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## A Gain-of-Function Mutation in the *HIF2A* Gene in Familial Erythrocytosis

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### SUMMARY

Hypoxia-inducible factor (HIF)  $\alpha$ , which has three isoforms, is central to the continuous balancing of the supply and demand of oxygen throughout the body. HIF- $\alpha$  is a transcription factor that modulates a wide range of processes, including erythropoiesis, angiogenesis, and cellular metabolism. We describe a family with erythrocytosis and a mutation in the *HIF2A* gene, which encodes the HIF-2 $\alpha$  protein. Our functional studies indicate that this mutation leads to stabilization of the HIF-2 $\alpha$  protein and suggest that wild-type HIF-2 $\alpha$  regulates erythropoietin production in adults.

A widely recognized example of an oxygen-regulated pathway is the erythropoietin system, in which the kidney senses decreased tissue oxygenation and, in turn, produces erythropoietin, thereby increasing the red-cell mass.<sup>1</sup> Studies of the regulation of the *EPO* gene led to the discovery of HIF, which consists of a labile  $\alpha$  subunit and a constitutively expressed  $\beta$  subunit.<sup>2</sup> Under normoxic conditions, the  $\alpha$  subunit, which consists of three isoforms — HIF-1 $\alpha$ , HIF-2 $\alpha$ , and HIF-3 $\alpha$  — is hydroxylated on two specific prolyl residues.<sup>3</sup> This hydroxylation targets HIF- $\alpha$  for degradation by the von Hippel–Lindau (VHL) tumor-suppressor protein.<sup>4</sup> Under hypoxic conditions, the hydroxylation is inhibited, thereby maintaining a stable HIF- $\alpha$  protein, which activates not only the *EPO* gene but also a broad range of other genes that orchestrate adaptation to hypoxia.<sup>2,5</sup>

Familial erythrocytosis provides an opportunity to study the oxygen-sensing mechanism.<sup>6</sup> This genetic disorder can be caused by a mutation in one of the proteins that hydroxylates HIF- $\alpha$  (prolyl hydroxylase domain protein 2 [PHD2])<sup>7,8</sup> or by a mutation in the VHL protein.<sup>9,10</sup> These findings raise the question of whether there are mutations in HIF- $\alpha$  itself.

The particular HIF isoform involved in erythropoietin regulation has been the subject of intensive investigation. Several results point to HIF-1 $\alpha$ ,<sup>11–13</sup> but mouse Hif-2 $\alpha$  (also known as endothelial PAS domain protein 1 [Epas1] or Hif-1 $\alpha$ -like factor [Hlf]) has been implicated as the principal regulator of erythropoietin in postembryonic mice.<sup>14–17</sup> We investigated the *HIF2A* gene in a family with erythrocytosis and found a missense mutation that impairs hydroxylation of the HIF-2 $\alpha$  protein, thereby allowing both for maintenance of its stable conformation and for its induction of erythrocytosis.

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## CASE REPORT

The index patient was a 43-year-old man who, at 23 years of age, had been found to have an elevated hemoglobin level (21.7 g per deciliter) and an elevated hematocrit (64%), with normal white-cell and platelet counts (Table 1). The hematocrit was maintained below 55% by means of venesection two to three times yearly. At 32 years of age, the patient's serum erythropoietin level was 40.0 mU per milliliter (normal range, 2.0 to 20.0), at which time the hemoglobin level was 18.9 g per deciliter, and the hematocrit was 54%. At 42 years of age, a deep venous thrombosis developed; as a consequence, more-frequent venesection was subsequently used to maintain his hematocrit below 45%. He remained well at 43 years of age, apart from gout. At the most recent testing (in December 2006), the hemoglobin level was 18.6 g per deciliter, the hematocrit was 55%, the white-cell count was 6300 per cubic millimeter, the platelet count was 178,000 per cubic millimeter, and the serum erythropoietin level was 31.1 mU per milliliter (normal range, 2.5 to 10.5 mU per milliliter).

