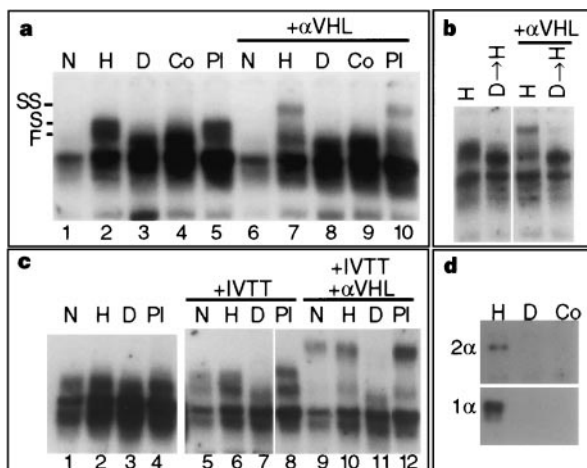
Normally, HIF  $\alpha$ -subunits are targeted for rapid degradation in normoxic cells by a proteasomal mechanism operating on an internal oxygen-dependent-degradation (ODD) domain<sup>5</sup>. Our data suggest that pVHL might be required for this process—a possibility which would be consistent with recent data that pVHL forms a multiprotein complex (containing Cul-2 and elongins B and C) which has homology with ubiquitin-ligase/proteasome-targeting complexes in yeast<sup>19,20</sup>. When cells were switched from

hypoxia to normoxia with addition of cycloheximide, HIF  $\alpha$ -subunits decayed with a half-life of about 5 min in wild-type VHL transfectants, compared with  $\sim$ 60 min in the VHL-defective RCC4 and 786-O cells, thus confirming a strong effect of pVHL on stability (Fig. 5a). Moreover, functional studies of Gal4 chimaeras containing the HIF-1 $\alpha$  ODD domain demonstrated a striking dependence of the isolated ODD domain on pVHL (Fig. 5b).

These experiments define a function for pVHL in the regulation of HIF-1. Given recent demonstrations of the importance of HIF-1 in tumour angiogenesis<sup>3,21</sup>, constitutive HIF-1 activation is clearly consistent with the angiogenic phenotype of VHL disease. Whether it is a sufficient explanation for oncogenesis is less clear. HIF-1 mediates gene activation not only by hypoxia, but also by growth factors such as insulin and insulin-like growth factor-1 (ref. 22). HIF-1 targets such as molecules involved in enhanced glucose metabolism and angiogenesis<sup>8,9,14</sup> are classically upregulated (by different mechanisms) in many forms of cancer, supporting an

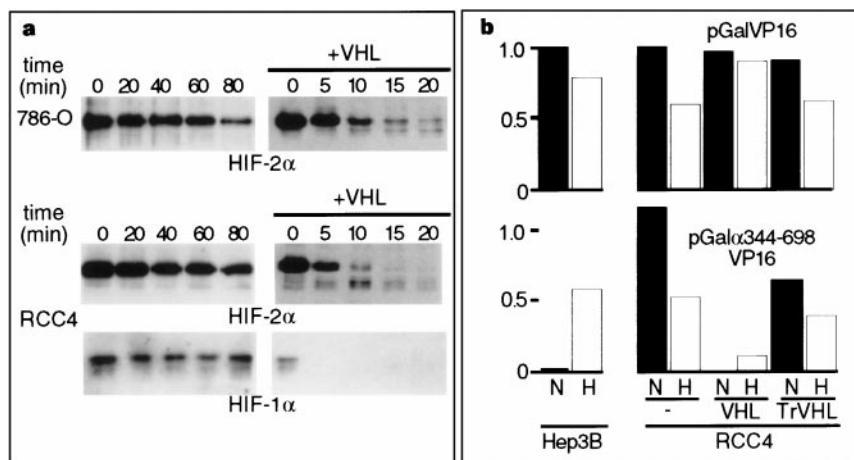
important role in tumour progression, although their role in the initiation of oncogenesis is less clear. pVHL is probably a multi-functional protein which could have other tumour suppressor actions<sup>11,23,24</sup>. One possibility is that other gene products could be targeted in a similar manner to HIF  $\alpha$ -subunits; however, comparison of anti-pVHL immunoprecipitates from metabolically labelled RCC4/VHL cells with and without proteasomal inhibitors has so far demonstrated only two species: HIF-1 $\alpha$  and HIF-2 $\alpha$  (G.-W.C., unpublished observations).

