Translational repression of HIF2α expression in mice with Chuvash polycythemia reverses polycythemia

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Chuvash polycythemia is an inherited disease caused by a homozygous germline \( VHL^{R200W} \) mutation, which leads to impaired degradation of HIF2α, elevated levels of serum erythropoietin, and erythrocytosis/polycythemia. This phenotype is recapitulated by a mouse model bearing a homozygous \( Vhl^{R200W} \) mutation. We previously showed that iron-regulatory protein 1–knockout (Irp1-knockout) mice developed erythrocytosis/polycythemia through translational derepression of Hif2α, suggesting that IRP1 could be a therapeutic target to treat Chuvash polycythemia. Here, we fed \( Vhl^{R200W} \) mice supplemented with Tempol, a small, stable nitroxide molecule and observed that Tempol decreased erythropoietin production, corrected splenomegaly, normalized hematocrit levels, and increased the lifespans of these mice. We attribute the reversal of erythrocytosis/polycythemia to translational repression of Hif2α expression by Tempol-mediated increases in the IRE-binding activity of Irp1, as reversal of polycythemia was abrogated in \( Vhl^{R200W} \) mice in which \( Irp1 \) was genetically ablated. Thus, a new approach to the treatment of patients with Chuvash polycythemia may include dietary supplementation of Tempol, which decreased Hif2α expression and markedly reduced life-threatening erythrocytosis/polycythemia in the \( Vhl^{R200W} \) mice.

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**Introduction**

Chuvash polycythemia is an autosomal recessive disorder that is endemic to the Chuvash population of the Russian Federation (1) and the Italian island of Ischia, but has also been found in patients around the world (2). Patients with Chuvash polycythemia die prematurely as a result of complications including thrombosis and cerebral hemorrhage (3). The major symptoms and signs found in these patients are headache, fatigue, abdominal pain, chest pain, plethora, hepatomegaly, and clubbing (3). Chuvash polycythemia is caused by a homozygous mutation in the von Hippel-Lindau (\( VHL \)) tumor-suppressor gene that results in a \( R200W \) amino acid substitution.

The \( VHL \) gene is involved in the hypoxia-sensing pathway, and it encodes a multifunctional protein that interacts with elongin B, elongin C, cullin-2, and RING-box protein 1 (\( Rbx1 \)) in a complex referred to as VCB-CUL2. This VCB-CUL2 complex functions as a ubiquitin ligase that promotes the degradation of \( \alpha \)-subunits of hypoxia-inducible factors (HIFs) (4, 5). More than 1,500 germline and somatic mutations have been identified in the \( VHL \) gene. All of these mutations cause \( VHL \) disease, except the 3 missense mutations \( VHLS^{200W}, VHL^{R200D}, \) and \( VHLS^{P191L} \) (6–8). These 3 homozygous mutations in the \( VHL \) gene cause congenital erythrocytosis/polycythemia, whereas patients with \( VHL \) disease develop vascularized tumors, including clear-cell renal cell carcinomas (CCCRCCs), cerebral hemangioblastoma, pheochromocytoma, endolymphatic sac tumors (ELSTs), epididymal or broad ligament cystadenomas, and pancreatic tumors (6, 9).

The \( VHL \) protein targets the \( \alpha \)-subunits of hypoxia-inducible factors (HIF1\(\alpha\) and HIF2\(\alpha\)) for proteasomal degradation after prolyl hydroxylase domain protein 2 (PHD2) site-specifically hydroxylates HIF\(\alpha\) proteins at normal oxygen concentrations. However, under low oxygen (hypoxic) conditions or when there is a loss of VHL function, HIF1\(\alpha\) and HIF2\(\alpha\) are stabilized. Since VHL is required for the degradation of HIF\(\alpha\) proteins (10), and its dysfunction promotes transcription of multiple HIF\(\alpha\) targets, including erythropoietin (\( EPO \)), a glycoprotein hormone essential for erythropoiesis (11), Chuvash polycythemia is associated with elevated levels of hypoxia-inducible factors HIF1\(\alpha\) and HIF2\(\alpha\) and higher levels of serum erythropoietin (12, 13). A mouse model of Chuvash polycythemia bearing the same homozygous \( Vh^{R200W} \) mutation that has been detected in patients with Chuvash polycythemia recapitulated the erythropoiesis/polycythemia phenotype by manifesting increased hematocrit, RBC, and hemoglobin levels and slightly elevated levels of serum erythropoietin and HIF2\(\alpha\) (14).

