Molecular Characterization of the von Hippel-Lindau Ubiquitin Ligase

by

Roxana Ioana Sufan

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology
University of Toronto

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2009

Abstract

Marking proteins for degradation by the proteasome is a classical function of ubiquitination. This process of covalent attachment of a chain of ubiquitin molecules to target proteins is governed by the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3). The von Hippel-Lindau (VHL) tumour suppressor protein forms an E3 ubiquitin ligase, ECV (Elongins BC/Cul2/VHL), which targets the α subunit of hypoxia-inducible factor (HIF) for ubiquitin-mediated destruction under normal oxygen tension. Tumour hypoxia promotes accumulation of HIFα, whose expression is associated with cancer progression, poor prognosis and resistance to conventional therapies, thus establishing HIF as a therapeutic target. Notably, VHL is functionally inactivated in VHL disease, a hereditary cancer syndrome characterized by the formation of tumours in multiple organs, as well as in the majority of sporadic clear-cell renal cell carcinomas (CCRCC) and haemangioblastomas. Recently, certain VHL mutations have been shown to cause the congenital disorder Chuvash polycythemia. Work contained in this thesis describes the temporally coordinated activation of the ECV, whereby oxygen-dependent recognition of HIFα by VHL triggers Cul2 modification by the ubiquitin-like molecule NEDD8, which enhances ECV ubiquitin ligase activity by recruiting the E2. In addition, the feasibility of ‘bio-tailored’ enzymes in the treatment of cancer is introduced by
creating a bioengineered VHL capable of targeting HIFα for degradation irrespective of oxygen tension, which leads to the dramatic inhibition of CCRCC tumour growth and angiogenesis in a xenograft model. Furthermore, a ubiquitin ligase composed of two F-box proteins, VHL and suppressor of cytokine signalling 1 (SOCS1), was identified and shown to be paramount for the negative regulation of erythropoiesis by targeting phosphorylated Janus kinase 2 (JAK2) for ubiquitin-mediated destruction. The malfunction of this ubiquitin ligase explains the excessive erythrocytosis observed in Chuvash polycythemia patients and reveals a novel genetic link between the seemingly distinct genes VHL and JAK2 in the development of polycythemia.
Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Michael Ohh for his support throughout my PhD, and for teaching me how to generate scientific questions and address these in an elegant, high-quality manner. With his guidance, I have been able to strive for perfection in every aspect of the projects I have performed. Members of my supervisory committee, Dr. Linda Penn, Dr. Fei-Fei Liu and Dr. David Malkin have provided very valuable suggestions and feedback on my work, and I would also like to thank them for their continued support. I am very grateful to both past and present members of the Ohh lab for their insight, suggestions and help; it has been a pleasure to work together with a group of such talented people. In particular, I would like to thank Ryan Russell for the great teamwork that helped us both bring our polycythemia project to successful completion. In addition, Dr. Olga Roche provided important experimental help and suggestions that I am thankful for. Collaborators, including Dr. Eduardo Moriyama, Adrian Mariampillai, Dr. Andrew Evans, Terri Richmond, Dr. Meredith Irwin, Dr. Fei-Fei Liu, Dr. Celeste Simon, Dr. Dwayne Barber, Dr. Alex Vitkin, Dr. Victor Yang and Dr. Brian Wilson have provided valuable expertise, experimental help and reagents that were indispensable for work contained in this thesis.

Importantly, I thank my parents, Nicoleta and Ion, for their invaluable continuous encouragement and understanding that has helped me perform to the best of my abilities. I would also like to thank my partner, Björn, for his ceaseless support and motivation.
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<td>ANG1/2</td>
<td>angiopoietin 1/2</td>
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<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
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<tr>
<td>AMF</td>
<td>autocrine motility factor</td>
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<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
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<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
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<tr>
<td>APP-BP1</td>
<td>APP binding protein 1</td>
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<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<tr>
<td>BFU-E</td>
<td>burst forming unit-erythroid</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>bHPAS</td>
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<td>BNIP3L</td>
<td>Bcl2/adenovirus E1B interacting protein 3L</td>
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<td>βTrCP</td>
<td>beta-transducin repeat-containing protein</td>
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<td>Card9</td>
<td>caspase recruitment domain 9</td>
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<td>CBP</td>
<td>CREB binding protein</td>
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<td>CCRCC</td>
<td>clear cell renal cell carcinoma</td>
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<td>Cdk2</td>
<td>cyclin-dependent kinase 2</td>
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<td>CFU-E</td>
<td>colony forming unit-erythroid</td>
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<td>CFU-G</td>
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<td>CIS</td>
<td>cytokine-inducible SH2-containing protein</td>
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<td>CK2</td>
<td>casein kinase 2</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>cyclooxygenase 2</td>
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<td>CREB</td>
<td>cAMP response-element binding protein</td>
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<td>cullin RING ligase</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>ELISA</td>
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<td>essential thrombocytopenia</td>
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FDA = food and drug administration
FH = fumarate hydratase
FIH1 = factor inhibiting HIF1
FN = fibronectin
G-CSF = granulocyte colony stimulating factor
G-CSFR = granulocyte colony stimulating factor receptor
GFP = green fluorescent protein
GLUT1/3 = glucose transporter 1/3
GSK3β = glycogen synthase kinase 3β
H&E = hematoxylin and eosin
HECT = homologous to E6-AP C-terminus
HEK = human embryonic kidney
HIF = hypoxia-inducible factor
HPAS = HLH, PASA, PASB
HPAC = HLH, PASA, PASB, PAC
HPACV = HLH, PASA, PASB, PAC, VHL α domain
HPACGV = HLH, PASA, PASB, PAC, Gly(6), VHL α domain
HRE = hypoxia-responsive elements
hTERT = human telomerase reverse transcriptase
IFNα = interferon α
IGF2 = insulin-like growth factor 2
IGFBP1-3 = insulin-like growth factor binding proteins 1-3
IL-2 = interleukin 2
iNOS = nitric oxide synthase
IRES = internal ribosomal entry site
JAK2 = Janus kinase 2
JH1/2 = JAK homology 1/2 domain
LDHA = lactate dehydrogenase A
LOX = lysyl oxidase
MAPK = mitogen-activated protein kinase
MDR-1 = multi-drug resistance-1
MMP 2 = matrix metalloproteinase
MPN = myeloproliferative neoplams
MT-MMP = membrane type-I matrix metalloproteinase
mTOR = mammalian target of rapamycin
NAE = NEDD8 activating enzyme
NCE = NEDD8 conjugating enzyme
NEDD8 = neural precursor cell expressed developmentally downregulated protein 8
NEDP1 = NEDD8 protease 1
NF-κB = nuclear factor κB
NGF = nerve growth factor
N-TAD = amino-terminal transactivation domain
OCT = optical coherence tomography
svOCT = speckle variance optical coherence tomography
Oct-4 = octomer binding factor 4
ODD = oxygen-dependent degradation domain
PAC = PAS-associated C-terminal domain
PAGE = polyacrylamide gel electrophoresis
PAI-1 = plasminogen activator inhibitor 1
PAS = PER/ARNT/SIM
PDGFβ = platelet-derived growth factor β
PEPCK = phosphoenolpyruvate carboxykinase
PFCP = primary familial and congenital polycythemia
PGE$_2$ = prostaglandin E$_2$
PGK = phosphoglycerate kinase
P-gp = P-glycoprotein
PHD = prolyl hydroxylase domain
PHZ = phenylhydrazine
PI3K = phosphatidylinositol-3-kinase
pJAK2 = phosphorylated JAK2
PMF = primary myelofibrosis
PML = promyelocytic leukemia
PTEN = phosphatase and tensin homolog
PV = polycythemia vera
RBC = red blood cell
Rbx1 = RING box 1
RING = really interesting new gene
Rpb1/7 = RNA polymerase II subunit 1/7
RTK = receptor tyrosine kinase
Rub1 = related to ubiquitin 1
SCF = Skp1/Cdc53/F-box protein
SCID = severe combined immunodeficiency
SDF-1 = stromal cell-derived factor 1
SDH = succinate dehydrogenase
SDS = sodium dodecyl sulfate
SH2 = src-homology 2
SIP1 = Smad-interacting protein 1
Skp1/2 = S phase kinase-associated protein 1/2
SOCS1-7 = suppressor of cytokine signalling 1-7
STAT5 = signal transducer and activator of transcription 5
SUMO = small ubiquitin-like modifier
Tcf4 = T-cell factor 4
TGFα = transforming growth factor α
Tie2 = angiopoietin 1 receptor
TNFα = tumour necrosis factor α
TPO = thrombopoietin
TPOR = thrombopoietin receptor
TSC1/2 = tuberous sclerosis complex 1/2
Ub = ubiquitin
Uba3 = ubiquitin-like modifier activating enzyme 3
Ubc = ubiquitin conjugating enzyme
Ubl = ubiquitin-like molecule
UCH = ubiquitin carboxyl-terminal hydrolase
uPAR = urokinase-type plasminogen activator receptor
VDU = VHL-interacting deubiquitinating enzyme
VEGF = vascular endothelial growth factor
VEGFR1/2 = vascular endothelial growth factor receptor 1/2
VHL = von Hippel-Lindau
Introduction

