NIH Public Access
Author Manuscript
J Mol Med (Berl). Author manuscript; available in PMC 2014 April 01.

Published in final edited form as:
J Mol Med (Berl). 2013 April ; 91(4): 507–512. doi:10.1007/s00109-012-0967-z.

A Novel *EPAS1/HIF2A* germline mutation in a Congenital Polycythemia with Paraganglioma

Felipe R. Lorenzo[1], **Chunzhang Yang**[2], **Mark Ng Tang Fui**[3], **Hariprasad Vankayalapati**[4], **Zhengping Zhuang**[2], **Thanh Huynh**[5], **Mathis Grossmann**[3], **Karel Pacak**[*,5], and **Josef T. Prchal**[*,1]

[1]Hematology Division, University of Utah School of Medicine and VAH, Salt Lake City, UT, 84132

[2]Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda

[3] Dept. of Endocrinology, and Dept. of Medicine Austin Health, University of Melbourne, Austin Health, Heidelberg, VIC 3084, Australia

[4]Center for Investigational Therapeutics. University of Utah and Huntsman Cancer Institute, Salt Lake City, UT.84112

[5]Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Abstract

Congenital polycythemias have diverse etiologies, including mutations in the hypoxia sensing pathway. These include *HIF2A* at exon 12, *VHL* gene (Chuvash polycythemia), and *PHD2* mutations, which in one family was also associated with recurrent pheochromocytoma/paraganglioma (PHEO/PGL). Over the past two decades, we have studied seven unrelated patients with sporadic congenital polycythemia who subsequently developed PHEO/PGL with, until now, no discernible molecular basis. We now report a polycythemic patient with a novel germline *HIF2A*^{F374Y} (exon 9) mutation, inherited from his mother, who developed PHEO/PGL.

We show that this is a gain-of-function mutation and demonstrate no loss-of-heterozygosity or additional somatic mutation of *HIF2A* in the tumor, indicating *HIF2A*^{F374Y} may be predisposing rather than causative of PHEO/PGL. This report, in view of 2 other concomitantly reported PHEO/PGL patients with somatic mutations of *HIF2A* and polycythemia, underscores the PHEO/PGL promoting potential of mutations of *HIF2A* that alone are not sufficient for PHEO/PGL development.

Correspondence: Josef T. Prchal M.D., Division of Hematology, 30N 1900E, 5C402 SOM University of Utah, Salt Lake City, UT 84132, USA. Phone +1-801-581-4220 Fax +1-801-585-3432 josef.prchal@hsc.utah.edu.
*both should be considered senior authors

Authors' contribution
Felipe R. Lorenzo performed study, identified mutation, interpreted data and wrote manuscript. Mark Ng Tang Fui identified paraganglioma and edited the manuscript.
Hariprasad Vankayalapati performed the computer studies of mutation effect and edited the manuscript.
Zhengping Zhuang performed the functional study of mutation effect and edited the manuscript.
Chunzhang Yang Zhuang performed the functional study of mutation effect and edited the manuscript.
Thanh Huynh studied paraganglioma and edited the manuscript
Mathis Grossmann studied paraganglioma and edited the manuscript.
Karel Pacak interpreted and supervised all experiments concerning paraganglioma and wrote the manuscript.
Josef T. Prchal JTP designed, interpreted and supervised all experiments and wrote the manuscript.

Conflict-of-interest disclosure: authors declare no competing financial interests.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Keywords

HIF2A; Paraganglioma; Familial erythrocytosis

INTRODUCTION

Elevated hemoglobin concentration is variably termed polycythemia or erythrocytosis. Based on the clinical history, polycythemias are divided into *acquired* and *congenital* categories[1]. In many instances, the principal cause is an elevated level of erythropoietin (EPO)[1, 2]. Hypoxia is a crucial stimulus for EPO production. One group of congenital polycythemias results from germline mutations of the hypoxia sensing pathway.