Iron is intimately associated with oxygen sensing in cells (15), and both erythropoiesis and proteasomal degradation of HIF2\(\alpha\) are regulated by iron. Two cytosolic iron regulatory proteins, IRP1 and IRP2, regulate many aspects of cellular iron homeostasis. IRPs posttranscriptionally control the expression of transcripts that contain iron-responsive element (IRE) sequences of iron metabolism proteins, including the iron import proteins transferrin receptor 1 (\( TTRIR1 \)) and divalent metal ion transporter 1 (\( DMT1 \)), the iron export protein ferroportin, the iron storage protein ferritin, and hypoxia-inducible factor 2\(\alpha\) (HIF2\(\alpha\)). IRP1 exists in a dynamic equilibrium between 2 forms: a cytosolic aconitase form with a 4Fe-4S cluster in its active site and an apoprotein form that displays IRE-binding activity. When cellular iron levels are low, IRP1 exists predominantly in the IRE-binding form that lacks an intact Fe-S cluster. When cellular iron levels are high, IRP1 regains its iron-sulfur cluster and exists mainly in the cytosolic aconitase form. The cytosolic aconitase form is also stabilized at low oxygen concentrations (16, 17).
Recently, we and others discovered that mice that lack Irp1 develop erythrocytosis/polycythemia (18–20). Hif2α, but not Hif1α, has an IRE in the 5′-UTR of its mRNA, and binding of Irp1 to this Hif2α IRE enables Irp1 to repress the translation of Hif2α and thus reduce the production of erythropoietin (18–21). Therefore, deletion of Irp1 causes erythrocytosis/polycythemia in mice through translational derepression of Hif2α. Hence, activation of Hif2α IRE binding of Irp1 is expected to reduce erythropoiesis. Here, we used Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a commercially available stable nitroxide radical that can readily permeate biological membranes (22, 23), to increase the IRE-binding activity of Irp1. Tempol has been shown to produce beneficial effects in cell and animal models of tumorigenesis, shock, hypertension, diabetes, ischemia-reperfusion injury, traumatic brain injury, and Parkinson’s disease (24–27) as well as to prevent alopecia induced by whole-brain radiotherapy in metastatic cancer patients when applied topically to the scalp (28). We previously showed that Tempol disassembles the cytosolic iron sulfur cluster of Irp1 and consequently activates its latent IRE-binding activity (29, 30). Combining these two unique molecular pathways of modulation of IRE-binding activity of Irp1 by Tempol and translational regulation of Hif2α by Irp1, we hypothesized that activation of Hif2α IRE binding of Irp1 in polycythemic VhlR200W mice by Tempol would inhibit Hif2α translation, reduce erythropoietin levels, and correct erythrocytosis/polycythemia.

## Results

**Tempol reverses plethora, splenomegaly, and erythrocytosis/polycythemia in VhlR200W mutant mice.** Erythrocytosis/polycythemia develops as early as 2 to 4 years of age in patients with Chuvash polycythemia (3). To elucidate whether VhlR200W mice develop erythrocytosis/polycythemia at such early ages, we measured the hematocrit levels of WT and R200W-mutant littermates at the ages of 42 to 112 days by capillary centrifugations as well as with a hematology analyzer, and we observed that the development of erythrocytosis/polycythemia started in VhlR200W mice at as early as 6 weeks of age (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI97684DS1), comparable to the temporal onset seen in very young human patients. The mutant mice also showed increased hemoglobin levels and RBC counts at early ages (Figure 1, B and C).