Inactivation of the von Hippel Lindau (VHL) tumour suppressor gene gives rise to the VHL hereditary cancer syndrome, characterized by the development of tumours in multiple organs. Functional inactivation of VHL is also observed in the majority of sporadic haemangioblastomas and clear cell renal cell carcinomas (CCRCC). In addition, certain VHL mutations have been recently shown to cause polycythaemia, a disorder characterized by excessive erythrocytosis.

Since its molecular cloning in 1993, much has been revealed about the vital tumour suppressor functions of VHL, but undoubtedly its best-characterized role is in the formation of an ubiquitin ligase. With the award of the Chemistry Nobel prize in 2004, ubiquitin-mediated protein degradation, an ingenious method of cells to precisely control protein half-life in response to a plethora of intracellular and extracellular stimuli, received its well-deserved recognition. Today, it is known that ubiquitination controls numerous cellular processes in addition to protein turnover, such as endocytosis, DNA repair and signal transduction. The focus in this thesis, however, is on the classical function of ubiquitin as a trigger of protein destruction. A few years after its discovery, a number of groups showed VHL to bind directly to the α subunit of hypoxia-inducible factor (HIF) and target it for ubiquitin-mediated degradation. HIF, a master regulator of oxygen homeostasis, is a transcription factor promoting cellular adaptation to reduced oxygen tension. However, high HIF activity in cancer has an oncogenic role, as it promotes tumour growth and is associated with poor prognosis in patients. The regulation of HIFα protein stability is part of a true oxygen-sensing mechanism, whereby HIFα is only recognized for degradation by VHL under normal oxygen tension, thus prohibiting its untimely activity.

However, defective VHL ubiquitin ligase function is intimately linked to VHL-associated neoplasms, in particular CCRCC, and has recently also been implicated in the development of polycythaemia. These findings exemplify the importance of finely-tuned ubiquitin ligase activity and provide the rationale for the study of the VHL ubiquitin ligase, which will ultimately lead to better therapeutic strategies for patients suffering from these diseases.
1.1 VHL disease

In 1894, a British surgeon by the name of Treacher Collins described his histopathological observations on bilateral vascular growths in the retinas of two siblings. Ten years later, Eugen von Hippel, a German ophthalmologist, described a second family presenting with similar blood vessel tumours of the retina and coined the term *angiomatosis retinae*. In 1927, Arvid Lindau, a Swedish neuropathologist, noted that these retinal lesions were associated with an increased risk of developing haemangioblastomas of the brain and spinal cord. In 1936, this familial cancer syndrome was termed von Hippel-Lindau (VHL) disease. Today, VHL disease is known to be associated with the development of tumours in multiple organs, such as the central nervous system (CNS; cerebellum, brainstem and spinal cord), retina, pancreas, adrenal gland, endolymphatic sac of the inner ear, epididymus (male), broad ligament (female) and kidney. While most of these tumours are benign, the kidney cancer is malignant and of the clear-cell type (Clear Cell Renal Cell Carcinoma, CCRCC), the most common form of kidney cancer. To date, CCRCC is the principal cause of morbidity and mortality in VHL patients.

VHL disease affects 1 in 36,000 individuals and does not display an ethnic, racial, cultural or gender bias. The cause of VHL disease has been linked to inheritance of a defective copy of the *VHL* tumour suppressor gene and tumours arise in VHL patients upon loss or inactivation of the remaining wild-type allele in susceptible cells. Molecularly, VHL disease has an autosomal recessive inheritance pattern. However, there is a 90% likelihood that the remaining wild-type *VHL* allele is mutated in a susceptible cell during the lifetime of a VHL germline heterozygote, accounting for the autosomal dominant inheritance pattern observed in the clinic. The requirement of biallelic inactivation of the VHL gene for development of VHL disease-associated tumours conforms to Knudson’s two-hit model of carcinogenesis by tumour suppressor genes. In accordance with this model, biallelic functional inactivation of VHL has also been associated with the majority of sporadic CCRCC (75%) and cerebellar haemangioblastoma (50-60%).

1.1.1 VHL disease subtypes

Clear genotype-phenotype correlations have emerged for VHL disease, allowing it to be classified into subcategories, depending on the patients’ likelihood of developing
phaeochromocytoma. Type 1 patients have a low risk of developing phaeochromocytoma, but present with CCRCC. Type 2 patients have a high risk of developing phaeochromocytoma, with type 2A patients having an additional low risk of developing CCRCC, whereas type 2B patients possess a high risk of CCRCC. Type 1, type 2A, and type 2B patients also develop the two cardinal features of VHL disease, namely, cerebellar and retinal haemangioblastomas. Notably, type 2C patients develop phaeochromocytoma exclusively. Typically, the mutations associated with type 1 disease are \( VHL \) gene deletions, microinsertions, and nonsense mutations, whereas the vast majority (>90%) of type 2 patients present with \( VHL \) missense mutations. The striking genotype-phenotype correlation observed for VHL disease has pointed to multiple different tumour suppressor functions for VHL.

