

# Mutation analysis of HIF prolyl hydroxylases (*PHD/EGLN*) in individuals with features of pheochromocytoma and renal cell carcinoma susceptibility

Dewi Astuti<sup>1,2\*</sup>, Christopher J Ricketts<sup>1,2\*</sup>, Rasheduzzaman Chowdhury<sup>3</sup>, Michael A McDonough<sup>3</sup>, Dean Gentle<sup>1,2</sup>, Gail Kirby<sup>1,2</sup>, Susanne Schlisio<sup>4,5</sup>, Rajappa S Kenchappa<sup>6</sup>, Bruce D Carter<sup>6</sup>, William G Kaelin Jr<sup>4</sup>, Peter J Ratcliffe<sup>7</sup>, Christopher J Schofield<sup>3</sup>, Farida Latif<sup>1,2</sup> and Eamonn R Maher<sup>1,2,8</sup>

<sup>1</sup>Centre for Rare Diseases and Personalised Medicine, University of Birmingham, Birmingham B15 2TT, UK

<sup>2</sup>CRUK Renal Molecular Oncology Group, Medical and Molecular Genetics, School of Clinical and Experimental Medicine, Institute of Biomedical Research, University of Birmingham College of Medical and Dental Sciences, Birmingham B15 2TT, UK

<sup>3</sup>Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Mansfield Road, Oxford OX1 3TA, UK

<sup>4</sup>Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Brigham and Women's Hospital, 44 Binney Street, Boston, Massachusetts 02115, USA

<sup>5</sup>Oxygen Sensing and Cancer Laboratory, Ludwig Institute for Cancer Research Ltd, Karolinska Institute, Nobelsvag 3, SE-171 77 Stockholm, Sweden

<sup>6</sup>Department of Biochemistry and Center for Molecular Neuroscience, Vanderbilt University Medical School, Nashville, Tennessee 37232, USA

<sup>7</sup>Henry Wellcome Building for Molecular Physiology, University of Oxford, Oxford OX3 9DU, UK

<sup>8</sup>West Midlands Regional Genetics Service, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK

(Correspondence should be addressed to E R Maher, Centre for Rare Diseases and Personalised Medicine, Institute of Biomedical Research, University of Birmingham School of Medicine, Birmingham B15 2TT, UK; Email: e.r.maher@bham.ac.uk)

\*(D Astuti and C J Ricketts contributed equally to this work)

## Abstract

Germline mutations in the von Hippel–Lindau disease (*VHL*) and succinate dehydrogenase subunit B (*SDHB*) genes can cause inherited pheochromocytoma and/or renal cell carcinoma (RCC). Dysregulation of the hypoxia-inducible factor (HIF) transcription factors has been linked to *VHL* and *SDHB*-related RCC; both HIF dysregulation and disordered function of a prolyl hydroxylase domain isoform 3 (*PHD3/EGLN3*)-related pathway of neuronal apoptosis have been linked to the development of pheochromocytoma. The 2-oxoglutarate-dependent prolyl hydroxylase enzymes *PHD1* (*EGLN2*), *PHD2* (*EGLN1*) and *PHD3* (*EGLN3*) have a key role in regulating the stability of HIF- $\alpha$  subunits (and hence expression of the HIF- $\alpha$  transcription factors). A germline *PHD2* mutation has been reported in association with congenital erythrocytosis and recurrent extra-adrenal pheochromocytoma. We undertook mutation analysis of *PHD1*, *PHD2* and *PHD3* in two cohorts of patients with features of inherited pheochromocytoma ( $n=82$ ) and inherited RCC ( $n=64$ ) and no evidence of germline mutations in known susceptibility genes. No confirmed pathogenic mutations were detected suggesting that mutations in these genes are not a frequent cause of inherited pheochromocytoma or RCC.

