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LOSS OF JAK2 REGULATION VIA VHL-SOCS1 E3 UBIQUITIN HETEROCOMPLEX UNDERLIES CHUVASH POLYCYTHEMIA

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SUMMARY

Chuvash polycythemia (CP) is a rare congenital form of polycythemia caused by homozygous R200W and H191D mutations in the *von Hippel-Lindau (VHL)* gene whose gene product is the principal negative regulator of hypoxia-inducible factor. However, the molecular mechanisms underlying some of the hallmark features of CP such as hypersensitivity to erythropoietin are unclear. Here, we show that VHL directly binds suppressor of cytokine signalling 1 (SOCS1) to form a heterodimeric E3 ligase that targets phosphorylated (p)JAK2 for ubiquitin-mediated destruction. In contrast, CP-associated VHL mutants have altered affinity for SOCS1 and fail to engage and degrade pJAK2. Systemic administration of a highly selective JAK2 inhibitor, TG101209, reverses the disease phenotype in *vhl*^{R200W/R200W} knock-in mice, a model that faithfully recapitulates human CP. These results reveal VHL as a SOCS1-cooperative negative

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AUTHOR CONTRIBUTIONS

R.C.R. designed and performed biochemical and mouse experiments. R.I.S. designed and performed biochemical experiments. B.Z. performed the mouse experiments. P.H., S.B., S.S.S., S.N.G., O.R., V.W.K.C., and L.M.B. performed and assisted with the biochemical experiments. S.A.H., T.D.R., and M.M.H. assisted with the mouse experiments. D.L.B., D.A.C. and M.C.S. provided the reagents associated with the mouse experiments. W.Y.K. designed the mouse experiments. M.S.I. conceptualised the model and designed the experiments. M.O. conceptualised the model, designed the experiments and wrote the manuscript.

regulator of JAK2 and provide compelling biochemical and preclinical evidence for JAK2-targeted therapy in CP patients.

Keywords

VHL; SOCS; JAK2; STAT5; polycythemia

INTRODUCTION

von Hippel-Lindau (VHL) protein is the substrate-conferring component of an E3 ubiquitin ligase ECV (Elongins BC/Cul2/VHL) that polyubiquitylates α subunits of hypoxia-inducible factor (HIF), a master transcription factor that governs adaptive cellular responses to hypoxia, for oxygen-dependent destruction^{1–4}. Under hypoxia, HIF α escapes ECV-mediated destruction and partners with HIF β to form an active transcription factor that engages hypoxia-responsive elements (HRE) in the promoters/enhancers of numerous hypoxia-inducible genes including *glucose transporter-1 (GLUT-1)*, *vascular endothelial growth factor (VEGF)* and *erythropoietin (EPO)* to promote anaerobic metabolism, angiogenesis and erythropoiesis, respectively⁵.

Inheritance of a mutated *VHL* gene causes VHL disease, an autosomal dominant cancer syndrome characterized by the development of cerebellar and retinal haemangioblastoma, pheochromocytoma and clear-cell renal cell carcinoma (CCRCC)⁵. Although the incidence of VHL disease is rare at 1 in 36,000 individuals, biallelic inactivation of *VHL* is frequently associated with sporadic haemangioblastoma and CCRCC⁶. Most tumour-associated VHL mutants have been shown or are predicted to compromise the ability of VHL to either bind prolyl-hydroxylated HIF α or form a proper ECV complex^{7,8}, and additional lines of investigation have demonstrated the critical oncogenic role of HIF α in CCRCC^{9–12}. Recently, Ang et al. identified a specific homozygous mutation 598C→T (R200W) within *VHL* that causes congenital autosomal recessive Chuvash polycythemia (CP) endemic to the Chuvash Autonomous Republic of the Russian Federation¹³. Subsequently, R200W and additional mutations (e.g., H191D) have been identified in a significant proportion of congenital polycythemia patients in diverse ethnic backgrounds without gender bias^{14,15–16,17}, suggesting that a defect in the ability of CP-VHL to maintain proper oxygen homeostasis to be the principal mechanism underlying CP^{13,16,18,19}. Curiously however, unlike classical VHL disease, CP is not associated with an increased risk of cancer despite a common defect in the HIF pathway, which illustrates a current inability to distinguish biochemical features between CP- and tumour-associated VHL mutants.

Polycythemia is a condition characterized by a net increase in the total number of red blood cells (RBCs) resulting in an elevated haematocrit (Hct), and is generally categorised as primary or secondary. Primary polycythemia, often called polycythemia vera (PV), is defined as excessive erythrocytosis arising from an intrinsic defect in erythroid progenitors rendering them hypersensitive to or independent of EPO stimulation²⁰. Secondary polycythemia is defined as excessive erythrocytosis arising from increased production of EPO²⁰, most often secondary to conditions of chronic hypoxia such as individuals with chronic obstructive pulmonary disease or smokers but also as paraneoplastic syndromes associated with certain malignancies including renal cell and hepatocellular carcinoma. Secondary polycythemia can also originate through mutations in PHD2 and HIF2 α that ultimately promote EPO production^{21–23}, recapitulated in mice with constitutive expression of HIF2 α ^{16,24}.