The index patient had a family history of erythrocytosis (Fig. 1A). When his mother was 35 years of age, her hemoglobin level was 16.3 g per deciliter, and the hematocrit was 50%, with normal white-cell and platelet counts. The red-cell mass was elevated (at 33 ml per kilogram). She was being treated by means of regular venesection. The most recent testing (in December 2006) revealed a hemoglobin level of 14.5 g per deciliter, a hematocrit of 48%, a white-cell count of 7000 per cubic millimeter, a platelet count of 187,000 per cubic millimeter, and a serum erythropoietin level of 63.3 mU per milliliter. At 63 years of age, she had a myocardial infarction, 1 year before the most recent testing.

The patient's 89-year-old maternal grandmother presented at 54 years of age with elevated values for hemoglobin, hematocrit, and red-cell mass (19.1 g per deciliter, 54%, and 35 ml per kilogram, respectively) and normal white-cell and platelet counts. Her bone marrow was normocellular. Venesection was performed two to three times yearly to maintain the hematocrit below 50%. Her medical history was otherwise notable only for hypertension and diverticulitis. Venesection has not been required since the age of 81 years, because the hemoglobin level has been between 14.0 and 15.5 g per deciliter, and the hematocrit has been between 45% and 52%. She has remained well. The most recent testing (in December 2006) revealed a hemoglobin level of 14.3 g per deciliter, a hematocrit of 46%, a white-cell count of 6700 per cubic millimeter, a platelet count of 186,000 per cubic millimeter, and a serum erythropoietin level of 118.6 mU per milliliter.

## METHODS

### PATIENTS

The index patient was from a group of 181 patients with erythrocytosis who were referred from hospitals throughout the United Kingdom and Ireland.<sup>10</sup> All the patients had an elevated hematocrit, and there was a wide range of erythropoietin levels. The patients did not fulfill the diagnostic criteria for polycythemia vera established by the British Committee for Standards in Haematology.<sup>18</sup> All patients gave written informed consent on entering the study, which was approved by the research ethics committee of Queen's University, Belfast, and was conducted according to the Declaration of Helsinki. On entering the registry, patients are routinely screened for defects in the erythropoietin receptor,<sup>19</sup> the *VHL* gene,<sup>9,10,20</sup> and the *PHD2* gene.<sup>7</sup>

### MUTATIONAL ANALYSIS OF *HIF2A*

Exon 12 of the *HIF2A* gene was amplified from DNA from peripheral-blood mononuclear cells by means of a polymerase-chain-reaction (PCR) assay with the forward primer 5'

TTGAGCAGCACTGTGAAACA'3, the reverse primer 5'ACATGGCTTGAGGTGATTCC' 3, and an annealing temperature of 55°C. The purified 773-bp PCR product was sequenced with the use of the BigDye Terminator kit (Applied Biosystems). For identification of the G1609→T mutation, amplification-refractory mutation-system (ARMS)-PCR primers were designed with the use of the primer-design program devised by Ye et al.<sup>21</sup> ([http://cedar.genetics.soton.ac.uk/public\\_html/primer1.html](http://cedar.genetics.soton.ac.uk/public_html/primer1.html)) (sequences available on request). DNA samples from peripheral-blood mononuclear cells from members of the patient's immediate family and 200 normal control samples from unrelated, ethnically matched blood donors from the United Kingdom (Human Random Control DNA panels, European Collection of Cell Cultures) were also screened for the G1609→T mutation, by means of ARMS-PCR.