### 1.1.2 Chuvash polycythemia

Recently, homozygous germline \( VHL \) point mutations have been shown to cause a distinct form of VHL disease, characterized by the exclusive development of polycythemia. Specifically, inheritance of \( VHL \) alleles homozygous for the \( 598C\rightarrow T \) (R200W) mutation has been associated with the development of autosomal recessive Chuvash polycythemia (CP), endemic to the Chuvash Autonomous Republic of the Russian Federation. R200W and additional CP-associated mutations (e.g., H191D) have since been identified in diverse ethnic backgrounds without gender bias.

Polycythemia is a condition characterized by a net increase in red blood cells (RBCs) resulting in elevated haematocrit, and is generally categorized as primary or secondary. Primary polycythemia is defined as excessive erythrocytosis arising from an intrinsic defect in erythroid progenitors, rendering them hypersensitive to, or independent of erythropoietin (EPO) stimulation. Secondary polycythemia is defined as excessive erythrocytosis arising from increased production of EPO. Chuvash Polycythemia has features of both primary and secondary polycythemia. CP patients and R200W/R200W mice that faithfully recapitulate the human CP condition (discussed in greater detail in chapter 1.7.2) have high EPO levels and an intrinsic hypersensitivity to EPO displayed by the erythroid progenitors burst forming units-erythroid (BFU-E) cells, prominent features of secondary and primary polycythemia, respectively. Specifically, laboratory values of CP patients reveal increased hemoglobin, hematocrit, EPO levels and a high RBC count, but no differences in white blood cell count when
compared to unaffected relatives (Table 1).\textsuperscript{35,36} Clinically, CP patients develop varicosities of the lower extremities and have a shorter lifespan due to cerebral vascular events and peripheral thrombosis. Interestingly, unlike VHL patients, CP patients do not have any increased risk of tumour development due to the \textit{VHL} mutation.\textsuperscript{13}

1.2 The VHL ubiquitin ligase ECV

1.2.1 VHL protein

In 1993, Latif and colleagues cloned the \textit{VHL} gene on chromosome 3p25, a region frequently deleted in sporadic kidney cancer.\textsuperscript{3} The \textit{VHL} gene consists of 3 exons and is conserved in rodents, flies and worms.\textsuperscript{3} \textit{VHL} mRNA is approximately 4.5 kB in size and its expression is ubiquitous throughout the organism.\textsuperscript{37-39} Due to an internal translation initiation site at codon 54, \textit{VHL} mRNA is translated into two proteins, the full-length VHL30 and a shorter isoform, VHL19.\textsuperscript{40-42} VHL30 is a 213 amino acid protein of approximately 24-30 kDa, while VHL19 contains 160 amino acids and has a molecular weight of 18-19 kDa. Reconstitution of CCRCC cells devoid of VHL with either VHL30 or VHL19 suppressed tumour development in nude mouse xenograft assays, suggesting the N-terminus of VHL to be dispensable for tumour suppressor function.\textsuperscript{40,42-44} However, in light of new data about the role of VHL N-terminal

<table>
<thead>
<tr>
<th></th>
<th>Affected</th>
<th>Unaffected</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)\textsuperscript{¶}</td>
<td>22.6 ± 1.4</td>
<td>14.5 ± 1.1</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Hematocrit (%)\textsuperscript{¶}</td>
<td>66.6 ± 3.9</td>
<td>42.7 ± 3.4</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Red blood cells ((\times 10^6/\text{µL}))\textsuperscript{¶}</td>
<td>7.5 ± 0.5</td>
<td>4.7 ± 0.5</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>White blood cells ((\times 10^3/\text{µL}))\textsuperscript{¶}</td>
<td>7.2 ± 1.6</td>
<td>7.3 ± 1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>EPO (mIU/ml), Females\textsuperscript{‡}</td>
<td>34 (11–102)</td>
<td>14 (8–41)</td>
<td>0.039</td>
</tr>
<tr>
<td>EPO (mIU/ml), Males\textsuperscript{†}</td>
<td>28 (19–43)</td>
<td>9 (7–12)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\textbf{Table 1. Laboratory values of CP-affected patients and their unaffected relatives.} Values are represented as mean ± SD, or mean (range). \textsuperscript{¶}Affected CP subjects, \(N=6\); Unaffected relatives, \(N=12\). \textsuperscript{‡}Affected CP subjects, \(N=9\); Unaffected relatives, \(N=21\). \textsuperscript{†}Affected CP subjects, \(N=7\); Unaffected relatives \(N=20\). Table adapted from references 35, 36.
phosphorylation in extracellular matrix deposition (discussed in more detail in Chapter 1.6.1), the question of whether VHL19 can support a tumour suppressor role should be revisited. For simplicity, the term VHL is used when referring to both isoforms generically. In addition to the full-length VHL mRNA, alternative splicing also gives rise to a second VHL mRNA, containing only exons 1 and 3. However, tumours exclusively producing this splice variant have been identified, suggesting that the protein encoded by this exon-2-less transcript is defective in tumour suppressor activity.

VHL is found to shuttle between the nucleus and cytoplasm and it appears that the steady-state distribution of VHL can be influenced by cell density. Furthermore, treatment of cells with RNA polymerase II inhibitors induces VHL accumulation in the nucleus. VHL protein shuttling has been shown to be important for VHL tumour suppressor function, since VHL mutants unable to shuttle due to fusion to strong heterologous nuclear import or export signals are functionally defective.

1.2.2 ECV

Upon isolation of the VHL gene, it was clear that neither the nucleotide nor the amino acid sequence of VHL resembled any proteins of known function. Therefore, in an effort to elucidate the molecular function of VHL, VHL-associated proteins were sought. It is now known that VHL forms a multiprotein complex with Elongin B, Elongin C and Cullin 2 (Cul2), termed ECV (Elongins BC/Cul2/VHL, Fig. 1.1). Clues to VHL function came from the observation that VHL-associated proteins Elongin C and Cul2 were similar to Saccharomyces cerevisiae yeast proteins Skp1 and Cdc53, respectively. Skp1 and Cdc53 are individual components of the yeast SCF complex (Skp1/Cdc53/F-box protein, Fig. 1.1), which is known to target specific proteins for ubiquitin-mediated degradation (the ubiquitin pathway is discussed in more detail in Chapter 1.2.3). The F-box protein associates with the rest of the complex through binding Skp1 via its F-box motif and also engages to substrate(s), thus conferring substrate specificity to the SCF. Indeed, the three-dimensional structure of VHL bound to Elongins BC confirmed that Elongin C resembled Skp1, and identified the region on VHL directly binding Elongin C as loosely resembling an F-box. In addition, VHL was shown to have two distinct domains, the α and the β domain. It is through the α domain that VHL engages Elongin C and thus the rest of the ECV complex, while the β domain is predicted to function as a putative substrate-docking site (Fig.
1.1). Notably, disease-associated mutations found in VHL patients map frequently to surface residues of α and β domains, pointing to the importance of both domains in the tumour suppressor function of VHL.