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## Introduction

Germline mutations in the von Hippel–Lindau (*VHL*) tumour suppressor gene and in the B, C and D subunits of succinate dehydrogenase (*SDHB*, *SDHC* and *SDHD*) are strongly linked with susceptibility to pheochromocytoma (Latif *et al.* 1993, Crossey *et al.* 1995, Woodward *et al.* 1997, Baysal *et al.* 2000, Gimm *et al.* 2000, Astuti *et al.* 2001a,b, Neumann *et al.* 2002, Schiavi *et al.* 2005, Mannelli *et al.* 2007). In addition, germline mutations in *VHL*, *SDHB* and *SDHD* are associated with susceptibility to renal cell carcinoma (RCC), though the risk of RCC is about five times higher in *VHL* disease (Maher *et al.* 1990, Latif *et al.* 1993, Vanharanta *et al.* 2004, Ong *et al.* 2007, Ricketts *et al.* 2008, 2010). Several mechanisms have been implicated in the development of *VHL*-related and *SDHB/D*-related pheochromocytomas. The *VHL* tumour suppressor gene product (pVHL) has multiple functions (see Frew & Krek 2007 references within) but its best-characterised function is the ability to regulate proteasomal degradation of hypoxia-inducible factor (HIF)-1 $\alpha$  and HIF-2 $\alpha$  (Maxwell *et al.* 1999), and overexpression of HIF-2 $\alpha$  in a RCC cell line counteracts pVHL tumour suppressor activity (Kondo *et al.* 2003). Inactivation of *SDHB/D* has been linked to accumulation of succinate that inhibits the prolyl hydroxylase enzymes necessary for proteasomal degradation of HIF- $\alpha$  subunits (Selak *et al.* 2005); pheochromocytomas from patients with germline *VHL*, *SDHB* and *SDHD* mutations demonstrate up-regulation of HIF-1 $\alpha$  and HIF-2 $\alpha$  and their downstream targets (Pollard *et al.* 2006). Although HIF dysregulation and a pseudohypoxic state are features of both *VHL*- and *SDHB/D*-associated tumours, another potential mechanism for pheochromocytoma susceptibility in these disorders is a failure of normal prolyl hydroxylase domain isoform 3 (PHD3) (EGLN3)-dependent developmental apoptosis of sympathetic neuronal cells causing persistence of ‘pheochromocytoma precursor cells’ (Lee *et al.* 2005). This latter process has also been linked to other pheochromocytoma susceptibility disorders (multiple endocrine neoplasia type 2 and neurofibromatosis) (Lee *et al.* 2005).

In normoxia, the HIF-1 and HIF-2  $\alpha$ -subunits are rapidly ubiquitinated and targeted for proteasomal degradation by a pVHL containing E3 ubiquitin ligase complex (Maxwell *et al.* 1999). The oxygen-dependent interaction of pVHL with HIF- $\alpha$  is determined by the hydroxylation status of key HIF- $\alpha$  proline residues (Pro-402 and Pro-564 in HIF-1 $\alpha$ ) (Ivan *et al.* 2001, Jaakkola *et al.* 2001, Masson *et al.* 2001, Yu *et al.* 2001): non-hydroxylated HIF- $\alpha$  binds to pVHL

approximately a 100-fold less tightly than hydroxylated HIF- $\alpha$  (Chan *et al.* 2002). Thus in the presence of oxygen, HIF- $\alpha$  hydroxylation is catalysed by prolyl hydroxylases that are members of the egg-laying-defective nine (EGLN) family (Bruick & McKnight 2001, Epstein *et al.* 2001). Under hypoxic conditions, the rate of prolyl hydroxylation, and hence the rate of proteasomal degradation of HIF- $\alpha$ , slows because pVHL binds only very weakly to the HIF- $\alpha$  subunits leading to stabilisation of HIF-1 and HIF-2 heterodimeric transcription factors and activation of the array of genes involved in the hypoxic response. In humans, three *EGLN* homologues have been implicated in HIF- $\alpha$  modification: PHD1/EGLN2/HIFPH1, PHD2/EGLN1/HIFPH2 and PHD3/EGLN3/HIFPH3 (Bruick & McKnight 2001, Epstein *et al.* 2001). To date, germline mutations in *PHD2* have been associated with congenital polycythaemia and, in one case/family, recurrent extra-adrenal pheochromocytoma (Percy *et al.* 2006, 2007, Al-Sheikh *et al.* 2008, Ladroue *et al.* 2008). We hypothesised that patients with features of inherited susceptibility to pheochromocytoma and/or RCC might harbour germline mutations in *PHD1*, *PHD2* or *PHD3*. To test this hypothesis, we analysed two large patient cohorts without mutations in known susceptibility genes.