## PLASMIDS, PROTEINS, PEPTIDES, AND CELL LINES

The coding sequence for human HIF-2 $\alpha$ , obtained from Integrated Molecular Analysis of Genomes and their Expression (IMAGE) Consortium clone 6305604 (American Type Culture Collection), was subcloned into the plasmid pcDNA5/FRT/TO, along with an N-terminal 3xFlag tag. Using standard methods, we constructed plasmids containing the Gly537→Trp or Pro531→Ala mutation, as well as the plasmid pcDNA3-GAL4-HIF-2 $\alpha$  (516–549) with or without the Gly537→Trp mutation. Peptides were synthesized by GenScript. The (His)<sub>6</sub>FlagPHD2 and GST-HIF-1 $\alpha$  (531–575) proteins were prepared as previously described.<sup>22</sup> In vitro translated proteins were prepared with the use of TNT kits (Promega). Stably transfected HEK293 Flp-In T-Rex cell lines (Invitrogen), in which the pcDNA5/FRT/TO vector is integrated into a single defined genomic locus, were generated using Flp recombinase (Promega), according to the manufacturer's instructions.

## ASSAYS

Binding assays, hydroxylase assays, real-time PCR, and Western blotting were performed as previously described.<sup>7,22,23</sup> P values of less than 0.05 were considered to indicate statistical significance.

Briefly, for binding assays, <sup>35</sup>S-labeled, in vitro translated wild-type or Gly537→Trp GAL4-HIF-2 $\alpha$  (516–549), prepared with the use of wheat-germ extracts, was incubated with or without 2  $\mu$ g of (His)<sub>6</sub>FlagPHD2. (His)<sub>6</sub>FlagPHD2 was immunoprecipitated with anti-Flag antibodies and washed, and the degree of binding was assessed. For competition experiments, GST-HIF-1 $\alpha$  (531–575) pre-bound to glutathione agarose was hydroxylated by recombinant PHD2, and we assessed the capacity of 0.75-nM wild-type hydroxylproline (Hyp)-HIF-2 $\alpha$  (527–542) peptide or Gly537→Trp Hyp-HIF-2 $\alpha$  (527–542) peptide to inhibit the binding of <sup>35</sup>S-labeled, reticulocyte lysate-translated VHL to hydroxylated GST-HIF-1 $\alpha$  (531–575).

For hydroxylase assays, wild-type HIF-2 $\alpha$  (527–542) peptide and Gly537→Trp HIF-2 $\alpha$  (527–542) peptide were treated with recombinant (His)<sub>6</sub>FlagPHD2 or were mock-treated with reaction buffer alone; the peptides were subjected to matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry, performed on a 4700 Proteomics Analyzer (Applied Biosystems).

For Western blotting, HEK293 Flp-In T-Rex cell lines stably expressing inducible wild-type, Gly537→Trp, or Pro531→Ala 3xFlagHIF-2 $\alpha$ , or the parental cell line, were treated with or without 0.1  $\mu$ g of doxycycline per milliliter for 16 hours. Cells were harvested, and equal quantities of cell lysate were examined by means of Western blotting with the use of anti-Flag or anti- $\beta$ -tubulin antibodies. Cell lines were exposed to hypoxic conditions (1% oxygen, 5% carbon dioxide, and 94% nitrogen) in the In Vivo 200 Hypoxia Workstation (Ruskin Technologies). For real-time PCR assays, HEK293 Flp-In T-Rex cell lines were treated with

1  $\mu\text{g}$  of doxycycline per milliliter for 16 hours. Total RNA was then extracted from cells and reverse transcribed. The messenger RNA (mRNA) levels of adrenomedullin (*ADM*), N-myc downstream regulated gene 1 (*NDRG1*), and vascular endothelial growth factor (*VEGF*) were measured with the use of real-time PCR on a Model 7300 real-time PCR machine (ABI). Relative quantification was performed by means of the  $\Delta\Delta\text{C}_T$  method, with  $\beta$ -actin as the endogenous control. SYBR Green primers were designed with the use of Primer Express software, version 3.0 (Applied Biosystems).

In a separate experiment, the HEK293 Flp-In T-Rex cell lines were treated with 0.1  $\mu\text{g}$  of doxycycline per milliliter for 16 hours under normoxic conditions. Cells were treated or mock-treated with 20  $\mu\text{g}$  per milliliter of cycloheximide for up to 45 minutes, to arrest protein synthesis. Cells were harvested, and equal quantities of lysates then examined by means of Western blotting with anti-Flag antibodies. Signals were quantified with the use of a ChemiDoc-It imaging system (UVP).