**VhlR200W mice developed erythema in their paws and snouts** (Figure 2A) as previously observed (14), analogous to the plethora seen in human patients with polycythemia. When these mutant mice were placed on a Tempol-supplemented diet at 6 to 8 weeks of age for a period of 3 to 6 months, the erythema in the paws and snouts disappeared, and they looked more like the WT animals. The spleens of VhlR200W mice were enlarged compared with those of their WT littermates (Figure 2B), indicating that splenomegaly and extramedullary erythropoiesis developed in these mutant mice. However, the spleens of VhlR200W mice fed a Tempol-supplemented diet looked normal and more like those of WT animals, suggesting that Tempol treatment reversed splenomegaly in these mutant mice.

We measured hematocrit levels of WT and VhlR200W mice maintained either on a control diet or on the same diet supplemented with Tempol and observed a significant increase in the hematocrit levels of control diet–fed VhlR200W mice in comparison with levels of WT mice on a control diet (Figure 2, C and D, and Supplemental Figure 2A), reconfirming that these mice had erythrocytosis/polycythemia. In addition, hemoglobin levels and RBC counts also significantly increased in the control diet–fed VhlR200W mice (Figure 2, E and F), as was observed earlier (14). Importantly, with Tempol treatment, the hematocrit, hemoglobin, and RBC levels of VhlR200W mice decreased significantly (Figure 2, C–F), consistent with our conclusion that erythropoiesis was reduced in Tempol-supplemented mutant mice. We did not observe any significant effect of either the mutation or the diet on RBC indices, reticulocytes, WBC counts, platelet counts, or serum glucose levels in VhlR200W mice (Supplemental Figure 2, B–H). We measured serum ferritin levels in control diet– and Tempol diet–fed WT and VhlR200W mice using the ELISA method and observed that serum ferritin levels were slightly but significantly higher in the control diet–fed VhlR200W mice compared with levels in WT mice (Supplemental Figure 2I), perhaps due to macrophage iron overload as a result of increased erythropagocytosis, as macrophages are the main source of serum ferritin (31).

**Decreased erythropoiesis in Tempol-treated Vhl-R200W mice results from the downregulation of EPO production caused by translational repression of Hif2α.** An earlier study showed that patients with Chuvash polycythemia had significantly higher serum erythropoietin concentrations (3). In previous work with VhlR200W mice (14), the researchers observed increased serum EPO, but the difference rela-
Downregulation of Hif2α in Tempol-fed VhR200W mice results from increased Hif2α IRE-binding activity of Irp1. Hif2α has an IRE in its 5′-UTR to which Irps can bind. To determine whether Tempol increased the Hif2α IRE-binding activity of Irp1 in these VhlR200W mice as previously observed in mouse embryonic fibroblast cells as well as in brain lysates from Irp2−/− animals (29), we performed an RNA mobility shift assay with kidney lysates from WT and VhlR200W mice using radiolabeled ferritin IRE and Hif2α IRE probes. Irp1 binding to both ferritin IRE and Hif2α IRE probes markedly increased in Tempol-supplemented mice (Figure 3, D–F), though the Irp1 protein levels remained unchanged (Figure 3G), indicating that increased binding of Irp1 with HIF2α IRE in its 5′-UTR in Tempol-treated VhlR200W mice contributed substantially to translational repression of HIF2α.

To confirm that Tempol ameliorated erythrocytosis/polycythemia in VhlR200W mice through activation of Irp1 binding to the Hif2α IRE, we fed Irp1−/− mice with either a control or Tempol-supplemented diet and then evaluated the complete blood counts (CBCs) in these mice. The hematocrit levels of Irp1−/− animals increased by more than 10% compared with those of WT mice on a control diet, as was the case with initial observations (18), but these elevated hematocrit levels did not decrease when Irp1−/− mice were fed a Tempol-supplemented diet (Figure 4A), demonstrating that Tempol supplementation ameliorated the extramedullary erythropoiesis.