## Materials and methods

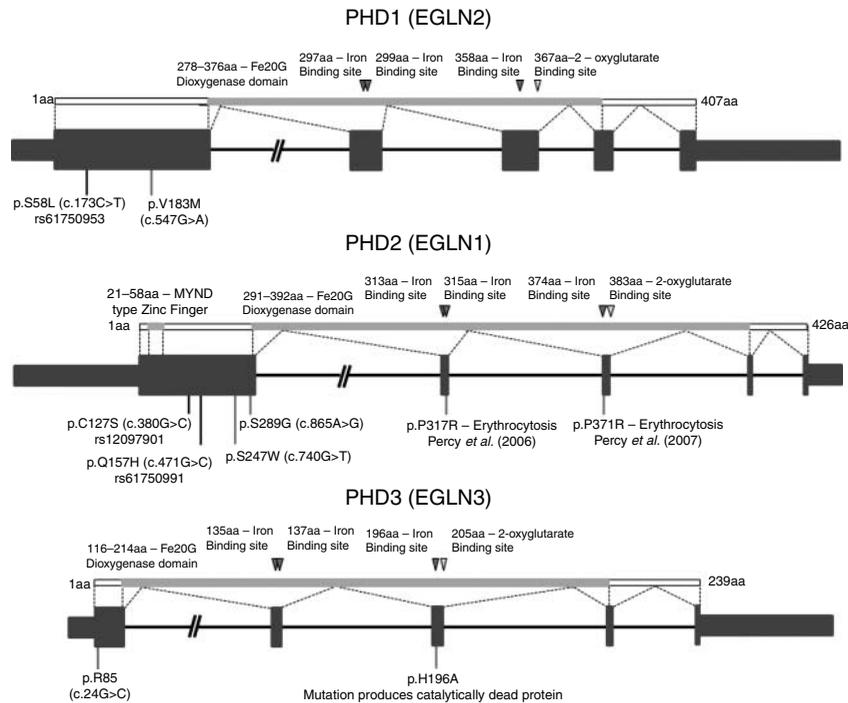
### Patients and samples

We analysed DNA from a) 82 individuals with features of inherited pheochromocytoma (extra-adrenal tumours in 23 cases) susceptibility (i.e. familial pheochromocytoma ( $n=6$ ), multiple tumours ( $n=30$ ) or young age at onset (diagnosed age 30 years or less, range 5–30 years, median 19 years ( $n=46$ )) but no detectable mutations in *VHL*, *SDHB*, *SDHD* or *RET* and b) 64 patients with features of RCC susceptibility (25 probands from familial RCC kindreds, 14 individuals with bilateral or multicentric RCC and 25 individuals with isolated unilateral early-onset RCC (diagnosis aged 40 years or less; range 17–40 years, median 28 years)) but no evidence of a germline mutation in a known RCC susceptibility gene (*VHL*, *SDHB*, *FH* and *FLCN*) (most cases had clear cell RCC) (Ricketts *et al.* 2008, Woodward *et al.* 2008).

### Molecular genetic analyses

*Mutation analysis of HIF prolyl hydroxylases (PHD/EGLN1–3)*

*PHD1/EGLN2* (exons 1–5), *PHD2/EGLN1* (exons 1–5) and *PHD3/EGLN3* (exons 1–5) (see Figure 1) were



**Figure 1** Schematic representation of the exon structure and location of genetic variants detected in PHD1, PHD2 and PHD3. The coding sequences for the *PHD* genes were obtained from Ensembl (<http://www.ensembl.org/index.html>), and protein domain annotations were obtained from the UniProt database (<http://www.uniprot.org/>).

examined by PCR–sequencing. DNA (50 ng) was preamplified using GenomiPhi DNA amplification kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer’s instructions, and 0.2 µl of GenomiPhi amplified DNA was used for PCR amplification. The PCR conditions were as follows: 95 °C for 15 min followed by 35 cycles of 95 °C, 55–58 °C and 72 °C for 30 s each and then 72 °C for 10 min. PCR was performed using HotStarTaq DNA polymerase (Qiagen). PCR and sequencing were repeated in the original DNA if sequence variation was observed in the GenomiPhi amplified DNA. Primer sequences for exons amplification are available upon request.

#### *CpG methylation analysis of PHD3*

*PHD3* CpG island methylation analysis was performed using combined bisulphite and restriction digest analysis (CoBRA). Sodium bisulphite modification of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. *PHD3* CpG island was amplified by nested PCR using primers: *PHD3*CoF1 5′-TTTTGGTTATAAATAATTAAGGTTGGYGTGGGT-3′; *PHD3*CoR1 5′-AATAACAAAACCCACCTCRTACAAACAAA-3′; *PHD3*CoF2 5′-TTYGGAGGYGGTTTYGGGGYGGAGTT-3′; *PHD3*CoR2 5′-AAAACATCTCRCCRCAAAATCRAAA-3′.

All amplification was done using HotStarTaq DNA polymerase (Qiagen).

#### **Functional analyses of the PHD3-Arg8Ser mutation**

##### *Plasmid construction*

The plasmid encoding wild-type *PHD3/EGLN3* (pcDNA3-*PHD3*) has been described previously (Lee *et al.* 2005). Mutant *PHD3/EGLN3* Arg8Ser was generated using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) with primers 5′-ACACATCATGAGCCTGGACCTGGAG-3′ (forward) and 5′-CTCCAGGTCCAGGCTCATGATGTGT-3′ (reverse) and verified by DNA sequencing.