Studies of hypoxic regulation of EPO led to the discovery of the master hypoxia transcription factor, HIF-1[3]. Polycythemia associated with mutations in the hypoxia sensing pathway include dominantly inherited gain-of-function mutations of HIF-2-alpha (*HIF2A*), all found in exon 12. There are also polycythemia-associated mutations of negative regulators of HIFs [4], such as VHL^{R200W} and VHL^{H191D} [5-7] mutations, and *PHD2* mutations [8], which in one family was associated with pheochromocytoma/paraganglioma (PHEO/PGL) [9].

HIFs are transcription factors, dimers composed of hypoxia inducible α subunits (HIF-1α, HIF-2α, and HIF-3α [10]) and the constitutively expressed β subunit, and are the key regulators of the hypoxia response pathway. In the presence of oxygen, α subunits are hydroxylated by PHDs and then interact with pVHL, which leads to ubiquitination and a rapid degradation in proteasome [11]. HIF2 rather than HIF1 is the principal regulator of EPO gene transcription [12] and HIFs also augment erythropoiesis by EPO-independent mechanism(s) [13, 14].

HIFs control vital functions via regulation of many functions and an array of genes [15], and HIFs' augmentation is responsible for the Warburg effect [15, 16], a unique feature of cancer metabolism. Germline and somatic mutations in the HIF pathway are a foundation of VHL tumor predisposition syndrome [17-19] that includes predisposition for PHEO/PGL. Typically, VHL syndrome is associated with dominantly-inherited mutations of the *VHL* gene, with a germline loss-of-function mutation of one allele and an acquired somatic mutation of the VHL allele in *trans*. In many instances, such as VHL syndrome, upregulated HIFs are tumor promoter genes, but in some contexts, HIFs can also have a role as tumor suppressors [20].

However, mutations of other PHEO/PGL-associated genes in the HIF pathway do not have associated polycythemia phenotype.

MATERIALS AND METHODS

Samples and screening for mutation

Propositus' blood and his mother's, and propositus' tumor were obtained with IRB approval, mRNA and gDNA extracted. Tumor and blood gDNA were subjected for PCR, likewise propositus tumor's mRNA was reverse transcribed, amplified and were sequenced for mutation.

Multiple Sequence Alignment of HIF-2α

Set sequences of HIF2α from 6 species were aligned (Genetyx v8.0, Shibuya, Japan).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Computational analysis

The structure of the HIF-2α^{F374Y} mutation was investigated using computational protein-protein docking and molecular dynamics (MD) simulations. The published HIF-2α X-ray structure [21] (PDB ID: 1P97-sequence 241-350) was used. A homology model was built using ICM modeling for remaining residues 351-375 (DVVFSMDQTESLFKPHLMAMNSIF), where HIF-2α^{F374Y} is located. Molecular mechanics based MMFF charges loop sampling and global energy optimization was performed. Docking against the crystal structure of the pVHL–ElonginB–ElonginC (VBC) complex bound to the 20-residue destruction sequence of HIF-1α (PDB ID: 1LQB) was utilized. ICM Optimal Docking Area (ODA) method [22] was used to predict the optimal surface with the lowest docking desolvation energy, 23.25 kcal/mol for the VBC structure.

HIF-2α protein stability assay

Stability of HIF-2α^{F374Y} was measured as described in article by Zhuang et al [23]. In brief, HeLa cells were transfected with *HIF2A*^{F374Y} vectors by Lipofectamine 2000 transfection reagent (Invitrogen). Medium was changed 4 hours after transfection and cells were maintained 48 hours before cycloheximide (20 μg/mL, Sigma-Aldrich) treatment. Cell lysate was resolved on NuPAGE Bis-Tris gel (Invitrogen), transferred to PVDF membrane (Millipore) and blotted with V5 antibody (Origene). HIF-2α protein residue was quantified through densiometric analysis.

Immunoprecipitation assay

HIF-2α ubiquitination and VHL binding was determined through immunoprecipitation assay as previously described [23] *HIF2A or HIF2A*^{F374Y} plasmids were co-transfected with Flag-tagged VHL plasmid into HeLa cells using Lipofectamine 2000 (Invitrogen). Cells were treated with MG-132 for 2 hrs (30 μM, EMD Millipore) before harvest. Cells were lysed in either NP40 lysis buffer for VHL binding, or RIPA lysis buffer supplemented with 1% SDS for HIF-2α ubiquitination assay. Lysate were immunoprecipitated using Dynabeads co-immunoprecipitation kit (Invitrogen) and anti-V5 antibody (Origene). Bound protein was eluted in loading buffer supplemented with 1% SDS. Sample lysate were subsequently analyzed by western blot.