Since EPO is a known specific transcriptional target of Hif2α (32, 33), we assessed Hif2α protein levels in nuclear fractions from renal lysates from WT mice on a control diet and VhR200W mice maintained on either a control diet or a Tempol-supplemented diet, after harvesting intact kidneys in a nitrogen chamber and lysing the tissues in an anaerobic chamber. We found that Hif2α protein levels were increased in VhR200W mouse kidney lysates but significantly reduced when the mutant mice were fed the Tempol-supplemented diet (Figure 3, B and C). We measured the mRNA levels of other HIF targets. Although Hickey et al. (14) found modest but significantly higher expression of the Glut1 gene in VhR200W kidney lysates, Glut1 mRNA levels remained unchanged in the kidneys of our VhR200W mice, nor did Tempol treatment alter the expression of Glut1 (Supplemental Figure 3A). Lung VEGF expression levels did not change in VhR200W mice (Supplemental Figure 3B), as was the case with initial observations (34).

Downregulation of Hif2α in Tempol-fed VhR200W mice prevented plethora, splenomegaly, and erythrocytosis/polycythemia in VhR200W mice. (A) The snouts and paws (indicated by white arrows) of VhR200W mice appeared more reddish in color than did those of their WT littermates. The redness disappeared when the mutant mice were fed a Tempol-supplemented diet for 3 months. (B) Splenomegaly of VhR200W mice was rescued by dietary Tempol supplementation, and the color of the spleens became lighter, suggesting that Tempol supplementation ameliorated the extramedullary erythropoiesis. (C and D) Hematocrit levels, as determined by capillary tube centrifugation, and (E) hemoglobin and (F) RBC levels of 5- to 8-month-old WT and VhR200W mice fed a control or Tempol-supplemented diet showed that with the control diet, these parameters were elevated in VhR200W mice, and Tempol supplementation significantly reduced hematocrit, hemoglobin, and RBC levels in the mutant mice. (D–F) ***P < 0.001, by ordinary 1-way ANOVA (multiple comparisons).

Figure 2. Dietary Tempol supplementation prevented plethora, splenomegaly, and erythrocytosis/polycythemia in VhR200W mice.
RBC and hemoglobin levels were also very high in Vhl R200W Irp1–/– mice, and these blood parameters were also not significantly decreased with Tempol treatment (Figure 4, B and C). In addition, Tempol did not significantly alter the RBC indices or reticulocyte, WBC, or platelet counts of Irp1–/– or Vhl R200W Irp1–/– mice.

Figure 3. Decreased erythropoiesis in Tempol-treated Vhl R200W mice was the result of EPO downregulation caused by translational repression of HIF2α, which was a consequence of the increased HIF2α IRE–binding activity of Irp1. (A) Serum EPO levels measured by ELISA were significantly increased in control diet–fed Vhl R200W mice, and Tempol treatment reduced EPO levels in these mutant mice. (B) Immunoblot analyses of nuclear fractions from kidney lysates showed increased HIF2α protein levels in Vhl R200W mice fed a control diet, and the HIF2α protein levels decreased when these mutant mice were fed a Tempol–supplemented diet. (C) Quantification of these HIF2α protein levels. (D) RNA mobility shift assays for ferritin IRE and HIF2α IRE binding of renal lysates in the absence or presence of β mercaptoethanol (ME) (which converts Irp1 from cytosolic aconitase to the IRE-binding form) showed that most of the IRE–binding activity was contributed by Irp1, and Tempol treatment significantly increased the IRE binding of Irp1 in Vhl R200W mice. Quantifications of the Irp1–binding activities of (E) ferritin IRE and (F) HIF2α IRE. (G) Western blot analyses of kidney lysates revealed that Irp1 protein levels remained unchanged in Tempol diet–fed Vhl R200W mice. Data in A, C, E, and F represent the mean ± SD. *P ≤ 0.05, **P ≤ 0.01, and ***P < 0.001, by unpaired, 2-tailed t test.