##### *PHD3 induced apoptosis assays*

Two different apoptosis assays were performed; an initial method to demonstrate differences and a second, more sensitive method. The first apoptosis assay was performed essentially as described by Lee *et al.* (2005). Undifferentiated rat PC12 cells were plated onto collagen-coated 6-well plates and were cotransfected the following day with 500 ng of GFP histone and either 1 µg of wild-type *PHD3*, *PHD3*-Arg8Ser, *PHD3*-His196Ala (catalytic dead mutant) or pcDNA3. Transfection was carried out using Lipofectamine 2000

(Invitrogen). Scoring of apoptotic cells (characterised as having condensed or fragmented nuclei) was done 72 h after transfection. Approximately, 400 cells were scored for each sample. Samples were blinded and assays were performed in duplicate.

For the second apoptosis assay, sympathetic neurons were isolated from the superior cervical ganglia (SCG) as previously described (Palmada *et al.* 2002). Briefly, SCG from Sprague–Dawley rats were isolated at postnatal day 4 (P4), and sympathetic neurons were dissociated with 0.25% trypsin and 0.3% collagenase for 30 min at 37 °C. After dissociation, the neurons were then cotransfected with GFP and either wild-type PHD3, PHD3-Arg8Ser, PHD3-His196Ala or pcDNA3 by electroporation on an Amaxa Nucleofector device as described previously (Kenchappa *et al.* 2006). Forty-eight hours later, cells were fixed in 4% paraformaldehyde (PFA) and stained with DAPI (Vector Laboratories, Peterborough, UK). The number of GFP-positive neurons with apoptotic or non-apoptotic nuclei was counted and ~50–80 neurons were evaluated for each sample. The assay was performed in triplicate with three different electroporations using different rat litters.

### Statistical analysis

Results for the apoptosis assays were compared using two-tailed Student's *t*-tests.

## Results

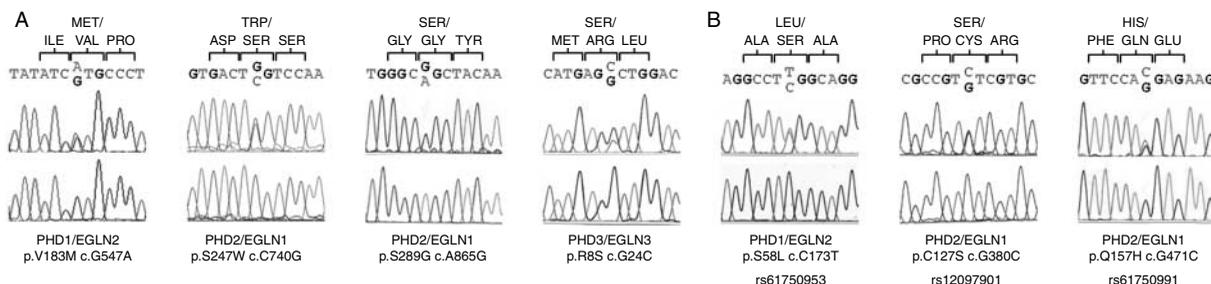
### Analysis of PHD3

The five exons and flanking sequences of *PHD3* were sequenced in a) 82 patients with features of non-syndromic inherited pheochromocytoma susceptibility (i.e. familial pheochromocytoma, multiple tumours or young age at onset) but no detectable mutations in *VHL*, *SDHB*, *SDHD* or *RET*, b) 22 sporadic pheochromocytoma and c) 64 patients with features

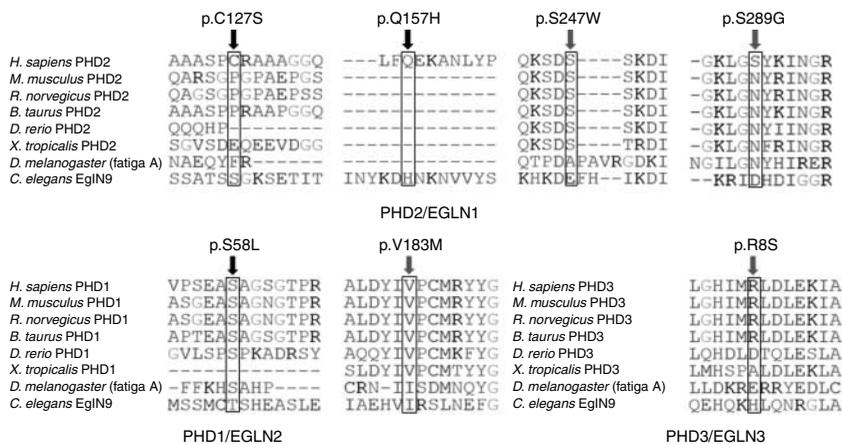
of RCC susceptibility. Sequence variants identified in patients were then tested for in normal controls.