JAK2 mutations, predominated by V617F that encodes constitutively active *JAK2*, have recently been identified in the vast majority of PV patients^{25–29}. *JAK2* binds most prominently to Signal Transducers and Activator of Transcription (STAT5) protein, which, upon phosphorylation by *JAK2*, dimerize and translocate to the nucleus to regulate expression of genes that control proliferation, differentiation and survival of haematopoietic cells³⁰. STAT5 also triggers a negative feedback mechanism by transactivating the expression of SOCS family members, which bind and inhibit activated *JAKs*³¹. Notably, SOCS1 directly binds and targets phosphorylated *JAK2* for ubiquitin-mediated degradation via E3 ubiquitin ligase ECS (Elongins BC/Cul2 or 5/SOCS1)^{32,33}. In addition, colony-forming units-erythroid (CFU-E) cells from the fetal livers of *SOCS1*^{−/−} mice were shown to be hyper-responsive to EPO³⁴. Moreover, *JAK2*(V617F) mutation induced PV phenotype in mouse bone marrow transplantation assays, and the introduction of *JAK2*(V617F) into cytokine-dependent cell lines promoted cytokine-independent signalling^{35–38}. Regardless of *JAK2*(V617F) mutation status, however, high STAT5 phosphorylation is detected in bone marrow biopsies of PV patients³⁹. These lines of evidence suggest that constitutive activation of *JAK2*-STAT5 signalling is a major causative determinant of PV, and that *JAK2*(V617F)-negative PV patients might harbour yet-identified mutations in genes encoding proteins in the *JAK2*-STAT5 pathway.

Most CP patients and *vhl*^{R200W/R200W} (*Vhl*^{R/R}) mice that faithfully recapitulate the human CP condition have elevated EPO levels, a hallmark feature of secondary polycythemia, due to the diminished capacity of CP-VHL(R200W) to bind HIF α ¹³, resulting in increased HIF-mediated transactivation of *EPO*. Intriguingly, there are also data from both mouse and human studies that suggest CP-associated VHL mutations mediate primary polycythemia. In particular, erythroid precursors from both CP patients and *VHL*^{R/R} mice exhibit an intrinsic hypersensitivity to EPO displayed by burst forming units-erythroid (BFU-E) cells^{13,18}. However, the molecular mechanism underlying this and other primary polycythemic features of CP is unknown.

RESULTS

CP-VHL mutants have reduced capacity to form ECV

A subset of tumour-associated VHL mutants is unable to form a proper ECV complex. We first asked whether CP-VHL mutants were similarly defective in binding to ECV components, and observed that CP-VHL (R200W and H191D; exhibits slightly faster migration under reducing conditions) mutants have diminished association with Elongins BC and Cul2 (Fig. 1a, b and Supplementary Fig. 1a). The tumour-associated VHL(C162F) mutant, which is known to be defective in forming an ECV complex⁴⁰, served as control. Thus, in addition to the previously reported defect in HIF α binding, CP-VHL mutants are compromised in ECV assembly, which also likely contributes to HIF α stabilization.

All tumour-associated VHL mutants tested-to-date have invariably shown a failure in binding to fibronectin (FN) and a concomitant defect in formation of FN fibrillar array in the extracellular space⁴¹. However, CP-VHL mutants showed robust extracellular FN matrix with distinct fibrillar array similar to wild-type VHL (Supplementary Fig. 1b and data not shown). Thus, VHL(R200W) and VHL(H191D) are the first naturally occurring disease-associated VHL mutants exhibiting proper FN matrix deposition, which is consistent with the absence of cancer predisposition in individuals with CP.

VHL binds to JAK2 in a proteasome- and EPO-dependent manner

In addition to reduced Cul2 binding, ³⁵S-metabolic labeling of 786-O (VHL^{−/−}) cells stably expressing VHL(R200W or H191D) revealed an associated protein of 120kDa in the

absence of proteasome inhibitor (Fig. 1b). JAK2 is approximately 120KDa and through its actions on STAT5 signalling is known to be a critical mediator of proliferation and survival of erythroid progenitor cells. Thus, we asked whether VHL interacts with JAK2. HA-VHL(WT) co-precipitated T7-JAK2 when co-expressed in HEK293 cells in a proteasome-dependent manner (Fig. 1c). Interestingly, HA-VHL(R200W and H191D) showed increased association with (p)JAK2 in comparison to HA-VHL(WT) in the absence of proteasome inhibitor (Fig. 1d). These results identify JAK2 as a proteasome-sensitive VHL-binding substrate and suggest that CP-VHL mutants have a diminished capacity to promote proteasome-dependent degradation of JAK2.

Notably, total level of JAK2 remained relatively unchanged upon HA-VHL(WT) expression (Fig. 1c and d, lower panels). We therefore sought to determine whether VHL bound to and degraded pJAK2. Introduction of HA-VHL(WT) in HEK293 cells resulted in a dramatic loss of pJAK2, while the total JAK2 expression remained relatively unchanged (Fig. 2a). In addition, the mouse haematopoietic cell line expressing EPO receptor (BaF3-EPOR) was stably infected with retrovirus encoding HA-human VHL to generate near-endogenous expression level of HA-VHL (Fig. 2b, lane 4, compare endogenous mouse (m)VHL level to slower migrating HA-human VHL). HA-VHL in BaF3-EPOR cells bound to endogenous JAK2 preferentially upon EPO stimulation in the presence of MG132, suggesting that the proteasome-sensitive substrate of VHL is pJAK2 (Fig. 2b).

VHL promotes ubiquitin-mediated degradation of pJAK2

We next asked whether VHL promotes ubiquitin-mediated degradation of activated JAK2, which is defined by Y1007/1008 phosphorylation⁴². HEK293 cells were co-transfected with plasmids encoding T7-JAK2 and EPOR and stimulated with EPO for 15 min to generate robust levels of pJAK2 (Fig. 2c, left panel). The whole cell extract was then immunoprecipitated with antibodies specific to T7 and the enriched T7-pJAK2 was then subjected to an in vitro ubiquitylation reaction using S100 extracts devoid of or reconstituted with VHL(WT) (Fig. 2c, right panel). While the total JAK2 expression was unaffected, the level of pJAK2 expression decreased dramatically in the presence of VHL(WT), which was accompanied by the appearance of pJAK2 polyubiquitylation (Fig. 2c, lane 4). The low level of VHL-dependent ubiquitylation observed in the absence of EPO is likely due to limited spontaneous JAK2 autophosphorylation, which is commonly observed upon ectopic JAK2 expression (Fig. 2c, right panel, lane 3). In addition, retrovirus driven shRNA-mediated knockdown of endogenous mVHL in BaF3-EPOR cells accentuated pJAK2 expression and downstream STAT5-responsive gene expression such as *PIM1* and *CISH* upon EPO treatment in comparison to BaF3-EPOR cells infected with retrovirus encoding non-targeting scrambled shRNA (Fig. 2d). VHL knockdown had negligible effect on SOCS1 expression, making non-specific changes in SOCS1 expression an unlikely explanation for the observed pJAK2 induction (Fig. 2d, lanes 1 and 2). These results suggest that VHL preferentially engages JAK2 upon EPOR activation to trigger ubiquitin-mediated down-regulation of pJAK2.