## RESULTS

### MUTATIONAL ANALYSIS

A heterozygous G $\rightarrow$ T change at base 1609 in exon 12 of the *HIF2A* gene was detected in the index patient (Fig. 1B). This change predicts the replacement of glycine by tryptophan at amino acid 537. We screened for the G1609 $\rightarrow$ T mutation, using ARMS-PCR analysis, in specimens from the mother and maternal grandmother of the index patient, who both had erythrocytosis, and from the unaffected father and an unaffected sibling. The results showed that this mutation segregated with the erythrocytosis phenotype (Fig. 1C). PCR-direct sequencing confirmed the presence of the G1609 $\rightarrow$ T mutation in affected family members (Fig. 1B). To test whether this mutation is a single-nucleotide polymorphism, the control panel of 200 DNA samples was screened by means of ARMS-PCR; it was found to be negative for the mutation (data not shown). Gly537, which is conserved in all known HIF-2 $\alpha$  proteins, is located in the vicinity of Pro531 (the primary hydroxylation site in HIF-2 $\alpha$ ) and is not present in HIF-1 $\alpha$ , in HIF-3 $\alpha$ , or in the Hif protein of *Drosophila melanogaster* or *Caenorhabditis elegans* (Fig. 1D).

### BINDING AND HYDROXYLASE ASSAYS

In vitro functional assays showed that PHD2, the hydroxylating protein, binds more weakly to the mutant Gly537 $\rightarrow$ Trp HIF-2 $\alpha$  (516–549) peptide than to the wild-type peptide (Fig. 2A). The mean ( $\pm$ SD) relative recovery of wild-type GAL4–HIF-2 $\alpha$  (516–549) from three replicates (expressed in arbitrary units) was  $100\pm 12$ , whereas that of Gly537 $\rightarrow$ Trp GAL4–HIF-2 $\alpha$  (516–549) was  $12\pm 7$  ( $P < 0.001$ ).

We also incubated PHD2 with wild-type or Gly537 $\rightarrow$ Trp HIF-2 $\alpha$  (527–542) peptide and assessed hydroxylation by using MALDI-TOF mass spectrometry. We found less hydroxylation with the mutant HIF-2 $\alpha$  (Fig. 2B). In addition, competition experiments showed that VHL binds to the prolyl-hydroxylated form of the mutant Gly537 $\rightarrow$ Trp HIF-2 $\alpha$  (527–542) weakly as compared with its wild-type counterpart (Fig. 2C). The mean degree of inhibition found with wild-type Hyp–HIF-2 $\alpha$  (527–542) was  $92\pm 1.5\%$ , whereas that of Gly537 $\rightarrow$ Trp Hyp–HIF-2 $\alpha$  (527–542) was  $1\pm 31\%$  ( $P = 0.008$ ). These data are evidence that the mutation affects the hydroxylation of HIF-2 $\alpha$  by PHD2, as well as the subsequent recognition of HIF-2 $\alpha$  by VHL.

### WESTERN BLOTTING AND REAL-TIME PCR

We generated isogenic, stably transfected HEK293 cells that can be induced to express Flag-tagged wild-type HIF-2 $\alpha$ , Gly537 $\rightarrow$ Trp HIF-2 $\alpha$ , or Pro531 $\rightarrow$ Ala HIF-2 $\alpha$  (as a hydroxylation-defective control). Real-time PCR involving primers specific for the nucleotide sequence