The significant structural homology between ECV and SCF complexes led to the hypothesis that VHL may play a role in targeting certain proteins for ubiquitin-mediated degradation. Indeed, Lisztwan et al. and Iwai et al. simultaneously showed that partially purified VHL complexes contained ubiquitin ligase activity. In addition, the Cullin binding partner RING finger protein Rbx1 (also known as ROC1 or Hrt1), known to enhance ubiquitin ligase activity of SCF complexes, was shown to associate with the ECV. This further strengthened the notion that VHL is an active component of an ubiquitin ligase complex (Fig. 1.1).
Figure 1.1. Similarities between SCF and ECV ubiquitin ligases. VHL forms an ubiquitin ligase complex, ECV, which is similar to the yeast SCF. Complexes recognize substrates through post-translational modifications, denoted here as circles on the target proteins. Substrates are tagged with a chain of ubiquitin molecules, leading to their degradation by the 26S proteasome. B, C: Elongin B, C; Ubc: ubiquitin conjugating enzyme; Ub: ubiquitin; F: F-box.
1.2.3 The ubiquitin-proteasome pathway

In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose received the Chemistry Nobel prize for their discovery of ubiquitin-mediated protein degradation. Ubiquitin, a small globular 76 amino acid protein, is highly conserved throughout eukaryotes, displaying only three amino acid changes from yeast to humans. Targeting cellular proteins for degradation by the ubiquitin-proteasome pathway involves covalent attachment of a chain of ubiquitin molecules to the target protein (ubiquitination), followed by proteolysis of the substrate by the 26S proteasome. Ubiquitination is a multistep process involving three types of enzymes, the E1 ubiquitin activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. Ubiquitin is first bound through its carboxy-terminal glycine to a cysteine residue on the E1. This reaction is ATP-dependent and forms a high-energy thioester bond between the two. Subsequently, ubiquitin is transferred to an active site cysteine of the E2 enzyme, again forming a thioester linkage. Finally, the E3 ubiquitin ligase catalyses the transfer of ubiquitin from the E2 to the substrate to be ubiquitinated. Ubiquitin is covalently attached to a lysine residue on the substrate, forming a stable isopeptide bond.

Polyubiquitination of substrates is achieved by repeated transfer of additional ubiquitin molecules to successive lysines on each previously conjugated ubiquitin, typically on Lys48. K48-linked ubiquitin chains are recognized by the 26S proteasome, which unfolds substrates in an ATP-dependent manner and directs them into its catalytic lumen. The 20S core catalytic component of the proteasome is a cylindrical chamber that includes subunits with trypsin, chymotrypsin and peptidylglutamyl peptidase-like activities, rapidly degrading substrate proteins into short polypeptides. Since ubiquitination is a dynamic and reversible process, de-ubiquitinating enzymes (DUBs) also exist. Some of the functions of DUBs include cleaving ubiquitin from proteins and from residual proteasome-associated peptides, disassembly of polyubiquitin chains and processing of immature ubiquitin.

While there are only two E1 enzymes, Ube1 and the recently identified Uba6, there are several E2 enzymes, Ubcs, and hundreds of E3 ubiquitin ligases, ultimately determining substrate specificity. Despite their large numbers, ubiquitin ligases can be classified into two categories: HECT and RING finger E3s. HECT (homologous to E6-AP C-terminus) domain ubiquitin ligases themselves also function as ubiquitin conjugating enzymes. In contrast, RING domain E3s do not have inherent catalytic activity, but instead use the Zn-binding RING finger
to recruit and direct an E2 enzyme towards specific substrates, which are bound by associated substrate-recruitment subunits. SCF-like complexes, such as the ECV, are prototypical RING ubiquitin ligases.

Figure 1.2. Ubiquitin and NEDD8 conjugation to substrates. (A) Ubiquitin is first conjugated to the E1 activating enzyme through a thioester bond. Subsequently, ubiquitin is transferred to the E2 ubiquitin conjugating enzyme, again forming a thioester bond. In the context of an E3 ubiquitin ligase, ubiquitin is conjugated by an isopeptide bond to a lysine residue on substrates. Polyubiquitination is necessary for substrate recognition and degradation by the 26S proteasome. (B) NEDD8 conjugation to substrates functions in a manner analogous to ubiquitination, involving the heterodimeric E1 enzyme, E2 and E3 enzymes. Ub: ubiquitin; N8: NEDD8.
1.2.4 NEDD8 modification of Cullins and functional consequences

Following the discovery of ubiquitin, a number of ubiquitin-like molecules (Ubls) have been identified, which share a common three-dimensional structure with ubiquitin, the ubiquitin fold, and also contain a C-terminal glycine residue. In a manner analogous to ubiquitination, Ubls are attached to target proteins through distinct E1, E2 and E3 enzymes (Fig. 1.2B). While the functional details of many Ubls still remain to be elucidated, the conjugation of substrates by the Ubls SUMO (small ubiquitin-related modifier) and NEDD8 (neural precursor cell-expressed developmentally downregulated-8), has been intensively studied. Numerous target proteins have been identified for both Ubls, with effects of conjugation varying between substrates, ranging from protein stability, transcriptional activation/repression and subcellular localization to modified interaction with other proteins. Interestingly, in the context of ECV and ubiquitination, NEDD8 conjugation to the Cul2 Cullin scaffolding component has been shown to increase the E3 ubiquitin ligase activity of the ECV.

The cullin gene family was identified in 1996 in *Caenorhabditis elegans* by Kipreos et al. There are at least three cullins in budding and fission yeast, five in nematodes and eight in humans. Indeed, NEDD8, known as Rub1 (related-to-ubiquitin 1) in yeast, has been shown to modify *C. elegans* Cul3 and all yeast and human cullins – *S. cerevisiae*: Cdc53, Cul3 and Rtt101, *Schizosaccharomyces pombe*: Pcu1, Pcu3 and Pcu4 and human: Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7 and PARC. Cullin NEDD8 modification, neddylation, occurs on a conserved lysine residue in the C-terminal domain.

NEDD8 conjugation to substrates involves the heterodimeric NEDD8 activating enzyme APP-BP1/Uba3 (NAE; E1) and Ubc12 as the NEDD8 conjugating enzyme (NCE; E2) (Fig. 1.2B). Furthermore, the RING finger protein Rbx1 has been proposed as the E3 NEDD8 ligase for Cullins. Similar to de-ubiquitinating enzymes, enzymes specific for Cullin de-neddylation have also been identified and include NEDP1 and the COP9 signalosome. In addition, UCH-L1, UCH-L3, Ataxin-3, PfUCH54, and USP21 act as both de-neddylation and de-ubiquitinating enzymes, however, their in vivo targets still remain to be determined.

Interestingly, the overall E3 ubiquitin ligase activity of yeast and mammalian SCF complexes is enhanced by covalent modification of cullin family proteins with NEDD8/Rub1. As such, Rub1 modification of the fission yeast cullin Pcu1 positively affects SCF function towards its...
ubiquitination substrate Rum1\textsuperscript{74}. In addition, the activity of the human SCF complexes containing F-box protein βTrCP or Skp2 (SCF\textsuperscript{βTrCP}, SCF\textsuperscript{Skp2}) is increased by the neddylation of Cul1. This facilitates the ubiquitination of substrates IkBα and p27, respectively\textsuperscript{96-100}. In accordance, it has recently been shown that NEDD8 modification of Cul2 enhances the E3 activity of the ECV complex in vivo\textsuperscript{70}, however, the underlying mechanism remains to be elucidated.