VHL patients rarely develop polycythemia despite harbouring VHL mutations that compromise HIF α degradation¹⁸. If deregulation of pJAK2 is involved in VHL-associated polycythemia, then tumour-associated VHL mutants incapable of binding or ubiquitylating HIF α are predicted to retain the ability to promote ubiquitin-mediated destruction of pJAK2. Consistent with our prediction, expression of a panel of tumour-associated VHL mutants, with the exception of F119S and L128F (discussed below), resulted in markedly lower pJAK2 expression in the absence of MG132 similar to wild-type VHL (Fig. 2e). In addition, a panel of tumour-associated VHL mutants retained binding to JAK2 in the presence of MG132 (Supplementary Fig. 2). Notably, well established α domain VHL mutants C162F and L158S, which cannot form an ECV^{40,43}, likewise decreased pJAK2 levels (Fig. 2e).

These results infer that a novel ECV-independent mechanism is responsible for VHL-mediated pJAK2 degradation.

To begin addressing the autonomy of HIF and JAK2 functions of VHL, we asked what the status of HIF and STAT5 responsive target genes were in microarray data available for RCC and matched normal kidney samples⁴⁴. The vast majority of RCC harbour VHL inactivating mutations, resulting in HIF stabilization. We have previously shown that the gene expression changes induced by VHL inactivation are highly correlated with those induced by HIF activation²⁴ and therefore hypothesized that RCCs would have high HIF-target gene expression. Indeed relative to matched normal kidney, the RCCs expressed a classic hypoxic signature including the upregulation of *VEGF* and *CA9* and downregulation of *IGFBP2* (Fig. 2f left panel). In contrast, the STAT5-responsive genes *SOCS1*, *CISH* and *RYK* showed no discernable changes in RCC (Fig. 2f right panel), reinforcing the notion that the oncogenic defects that give rise to RCC are unlikely to involve JAK2 signalling.

VHL binds to and requires SOCS1 to promote pJAK2 degradation

VHL, as well as certain F-box proteins that confer substrate specificity, have been shown recently to homodimerize^{45–49}. Moreover, homodimerization of entire E3 enzymes such as the SCF (Skp1/Cdc53 or Cul1/F-box protein) have been shown to increase the efficiency of ubiquitylation by improving spatial orientation of substrate to active site⁴⁹. We asked whether an F-box-like protein SOCS1 interacts with VHL to promote ECV-independent degradation of pJAK2. T7-VHL co-precipitated HA-SOCS1 when ectopically expressed in HEK293 cells (Fig. 3a, left panel), and similar results were obtained by reciprocal immunoprecipitation (Fig. 3a, right panel). Co-expression of VHL with SOCS1, 2 and 3 revealed preferential interaction between VHL and SOCS1 (Fig. 3b), which is in keeping with the known role of SOCS1, but not SOCS2 or 3, in the ubiquitin-mediated pJAK2 degradation^{32,50}. Notably, endogenous VHL co-precipitated endogenous SOCS1 in BaF3-EPOR cells treated with EPO (Fig. 3c) and affinity-purified recombinant HA-VHL(C162F), which cannot bind elongins B/C, Rbx1 and Cul2, bound specifically to re-natured FLAG-SOCS1 resolved on membrane (Fig. 3d). These results suggest a direct interaction between SOCS1 and VHL under physiologic conditions.

VHL(F119S) and VHL(L128F) mutants fail to promote pJAK2 degradation (Fig. 3e and see Fig. 2f), but are capable of forming an intact ECV to properly target HIF α for degradation (Fig. 3f), which further support the notion that the defect in pJAK2 regulation is independent of ECV function. Both F119S and L128F mutants were, however, severely compromised in binding SOCS1 (Fig. 3g) despite retaining the ability to bind JAK2 (data not shown), underscoring the potential requirement of SOCS1, rather than ECV complex formation, in VHL-mediated pJAK2 degradation. Moreover, analogous to the α domain of VHL, the SOCS-box of SOCS1 facilitates the recruitment of the ECS components including Cul5, Elongins BC and Rbx1^{33,51,52}. While both VHL(WT) and VHL(C162F; α domain mutant that cannot form an ECV) mutant promoted pJAK2 degradation when co-expressed with wild-type SOCS1, co-expression of SOCS1 Δ SOCS-box mutant abrogated pJAK2 degradation (Fig. 3h). These results suggest that the SOCS-box is necessary for enzymatic activity of the VHL/SOCS1 heterocomplex for the degradation of pJAK2.