encoding the epitope tag showed similar levels of messenger RNA in the wild-type and Gly537→Trp constructs (data not shown). Western blotting showed that under normoxic conditions, levels of mutant Gly537→Trp HIF-2 $\alpha$  are higher than levels of the wild-type protein, though not as high as Pro531→Ala HIF-2 $\alpha$  levels (Fig. 2D). Hypoxic conditions (1% oxygen), which increase the steady-state levels of wild-type HIF-2 $\alpha$ , diminish but do not eliminate the difference in protein levels between wild-type and Gly537→Trp HIF-2 $\alpha$  (data not shown). Under normoxic conditions, as compared with the expression of wild-type HIF-2 $\alpha$ , the expression of Gly537→Trp HIF-2 $\alpha$  induced significantly increased mRNA transcript levels from the *ADM* gene, the *NDRG1* gene, and the *VEGF* gene, all targets of *HIF2A*<sup>5</sup> (Fig. 2E). Similar results were found for a transfected reporter gene containing three copies of the human erythropoietin enhancer upstream of the transcription start site (data not shown). After treatment of the HEK293 cells with cycloheximide to arrest protein synthesis, the Gly537→Trp mutant of HIF-2 $\alpha$  was degraded more slowly than wild-type HIF-2 $\alpha$ , though not as slowly as the Pro531→Ala mutant (Fig. 2F). Taken together, the results of the functional assays indicate that the Gly537→Trp mutation causes a gain of function in HIF-2 $\alpha$ .

## DISCUSSION

Our findings are evidence that HIF-2 $\alpha$  is a transcription factor that regulates erythropoietin levels in humans; the results also support previous findings that indicated the same role for Hif-2 $\alpha$  in postembryonic mice.<sup>14-17</sup> Our data do not rule out a role of HIF-1 $\alpha$  in erythropoietin regulation under certain circumstances; there is evidence that this isoform plays a role in erythropoietin production during embryogenesis and in the adult retina.<sup>12,13</sup>

Previous in vitro studies have shown tolerance for an unexpectedly wide range of amino acid substitutions in the vicinity of the primary hydroxylation site of HIF- $\alpha$ .<sup>22,24</sup> Our study shows that, in a physiologic context, the range of tolerated amino acid substitutions is likely to be substantially narrower than expected, which provides a rationale for the conservation of amino acids in the vicinity of the hydroxylation site of HIF- $\alpha$ . Gly537 occupies a position unique to the HIF-2 $\alpha$  isoform; the mutation separates highly conserved amino acid sequences that, at least in the case of the HIF-1 $\alpha$ -VHL interaction, make critical contacts.<sup>4</sup>

Our study, in conjunction with previous studies, identifies the PHD2-HIF-2 $\alpha$ -VHL pathway as the core molecular machinery that regulates erythropoietin production in humans. Intriguingly, a partial as opposed to complete loss or gain of function at each step of the pathway is sufficient to induce the erythrocytosis phenotype. Erythrocytosis can be caused by near-haploinsufficiency with regard to PHD2<sup>7,8</sup> or two copies of a hypomorphic allele encoding VHL.<sup>9,10</sup> In the family we studied, it is due to one copy of a hypermorphic allele encoding HIF-2 $\alpha$ . Collectively, these findings suggest that each of the three proteins contributes critically to the maintenance of erythropoietin at appropriate levels.

In mice, a liver-specific *Hif2A* gain-of-function mutation and a loss of function of VHL each causes erythrocytosis and hepatic hemangiomas,<sup>15,25</sup> raising the possibility that the patients we studied may share features of the von Hippel-Lindau syndrome. However, these patients had no history of renal-cell carcinoma, pheochromocytoma, or central nervous system hemangioblastoma, the hallmarks of the von Hippel-Lindau syndrome. This may be due to HIF-independent functions of VHL. It may also be due to the fact that the present case represents a partial, as opposed to complete, gain of HIF-2 $\alpha$  function, as suggested by the activity of the Gly537→Trp mutant as compared with that of the Pro531→Ala mutant (Fig. 2D, 2E, and 2F), thereby complicating the comparison of results in humans and results in animals. Although the specific erythrocytosis phenotype we observed in our patients points to the erythropoietin gene as a HIF-2 $\alpha$ -specific target in adults, we must acknowledge the evidence of a broader spectrum of HIF-2 $\alpha$  target genes.<sup>5</sup>