Reverse Transcription and Gene Expression

RNA from patient's tumor and three normal adrenal tissues were reverse transcribed using SuperScript VILO (Life Technology) and analyzed by hydrolysis probes (Applied Biosystem, Life Technology).

The amount of each target mRNA was normalized using PPIA and GUSB mRNA using geNorm Plus (Biogazelle, Zwijnaarde, Belgium) in determining the most stable reference gene.

Copy Number Assay

Copy number determination for *EGLN1* and *VHL* genes were carried out using the propositus' and his mother's blood gDNA and propositus' tumor DNA and compared with 3 unaffected controls. Taqman hydrolysis probe (Applied Biosystem, Life Technology) for quantifying *EGLN1* and *VHL* copy number were normalized using *RNAse P* as endogenous reference gene, samples done in quadruplicate and run in 2 separate experiments.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RESULTS AND DISCUSSION

The propositus is a 50 year-old male, polycythemic with high EPO documented since early childhood (Hb 22.4 g/dl, red cell mass 5338 ml, 69ml/kg) and treated by phlebotomies. At age 35 he developed high blood pressure and elevated urine norepinephrine. A lesion was found at the organ of Zuckerkandl; the tumor was removed and PGL was found. His catecholamines normalized; however, EPO remained elevated. Two years later, on a routine follow-up a new lesion was found in the paraaortic region, and thoracic masses were detected again but patient was asymptomatic. At age 47, he developed recurrence of adrenergic symptoms with elevated norepinephrine and new vertebral, paraaortic region, celiac axis and supramesenteric axis lesions found, with ^{68}Ga Octreotate PET/CT uptake and he remained asymptomatic. He underwent ^{131}I-metaiodobenzylguanidine therapy; his urine catecholamine normalized, but plasma normetanephrine, Hb% and EPO remain elevated.

His 83-year-old mother agreed to genetic testing but initially refused to give further information. On subsequent request she granted us a permission to access only her laboratory data available which included hemoglobin 148 g/L (normal range: 110-160) hematocrit 0.44 (normal range: 0.35-0.47) white blood count 4.8 $\times 10^9$/L (normal range: 4-11) platelets: 180 $\times$ 10^9/L (normal range: 150-450) and normal iron studies. While these results are within normal range, it should be noted that prevalence of anemia in this population is reported to exceed 30% and in our experience we have encountered several examples wherein an initial testing for polycythemia was negative in the affected person only to be repeatedly positive in subsequent testing; we have published one such example [24].

Mutation screening

His 83-year-old mother agreed to genetic testing but refused to give further information. Blood gDNA of the propositus and mother and PGL tissue gDNA and cDNA had a heterozygous missense mutation NM_001430: c.1121T>A at exon 9, codon 374 (*HIF2A*^{F374Y}) (Figure 1A). These results rule out loss-of-heterozygosity as a contributing cause of PGL genesis. We have previously not encountered this *HIF2A*^{F374Y} mutation in screening referred to us with unexplained congenital polycythemia (>30 at Baylor college of Medicine in Houston and 17 polycythemic and 41 normal controls at University of Utah). Other genes of the HIF pathway (*EGLN1/PHD2*, *VHL, SDHB, SDHC, SDHD*) and those causing polycythemia by different mechanisms (*JAK2*, *EPOR*, α,β-globins) had no mutations.

Multiple sequence alignment

Multiple sequence alignment of HIF-2α protein was performed and F374 was found to be highly conserved among man, chimpanzee, mouse, horse, cattle, chicken and zebrafish (Figure 1B), suggesting this serves an important function.