CP-VHL has altered affinity for SOCS1, attenuating pJAK2 engagement

We asked whether the observed defect in pJAK2 degradation via CP-VHL was due to a failure in binding SOCS1. Unexpectedly, both VHL(R200W and H191D) mutants showed a dramatic increase in SOCS1 binding in comparison to their wild-type VHL counterpart (Fig. 4a), which suggests that CP-causing mutations confer significantly higher affinity for SOCS1. We next asked whether this altered affinity of CP-VHL for SOCS1 affected pJAK2

recruitment. pJAK2 co-precipitated significantly lower amounts of CP-VHL mutants in comparison to VHL(WT), suggesting that the abnormal association between CP-VHL and SOCS1 hinders pJAK2 substrate binding (Fig. 4b). We next directly compared the efficiency of VHL(WT)/SOCS1 against CP-VHL/SOCS1 in promoting pJAK2 degradation. T7-pJAK2 was first generated by ectopic expression of EPOR and T7-JAK2 in HEK293 cells followed by EPO stimulation. Cells were lysed and immunoprecipitated with an anti-T7 antibody. T7-pJAK2 enriched on beads were washed and equally distributed into 4 reaction tubes, as confirmed by comparable levels of anti-T7 IgG_L (Fig. 4c, bottom panel), and mixed with HEK293 cell lysates expressing empty plasmid (Mock), HA-VHL(WT, R200W or H191D) in combination with HA-SOCS1. VHL(WT)/SOCS1 containing lysate markedly reduced the level of pJAK2 in comparison to CP-VHL/SOCS1 or SOCS1 only containing lysates (Fig. 4c). Consistent with this observation, EPO-induced pJAK2 levels persisted longer in BaF3-EPOR-shVHL cells reconstituted with lentivirus-mediated HA-VHL(R200W) in comparison to HA-VHL(WT) reconstitution (Fig. 4d). These results demonstrate that the CP-VHL/SOCS1 heterocomplex is defective in promoting pJAK2 degradation.

VHL negatively regulates EPOR-mediated JAK2-dependent colony growth

Stable knockdown of VHL in BaF3-EPOR cells increased the number of colonies in 1% methylcellulose at sub-saturating EPO concentrations (Fig. 4e, top panel, 50 and 100mU). In contrast, no significant changes were observed in colony number between VHL knockdown and non-targeting control BaF3-EPOR cells in increasing IL3 concentrations (Fig. 4e, bottom panel), which suggests a yet-defined complexity of regulation at the level of receptor specificity. We asked whether the increased EPOR-specific colony formation upon VHL loss involved JAK2. The molar concentration of highly specific JAK2 inhibitor (TG101209; 0.6μM) that resulted in a mild reduction in colony formation of BaF3-EPOR cells plated on methylcellulose containing a sub-saturating EPO concentration was first determined (Supplementary Fig. 3). At this concentration of TG101209, the percent inhibition of colony formation was more pronounced in BaF3-EPOR with stable VHL knockdown than in non-targeting control cells (Fig. 4f). In addition to a quantitative increase in colony number, VHL loss increased the actual size of the colonies, which was reduced upon TG101209 treatment (Fig. 4g). These results suggest that VHL negatively regulates EPOR-mediated colony growth and size in a JAK2-dependent manner.

JAK2 inhibitor treatment corrects CP phenotypes *in vivo*

Vhl^{R/R} mice harbouring a homozygous R200W mutation have been shown to develop age-dependent polycythemia with significant elevations in Hct starting at 14–16 weeks of age¹⁸. We generated a cohort of *Vhl*^{R/R} mice, at least 20 weeks of age, and subjected them to treatment with TG101209 (100mg/kg) or vehicle by twice daily oral gavage⁵³. The Hct levels of TG101209-treated *Vhl*^{R/R} mice steadily declined from baseline while the Hct of vehicle-treated mice were relatively unchanged (mean Hct at 5 weeks of treatment: 35.4% and 49.4% respectively, *P* < 0.05) (Fig. 5a). The Hct levels of TG101209-treated wild-type mice declined as expected (mean Hct decreased from approximately 42% to 33% over 5 weeks). The difference in Hct was clearly evident by comparison of the plantar footpads of the *Vhl*^{R/R} mice but not wild-type mice (Fig. 5b and Supplementary Fig. 4).

Splenomegaly is a common distinguishing feature of primary polycythemia and evident in *Vhl*^{R/R} mice¹⁸. At the time of necropsy, the spleens of vehicle-treated *Vhl*^{R/R} mice were noticeably larger than those of TG101209-treated counterparts (Fig. 5c). In contrast, the spleens of wild type mice treated with TG101209 or vehicle were indistinguishable (Supplementary Fig. 4). Previous reports have shown that the spleens of *Vhl*^{R/R} mice have an increased number of megakaryocytes, another feature of primary polycythemia not evident

in secondary polycythemia¹⁸. Evaluation of H&E stained sections of the spleens revealed a significantly increased number of megakaryocytes in vehicle-treated *Vhl*^{R/R} mice relative to TG101209-treated mice (Fig. 5c, arrows, and Fig. 5d, $P < 0.005$). These results suggest that splenomegaly and the increased proliferation of megakaryocytes seen in *Vhl*^{R/R} mice are JAK2-dependent.

We next asked to what extent our *in vivo* findings were mediated in a cell autonomous manner. Erythroid progenitors from PV patients are hypersensitive to EPO due to JAK2 activating mutations associated with increased levels of phosphorylated JAK2 and STAT5³⁹. Consistent with previous reports showing that erythroid progenitors from CP patients and *Vhl*^{R/R} mice are hypersensitive to EPO^{13,18}, we observed an increased number of CFU-E colonies in EPO-treated erythroid progenitors from *Vhl*^{R/R} mice compared to WT mice (Fig. 5e). In order to determine whether the primary hypersensitivity was mediated in a JAK2-dependent manner we first asked whether pJAK2 was upregulated in the erythroid precursors of *Vhl*^{R/R} mice. To this end, single cell suspensions enriched with erythroid progenitors were generated from spleens of phenylhydrazine-treated *Vhl*^{R/R} or WT mice and residual cytokines were removed by washes in cytokine-free media. Cells were cytokine starved for an additional 4 h to purge any pre-existing stimulation of the JAK2-STAT5 pathway and subsequently treated with increasing concentrations of exogenous EPO for 15 min. Expression levels of pJAK2 and pSTAT5 were markedly higher in *Vhl*^{R/R} compared to the WT erythroid progenitor-enriched cell lysates (Fig. 5f), suggesting that their hypersensitivity to EPO might be mediated in a JAK2-dependent manner. Moreover, consistent with observations made in cell lines, VHL(R200W) isolated from *Vhl*^{R/R} splenic cells co-precipitated endogenous SOCS1 and JAK2 (Fig. 5g).