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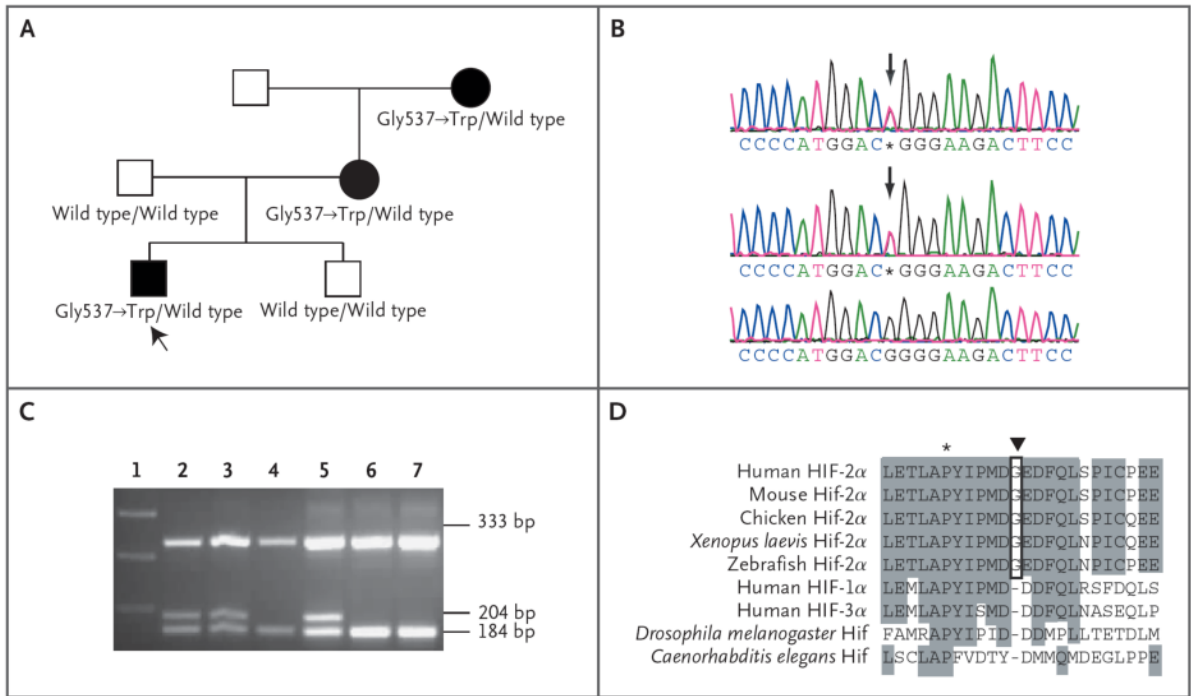
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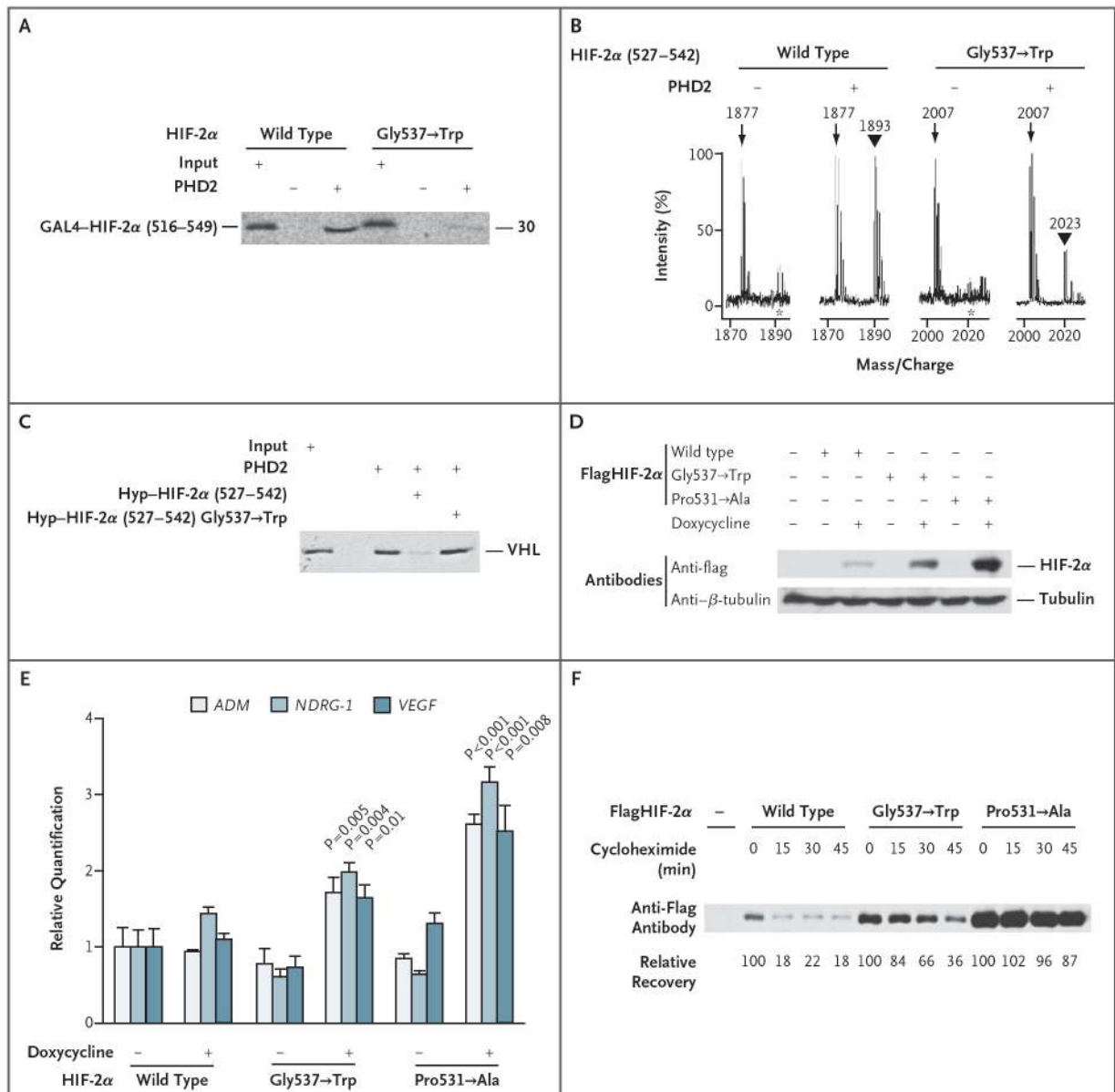
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**Figure 1. *HIF2A* Genotypes in a Family with Hereditary Erythrocytosis and Identification of the G1609→T Mutation**