Molecular dynamics simulation

To further test HIF-2α^{F374Y} protein function , simulated structural analysis and molecular dynamics were performed using the HIF-2α^{F374Y} to determine residue binding site identified for ODA[22, 25], which was predicted at the Pro103, Gly104 and Thr105 hairpin loop region of b domain of pVHL (Fig. 1C). To define the overall effects of the HIF-2α^{F374Y} mutation, MD simulations of ElonginB-ElonginC (VBC-HIFs-a degradation pathway) in complex with HIF-2α^{F374Y} model was performed and it confirmed that HIF-2α^{F374Y} causes conformational changes at Pro103, Gly104 and Thr105 sites of pVHL b domain, and exhibited significant changes at ElonginC Ser87, Thr88 and Glu88 contact sites

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(figure 1C). This suggests a potential model of functional effect of HIF-2α^{F374Y} to be the impairment of ElonginC interaction leading to gain-of-function of HIF-2α.

Stability of mutant HIF-2α

We determined the stability of HIF-2α^{F374} using cycloheximide treatment. Wild type HIF2α protein had an 11.3 min. lifespan. In contrast, HIF-2α^{F374} was more stable, with a half-life of 51.2 min. (Figs. 2A and 2B). To further examine the HIF-2α^{F374} signaling, we investigated the HIF-2α VHL binding and ubiquitination. After incubating His-tagged HIF-2α proteins with VHL, comparable immunoprecipitation was performed with an anti-HA antibody, as shown in the middle blot (Fig. 2C). This shows that the mutant HIF-2α protein has decreased binding to VHL protein and was less efficiently ubiquitinated compared to wild-type HIF-2α (Fig. 2C). This further indicates that HIF-2α^{F374} results in its gain-of-function with decreased E3 ligase binding and delayed proteasomal degradation.

Real-time PCR

We analyzed the activity of hypoxia related pathway by quantifying HIF-regulated genes expressed in the tumor. We found marked up-regulation of *GLUT1* and *VEGFA* and down-regulation of *EDN1* transcript (Fig.2 D), consistent with *HIF2A* gain-of-function mutation. Structural variation within the gene such as copy number can influence gene expression and be associated with specific phenotype, despite normal sequence of the *EGLN1* and *VHL* genes. Thus, we further evaluated the copy number of these two genes and both genes were diploid in both tumor and germline DNA. These data were identical to concomitantly tested three controls that were analyzed at the same time. These data indicate that the copy number of variations of *EGLN1* and *VHL* gene were not contributory to the phenotype we observed in the propositus.

This is the first report of a *germline* gain-of-function *EPAS1/HIF2A* mutation associated with congenital polycythemia that is also associated with multiple PGL tumors. Unlike other *HIF2A* genes that are located in exon 12, the *HIF2A^{F374}* mutation is located in a uniquely conserved site at exon 9. We found no evidence of *HIF2A* loss-ofheterozygosity, additional mutations of *HIF2A* in tumor tissue, or copy number variations in both germline and tumor DNAs. This is in contrast to that found in von Hippel-Lindau PHEO/PGL syndrome and in a family with polycythemia and PHEO/PGL associated with a PHD2 mutation [25]. However, the failure to find polycythemia in propositus‘ mother whose blood counts were at the time of testing at her age of 83 years within normal range, is not in keeping with this mutation being fully penetrant for polycythemia phenotype. It should be noted that prevalence of anemia in her age group is reported to exceed 30% that can obscure the polycythemia phenotype. Further, in our experience, we have encountered several examples wherein an initial evaluation for polycythemia phenotype was negative in the affected person bearing dominantly inherited polycythemia-causing gene and only to be repeatedly positive in subsequent evaluations. Indeed, we previously published one such an example [24].

The fact that two unrelated, concomitantly reported PHEO/PGL patients with polycythemia who had *somatic* mutations in tumor tissues of *HIF2A,* albeit at different locations than *HIF2A*^{F374} (G1588A and C1589T exon 12 [23]), underscores the PHEO/PGL promoting potential of gain-of-function mutations of *HIF2A* that alone, either as somatic or germline mutations, can contribute to but are not sufficient for PHEO/PGL development.