1.3 HIFα is a substrate of the VHL ubiquitin ligase

1.3.1 ECV-mediated, oxygen-dependent degradation of HIFα

Several putative substrates of the ECV complex have been identified, including the seventh (Rpb7) and the large (Rpb1) subunit of RNA polymerase II, atypical protein kinase C (aPKC) and VHL-interacting deubiquitinating enzyme (VDU)\textsuperscript{101-104}. However, undoubtedly the best-characterized substrate of the VHL ubiquitin ligase is the hypoxia-inducible factor (HIF) α subunit\textsuperscript{5,6,8} (Fig. 1.3).

HIF, a heterodimeric transcription factor, is a master regulator of oxygen homeostasis, controlling the transcription of over 60 hypoxia-inducible genes (discussed in more detail in chapter 1.4), including vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) and erythropoietin (EPO)\textsuperscript{10} (Table 2). HIF is composed of two subunits, HIFα and HIFβ, also known as Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)\textsuperscript{10,105}. Under low oxygen tension or hypoxia, HIFα and ARNT heterodimerize to form the active transcription factor HIF, which binds to promoters of hypoxia-inducible genes, leading to their transactivation\textsuperscript{10,105} (Fig. 1.3). Under normal oxygen tension or normoxia, HIFα is hydroxylated on conserved proline residues by prolyl-4-hydroxylases, members of the prolyl hydroxylase domain (PHD) family (also known as EglN or HPH family)\textsuperscript{11,12,106-111}. In addition to molecular oxygen, these enzymes require Fe\textsuperscript{2+} and 2-oxoglutarate for HIFα hydroxylation. Notably, proline hydroxylation of HIFα is necessary and sufficient for its recognition by VHL, specifically by the VHL substrate-docking β domain\textsuperscript{8,11,12} (Fig. 1.3). This leads to ECV-mediated HIFα polyubiquitination and subsequent degradation by the 26S proteasome\textsuperscript{5-9,11,12}. Unlike HIFα,
ARNT is constitutively expressed and stable, thus HIF regulation occurs at the level of HIFα stability. In VHL disease and cells lacking wild-type VHL, ubiquitination of prolyl-hydroxylated HIFα does not occur. Thus, under both normoxic and hypoxic conditions, the active HIF heterodimer is formed, resulting in overexpression of hypoxia-inducible genes.
Figure 1.3. ECV targets HIFα for ubiquitin-mediated degradation under normal oxygen tension. Under hypoxia, HIFα is bound by the transcriptional coactivators p300/CBP and dimerizes with ARNT. The resulting HIF transcription factor binds to hypoxia-responsive elements (HREs) in target gene promoters, initiating the transcription of numerous hypoxia-inducible genes, known as the hypoxic response. In the presence of oxygen, HIFα is hydroxylated on conserved proline residues by prolyl hydroxylases (PHDs) and on asparagine by the factor-inhibiting HIF1 (FIH1) enzyme. Prolyl hydroxylation promotes HIFα recognition by the β domain of VHL and subsequent ubiquitination by the ECV, resulting in HIFα degradation via the 26S proteasome. In addition, the asparaginyl hydroxylation prevents p300/CBP from binding HIFα, thus also prohibiting transcription of hypoxia-inducible genes. B, C: Elongin B, C; UbcH5: ubiquitin conjugating enzyme shown to promote HIF1α ubiquitination; OH: hydroxyl group, Ub: ubiquitin.
1.3.2 HIF proteins

There are three HIFα genes in humans, HIF1α, HIF2α and HIF3α. HIF1α and HIF2α display 48% sequence identity, while HIF3α is only 34% identical to HIF1α and HIF2α. HIF3α gives rise to six splice isoforms, one of which, HIF3α4 has been recently shown to act as a dominant negative HIFα, inhibiting both HIF1- and HIF2-mediated transcription. However, while HIF1 and HIF2 transcription factors have been well-characterized, the function of full-length HIF3α1 and of the other splice variants remains largely unexplored.

The HIF1α, HIF2α and ARNT proteins are 826 amino acids and approximately 120 kDa, 874 amino acids and 115 kDa, and 789 amino acids and 87 kDa in size, respectively. Whereas HIF1α and ARNT are ubiquitously expressed, HIF2α expression is restricted to vascular endothelium, liver parenchyma, lung type II pneumocytes and kidney epithelial cells. HIFα and ARNT are members of the bHLH/PAS (basic helix-loop-helix/PER/ARNT/SIM) transcription factor family. bHLH/PAS regions are present in the N-termini of HIF1α, HIF2α and ARNT and are required for HIFα and ARNT heterodimerization and DNA-binding. Even though the bHLH region is an established DNA-interaction motif, HIF1α PAS and PAS-associated C-terminal (PAC) domains are also required for optimal DNA-binding of the HIF1 heterodimer.

C-terminal to the bHLH/PAS/PAC regions of HIFα is the oxygen-dependent degradation domain (ODD), the minimal region conferring instability to HIFα. The ODD harbours the proline residue hydroxylated by PHD enzymes within the well-conserved LAPYIXMD motif. Proline hydroxylation of P564 in HIF1α and P531 in HIF2α results in VHL-mediated ubiquitination. In addition, a second proline, P402, located N-terminal of the HIF1α ODD can also be hydroxylated, allowing for HIF1α ubiquitination. The human PHD family consists of three enzymes, PHD1-3, with PHD2 being the primary HIF1α prolyl hydroxylase in most tissues, setting normoxic HIF1α levels. In contrast, PHD3 is more effective in controlling HIF2α levels. While all PHDs are capable of hydroxylating HIF1α P564, PHD2 is most active in this regard. In addition, only PHD1 and PHD2 are capable of hydroxylating HIF1α P402. Notably, while PHD1 expression is high irrespective of oxygen
tension, PHD2 and PHD3 mRNA levels are increased under hypoxia, which may prime cells for rapid HIFα degradation upon reoxygenation\textsuperscript{106,130-132}.

![Figure 1.4. Domain structure of HIFα.](image)

**Figure 1.4. Domain structure of HIFα.** In this schematic, HIFα represents HIF1α and HIF2α. b: basic; HLH: helix-loop-helix; PAS: Per/ARNT/Sim; PAC: PAS-associated C-terminal domain; ODD: oxygen-dependent degradation domain; N-TAD: N-terminal transactivation domain; C-TAD: C-terminal transactivation domain. Triangles indicate residues that are hydroxylated under normal oxygen tension.