To further ascertain whether the hypersensitivity of *Vhl*^{R/R} erythroid precursors to EPO was mediated in a JAK2-dependent manner, we evaluated the effects of JAK2 inhibition by performing CFU-E assays using haematopoietic precursors isolated from the spleens of *Vhl*^{R/R} mice in the presence or absence of exogenous EPO and TG101309 or vehicle. No CFU-E colonies were appreciable in the absence of EPO in either vehicle- or TG101209-treated mice (data not shown). In the presence of EPO, the number of CFU-E formed was significantly lower in haematopoietic precursors cultured in the presence of TG101209 relative to vehicle (Fig. 5h), suggesting that the hypersensitivity of *Vhl*^{R/R} haematopoietic precursors to EPO is mediated in a JAK2 dependent-manner. These results demonstrate that homozygous R200W mutation increases JAK2-STAT5 signalling and impart hypersensitivity to EPO in a JAK2-dependent manner.

DISCUSSION

Mapping of VHL disease-associated mutations on VHL/Elongin B/Elongin C (VBC) crystal structure engaged with HIF1 α peptide has revealed two major domains α and β required for Elongin C and HIF1 α binding, respectively^{7,8,54}. VHL mutations that disrupt (F119S and L128F) or enhance (R200W and H191D) SOCS1 binding interestingly clustered to a unique third region of VHL, revealing a likely protein-protein interaction interface or 'SOCS groove' required for the engagement of SOCS1 (Fig. 6a). Notably, the SOCS groove does not overlap with Elongin C or HIF1 α binding interface. This is consistent with the observed autonomy of HIF- and JAK2- associated functions of VHL clearly revealed by F119S and L128F mutants, which retain the ability to degrade HIF α but fail to degrade pJAK2 despite their ability to form ECV. Conversely, α domain C162F mutant retains the ability to degrade pJAK2 despite its inability to form ECV and degrade HIF α .

The present findings support the following revised model of CP (Fig. 6b). In normal individuals, VHL forms a proper ECV complex and negatively regulate HIF α via the

ubiquitin pathway. In contrast, CP-associated mutations attenuate HIF α binding and ECV complex formation, causing the reported mild stabilization of HIF α , which leads to the overproduction of HIF-target EPO in the kidney and secondary polycythemia. In normal individuals, VHL also binds SOCS1 through its SOCS groove and trigger ubiquitin-mediated pJAK2 degradation, and thus negatively regulate the JAK2-STAT5 pathway. CP-associated mutations cause conformational change within the SOCS groove, leading to an inordinately tight CP-VHL/SOCS1 association and impede pJAK2 recruitment and degradation. Resulting pJAK2 stabilization promotes hyperactivation of the JAK2-STAT5 pathway in erythroid progenitors, causing hypersensitivity to EPO and primary polycythemia.

PV-associated JAK2(V617F) mutation causes uncontrolled expansion of RBCs, but also gives rise to pleomorphic and clustered megakaryocytes hypersensitive to thrombopoietin, which, similar to EPO, signals through JAK2 via the thrombopoietin receptor³⁵. Abnormal megakaryocyte function is thought to be critical in thrombotic complications frequently observed in PV patients⁴². Strikingly, *Vhl*^{R/R} mice exhibit increased number of megakaryocytes that cluster and CP patients, like PV patients, often present with thrombotic complications^{13,18}. In contrast, secondary polycythemia associated with elevated serum EPO does not give rise to megakaryocyte defects; an observation faithfully supported in mice with constitutive overexpression of EPO that do not develop thrombotic complications despite inordinately high RBC count⁵⁵. Moreover, some CP patients appear not to exhibit elevated serum EPO levels^{15,17,56}. These observations suggest that the hyperactive JAK2-STAT5 signalling, rather than the increased EPO production due to a mild defect in HIF regulation, is the principal mechanism underlying thrombotic complications observed in CP patients. Perhaps more importantly, the ability of pharmacologic JAK2 inhibition to normalize the number of splenic megakaryocytes in *Vhl*^{R/R} mice suggests that not only will JAK2 inhibition be useful to lower the Hct of CP patients but might also be able to lower the rate of thrombotic complications.

The discovery of JAK2 mutations in PV patients has certainly expedited the clinical trials of JAK2 inhibitors in the management of PV. However, despite clinical features shared between PV and CP, including hypersensitivity to EPO and megakaryocyte defects associated with thrombotic complications, JAK2 inhibitors have not been considered for CP patients since the critical role of JAK2 in the pathogenesis of CP has not been, until now, realized. Thus, the present findings not only uncover a molecular cooperativity between VHL and SOCS1 in the negative regulation of JAK2-STAT5 pathway, but also provide compelling biochemical and preclinical evidence for JAK2-targeted therapy in CP patients.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Supplementary Material