As both pVHL and HIF-1 are widely expressed, it is likely that the physiological role of pVHL in HIF-1 regulation is general. It is not yet clear whether pVHL has actions on oxygen-regulated gene expression other than through HIF-1. Stabilization of hypoxia-inducible mRNAs has been reported in VHL-deficient cells<sup>12,13</sup>. This might represent an independent action of pVHL. However, regulation of these RNAs is commonly abolished in HIF-1-deficient cells<sup>3,25</sup>, so the mRNA stability factors could lie downstream of



**Figure 4** Effect of cobaltous ions and iron chelation on the pVHL/HIF-1 interaction. **a**, EMSA and supershift analysis of RCC4/VHL cells exposed (4 h) to normoxia (N), hypoxia (H; 1% O<sub>2</sub>), DFO (100  $\mu$ M), cobaltous chloride (Co, 100  $\mu$ M) or proteasomal inhibition (PI). In lanes 6–10, IG32 was added (+ $\alpha$ VHL). **b**, EMSA and supershift analysis of RCC4/VHL cells subjected to hypoxia (H; 1% O<sub>2</sub> for 8 h) or DFO (100  $\mu$ M) with hypoxia (D  $\rightarrow$  H; cells exposed to DFO for 8 h, 4 h

normoxia, then 4 h hypoxia). **c**, EMSA of RCC4 cells showing only faster mobility HIF-1 (lanes 1–4). Addition of *in vitro* transcribed/translated pVHL alone (lanes 5–8, +IVTT), or together with IG32 (lanes 9–12, +IVTT+ $\alpha$ VHL); slower mobility HIF-1 forms and supershifts in cells exposed to normoxia, hypoxia and PI, but not DFO. **d**, HIF $\alpha$  immunoblots of anti-pVHL immunoprecipitates of RCC4/VHL cells.



**Figure 5** Effect of pVHL on HIF $\alpha$  stability and ODD domain function. **a**, Western analysis of HIF  $\alpha$ -subunit stability in cells lacking VHL (RCC4, 786-O) and stable transfectants expressing pVHL (+VHL). Cells were incubated in hypoxia (4 h), then moved to normoxia (time 0) with addition of cycloheximide (100  $\mu$ M) and harvested up to 80 min later. **b**, Representative functional assay of the HIF-1 $\alpha$  ODD domain. Hep3B or RCC4 cells were transfected with Gal4 reporter pUAS-tk-Luc, and either pGalVP16 (upper panel) encoding the Gal4 DNA-binding domain fused to the VP16 activation domain, or pGal $\alpha$ 344-698VP16 (lower panel) which includes

HIF-1 $\alpha$  amino acids 344–698 (containing the entire ODD domain<sup>6</sup>). RCC4 cells were co-transfected with pcDNA3 (–), pcDNA3-VHL (VHL) or pcDNA3-VHL.103FS (TrVHL). After transfection, cells were divided for 24 h incubation in normoxia (N) or hypoxia (H; 0.1% O<sub>2</sub>). Corrected luciferase counts are shown, normalized to the normoxic value with pGalVP16 or pGalVP16+pcDNA3. The HIF-1 $\alpha$  domain confers suppression and hypoxic regulation in Hep3B cells but not RCC4 cells, where re-expression of pVHL restores these properties.



HIF-1. Positive and negative effects on HIF-1 activation by iron chelators, hydrogen peroxide and redox active agents have indicated that the underlying oxygen sensing mechanism may involve oxygen-dependent generation of partially reduced oxygen radical species by redox active iron centre(s)<sup>2,6,7,15,26</sup>. Such system(s) could act distantly on a transduction pathway or might directly modify the ODD domains of HIF $\alpha$  through a caged radical system, as proposed for iron sensing in the regulation of IRP2 (ref. 27). Cobaltous ions activate HIF-1 in inverse relation to iron availability, indicating that there may be competition for incorporation into a metal centre<sup>28</sup>.

Our data support a model in which VHL/HIF complexes form in normoxic cells and target HIF $\alpha$  subunits for destruction. In hypoxia, degradation is suppressed despite complex formation, perhaps because a critical targeting modification of the HIF $\alpha$  ODD domain cannot occur without oxygen. Cobaltous ions and desferrioxamine prevent formation of the VHL complex, providing a different mechanism for stabilization of HIF-1 and potentially explaining why activation by these stimuli is relatively resistant to oxygen and certain other radical-generating processes<sup>6,16,29</sup>. The iron dependence of complex formation could indicate that an iron-containing protein is an essential component of the complex, perhaps involved in local generation of an oxygen-sensing signal. □