The pedigree of the family with hereditary erythrocytosis and mutant *HIF2A* is shown in Panel A. Squares represent male family members, circles represent female family members, and solid symbols represent family members with erythrocytosis; genotypes are shown under each symbol. The index patient is indicated with an arrow. Not shown are an unaffected younger sister of the index patient whose genotype was unavailable and two maternal aunts and two maternal uncles of the index patient, from whom neither medical histories nor genotypes could be obtained. Panel B shows nucleotides 1599 to 1621 in exon 12 of *HIF2A*. On sequencing, a heterozygous G→T change at base 1609 was detected in the index patient (top plot, asterisk and arrow), as compared with the wild-type sequence (bottom). The maternal grandmother of the index patient also had the mutation (middle plot, arrow). Panel C shows the results of amplification-refractory mutation-system (ARMS)–polymerase chain reaction (PCR) performed on peripheral-blood mononuclear cells for detection of the G1609→T mutation. Samples with a G, or wild-type, allele yielded a PCR product of 184 bp, whereas the T, or mutant, allele is 204 bp. The affected index patient and his mother and grandmother had both the wild-type and mutant alleles (lanes 2, 3, and 5, respectively). The T allele is absent in the unaffected father and brother of the index patient (lanes 4 and 6, respectively). Lane 1 contains a 100-bp DNA size marker, and all other lanes contain the PCR control band of 333 bp. Lane 7 contains a control sample that is negative for the G1609→T mutation. Panel D lists the amino acid sequences (shown with single-letter symbols) of the hypoxia-inducible factor (HIF)  $\alpha$  residues 526 to 549 (human HIF-2 $\alpha$  nomenclature) in human, mouse, chicken, *Xenopus laevis*, and zebrafish, as well as the single HIFs from *Drosophila melanogaster* and *Caenorhabditis elegans*. Shading indicates conserved residues, the asterisk indicates the hydroxyl acceptor proline (Pro531 in HIF-2 $\alpha$ ), and the inverted triangle indicates Gly537 of human HIF-2 $\alpha$ .