### 1.3.3 HIF transactivation of hypoxia-inducible genes

Upon HIFα heterodimerization with ARNT, the HIF transcription factor binds to hypoxia-responsive elements (HREs) containing the core sequence 5’-RCGTG-3’ in the promoters/enhancers of hypoxia-inducible genes\textsuperscript{113,114,133,134} (Fig. 1.3). Both HIF1α and HIF2α contain two transactivation domains – the amino-terminal transactivation domain (N-TAD), which partially overlaps with the ODD, and the carboxy-terminal transactivation domain (C-TAD) (Fig. 1.4). The N-TAD and C-TAD are separated by an inhibitory region, the removal of which augments HIF1α and HIF2α transcriptional activity\textsuperscript{127,135}. A number of genes can be induced by both HIF1α and HIF2α, while a few target genes are only induced by HIF1α or by HIF2α (further discussed in chapter 1.4.1). Both the N-TAD and C-TAD are important for transactivation of target genes common to HIF1α and HIF2α, however, induction of HIF1α- or HIF2α-specific target genes depends on the respective HIFα N-TAD. Domain-swapping analysis of bHLH/PAS, N-TAD and C-TAD between HIF1α and HIF2α showed that the N-TAD, and not the bHLH/PAS or C-TAD, triggered HIF1α- or HIF2α-specific transcription\textsuperscript{136}. Thus, irrespective of the origin of bHLH/PAS and C-TAD domains, the transcriptional specificity of chimeric HIF1α/HIF2α proteins was controlled by the N-TAD they possessed. Therefore, a HIF1α N-TAD led to transcription of HIF1α-inducible genes, while transcription of
HIF2α-responsive genes was induced by a HIF2α N-TAD\textsuperscript{136}. Interaction of the N-TAD with HIF1α- or HIF2α-, cell-type- and promoter-specific transcriptional coactivators may thus underlie the observed differences in HIF1α/HIF2α transcriptional profiles.

The C-TAD of HIF1α and HIF2α is known to bind the transcriptional coactivator p300/CBP (CREB binding protein), which promotes transcription\textsuperscript{137-140} (Fig. 1.3). Interestingly, in the presence of molecular oxygen, a conserved asparagine residue in the C-TAD of HIF1α, N803, is hydroxylated by the factor-inhibiting HIF1 (FIH1) enzyme\textsuperscript{141-147}. FIH1, similar to the PHD family enzymes, is a Fe\textsuperscript{2+} and 2-oxoglutarate-dependent dioxygenase\textsuperscript{143,146,147}. Notably, HIF1α asparagine hydroxylation prevents association with p300/CBP, thus inhibiting HIF1-mediated transcription in the presence of oxygen (Fig. 1.3). Even though HIF2α also contains the conserved asparagine residue in its C-TAD (N851), it is not hydroxylated as efficiently as HIF1α by FIH1 and is thus relatively resistant to FIH1-mediated transcriptional inhibition\textsuperscript{148}.

### 1.4 HIF-regulated genes and their role in tumourigenesis

#### 1.4.1 Transcriptional targets of HIF1 and HIF2

HIFs transactivate genes that allow cells to adapt to hypoxic environments. Importantly, HIF-induced genes also promote survival, growth, invasion and metastasis of tumour cells\textsuperscript{10}. For this reason, the HIF transactivation profile has been extensively studied, with particular emphasis on HIF1, the first HIF identified. Today the list of HIF1-regulated genes in cancer is extensive, comprising over 60 genes involved in proliferation, cell viability, anaerobic metabolism, angiogenesis, erythropoiesis, invasion, metastasis as well as radiotherapy and chemotherapy treatment resistance\textsuperscript{10} (Fig. 1.3 and Table 2). HIF2 is capable of binding to the same HREs recognized by HIF1 and thus has been shown to transactivate a number of genes previously thought to be exclusive HIF1 targets. In addition to the overlapping activity with HIF1, HIF2 also transactivates a specific subset of genes.

HIF1 has been shown to uniquely drive transcription of glycolytic enzymes, such as \textit{phosphoglycerate kinase (PGK)} and \textit{lactate dehydrogenase A (LDHA)}, while HIF2 uniquely transactivates \textit{octomer binding transcription factor-4 (Oct-4)}, a transcription factor essential for
maintaining stem cell pluripotency. In addition, HIF2-predominant targets include vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), the angiopoietin-1 receptor Tie2, erythropoietin (EPO), transforming growth factor α (TGFα) and membrane type-1 matrix metalloproteinase (MT-MMP).
### Anaerobic metabolism
- Glucose transporters 1 and 3 (GLUT1, GLUT3)
- Phosphoglycerate kinase (PGK)
- Lactate dehydrogenase A (LDHA)

### Cell proliferation and survival
- Transforming growth factor α (TGFα)
- Platelet-derived growth factor β (PDGFβ)
- Cyclooxygenase 2 (COX2)
- Insulin-like growth factor 2 (IGF2)
- Insulin-like growth factor binding protein 1 (IGFBP1)
- Survivin
- Vascular endothelial growth factor A (VEGF-A)
- Erythropoietin (EPO)

### Angiogenesis and erythropoiesis
- Vascular endothelial growth factor A (VEGF-A)
- VEGF receptor 1 and 2 (VEGFR1, VEGFR2)
- Platelet-derived growth factor β (PDGFβ)
- Angiopoietin 1 and 2 (ANG1, ANG2)
- ANG1 receptor (Tie-2)
- Plasminogen activator inhibitor (PAI-1)
- Matrix metalloproteinase 2 (MMP-2)
- Nitric oxide synthase (iNOS)
- Adrenomedullin (ADM)
- Endothelin 1 (ET-1)
- Erythropoietin (EPO)

### Invasion and metastasis
- Matrix metalloproteinase 2 (MMP-2)
- Membrane type-I matrix metalloproteinase (MT-MMP)
- Urokinase-type plasminogen activator receptor (uPAR)
- Autocrine motility factor (AMF)
- Lysyl oxidase (LOX)
- Chemokine (C-X-C motif) receptor 4 (CXCR4)
- E-cadherin (negative regulation)

### Treatment resistance
- P-glycoprotein (P-gp)/multi-drug resistance-1 (MDR-1)
- Human telomerase reverse transcriptase (hTERT)

### Early endosome fusion
- Rabaptin-5 (transcriptional repression)

### Stem cell pluripotency
- Octomer-binding transcription factor 4 (Oct-4)

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| Table 2. Selected HIF transcriptional targets. See text for corresponding references. | †Predominant HIF1-target genes. ‡Predominant HIF2-target genes. |
1.4.2 Anaerobic metabolism

ATP production by oxidative phosphorylation is severely limited in cells experiencing reduced oxygen tension. This leads to HIF-mediated induction of glycolysis enzymes and glucose transporters GLUT1 and GLUT3, in an effort to maintain cellular ATP levels\textsuperscript{149,159,160}. The switch to anaerobic metabolism is a trademark of solid tumour biology and is commonly referred to as the Warburg effect\textsuperscript{161}. However, cancer cells are also known to display high rates of glycolysis under normal oxygen tension. Interestingly, the glycolysis metabolites pyruvate and oxaloacetate can inhibit PHD activity by binding to the 2-oxoglutarate site of the enzymes\textsuperscript{162,163}. This was shown to prevent HIF1\(\alpha\) hydroxylation and thus stabilize HIF1\(\alpha\) under normoxia, inducing an oxygen-independent positive feedback loop for HIF transcriptional activity and ATP production by glycolysis.