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Acknowledgments

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References

1. Ivan M, et al. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science*. 2001; 292:464–8. [PubMed: 11292862]
2. Jaakkola P, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science*. 2001; 292:468–72. [PubMed: 11292861]
3. Maxwell P, et al. The von Hippel-Lindau gene product is necessary for oxygen-dependent proteolysis of hypoxia-inducible factor α subunits. *Nature*. 1999; 399:271–5. [PubMed: 10353251]
4. Ohh M, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol*. 2000; 2:423–7. [PubMed: 10878807]
5. Roberts AM, Ohh M. Beyond the hypoxia-inducible factor-centric tumour suppressor model of von Hippel-Lindau. *Curr Opin Oncol*. 2008; 20:83–9. [PubMed: 18043261]
6. Kim WY, Kaelin WG. Role of VHL gene mutation in human cancer. *J Clin Oncol*. 2004; 22:4991–5004. [PubMed: 15611513]
7. Stebbins CE, Kaelin WG, Pavletich NP. Structure of the VHL-ElonginC-elonginB complex: implications for VHL tumor suppressor function. *Science*. 1999; 284:455–61. [PubMed: 10205047]
8. Min JH, et al. Structure of an HIF-1 α -pVHL complex: hydroxyproline recognition in signaling. *Science*. 2002; 296:1886–9. [PubMed: 12004076]
9. Clifford SC, et al. Contrasting effects on HIF-1 α regulation by disease-causing pVHL mutations correlate with patterns of tumorigenesis in von Hippel-Lindau disease. *Hum Mol Genet*. 2001; 10:1029–38. [PubMed: 11331613]
10. Kondo K, Kim WY, Lechpammer M, Kaelin WG Jr. Inhibition of HIF2 α is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol*. 2003; 1:E83. [PubMed: 14691554]
11. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. *Cancer Cell*. 2002; 1:237–246. [PubMed: 12086860]
12. Hoffman MA, et al. von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet*. 2001; 10:1019–27. [PubMed: 11331612]
13. Ang SO, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet*. 2002; 32:614–621. [PubMed: 12415268]
14. Perrotta S, et al. Von Hippel-Lindau-dependent polycythemia is endemic on the island of Ischia: identification of a novel cluster. *Blood*. 2006; 107:514–9. [PubMed: 16210343]
15. Pastore Y, et al. Mutations of von Hippel-Lindau tumor-suppressor gene and congenital polycythemia. *Am J Hum Genet*. 2003; 73:412–9. [PubMed: 12844285]
16. Pastore YD, et al. Mutations in the VHL gene in sporadic apparently congenital polycythemia. *Blood*. 2003; 101:1591–5. [PubMed: 12393546]
17. Bento MC, et al. Congenital polycythemia with homozygous and heterozygous mutations of von Hippel-Lindau gene: five new Caucasian patients. *Haematologica*. 2005; 90:128–9. [PubMed: 15642680]
18. Hickey MM, Lam JC, Bezman NA, Rathmell WK, Simon MC. von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2 α signaling and splenic erythropoiesis. *J Clin Invest*. 2007; 117:3879–89. [PubMed: 17992257]
19. van Rooijen E, et al. Zebrafish mutants in the von Hippel-Lindau (VHL) tumor suppressor display a hypoxic response and recapitulate key aspects of Chuvash polycythemia. *Blood*. 2009
20. Messinezy M, Pearson TC. The classification and diagnostic criteria of the erythrocytoses (polycythaemias). *Clin Lab Haematol*. 1999; 21:309–16. [PubMed: 10646073]
21. Percy MJ, et al. A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. *Blood*. 2007; 110:2193–6. [PubMed: 17579185]
22. Percy MJ, et al. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med*. 2008; 358:162–8. [PubMed: 18184961]
23. Percy MJ, et al. A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci USA*. 2006; 103:654–9. [PubMed: 16407130]

24. Kim WY, et al. Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. *EMBO J.* 2006; 25:4650–62. [PubMed: 16977322]
25. Baxter EJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005; 365:1054–61. [PubMed: 15781101]
26. James C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature.* 2005; 434:1144–8. [PubMed: 15793561]
27. Kralovics R, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med.* 2005; 352:1779–90. [PubMed: 15858187]
28. Levine RL, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell.* 2005; 7:387–97. [PubMed: 15837627]
29. Zhao R, et al. Identification of an acquired JAK2 mutation in polycythemia vera. *J Biol Chem.* 2005; 280:22788–92. [PubMed: 15863514]
30. Watowich SS, et al. Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu Rev Cell Dev Biol.* 1996; 12:91–128. [PubMed: 8970723]
31. Constantinescu SN, Girardot M, Pecquet C. Mining for JAK-STAT mutations in cancer. *Trends Biochem Sci.* 2008; 33:122–31. [PubMed: 18291658]
32. Ungureanu D, Saharinen P, Junttila I, Hilton DJ, Silvennoinen O. Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. *Mol Cell Biol.* 2002; 22:3316–26. [PubMed: 11971965]
33. Kamizono S, et al. The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. *Journal of Biological Chemistry.* 2001; 276:12530–8. [PubMed: 11278610]
34. Sarna MK, et al. Differential regulation of SOCS genes in normal and transformed erythroid cells. *Oncogene.* 2003; 22:3221–30. [PubMed: 12761492]
35. Wernig G, et al. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood.* 2006; 107:4274–81. [PubMed: 16478879]
36. Lacout C, et al. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood.* 2006; 108:1652–60. [PubMed: 16670266]
37. Bumm TG, et al. Characterization of murine JAK2V617F-positive myeloproliferative disease. *Cancer Res.* 2006; 66:11156–65. [PubMed: 17145859]
38. Tiedt R, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood.* 2008; 111:3931–40. [PubMed: 18160670]
39. Teofili L, et al. Different STAT-3 and STAT-5 phosphorylation discriminates among Ph-negative chronic myeloproliferative diseases and is independent of the V617F JAK-2 mutation. *Blood.* 2007; 110:354–9. [PubMed: 17376889]
40. Ohh M, et al. Synthetic peptides define critical contacts between elongin C, elongin B, and the von Hippel-Lindau protein. *J Clin Invest.* 1999; 104:1583–91. [PubMed: 10587522]
41. Ohh M, et al. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell.* 1998; 1:959–68. [PubMed: 9651579]
42. Bellucci S, Michiels JJ. The role of JAK2 V617F mutation, spontaneous erythropoiesis and megakaryocytopoiesis, hypersensitive platelets, activated leukocytes, and endothelial cells in the etiology of thrombotic manifestations in polycythemia vera and essential thrombocythemia. *Semin Thromb Hemost.* 2006; 32:381–98. [PubMed: 16810614]
43. Lonergan KM, et al. Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. *Mol Cell Biol.* 1998; 18:732–41. [PubMed: 9447969]
44. Lenburg ME, et al. Previously unidentified changes in renal cell carcinoma gene expression identified by parametric analysis of microarray data. *BMC Cancer.* 2003; 3:31. [PubMed: 14641932]
45. Chung J, Roberts AM, Chow J, Coady-Osberg N, Ohh M. Homotypic association between tumour-associated VHL proteins leads to the restoration of HIF pathway. *Oncogene.* 2006; 25:3079–83. [PubMed: 16407835]