## Methods

**Cells and transfections.** 786-O cells expressing pVHL, truncated pVHL (amino acids 1–115), or empty vector<sup>17</sup> were a gift from W. G. Kaelin. RCC4 cells were a gift from C. H. C. M. Buys. Other RCC lines were provided by M. Lerman. HeLa and Hep3B cells were from ECACC. RCC4/VHL was obtained by transfection with pCDNA3-VHL and G418 selection. Cells were plated in medium lacking G418 24 h before experiments, which were performed on 75 cm<sup>2</sup> dishes approaching confluence. Proteasomal inhibition was with 100  $\mu$ M calpain inhibitor I and 10  $\mu$ M N-carbobenzoxyl-L-leucyl-L-norvalinal. Transient transfections were by electroporation. Transfected cells were split for parallel normoxic and hypoxic incubation (Napco 7001, Precision Scientific). Luciferase reporter gene activity was corrected for transfection efficiency by assay of  $\beta$ -galactosidase expression from the co-transfected control plasmid pCMV- $\beta$ Gal.

**RNA analysis.** Total RNA was extracted and analysed by ribonuclease protection. Riboprobe details are given in Table S1 of the Supplementary Information.

**Plasmid constructions.** pCDNA3-VHL contained nucleotides 214–855 of GenBank accession no. L15409 in pcDNA3 (Invitrogen). pcDNA3-VHL.103FS was made using site-directed mutagenesis to delete nucleotides 522–523. HRE reporter genes were based on pGL3-basic (Promega) or pPUR (Clontech) and contained either a minimal SV40 promoter or a minimal (–40 bp) thymidine kinase promoter linked to a firefly luciferase gene (details of HREs are in Supplementary Information S3). pGalVP16 encoded the Gal4 DNA-binding domain (amino acids 1–147) linked in-frame to the activation domain (amino acids 410–490) from herpes simplex virus protein 16; pGal $\alpha$ 344–698VP16 encoded the indicated amino acids of HIF-1 $\alpha$  between those domains. Plasmid pUAS-tk-Luc contained two copies of the Gal4 binding site linked to a thymidine-kinase-promoted luciferase reporter gene.

**Cell lysis, immunoblotting and immunoprecipitation.** Whole cell extracts were prepared by homogenization in denaturing conditions and aliquots immunoblotted for HIF  $\alpha$ -subunits with 28b (anti-HIF-1 $\alpha$ ) and 190b (anti-HIF-2 $\alpha$ ) as described<sup>16</sup>, or using clone 54 (anti-HIF-1 $\alpha$ , Transduction Laboratories). For immunoprecipitation, lysis was performed in 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub> and 1 mM sodium orthovanadate with aprotinin (10  $\mu$ g ml<sup>-1</sup>), 'Complete' protease inhibitor (Boehringer) and 1.0 mM 4-(2-aminoethyl)benzene sulphonyl fluoride for 30 min on ice. After clearance by centrifugation, 120  $\mu$ g aliquots of lysate were incubated for 2 h at 4 °C with 4  $\mu$ g affinity-purified anti-HIF-2 $\alpha$  polyclonal antibodies (raised against a bacterially expressed fusion protein including amino acids 535–631) or 4  $\mu$ g ammonium sulphate precipitated anti-HIF-1 $\alpha$  polyclonal antibodies (raised against an immunogen including amino acids 530–652) in parallel with normal rabbit immunoglobulin (control), or alternatively with 0.7  $\mu$ g anti-pVHL antibody (IG32, Pharmingen) or control (antibody to SV40 T antigen, pAb419, a gift from E. Harlow or antibody to

VEGF, VG-7be, a gift from H. Turley). 10  $\mu$ l conjugated agarose beads pre-blocked with 20 mg ml<sup>-1</sup> BSA was added and lysates incubated for 2 h with rocking. Pellets were washed five times, eluted with sample buffer, and divided into 2–6 aliquots for immunoblotting.

**Electrophoretic mobility shift and supershift assays.** We prepared nuclear extracts using a modified Dignam protocol and incubated 5  $\mu$ g (HeLa) or 7.5  $\mu$ g (RCC4) with a <sup>32</sup>P-labelled 24-bp oligonucleotide probe (sense strand; 5'-GCCCTACTGCTGCTGCCTCGCATGGC-3') from the mouse Epo 3' enhancer as described<sup>25</sup>. For supershift assays, 0.5  $\mu$ g IG32, VG-7be (isotype and subclass matched control for IG32) or clone 54 (anti-HIF-1 $\alpha$ ) was added and reactions were incubated for 4 h at 4 °C before electrophoresis. *In vitro* transcription translations of pCDNA3-VHL and pCDNA3-VHL.103FS were done using reticulocyte lysate (Promega); 1  $\mu$ l of a 1:5 dilution in PBS was added to binding reactions 2 h before electrophoresis or addition of antibody.

Received 11 March; accepted 13 April 1999.