1.4.3 Tumour cell proliferation and survival

Cell growth and survival is promoted by HIF-induced genes such as TGF\(\alpha\), VEGF-A, EPO, platelet-derived growth factor \(\beta\) (PDGF\(\beta\)), cyclooxygenase 2 (COX2), insulin-like growth factor 2 (IGF2) and insulin-like growth factor binding protein 1 (IGFBP1)\textsuperscript{114,152-156,164-167}. Notably, the suggested cells of origin of CCRCC, renal proximal tubule epithelial cells, are particularly sensitive to the mitogenic effects of TGF\(\alpha\), and both TGF\(\alpha\) and its receptor, epidermal growth factor receptor (EGFR), are commonly upregulated in VHL \(-/-\) cell lines and CCRCC\textsuperscript{168-170}. In addition, binding of TGF\(\alpha\) to EGFR is also known to induce the PI3K signalling pathway, which leads to increased HIF1\(\alpha\) expression, thus creating a positive feedback loop for CCRCC cell survival and proliferation\textsuperscript{156}. EGFR-mediated HIF1\(\alpha\) expression has also been shown to induce the anti-apoptotic HIF-target gene survivin in breast cancer\textsuperscript{171}. An additional positive feedback loop for tumour cell survival is observed in colorectal carcinoma, where HIF1 induces COX2 expression, which is accompanied by increased levels of prostaglandin E\(_2\) (PGE\(_2\))\textsuperscript{166}. PGE\(_2\) not only directly promotes tumour cell survival, but also enhances HIF1\(\alpha\) transcriptional activity by activating the MAPK pathway, previously shown to increase HIF1\(\alpha\)-mediated transactivation by facilitating the interaction between p300 and HIF1\(\alpha\) C-TAD\textsuperscript{166,172}.
1.4.4 Angiogenesis and erythropoiesis

Angiogenesis is critical for tumour growth, as the oxygen and nutrient supply of tumour cells becomes limited with increasing distance from the nearest blood vessel. HIF itself directly transactivates a number of pro-angiogenic factors, the most noteworthy of which is VEGF-A\textsuperscript{114,149,154,155}. VEGF-A, expressed in a large number of human tumours, has potent angiogenic properties due to its induction of endothelial cell survival, proliferation and migration\textsuperscript{173}. Interestingly, one of the early observations that VHL disease-associated tumours such as haemangioblastoma and CCRCC are hypervascular and produce high levels of VEGF led to the discovery of VHL-mediated HIF\textsubscript{α} degradation\textsuperscript{174-177}. In addition to VEGF-A activation, HIF upregulates other pro-angiogenic factors, such as $VEGFR\,1$ and -2, $PDGF\,\beta$, angiopoietins ANG-I and -2, $ANG1$ receptor Tie-2, plasminogen activator inhibitor-1 (PAI-1) and matrix metalloproteinase MMP-2, allowing survival, proliferation and migration of endothelial cells, as well as recruitment of pericytes and smooth muscle cells to primitive blood vessels\textsuperscript{113,114,151,158,167,178-182}. $VEGF\,A$ and other HIF-regulated genes such as nitric oxide synthase (iNOS), adrenomedullin (ADM) and endothelin-1 (ET-1) increase vascular permeability for efficient nutrient delivery\textsuperscript{183-185}.

In addition to stimulating new blood vessel growth, HIF can also enhance oxygen delivery to tumour cells by stimulating erythropoiesis. EPO, the first HIF-responsive gene identified, is strongly induced by HIF2 in the kidney and acts on erythroid progenitors to drive production of red blood cells, thus increasing oxygen delivery to tissues\textsuperscript{134,152,153}. Inordinately high production of EPO can also lead to polycythemia, a condition of excessive erythrocytosis, observed in a number of patients with sporadic CCRCC or in mice with constitutive overexpression of HIF2\textsubscript{α}\textsuperscript{186,187}.

1.4.5 Invasion and metastasis

Tumour metastasis is the primary cause of death in cancer patients and thus constitutes a pivotal step in tumour pathogenesis. HIF expression correlates with metastasis in multiple tumour types and promotes an invasive and migratory phenotype by inducing the expression of $MMP\,2$, urokinase-type plasminogen activator receptor (uPAR), autocrine motility factor (AMF), lysyl oxidase (LOX) and chemokine receptor $CXCR\,4$\textsuperscript{188-192}. Loss of E-cadherin, a major constituent of cell junctions in polarized epithelial cells, is associated with epithelial to mesenchymal transition
(EMT), a hallmark of many epithelial cancers, leading to increased tumour invasion and metastasis. Recently, loss of VHL in CCRCC was shown to result in loss of E-cadherin expression, likely achieved by HIF-mediated upregulation of the E-cadherin transcriptional repressors Smad-interacting protein-1 (SIP1) and Snail. LOX, an amine oxidase involved in extracellular matrix formation, has been recently shown to promote metastasis from breast tumours by increasing the activity of focal adhesion kinase (FAK) and cell-extracellular matrix interactions. Genetic and pharmacological inhibition of the HIF-regulated LOX was sufficient to prevent hypoxia-induced cell invasion and metastasis in vivo. CXCR4, the most common chemokine receptor expressed in human tumours, has been shown to promote the directional migration of metastatic tumour cells. The stromal cell-derived factor-1 (SDF-1) ligand for CXCR4 is highly expressed in the common metastatic sites lung, liver, bone marrow and lymph nodes, explaining tumour metastasis to these sites. Notably, VHL knock-down in a murine CCRCC xenograft resulted in high expression of HIF1α-induced CXCR4 and increased tumour metastasis. The metastatic potential of the primary tumour was linked to CXCR4 expression, as neutralizing antibodies against SDF-1 significantly reduced tumour metastases.

1.4.6 Radiotherapy and chemotherapy treatment resistance

The mechanism of action of radiation therapy relies on DNA damage induced by oxygen free radicals. However, it is well-known that tumour hypoxia itself inhibits radiation therapy efficacy due to the absence of molecular oxygen. Additionally, HIF expression has also been correlated with lack of efficacy of chemotherapy, which can be at least partially attributed to HIF-mediated expression of the P-glycoprotein (P-gp)/multi-drug resistance-1 (MDR-1) drug efflux pump. HIF has also been implicated in the upregulation of double-strand break repair enzymes that render cells resistant to the DNA-damaging effects of radiation- and chemotherapy. Human telomerase reverse transcriptase (hTERT) has also been shown to be a HIF-target gene and is associated with increased tumour growth and resistance to several cancer therapeutics.

1.4.7 VHL-mediated regulation of endocytosis

Recently, ligand-engaged EGFR turnover rate by endocytosis was shown to be significantly prolonged in VHL-/- CCRCC cells. This was linked to high HIF activity, specifically to HIF-mediated transcriptional repression of rabaptin-5, a critical effector of Rab5, which controls early endosome fusion. Endocytosis is a major mechanism for receptor inactivation and involves a
sequence of early and late endosomal fusion events, ultimately leading to degradation of internalized receptors in lysosomes\textsuperscript{205}. Rab proteins, a group of small Ras-like GTPases, control the early and late endosome fusions, with Rab5 being a critical activator of early endosome fusion\textsuperscript{206,207}. HIF was shown to repress transcription of the Rab5 effector rabaptin-5, by directly engaging the HRE of the rabaptin-5 gene, \textit{RABEP1}, which correlated with decreased RNA polymerase II recruitment to the \textit{RABEP1} promoter\textsuperscript{204}. This resulted in decreased early endosome fusion and thus delayed ligand-bound EGFR turnover. In agreement, \textit{RABEP1} mRNA and rabaptin-5 protein levels were significantly decreased in CCRCC tumour samples with strong hypoxic signatures, compared to normal renal tissue\textsuperscript{204}. As ligand-engaged internalized EGFR is known to be autophosphorylated and catalytically active, these findings explain the increased EGFR signalling observed in VHL\textsuperscript{-/-} CCRCC cells, which is known to promote cell proliferation and survival.