that Tempol did not lead to the suppression of hematocrit levels in the absence of Irp1. Moreover, we crossed Vhl R200W heterozygous (Vhl R/+) mice with Irp1–/– animals and bred the Vhl R/– Irp1–/– mice to generate Vhl R200W Irp1–/– mice. We maintained these mice on either a control or Tempol–supplemented diet after weaning. Strikingly, hematocrit levels were elevated to as high as 84% in these Vhl R200W Irp1–/– mice, indicating that impaired degradation of Hif2α caused by the homozygous R200W mutations in the Vhl gene, combined with translational derepression of Hif2α caused by the targeted deletion of Irp1, led to markedly increased erythropoiesis in these mice. These highly elevated hematocrit values did not significantly decrease upon Tempol treatment (Figure 4A), further confirming that Tempol attenuates erythrocytosis/polycthemia through Irp1 activation and demonstrating that deletion of Irp1 in mice blocks the beneficial effects of Tempol.

RBC and hemoglobin levels were also very high in Vhl R200W Irp1–/– mice, and these blood parameters were also not significantly decreased with Tempol treatment (Figure 4, B and C). In addition, Tempol did not significantly alter the RBC indices or reticulocyte, WBC, or platelet counts of Irp1–/– or Vhl R200W Irp1–/– mice. (Supplemental Figure 4, A–F). EPO levels of the control diet–fed Vhl R200W mice were 3.3-fold higher than those of WT mice maintained on the control diet (Figure 4D), whereas we detected a significant 1.5-fold increase of EPO levels in Vhl R200W mice, which indicated that Irp1 is a physiologic regulator of Hif2α and erythropoietin expression levels.

Tempol represses hypoxia-induced erythrocytosis/polycthemia in WT mice. Humans living at high altitudes face survival challenges because of erythrocytosis/polycthemia and ensuing complications caused by hypobaric hypoxia (35, 36). To determine whether Tempol
could attenuate erythrocytosis/polycythemia in mice that developed erythrocytosis/polycythemia caused by hypoxia, we placed WT mice fed a control or Tempol-supplemented diet into a hypoxia chamber containing 10% oxygen for 23 days and then measured RBC parameters. We found that the hematocrit, hemoglobin, and RBC levels of these mice increased significantly under hypoxic conditions (Figure 4, E–G). Tempol diet feeding significantly reduced the levels of hematocrit, hemoglobin, and RBC levels in hypoxic mice compared with control diet–fed mice (Figure 4, E–G), indicating that Tempol partially protected these hypoxic WT mice from developing erythrocytosis/polycythemia. Importantly, 2 of the 5 mice fed a control diet died while they were in the hypoxic chamber, whereas all 5 Tempol-fed hypoxic mice survived, suggesting that Tempol prevented mortality in mice exposed to hypoxic environments.

**Tempol increases the survival of VhlR200W mice.** We studied 1-year survival rates of WT, VhlR200W, and VhlR200W Irp1−/− mice maintained on a control or Tempol-supplemented diet and observed that the mortality rates of VhlR200W mice were significantly higher than those of WT mice and that Tempol supplementation changed the survival rates of VhlR200W mice from 87% to 93% (Figure 5), again suggesting a beneficial effect of Tempol on these mutant mice. However, survival rates did not increase with Tempol supplementation when Irp1 was deleted from these VhlR200W mice, reconfirming that Tempol acts primarily through Irp1 activation (Figure 5).
Discussion

In this study, we showed that activation of Irp1 in VhlR200W mice by dietary Tempol supplementation repressed Hif2α expression, decreased EPO production, ameliorated erythrocytosis/polycythemia, and increased the lifespans of these mice (Figure 6). Until recently, phlebotomy therapy had been the only known treatment for patients with Chuvash polycythemia, but it is not clear whether phlebotomy actually attenuates complications or reduces the mortality of these patients (13). On the contrary, frequent phlebotomies cause iron deficiency that can lead to two opposing effects on HIF2α expression. Iron deficiency exerts a direct stabilizing effect on HIF2α by inhibiting PHD2 activity and subsequently reducing its proteasomal degradation, whereas increased binding of IRP1 to the HIF2α IRE at low iron concentrations leads to translational repression of HIF2α. Overall, phlebotomies may increase HIF2α expression, induce iron deficiency in essential nonerythropoietic tissues, and fail to sufficiently repress erythrocytosis/polycythemia. It is important to note here that both HIF1α and HIF2α are expected to increase in many iron deficiency conditions, but the IRE present in the HIF2α transcript, but not in the HIF1α transcript, dictates that its translation will be repressed in iron-depleted cells that are rich in IRP1 (16, 18).