Acknowledgments

JTP is supported by NIH-P01CA108671, VA Merit Review Award and University of Utah Seed Grant Program for studies of hypoxic adaptation

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

MG is supported by a Career Development Fellowship from the National Health and Medical Research Council of Australia (#1006407).

KP, ZZ, CY, TH are supported, in part, by the Intramural Research Program of the NIH, NICHD and NINDS.

References

1. Prchal JT. Classification and molecular biology of polycythemias (erythrocytoses) and thrombocytosis. Hematol Oncol Clin North Am. 2003; 17:1151–1158. vi. [PubMed: 14560779]
2. Semenza GL. HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol. 2001; 13:167–171. [PubMed: 11248550]
3. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA. 1993; 90:4304–4308. [PubMed: 8387214]
4. Percy MJ, Furlow PW, Lucas GS, Li X, Lappin TR, McMullin MF, Lee FS. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. N Engl J Med. 2008; 358:162–168. [PubMed: 18184961]
5. Ang SO, Chen H, Gordeuk VR, Sergueeva AI, Polyakova LA, Miasnikova GY, Kralovics R, Stockton DW, Prchal JT. Endemic polycythemia in Russia: mutation in the VHL gene. Blood Cells Mol Dis. 2002; 28:57–62. [PubMed: 11987242]
6. Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, Liu E, Sergueeva AI, Miasnikova GY, Mole D, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. Nat Genet. 2002; 32:614–621. [PubMed: 12415268]
7. Pastore Y, Jedlickova K, Guan Y, Liu E, Fahner J, Hasle H, Prchal JF, Prchal JT. Mutations of von Hippel-Lindau tumor-suppressor gene and congenital polycythemia. Am J Hum Genet. 2003; 73:412–419. [PubMed: 12844285]
8. Percy MJ, Furlow PW, Beer PA, Lappin TR, McMullin MF, Lee FS. A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. Blood. 2007; 110:2193–2196. [PubMed: 17579185]
9. Ladroue C, Hoogewijs D, Gad S, Carcenac R, Storti F, Barrois M, Gimenez-Roqueplo AP, Leporrier M, Casadevall N, Hermine O, et al. Distinct deregulation of the hypoxia inducible factor by PHD2 mutants identified in germline DNA of patients with polycythemia. Haematologica. 2012; 97:9–14. [PubMed: 21933857]
10. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev. 1997; 11:72–82. [PubMed: 9000051]
11. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda). 2009; 24:97–106. [PubMed: 19364912]
12. Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal ablation of Hif-2alpha results in anemia. Proc Natl Acad Sci U S A. 2007; 104:2301–2306. [PubMed: 17284606]
13. Yoon D, Pastore YD, Divoky V, Liu E, Mlodnicka AE, Rainey K, Ponka P, Semenza GL, Schumacher A, Prchal JT. Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. J Biol Chem. 2006; 281:25703–25711. [PubMed: 16787915]
14. Yoon D, Ponka P, Prchal JT. Hypoxia. 5. Hypoxia and hematopoiesis. Am J Physiol Cell Physiol. 2011; 300:C1215–1222. [PubMed: 21368293]
15. Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell. 2012; 148:399–408. [PubMed: 22304911]
16. Semenza GL. HIF-1 mediates the Warburg effect in clear cell renal carcinoma. J Bioenerg Biomembr. 2007; 39:231–234. DOI 10.1007/s10863-007-9081-2. [PubMed: 17551816]
17. Schimke RN, Collins DL, Stolle CA. Von Hippel-Lindau Syndrome. 1993 DOI NBK1463 [bookaccession].
18. Friedrich CA. Genotype-phenotype correlation in von Hippel-Lindau syndrome. Hum Mol Genet. 2001; 10:763–767. [PubMed: 11257110]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

19. Richards FM, Webster AR, McMahon R, Woodward ER, Rose S, Maher ER. Molecular genetic analysis of von Hippel-Lindau disease. J Intern Med. 1998; 243:527–533. [PubMed: 9681854]

20. Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, Losman JA, Joensuu P, Bergmann U, Gross S, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. Nature. 2012; 483:484–488. [PubMed: 22343896]