46. Dixon C, et al. Overproduction of polypeptides corresponding to the amino terminus of the F-box proteins Cdc4p and Met30p inhibits ubiquitin ligase activities of their SCF complexes. *Eukaryot Cell*. 2003; 2:123–33. [PubMed: 12582129]
47. Kominami K, Ochotorena I, Toda T. Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. *Genes Cells*. 1998; 3:721–35. [PubMed: 9990507]
48. Suzuki H, et al. Homodimer of two F-box proteins betaTrCP1 or betaTrCP2 binds to IkappaBalpha for signal-dependent ubiquitination. *J Biol Chem*. 2000; 275:2877–84. [PubMed: 10644755]
49. Tang X, et al. Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination. *Cell*. 2007; 129:1165–76. [PubMed: 17574027]
50. Song MM, Shuai K. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *J Biol Chem*. 1998; 273:35056–62. [PubMed: 9857039]
51. Kamura T, et al. Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase. *J Biol Chem*. 2001; 276:29748–53. [PubMed: 11384984]
52. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J Biol Chem*. 2002; 277:42394–8. [PubMed: 12228220]
53. Pardanani A, et al. TG101209, a small molecule JAK2-selective kinase inhibitor potently inhibits myeloproliferative disorder-associated JAK2V617F and MPLW515L/K mutations. *Leukemia*. 2007; 21:1658–68. [PubMed: 17541402]
54. Hon WC, et al. Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. *Nature*. 2002; 417:975–8. [PubMed: 12050673]
55. Shibata J, et al. Hemostasis and coagulation at a hematocrit level of 0.85: functional consequences of erythrocytosis. *Blood*. 2003; 101:4416–22. [PubMed: 12576335]
56. Cario H, et al. Mutations in the von Hippel-Lindau (VHL) tumor suppressor gene and VHL-haplotype analysis in patients with presumable congenital erythrocytosis. *Haematologica*. 2005; 90:19–24. [PubMed: 15642664]

Figure 1. CP-VHL exhibits altered binding to ECV components and JAK2

(A) HEK293 cells transfected with the indicated plasmids were immunoprecipitated and immunoblotted with the indicated antibodies. (B) ^{35}S -radiolabelled 786-O subclones stably expressing indicated HA-VHL were immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE and visualized by autoradiography. (C) HEK293 cells transfected with the indicated combination of plasmids were treated with (+) or without (–) MG132. Equal amounts of cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (D) HEK293 cells transfected with the indicated plasmids were lysed in the absence of MG132, immunoprecipitated and immunoblotted with the indicated antibodies. WCE: whole cell extract; IP: immunoprecipitation; IB: immunoblot; AR: autoradiography; Asterisk: non-specific protein bands.

Figure 2. VHL promotes ubiquitin-mediated destruction of pJAK2

(A) HEK293 cells transfected with the indicated plasmids were lysed and immunoblotted with the indicated antibodies. (B) BaF3-EPOR cells infected with retrovirus encoding HA-VHL were lysed, immunoprecipitated with anti-HA or isotype-matched control antibody and immunoblotted with anti-VHL antibody (right panel). The same HA-VHL-expressing BaF3-EPOR cells treated with (+) or without (-) EPO were immunoprecipitated and immunoblotted with the indicated antibodies (left panel). (C) HEK293 cells transfected with the indicated plasmids were treated with (+) or without (-) 20U ml⁻¹ of EPO and pJAK2 isolated via anti-T7 immunoprecipitation (left panel), which was then added to an in vitro ubiquitylation reaction containing proteasome-depleted S100 fractions with (+) or without (-) VHL (right panels). Reaction mixtures were re-immunoprecipitated with anti-T7 antibody and immunoblotted with the indicated antibodies. (D) BaF3-EPOR-shVHL or -shScr cells stimulated with EPO for indicated time periods were lysed and immunoblotted with the indicated antibodies. RNA was also extracted and indicated STAT5- responsive mRNA levels were measured by quantitative real-time PCR normalized to *GAPDH* and EPO-stimulated levels of transcripts in BaF3-EPOR-shScr cells arbitrarily set to 1.0 (graph). Error bars represent standard deviations of the fold-changes over three independent experiments. (E) HEK293 cells transfected with indicated plasmids were lysed and immunoblotted with the indicated antibodies. (F) Expression profiles of known HIF- (left panel) and STAT5- (right panel) responsive genes were generated by analysis of 18 RCC

samples in comparison to normal matched tissue control run on the Affymetrix Human Genome U133B Array (GEO GDS507)⁴⁴. Median expression is indicated by horizontal bar. Error bars represent the standard deviation in the levels for each sample set. Asterisk denotes non-specific protein bands.