HIF2α is also associated with many other VHL diseases including CCRCCs, and targeting HIF2α is of tremendous interest for the treatment of patients with such diseases (37, 38). Small-molecule Hif2α inhibitors have been found to improve VHL disease–associated phenotypes in zebrafish (39) and in mouse models (37, 40), and some of these inhibitors are now in clinical trials. Thus, Tempol therapy can be very useful to treat the diseases associated with upregulation of HIF2α expression or mutation in the VHL or PHD2 gene. Tempol was also previously found to protect Irp2−/− mice from neurodegeneration through disassembly of the iron-sulfur cluster of Irp1, activating its IRE-binding activity and thus partially compensating for the loss of Irp2 in those cells and tissues in which Irp2 was the main functional Irp (29).

Tibetans and people living in high-altitude regions for generations have developed genetic adaptations and shown improved survival, despite living with decreased oxygen availability (41, 42). A high-frequency missense mutation in the EGLN1 gene encoding PHD2 was recently found to be responsible for the adaptive response in Tibetans (43). However, for most of the nonadapted people, exposure to high-altitude hypoxia causes erythrocytosis/polycythemia characterized by increased erythropoiesis and elevated hematocrit levels. Our results showed that Tempol therapy ameliorates hypoxic erythrocytosis/polycythemia, analogous to human hypoxia, indicating that Tempol could constitute a therapeutic approach to treat high-altitude erythrocytosis/polycythemia in nonadapted humans.

Our results showed hypersensitivity to EPO, which is a hallmark of Chuvash polycythemia patients (44, 45) and the Chuvash polycythemia VhlR200W mouse model (14). In normal individuals, VHL binds to the suppressor of cytokine signaling 1 (SOCS1) protein and promotes ubiquitin-mediated degradation of phosphorylated Janus kinase 2 (p-JAK2), thus blocking the JAK2/STAT5 pathway (46). In VhlR200W mice, mutated VHL was reported to bind to SOCS1 more tightly, thus reducing the degradation of p-JAK2 (46). As a result, stabilized p-JAK2 was proposed to hyperactivate the JAK2/STAT5 pathway, leading to EPO hypersensitivity in erythroid progenitors (46). Treatment of 3 Chuvash polycythemia patients with ruxolitinib, an inhibitor of JAK1 and JAK2, was recently shown to reduce the symptoms of erythrocytosis/polycythemia and the need for frequent phlebotomy treatments (47). However, our results suggest that increased HIF2α expression is the more important factor in causing erythrocytosis/polycythemia in VhlR200W mice.

Our present study shows that Irp1 functions as a critical regulator of erythropoietin expression through its ability to repress Hif2α translation when it is in its apo form, which lacks a Fe-S cluster, by binding to the Hif2α IRE. We have discovered that the use of Tempol, a stable nitroxide known to disassemble the Fe-S cluster in Irp1, leads to translational repression of Hif2α in a Chuvash polycythemia mouse model. Other drug therapy approaches have involved interfering with the functions of HIF2α itself or with downstream sensitivities to HIF2α. Our proposed treatment involves the repression of HIF2α translation, and it could be of great therapeutic value alone or in conjunction with other proposed treatments, since it acts upstream of other therapies. Since manipulations of iron status cause competing effects on HIF2α expression, we propose that targeting the upstream translational regulator IRP1 may offer a more straightforward treatment and better outcomes in patients whose prognosis remains relatively unimproved by current treatment strategies. Our proposed approach is also feasible and potentially cost effective. In summary, the results of our study, which demonstrate an important role for translational regulation of
HIF2α in these disease settings, suggest that oral Tempol administration should be explored as a therapeutic intervention to prevent erythrocytosis/polycythemia in patients with Chuvash polycythemia and in nonadapted individuals living at high altitude who suffer from erythrocytosis/polycythemia.