A germline missense substitution, c.24G>C (p.Arg8Ser), was detected in 1 of 82 patients with features of non-syndromic inherited pheochromocytoma susceptibility (see Fig. 2A) (but not in 222 control chromosomes). The substitution was predicted to be benign and tolerated by bioinformatic analysis with the Polyphen and SIFT programs, and occurred in the N-terminal region of the protein which, by analogy with structural and biochemical work on the catalytic domain of PHD2 (residues 181–426, tPHD2 hereafter), is unlikely to be directly involved in catalysis (McDonough *et al.* 2006, Flashman *et al.* 2008, Chowdhury *et al.* 2009; see Figs 1, 3 and Table 1). The female patient had bilateral adrenal pheochromocytomas at age 22. She showed no clinical evidence of a known familial susceptibility syndrome and no family history of pheochromocytoma. The missense substitution was not detected in her clinically unaffected father and sister, but it was not possible to determine whether it had arisen *de novo* because no DNA was available from her mother who had died from an unrelated cause. No novel sequence variants were detected in the 64 patients with RCC susceptibility.

Previously, wild-type *PHD3* has been shown to induce apoptosis of rat pheochromocytoma (PC12) cells and rat sympathetic neurons. To investigate whether the *PHD3* p.Arg8Ser missense substitution might alter pro-apoptotic activity, PC12 cells and rat sympathetic neurons were transfected with either wild-type *PHD3*, p.Arg8Ser mutant *PHD3*, empty vector control or a catalytically inert p.His196Arg *PHD3* mutant (Lee *et al.* 2005, Schlisio *et al.* 2008). For the PC12 cells, a baseline apoptosis rate of 2–4% was seen with the empty vector and the inert mutant *PHD3* were associated with an increase in apoptosis



**Figure 2** Sequence chromatographs of variants of PHD1, PHD2 and PHD3. (A) Novel variants of PHD1, PHD2 and PHD3 with coding region and amino acid changes. (B) Known variants of PHD1 and PHD2 with SNPdb reference (<http://www.ncbi.nlm.nih.gov/projects/SNP>).



**Figure 3** Conservation of variants of PHD1, PHD2 and PHD3. Conservation of the *PHD* genes performed using sequences obtained from Ensembl (<http://www.ensembl.org/index.html>) and aligned using clustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

(12.7 and 14% respectively) (data not shown). For the more sensitive rat sympathetic neuron assay, a similar baseline apoptosis rate of 3–4% was seen with the empty vector and the inert mutant control. Again wild-type and p.Arg8Ser mutant PHD3 were associated with an increase in apoptosis (30.6 and 22.8% respectively) that was highly statistically significant compared to the catalytically inert mutant ( $P=0.0001$  and  $P=0.0003$ ), though the p.Arg8Ser mutant demonstrated a mildly, yet statistically significant, lower level of apoptosis ( $P=0.016$ ) than that of the wild type (see Fig. 4).

We also analysed up to 22 sporadic pheochromocytomas for somatic mutations and for *de novo* promoter region methylation of PHD3. No somatic mutations were detected in 22 tumours, and analysis of a *PHD3* 5' CpG island (nt –345 to –160 relative to the ATG start codon; NM 022073.2 Genome Browser on Human, May 2004 assembly) by CoBRA and bisulphite sequencing in 17 sporadic pheochromocytoma tumours demonstrated partial promoter methylation (that was not detected in matched normal (blood) tissue) in only one of 17 tumours analysed.

### Analysis of *PHD2*

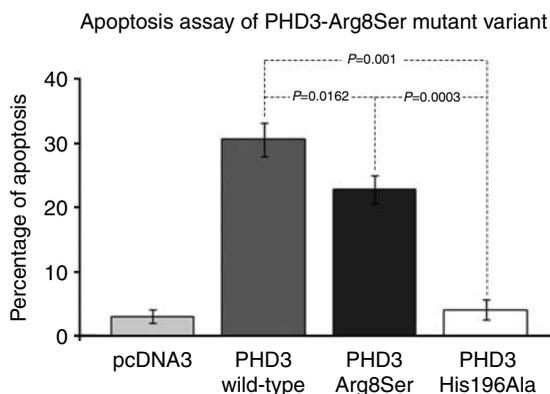
Of 82 patients with features of pheochromocytoma susceptibility, 10 patients (12.2%) had a germline non-synonymous missense substitution, c.471G>C (p.Q157H) (see Fig. 2B), but this was also detected in 3/111 (2.7%) of normal controls ( $P=0.017$ ).

A novel missense p.Ser247Trp (c.740 C>G) substitution was detected in a patient with unilateral RCC at age 55 years and a contralateral RCC at age 56 years (see Fig. 2A). The substitution was not detected in 222 chromosomes from normal subjects and occurred in a region of the protein with no predicted function (see Fig. 1). Bioinformatic analysis with the Polyphen and SIFT programs suggests that this is possibly damaging and intolerant, and the S247 residue is conserved down to *Danio rerio* and *Xenopus tropicalis*, but not to *Drosophila melanogaster* or *Caenorhabditis elegans* (Fig. 3 and Table 1). However, there were no relatives with RCC to test segregation in the family.