### Figure 2. Functional Characterization of the Gly537 $\rightarrow$ Trp Hypoxia-Inducible Factor (HIF) 2 $\alpha$ Mutant

Panel A shows the results of binding assays. Input represents 1% of the total amount of wild-type Gly537 $\rightarrow$ Trp GAL4-HIF-2 $\alpha$  (516-549) incubated with (His)<sub>6</sub>FlagPHD2. Panel B shows the results of hydroxylase assays, involving matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry. The positions of the unmodified peptides were 1877 amu for wild type and 2007 amu for Gly537 $\rightarrow$ Trp and for hydroxylated peptides were 1893 atomic mass units for wild type and 2023 amu for Gly537 $\rightarrow$ Trp (as potassium adducts). The small peaks at 1893 and 2023 in the untreated samples (asterisks) probably represent the oxidation of methionine in the peptide. Panel C shows the results of experiments examining the capacity of wild-type or Gly537 $\rightarrow$ Trp HIF-2 $\alpha$  (527-542) peptide to compete with hydroxylated GST-HIF-1 $\alpha$  (531-575) for VHL binding. Input represents 25% of the total amount of VHL added in these experiments. Panels D and F show the results of Western blotting. Panel D shows the steady-state protein levels of wild-type and mutant HIF-2 $\alpha$ ,

whereas Panel F shows the protein levels after protein synthesis has been arrested with the use of cycloheximide. Panel E shows the results of real-time PCR assays. The levels of messenger-RNA transcripts from the adrenomedullin gene (*ADM*), N-myc downstream regulated gene 1 (*NDRG1*), and the vascular endothelial growth factor gene (*VEGF*) were measured with the use of real-time PCR. Means from three separate experiments are shown. T bars indicate standard deviations. The P values are for the comparison with doxycycline-induced levels of wild-type HIF-2 $\alpha$ . In Panel F, for each construct, the relative recovery was normalized to the value for 0 minutes, and the first lane shows results for the parental cell line. Hyp denotes hydroxyproline, and PHD2 prolyl hydroxylase domain protein 2.

**Table 1**  
 Age at Presentation and Hematologic Values in a Family with HIF-2 $\alpha$ -Associated Erythrocytosis.\*

| Value                                     | Index Patient | Unaffected Brother | Mother | Father  | Maternal Grandmother |
|---|---------------|--------------------|--------|---------|----------------------|
| Age at presentation (yr)                  | 23            | —                  | 35     | —       | 54                   |
| Hemoglobin (g/dl)                         | 21.7          | 15.6               | 16.3   | 13.4    | 19.1                 |
| Hematocrit (%)                            | 64            | 46                 | 50     | 39      | 54                   |
| White-cell count (per mm <sup>3</sup> )   | 7200          | 6100               | Normal | 6900    | 6900                 |
| Platelet count (per mm <sup>3</sup> )     | 226,000       | 193,000            | Normal | 183,000 | 106,000              |
| Serum erythropoietin (mU/ml) <sup>†</sup> | 31.1          | 10.9               | 63.3   | 16.2    | 118.6                |

\* Erythropoietin data were obtained in December 2006; all other laboratory data were obtained at presentation.

<sup>†</sup> The normal range for erythropoietin is 2.5 to 10.5 mU per milliliter.