**Methods**

**Study design.** On the basis of our previous findings (18, 29), we hypothesized that Tempol would activate the IRE-binding activity of Irp1, which would thereby inhibit Hif2α translation and diminish polycythemia in VhlR200W mice. We measured hematocrit levels, which are the hallmark for erythrocytosis/polycythemia, using the capillary spin method. We also used an IDEXX hematoanalyzer to obtain hematocrit values for WT and VhlR200W mice on a control or Tempol-supplemented diet. Next, we used ELISA assays to determine serum EPO levels, since increased EPO production was found to cause erythrocytosis/polycythemia in VhlR200W mice. We measured Hif2α levels by Western blotting, because EPO is a target of Hif2α.

Since HIF2α has an IRE in its 5′-UTR, we assessed whether the Hif2α IRE-binding activity of Irp1 was increased in Tempol-supplemented VhlR200W mice by performing RNA mobility shift assays, also known as RNA gel shift or RNA bandshift assays, after incubating the renal cell lysate with 32P-labeled Hif2α IRE. We did not exclude any data. In all experiments, animals were randomized to different treatment groups. For the survival study experiment (Figure 5), sample sizes are specified in the figure legend, and for all other experiments, sample sizes are indicated in the figures. For Supplemental Figures 2–4, the sample sizes are specified in each figure legend.

**Animals.** The VhlR200W mice were originally generated by Hickey et al. (14). Heterozygous breeding pairs (C57BL/6 background) (48) were provided by Mary Slingo and Peter A. Robbins (University of Oxford, Oxford, United Kingdom). The mice were weaned at 3 to 4 weeks of age, genotyped as described by Hickey et al., and maintained on a normal NIH-07 diet unless otherwise noted. For diet experiments, WT and VhlR200W littermates were placed on a control diet or on the same diet supplemented with Tempol at 6 to 8 weeks of age and were maintained on these diets until used for experiments. Irp1+/− mice were generated as previously described (49, 50) and backcrossed with C57BL/6 mice for 10 generations. VhlR200WIrp1−/− mice were generated by first crossing VhlR200W heterozygous (VhlR/+ ) mice with Irp1−/− mice, and then breeding the VhlR+/−/+ mice. All the experiments were performed with 5- to 8-month-old mice unless noted otherwise. Mice of different genotypes and on different diets were age matched.
Diet description. The Tempol diet was prepared by uniformly mixing powdered Tempol (Sigma-Aldrich) with bacon-flavored mouse chow (Bio-Serv) by a cold-press technique at a concentration of 10 mg/g food. Bacon-flavored chow without Tempol was used as the control diet (29).

Hematocrit measurement (capillary spin method). Approximately 100 μl blood was harvested from each mouse by mandibular bleeding (from the facial vein) into a heparin-coated BD microtainer tube with lithium heparin. Hematocrit tubes were 80% filled with blood by capillary action, sealed, and centrifuged in a microhematocrit centrifuge (LW Scientific). Hematocrit values were measured with a hematocrit reader. Unless otherwise noted, all the hematocrit data presented were measured using this capillary spin method.

CBC measurement. Approximately 100 μl blood was harvested from each mouse by mandibular bleeding and placed into a heparin-coated tube. Hemoglobin and RBC levels of mice for the hypoxia experiment (Figure 4, F and G) were obtained from the CBC measurement on a Hemavet 1500 Analyzer (Drew Scientific). All other CBC measurements were done with an IDEXX ProCyte Dx Hematology Analyzer.

Serum erythropoietin and serum ferritin measurements by ELISA. Erythropoietin and ferritin were measured in serum samples with ELISA kits from R&D Systems. Hematocrit tubes were 80% filled with blood by capillary action, sealed, and centrifuged in a microhematocrit centrifuge (LW Scientific). Hematocrit values were measured with a hematocrit reader. Unless otherwise noted, all the hematocrit data presented were measured using this capillary spin method.