Mutation analysis of 22 sporadic pheochromocytomas revealed a novel missense variant (p.Ser289Gly; c.865A>G) in a single tumour (see Fig. 2A). Germline

**Table 1** Bioinformatic analysis of the *PHD* genes using the PolyPhen and SIFT programs (<http://genetics.bwh.harvard.edu/pph/> and <http://sift.jcvi.org/>)

Genes	AA change	PolyPhen	SIFT		
<i>PHD1</i>	S58L	1.773	Possibly damaging	0.03	Intolerant
<i>PHD1</i>	V183M	1.361	Benign	0.02	Intolerant
<i>PHD2</i>	C127S	–	Benign	0.00	Intolerant
<i>PHD2</i>	Q157H	1.538	Possibly damaging	0.00	Intolerant
<i>PHD2</i>	S247W	1.529	Possibly damaging	0.03	Intolerant
<i>PHD2</i>	S289G	0.376	Benign	0.20	Tolerant
<i>PHD3</i>	R8S	1.297	Benign	1.00	Tolerant



**Figure 4** Apoptosis assay of PHD3-Arg8Ser mutant variant. Rat sympathetic neurons were cotransfected with GFP and with either wild-type PHD3, PHD3-Arg8Ser, PHD3-His196Ala or pcDNA3. The number of GFP-positive neurons with apoptotic nuclei was counted, and ~70–100 neurons were evaluated for each sample from three separate experiments. Error bars demonstrate s.d., and *P* values from Student's *t*-tests are shown.

DNA was not available, and the substitution was not detected in 222 chromosomes from normal subjects. Structurally informed bioinformatic analysis with the Polyphen and SIFT programs suggested that this substitution is likely to be a benign variant, and Ser289 residue is not conserved in rodents or in human PHDs (see Fig. 3 and Table 1). Crystallographic analyses (Chowdhury et al. 2009) reveal that Ser289 is not involved with the active site (see Figs 1 and 5).

### Analysis of PHD1

Mutation analysis of *PHD1* in 82 patients with evidence of inherited pheochromocytoma susceptibility revealed a novel non-synonymous p.Ser58Leu (c.173C>T) germline missense substitution (see Fig. 2B) in 5 cases (6%) and 3/116 (2.6%) normal controls (*P*=0.29).

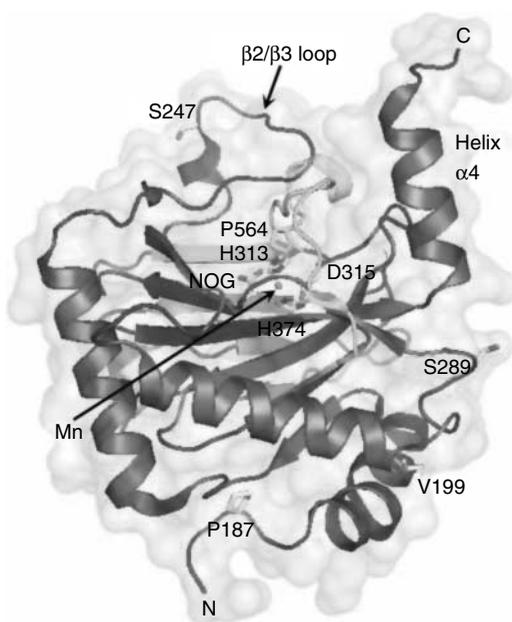
A novel missense p.Val183Met (c.547 G>A) substitution was detected in a patient with familial RCC (see Fig. 2A). Both the proband and her father had been affected with RCC in the sixth decade, but germline DNA was not available from other relatives. The substitution was not detected in 222 chromosomes from normal subjects, and bioinformatic analysis gave conflicting results with Polyphen suggested that this was a benign substitution but the SIFT program reporting it to be intolerant (see Table 1). Val183 is conserved down to *D. rerio*, but not to *D. melanogaster* or *C. elegans* (see Fig. 3).

No potential pathogenic variants were detected in 22 sporadic pheochromocytoma tumours.

## Discussion

We did not find any evidence that germline mutations in *PHD1*, *PHD2* or *PHD3* are frequent causes of inherited susceptibility to pheochromocytoma or RCC. Thus, although Ladroue et al. (2008) reported a patient with recurrent paraganglioma and a germline *PHD2* missense mutation (c.1121A→G, p.H374R, with loss of the wild-type allele in paraganglioma tissue), our findings suggest that *PHD2* mutations are rare in patients with features of inherited pheochromocytoma (95% confidence interval 0–4.4%). *PHD2* mutations were initially described in association with familial erythrocytosis (Percy et al. 2006, 2007, Al-Sheikh et al. 2008) and that the patient reported by Ladroue et al. (2008) had both recurrent paraganglioma and congenital erythrocytosis. Although it might be hypothesised that germline *PHD2* mutations might be restricted to pheochromocytoma patients with congenital erythrocytosis, we did not identify a *PHD2* mutation in a patient with early onset pheochromocytoma and erythrocytosis.