21. Erbel PJ, Card PB, Karakuzu O, Bruick RK, Gardner KH. Structural basis for PAS domain heterodimerization in the basic helix--loop--helix-PAS transcription factor hypoxia-inducible factor. Proc Natl Acad Sci U S A. 2003; 100:15504–15509. [PubMed: 14668441]

22. LLC M ICM 3.4 Manual. Molsoft LLC; San Diego: For energy Minimization,Docking and Molecular Dynamics calculations. (http://www.molsoft.com)

23. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, Popovic V, Stratakis CA, Prchal JT, Pacak K. Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia. N Engl J Med. 2012; 367:922–930. [PubMed: 22931260]

24. Kralovics R, Sokol L, Prchal JT. Absence of polycythemia in a child with a unique erythropoietin receptor mutation in a family with autosomal dominant primary polycythemia. J Clin Invest. 1998; 102:124–129. [PubMed: 9649565]

25. Ladroue C, Carcenac R, Leporrier M, Gad S, Le Hello C, Galateau-Salle F, Feunteun J, Pouyssegur J, Richard S, Gardie B. PHD2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med. 2008; 359:2685–2692. [PubMed: 19092153]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

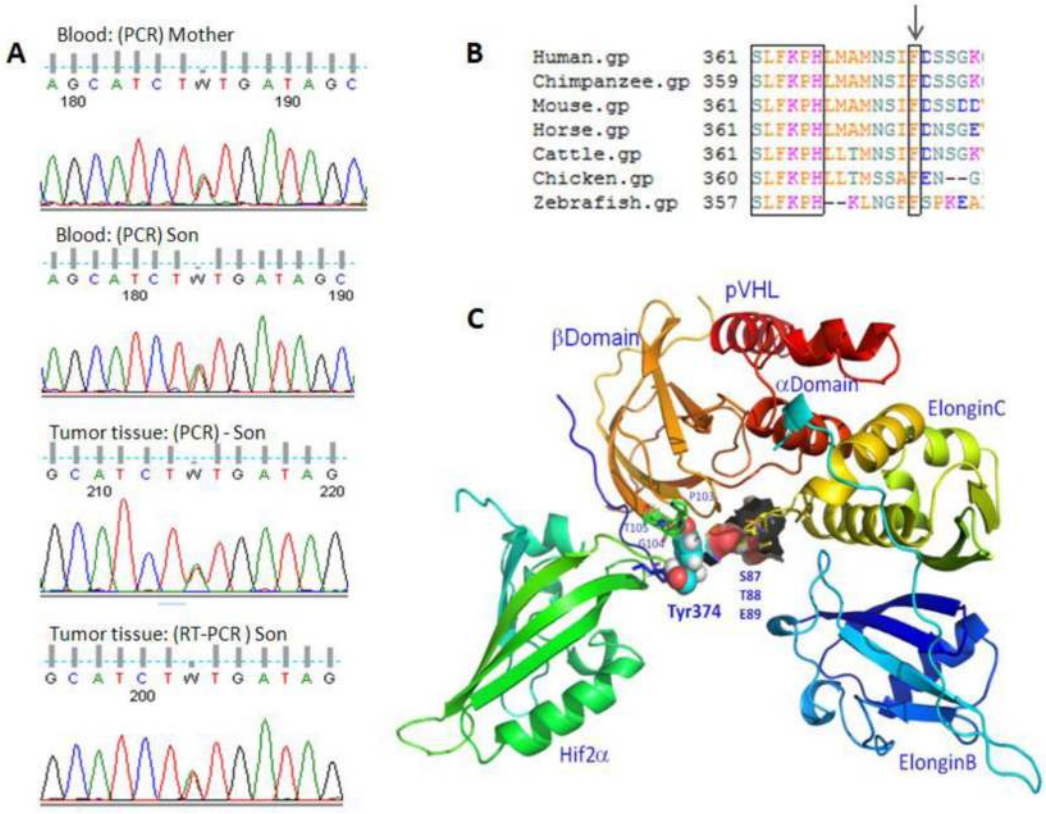

Figure 1A-C. *HIF2A* mutation screening and structural modeling analysis