- Wang, G. L., Jiang, B.-H., Rue, E. A. & Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc. Natl Acad. Sci. USA* **92**, 5510–5514 (1995).
- Bunn, H. F. & Poyton, R. O. Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885 (1996).
- Carmeliet, P. et al. Role of HIF-1 $\alpha$  in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**, 485–490 (1998).
- An, W. G. et al. Stabilization of wild-type p53 by hypoxia-inducible factor 1 $\alpha$ . *Nature* **392**, 405–408 (1998).
- Huang, L. E., Gu, J., Schau, M. & Bunn, H. F. Regulation of hypoxia-inducible factor 1 $\alpha$  is mediated by an oxygen-dependent domain via the ubiquitin-proteasome pathway. *Proc. Natl Acad. Sci. USA* **95**, 7987–7992 (1998).
- Goldberg, M. A., Dunning, S. P. & Bunn, H. F. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**, 1412–1415 (1988).
- Wang, G. L. & Semenza, G. L. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* **82**, 3610–3615 (1993).
- Warburg, O. *The Metabolism of Tumours* (Arnold Constable, London, 1930).
- Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353–364 (1996).
- Shweiki, D., Itin, A., Soffer, D. & Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843–845 (1992).
- Kaelin, W. G. & Maher, E. R. The VHL tumour-suppressor gene paradigm. *Trends Genet.* **14**, 423–426 (1998).
- Gnarra, J. R. et al. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. *Proc. Natl Acad. Sci. USA* **93**, 10589–10594 (1996).
- Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G. Jr & Goldberg, M. A. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc. Natl Acad. Sci. USA* **93**, 10595–10599 (1996).
- Dang, C. V. & Semenza, G. L. Oncogenic alterations of metabolism. *Trends Biochem. Sci.* **24**, 68–72 (1999).
- Huang, L. E., Arany, Z., Livingston, D. M. & Bunn, H. F. Activation of hypoxia-inducible transcription factor depends primarily on redox-sensitive stabilization of its  $\alpha$  subunit. *J. Biol. Chem.* **271**, 32253–32259 (1996).
- Wiesener, M. S. et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1 $\alpha$ . *Blood* **92**, 2260–2268 (1998).
- Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W. G. Jr Tumour suppression by the human von Hippel-Lindau gene product. *Nature Med.* **1**, 822–826 (1995).
- Salceda, S. & Caro, J. Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. *J. Biol. Chem.* **272**, 22642–22647 (1997).
- Pause, A. et al. The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. *Proc. Natl Acad. Sci. USA* **94**, 2156–2161 (1997).
- Loneragan, K. M. et al. Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. *Mol. Cell Biol.* **18**, 732–741 (1998).
- Maxwell, P. H. et al. Hypoxia inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc. Natl Acad. Sci. USA* **94**, 8104–8109 (1997).
- Zelzer, E. et al. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1 $\alpha$ /ARNT. *EMBO J.* **17**, 5083–5094 (1998).
- Duan, D. R. et al. Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* **269**, 1402–1406 (1995).
- Ohh, M. et al. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol. Cell* **1**, 959–968 (1998).
- Wood, S. M. et al. Selection and analysis of a mutant cell line defective in the hypoxia-inducible factor-1  $\alpha$ -subunit (HIF-1 $\alpha$ ). *J. Biol. Chem.* **273**, 8360–8368 (1998).
- Fandrey, J., Frede, S. & Jelkmann, W. Role of hydrogen peroxide in hypoxia-induced erythropoietin production. *Biochem. J.* **303**, 507–510 (1994).
- Iwai, K. et al. Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. *Proc. Natl Acad. Sci. USA* **95**, 4924–4928 (1998).
- Ho, V. T. & Bunn, H. F. Effects of transition metals on the expression of the erythropoietin gene: further evidence that the oxygen sensor is a heme protein. *Biochem. Biophys. Res. Commun.* **223**, 175–180 (1996).
- Chandel, N. S. et al. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc. Natl Acad. Sci. USA* **95**, 11715–11720 (1998).
- Gnarra, J. R. et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nature Genet.* **7**, 85–90 (1994).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

**Acknowledgements.** We thank W. Kaelin, C. Buys and N. Lerman for cell lines, and N. Proudfoot, A. Harris, D. Gillespie, J. O'Rourke, Y.-M. Tian and L. Nicholls. Financial support was from the Wellcome Trust, the Barnes Trust, the Deutsche Forschungsgemeinschaft, the Cancer Research Campaign, Action Research and the Medical Research Council.

Correspondence and requests for materials should be addressed to P.J.R. (e-mail: peter.ratcliffe@imm.ox.ac.uk).