Tissue and lysate preparation. Animals were euthanized using CO2, and the tissues were harvested immediately inside a nitrogen chamber. Fresh tissues were lysed inside the anaerobic chamber in lysis buffer that was de-aerated by cyclic freeze-thawing and air removal with argon to stabilize Hif2α.

RNA mobility shift assay. The RNA mobility shift assay was performed as previously described (49). Tissue lysates were prepared in an anaerobic chamber, as described in the Tissue and lysate preparation section, in oxygen-depleted lysis buffer containing 10 mM HEPES (pH 7.2), 3 mM MgCl2, 40 mM KCl, 5% glycerol, 0.2% nonidet P-40, 5 mM DTT, 1 mM AEBSF, 10 μg/ml leupeptin, and Complete EDTA-Free Protease Inhibitor Mixture (Roche Applied Science). Lysate was added at a volume containing 10 μg total protein to bandshift buffer containing 25 mM Tris-HCl (pH 7.5) and 40 mM KCl, with or without 2% β-mercaptoethanol, a chemical that activates Irp1 in vitro. The resulting sample solutions were incubated for 5 minutes at room temperature with 12.5 μl reaction mixture containing 20% glycerol, 0.2 units/μl Super RNAsine, 0.6 μg/μl yeast transfer RNA (tRNA), 5 mM DTT, and 20 nM 32P-labeled IRE from the human ferritin H chain gene (51) or 60 nM 32P-labeled IRE from the Hif2α gene (52) in 25 mM Tris-HCl (pH 7.5) and 40 mM KCl. A 20-μl amount of the resulting reaction mixture was loaded into 10% acrylamide/TBE gel, which was run at 200 V for 2.15 hours, and the gel was fixed, dried, and exposed for autoradiography. Quantitation was performed with the ImageQuant program for Macintosh (GE Healthcare).

Western blot analysis and antibodies. Tissue lysates were prepared in an anaerobic chamber in oxygen-depleted lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, and Complete EDTA-Free Protease Inhibitor Mixture. Protein analysis with the anaerobic lysates was carried out as previously described (53). The lysates were added to sample buffers inside an anaerobic chamber for Western blot experiments. Equal amounts of protein (20–50 μg/lane) were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membrane was blocked with 5% nonfat milk and 0.1% Triton X-100 in PBS and probed at room temperature in the same blocking buffer. IRP1 antibody was prepared against purified human IRP1 and used at a 1:5,000 dilution. Hif2α Western blotting was done with nuclear fractions from kidney lysates after harvesting intact kidneys in a nitrogen chamber and lysing the tissues in an anaerobic chamber. Hif2α antibody (affinity-purified goat anti-human) was obtained from R&D Systems (catalog AF2886), and a final concentration of 1.0 μg/ml was used as described in the R&D Systems protocol. Monoclonal anti-β-actin antibody (Sigma-Aldrich) was used at a 1,000 dilution. Western blots were treated with secondary peroxidase-conjugated goat anti-rabbit IgG or sheep anti-mouse IgG antibodies (GE Healthcare) at 1:5,000 and 1:2,000 dilutions, respectively.

Statistics. Data are expressed as the mean ± SD. Comparisons between 2 groups were analyzed using an unpaired, 2-tailed t test. Analyses of multiple groups were performed using an ordinary 1-way ANOVA (multiple comparisons). P values of less than 0.05 were considered statistically significant. Statistical analyses of mouse survival curves were performed with a log-rank (Mantel-Cox) test (curve comparison) using GraphPad Prism 7.0 (GraphPad Software).

Study approval. All procedures involving mice were performed following protocols approved by the IACUC of the NICHD (protocol 15-038) and met NIH guidelines for the humane care of animals.

Author contributions. MCG designed the project, performed most of the experiments, analyzed the data, and wrote the manuscript. DLZ performed experiments, analyzed data, and reviewed the manuscript. HO performed experiments and reviewed the manuscript. MAE performed experiments and reviewed the manuscript. TAR designed and supervised the project and wrote the manuscript.

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