All three PHD isoforms regulate both HIF-1 $\alpha$  and HIF-2 $\alpha$ , but differ in expression patterns, specificities for the two different prolyl hydroxylation sites within the HIF-1 $\alpha$  and 2 $\alpha$  subunits, and a degree of selectivity between HIF- $\alpha$  isoforms (Appelhoff et al. 2004, Raval et al. 2005). *PHD2* appears to be the major regulator of HIF- $\alpha$  prolyl hydroxylation in normoxia and mild hypoxia, and we postulated that *PHD2* mutations (that led to HIF dysregulation) might present with inherited RCC (previously, a similar approach had revealed unsuspected germline *SDHB* and *FLCN* mutations in ~10% of patients with apparently non-syndromic inherited RCC (Ricketts et al. 2008, Woodward et al. 2008)). Although we detected a novel *PHD2* missense variant (c.840 C>G, p.S247W) in a proband with two independent RCC, the significance of this finding is uncertain. While the absence of the variant in 222 chromosomes from normal subjects and bioinformatic analysis were suggestive of pathogenicity (the S247 residue is conserved in *D. rerio*), we are unable to prove a loss of function effect. S247 is located in the variable region (variable with respect to *PHD1*, *PHD2* and *PHD3* sequences) between strands  $\beta$ 2 and  $\beta$ 3 (referred to as 'β2β3 loop') in the published crystal structures of *PHD2* (McDonough et al. 2006, Chowdhury et al. 2009). Subsequent biophysical analyses have shown that this region is flexible and involved in determining the prolyl hydroxylation site selectivity of the PHDs (Villar et al. 2007, Flashman et al. 2008, Chowdhury et al. 2009). However, a point mutation at this position (Ser289Lys) that could be



**Figure 5** Ribbon representation of tPHD2.CODD complex structure showing the germline mutation sites (highlighted in white) in PHD2 (Chowdhury *et al.* 2009). The tPHD2 fold comprises four  $\alpha$  helices and ten  $\beta$  strands of which eight strands form a double-stranded  $\beta$  helix (DSBH) (McDonough *et al.* 2006, Chowdhury *et al.* 2009). The recognition of HIF- $\alpha$  by PHDs involves interactions that are discrete from the hydroxylation site involving the  $\beta$ 2/ $\beta$ 3 loop and the C-terminal  $\alpha$ 4 helix among others. Although the  $\beta$ 2/ $\beta$ 3 loop of PHD2 makes hydrophobic contacts with the conserved LXXLAP motif, residue S247 is located on a part of  $\beta$ 2/ $\beta$ 3 loop that does not interact with HIF- $\alpha$ . The figure also shows that residues S289, V199 (analogous to V183 in PHD1) and P187 (analogous to R8 in PHD3) are far from the HIF- $\alpha$ -binding sites or the catalytic Fe/2OG-binding motifs in PHD2.

produced in the soluble form was as active as wild-type PHD2 (in studies using recombinant tPHD2) with respect to hydroxylation of the peptide fragments of the N- and C-terminal oxygen-dependent degradation domains of HIF-1 $\alpha$  when these were tested either separately or simultaneously in competition revealing that (at least) under standard incubation conditions, mutations at Ser289 are unlikely to affect absolute activity or selectivity with respect to the hydroxylation sites on HIF- $\alpha$  (data not shown).

Distinguishing mutations from non-pathogenic sequence variants and predicting the functional effect of rare missense variants on protein function can be challenging, but while the pathogenicity of the p.S247W missense substitution cannot be unequivocally established or disproved, our results do suggest that germline *PHD2* mutations are not a frequent cause of inherited RCC. Similarly, a possible *PHD1* missense mutation (p.Val183Met) was detected in only one

proband with features of non-syndromic inherited RCC. While this variant was not detected in >200 control chromosomes and the V183 residue is conserved in *D. rerio*, bioinformatic predictions of likely pathogenicity (Polyphen and SIFT programs) were equivocal and the substitution occurs outside of the prolyl hydroxylation catalytic domain (AA 189–375).