(A) Sanger sequence of the blood and tumor tissue gDNA and cDNA. (B) Alignment of the HIF-2α amino acid sequence from 361 to 379 showing F374 residue in human against other known species. (C) Computational insight into HIF-2α^{F374Y} mutation by Optimal Docking and Molecular Dynamic Studies. Y374 mutation - structural changes at the Pro103, Gly104 ND Thr105 site of pVHL βdomain & exhibit significant conformational changes both at the βdomain and ElonginC Ser87, Thr88 and Glu89 site (Significant conformational changes can be observed mainly at ElonginC).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

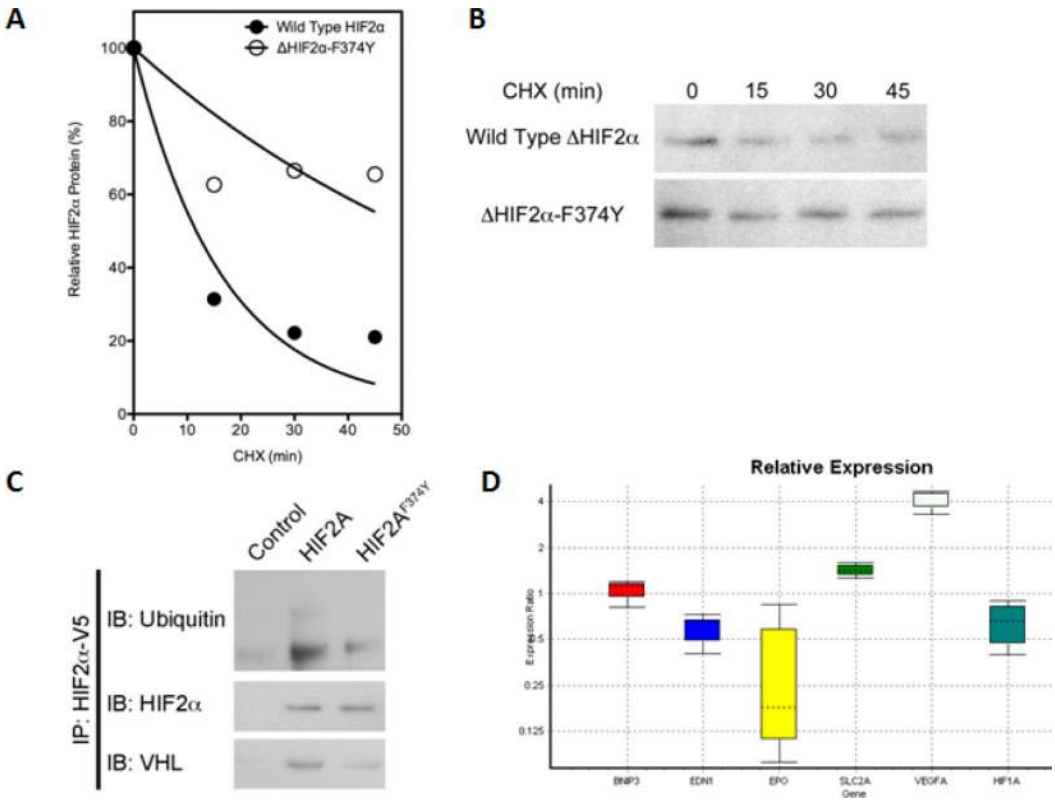

Figure 2 A-C. Protein assay and expression study

(A) Enhanced stability of mutant HIF-2α proteins evidenced by the higher levels seen on Western blot analysis over time following the addition of cycloheximide (CHX). (B) Quantification and half-life of the HIF-2α mutants based on data in figure 2A. (C) Binding and ubiquitination of the HIF-2α^{F374Y} were analyzed by immunoprecipitation assay. (D) Quantitative gene expression ratio of tumor to normal tissue of HIF and some target genes by qPCR. qPCR assay consist of 2 tumors and 3 normal adrenal tissues of two independent experiments assayed in duplicates.