Figure 3. VHL binds to and cooperates with SOCS1 to negatively regulate pJAK2

(A,B) HEK293 cells transfected with the indicated plasmids were lysed, immunoprecipitated and immunoblotted with the indicated antibodies. (C) BaF3-EPOR cells stimulated with (+) or without (–) EPO were lysed, immunoprecipitated with anti-VHL or isotype-matched control antibody and immunoblotted with the indicated antibodies. (D) Affinity-purified HA-VHL(C162F) and cell lysates generated from HEK293 cells expressing FLAG-SOCS1 or HA-VHL(C162F) were resolved on SDS-PAGE and immunoblotted with anti-HA antibody (lanes 1–3). HEK293 cells transfected with empty plasmid (Mock) or plasmid encoding FLAG-SOCS1 were lysed and immunoblotted with anti-FLAG antibody (lanes 4 and 5) or immunoprecipitated with anti-FLAG antibody and far-Western blotted (FB) with purified recombinant HA-VHL(C162F) followed by anti-HA antibody (lanes 6 and 7). (E) Whole cell extracts prepared from HEK293 cells transfected with the indicated plasmids were immunoblotted with the indicated antibodies. (F) 786-O subclones stably expressing the indicated HA-VHL were lysed and immunoblotted with indicated antibodies (right panel) or immunoprecipitated with anti-HA antibody and immunoblotted with indicated antibodies (left panel). (G) HEK293 cells transfected with the indicated plasmids in combination with a plasmid encoding HA-SOCS1 were lysed, immunoprecipitated and immunoblotted with anti-VHL antibody. (H) Whole cell extracts prepared from HEK293 cells transfected with the indicated plasmids and stimulated with EPO were immunoblotted with the indicated antibodies. Asterisk: non-specific protein bands.

Figure 4. CP-VHL mutants are defective in pJAK2 degradation and pharmacologic JAK2 inhibition attenuates VHL-dependent BaF3-EPOR colony formation

(A) HEK293 cells transfected with the indicated plasmids were lysed, immunoprecipitated and immunoblotted with anti-HA antibody. (B) HEK293 cells transfected with the indicated plasmids were treated with EPO and MG132, lysed, immunoprecipitated and immunoblotted with the indicated antibodies. (C) Equal amounts of T7-pJAK2 isolated from EPO-treated HEK293 cells were mixed with HEK293 cell lysates expressing indicated plasmids. Reaction mixtures were immunoprecipitated with anti-T7 antibody and immunoblotted with anti-pJAK2 antibody. (D) BaF3-EPOR-shVHL cells infected with lentivirus encoding HA-VHL(R200W and WT) were treated with EPO for indicated time periods and immunoblotted with the indicated antibodies. (E) Polyclonal BaF3-EPOR cells stably expressing shVHL or shScr were plated in 1% methylcellulose containing the indicated levels of EPO (top graph) or IL3 (bottom graph). Colony numbers were generated from a single-blind analysis and are representative of four (top graph) or three (bottom graph) independent experiments performed in duplicate. Error bars represent standard deviation between replicate wells. (F) BaF3-EPOR cells were plated in 1% methylcellulose containing EPO in combination with the indicated concentrations of JAK2 inhibitor TG101209. Colony numbers were generated from a single-blind analysis and are representative of two independent experiments performed in triplicate. Error bars represent standard deviation between replicate wells. (G) BaF3-EPOR-shVHL and -shScr cells were plated in 1% methylcellulose containing 100mU EPO in combination with the indicated concentrations of TG101209. Eighty colonies were counted for each condition and average colony size was

determined by digitization of microscope photographs followed by measurement of pixel count for each colony, generating an arbitrary two-dimensional area directly proportional to colony size. Scale bar = 0.2mm. Colonies extracted from methylcellulose were lysed and immunoblotted with the indicated antibodies (right panels).

Figure 5. TG101209 treatment rescues polycythemia features in $Vhl^{R/R}$ mice

(A) Hct levels of vehicle or TG101209 treated $Vhl^{R/R}$ mice. Normal Hct range highlighted in grey. $P < 0.05$ after 5 weeks of treatment. Error bars = standard error of the mean (SEM). (B) Photographs of the plantar footpads of vehicle and TG101209 treated $Vhl^{R/R}$ mice. Scale bar = 3.0mm (C) Photographs of spleens (scale bar = 4.0mm) and photomicrographs of H&E stained sections of spleens (scale bars = 10µm) from $Vhl^{R/R}$ mice treated with vehicle or TG101209. Yellow arrows indicate megakaryocytes. (D) Quantification of average number of megakaryocytes per high power field in spleens of vehicle or TG101209 treated $Vhl^{R/R}$ mice, $P < 0.005$. Error bars = SEM. (E) Numbers of CFU-E colonies formed from the splenic haematopoietic precursors of $Vhl^{R/R}$ versus WT mice when cultured in the presence of $3U\ ml^{-1}$ of EPO, $P < 0.005$. Error bars = SEM. (F) Single cell suspensions enriched with erythroid progenitors generated from spleens of phenylhydrazine-treated $Vhl^{R/R}$ or WT mice were washed, starved in cytokine-free media and treated with increasing concentrations of exogenous EPO for 15 min. Cell lysates were then immunoblotted with the indicated antibodies. (G) $Vhl^{R/R}$ splenic cells were lysed and sonicated in the presence of phosphatase inhibitors and MG132. Lysates were equally divided, immunoprecipitated with anti-VHL or isotype-matched control antibody, and immunoblotted with the indicated antibodies. (H) Numbers of CFU-E colonies formed from the splenic haematopoietic precursors of $Vhl^{R/R}$

mice when cultured in the presence of vehicle or TG101209, $P < 0.05$. Error bars = SEM. Asterisk denotes non-specific protein bands.

Figure 6. The 'SOCS groove' and the revised molecular model of CP

(A) Mutations that influence SOCS1 binding are clustered within the 'SOCS-groove' as indicated (red) on the VHL/Elongin B/Elongin C crystal structure bound to HIF1 α peptide. Analyzed using DeppView/Swiss-PdbViewer v4.0. (B) Molecular model of CP. See text for details.