Functional studies of mutant pVHL protein associated with differing VHL disease phenotypes (e.g. with differential risks of RCC and pheochromocytoma) suggest that whereas impairment of the ability to regulate HIF is necessary for predisposition to RCC, it is not sufficient, and impairment of non-HIF-related pVHL functions is also required (Clifford *et al.* 2001). Hence, mutations that lead only to dysregulation of HIF (e.g. germline mutations in *PHDs*, *HIF-1* or *HIF-2* genes) might not be sufficient to cause an inherited RCC phenotype. Interestingly, functional analysis of pVHL mutants has also suggested that some VHL mutations associated with a high risk of pheochromocytoma do not compromise pVHL regulation of HIF, so implicating HIF-independent mechanisms in the development of VHL-associated pheochromocytoma (Clifford *et al.* 2001, Hoffman *et al.* 2001). Consistent with this hypothesis, Lee *et al.* (2005) described a HIF-independent pathway linked to inherited causes of pheochromocytoma (including those associated with germline *VHL*, *SDHB* and *SDHD* mutations). They suggested that inherited pheochromocytomas originate from sympathetic neuronal precursor cells that usually undergo c-Jun-dependent apoptosis during embryogenesis when growth factors such as nerve growth factor (NGF) become limiting (Estus *et al.* 1994, Schlingensiepen *et al.* 1994, Ham *et al.* 1995, Xia *et al.* 1995). Germline *VHL* and *SDHB* mutations compromise this pathway leading to a failure of normal developmental apoptosis and survival of cells that would give rise to pheochromocytoma. Intriguingly, *PHD3* (but not *PHD1* or *PHD2*) is a key mediator of this apoptotic pathway, and germline mutations in *KIF1BBeta* (that encodes a target of *PHD3/EGLN3*) have been associated with familial susceptibility to neural crest tumours (Schlisio *et al.* 2008). Furthermore, *PHD3* knockout mice demonstrated increased numbers of cells in the superior cervical ganglion, adrenal medulla and carotid body (Bishop *et al.* 2008). However, we identified only one potential *PHD3* mutation in our cohort of inherited pheochromocytoma probands and, although the missense variant was not detected in normal controls, we did not detect any evidence that the p.Arg8Ser impaired neuronal apoptosis to a substantial degree (as compared to a catalytically inert mutant). Although the

partial loss of functional effect of the p.Arg8Ser mutant in the sensitive rat sympathetic neuron apoptosis assay suggests that the variant might contribute to pheochromocytoma susceptibility, we suggest that germline *PHD3* mutations are not a frequent cause of inherited pheochromocytoma. Germline mutations in both *VHL* and *SDHB/D* mutations are important causes of familial pheochromocytoma; however, somatic mutations of these genes are infrequent in sporadic tumours (Eng et al. 1995, Astuti et al. 2001a,b). Hence, we concentrated our efforts on testing for germline *PHD1*, *PHD2* and *PHD3* mutations in patients with features of inherited pheochromocytoma. In our preliminary analysis of sporadic pheochromocytoma, we did not find any frequent evidence for somatic inactivation of *PHD1*, *PHD2* or *PHD3*. In contrast to pheochromocytoma, somatic VHL mutations are very frequent in sporadic RCC (Foster et al. 1994, Gnarr et al. 1994, Banks et al. 2006) (though somatic SDHB inactivation is rare in RCC (Morris et al. 2004)). However, large-scale resequencing of RCC samples ( $n=101$ ) did not detect mutations in *PHD1*, *PHD2* or *PHD3* (Dalglish et al. 2010), and we have not detected frequent evidence of up-regulation of any of the three prolyl hydroxylase genes in RCC cell lines treated with 5-azacytidine (Morris et al. 2010 and unpublished studies). The absence of germline *PHD1*, *PHD2* or *PHD3* mutations in inherited pheochromocytoma or RCC might suggest that inactivation of one of these genes is insufficient to replicate the range of pro-oncogenic effects associated with pathogenic germline *VHL* or *SDHB/D* mutations. pVHL has been reported to have multiple functions (Frew & Krek 2007), and while inactivation of SDH has been linked to accumulation of succinate and resulting inhibition of prolyl hydroxylase enzymes that are necessary for proteasomal degradation of HIF- $\alpha$  subunits (Selak et al. 2005), succinate accumulation may also inhibit other 2-oxoglutarate-dependent enzyme superfamily members which may also contribute to the development of SDHB/D-associated tumours (e.g. histone demethylases, some of which are implicated in the epigenetic regulation of oncogenes and tumour suppressor genes) (Smith et al. 2008). Nevertheless, a significant fraction of patients with features of inherited pheochromocytoma or RCC do not have a detectable mutation in a known susceptibility gene. The advent of comprehensive genomic analysis techniques (e.g. whole exome resequencing) (Kryukov et al. 2009, Summerer et al. 2010) should expedite the identification of novel pheochromocytoma/RCC predisposition genes and so provide further insights into the mechanism of tumourigenesis in these disorders.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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