1.5 HIF as a therapeutic target

1.5.1 HIF overexpression is associated with tumour progression

In light of the myriad of genes transactivated by HIF that allow tumour cells to thrive in the otherwise inhospitable hypoxic environment, it is not surprising that HIF overexpression is frequently associated with increased phenotypic aggressiveness and poor prognosis in numerous tumour types. A recent study comparing various normal and malignant tissues by immunohistochemistry revealed that protein expression of both HIF1\(\alpha\) and HIF2\(\alpha\) was frequently increased in brain, breast, kidney, liver, bladder, colon, pancreatic, prostate and ovarian tumours\textsuperscript{208}. Specifically, analyses of various clinical specimens revealed that increased HIF1\(\alpha\) protein expression correlates with poor patient prognosis in head and neck cancer, glioblastoma, osteosarcoma and nasopharyngeal, colorectal, gastric, pancreatic, bladder, breast, cervical, endometrial and ovarian carcinomas\textsuperscript{209-219}. In contrast, elevated HIF2\(\alpha\) expression was shown to correlate with tumour aggressiveness in hepatocellular, colorectal carcinoma, melanoma, ovarian and non-small cell lung cancers\textsuperscript{213,220-222}. Together, these studies highlight that HIF activation is a common event in cancer and suggest that HIF may play a role in tumourigenesis.
Several studies have pointed to the significance of VHL-mediated HIFα regulation in cancer, especially in the development of CCRCC. Reintroduction of VHL into VHL−/- CCRCC cancer cell lines suppressed their ability to form tumours in nude mouse xenograft assays, establishing VHL as a gatekeeper of the renal epithelium, whose inactivation is an early and requisite step in CCRCC tumourigenesis44. In particular, HIF2α has been established as the critical oncogenic player in VHL−/- CCRCC. Expression of a non-degradable form of HIF2α, but not of HIF1α, in VHL-reconstituted CCRCC cell lines restored their ability to form tumours in murine xenograft assays223,224. Conversely, shRNA-mediated knockdown of HIF2α in VHL−/- CCRCC cells inhibited tumour formation in a nude mouse xenograft assay225. Furthermore, conditional inactivation of VHL in the renal cortex through phosphoenolpyruvate carboxykinase (PEPCK)-driven Cre recombinase gives rise to glomerular and tubular renal cysts226. In agreement, mouse renal cyst development can only be rescued by inactivation of ARNT, not of HIF1α, suggesting that another ARNT binding partner, such as HIF2α drives kidney transformation226,227.

1.5.2 Oxygen-dependent and -independent mechanisms for high HIF expression in solid tumours

Solid tumours frequently contain regions of hypoxia, as the diffusion capacity of oxygen from the nearest blood vessel is surpassed. In addition, tumour cells close to a blood vessel can experience hypoxia due to disruptions in blood flow, a common characteristic of malformed tumour vasculature. In these hypoxic regions, HIFα is not prolyl hydroxylated and thus not recognized by VHL, which prevents its ubiquitin-mediated degradation and ultimately results in high HIF transcriptional activity1,10. As previously mentioned, functional inactivation of VHL found in VHL disease and sporadic haemangioblastoma and CCRCC leads to inappropriately high levels of HIFα, resulting in overexpression of hypoxia-inducible genes under both normoxic and hypoxic conditions1,5,10,228. Interestingly, mutations in tumour suppressors other than VHL, such as enzymes of the tricarboxylic acid cycle succinate dehydrogenase (SDH) or fumarate hydratase (FH), can also cause inappropriate HIFα stabilization through inhibiting recognition by VHL229,231. Specifically, increased levels of succinate and fumarate have been shown to inhibit PHD activity, preventing prolyl-hydroxylation of HIFα229,231.
Growth factors binding to their cognate receptor tyrosine kinases (RTKs) and ensuing activation of the PI3K and MAPK signalling pathways can also regulate HIF1α protein levels in an oxygen-independent manner\textsuperscript{10}. Both pathways can activate mammalian target of rapamycin (mTOR)-mediated cap-dependent translation of \textit{HIF1α} mRNA\textsuperscript{10}. For example, mutations resulting in inactivation of the tumour suppressor genes \textit{phosphatase and tensin homolog (PTEN), promyelocytic leukaemia (PML)} or \textit{tuberous sclerosis (TSC1, TSC2)}, or activation of the oncogenes \textit{HRAS} or \textit{ERBB2} cause increased HIF1α expression that can be blocked by rapamycin, an mTOR inhibitor\textsuperscript{232-238}. Interestingly, \textit{HIF2α} mRNA translation has also been recently shown to be mediated by mTOR\textsuperscript{239}. In addition to enhancing mTOR-mediated \textit{HIF1α} translation, signalling through PI3K and MAPK pathways can also promote mTOR-independent HIF1α expression, possibly though internal ribosomal entry site (IRES)-dependent mechanisms, the details of which remain to be elucidated\textsuperscript{240-242}.

1.5.3 Current agents inhibiting HIF activity

As discussed previously, high levels of HIF1α or HIF2α have been strongly linked with tumour progression and poor prognosis in a variety of solid tumours. In animal models, HIFα overexpression is associated with increased tumour growth, vascularization and metastasis, while HIF loss-of-function has the opposite effect, implicating HIF1α and HIF2α as compelling therapeutic targets for anti-cancer therapy\textsuperscript{160,243-246}. Currently, a number of compounds that have been shown to block HIF1α activity are under investigation and some of these have entered clinical trials or have been approved by regulatory authorities for use in the clinic\textsuperscript{247}. HIF1 inhibition is achieved either through reduction of HIF1α protein levels, by decreasing HIF1 DNA-binding activity, or through interfering with HIF1-mediated transactivation of target genes.

HIFα protein levels are controlled by VHL-mediated degradation and by \textit{HIFα} mRNA translation. As activation of PI3K and MAPK signalling pathways can increase HIF1α protein synthesis, a number of inhibitors of oncogenic signalling pathways, such as the EGFR inhibitors gefinitib (Iressa), erlotinib (Tarceva) and cetuximab (C225) and the HER2/Neu inhibitor trastuzumab (Herceptin) have been shown to inhibit HIF1α protein expression\textsuperscript{248-250}. This is likely due to inhibition of mTOR-mediated HIF1α mRNA translation, which can also be achieved directly with mTOR inhibitors temsirolimus (CCI-779) and everolimus (RAD-001)\textsuperscript{251-252}.
Other molecules, such as the topoisomerase I inhibitor topotecan, the cyclin-dependent kinase inhibitor flavopiridol and the microtubule polymerization inhibitor 2-methoxyestradiol, can also inhibit HIF1α protein expression, but their mechanisms of action have not yet been identified\textsuperscript{254-256}. In addition, HSP90 chaperone inhibitors 17-AAG and 17-DMAG, and HDAC inhibitor LAQ824 have been shown to induce HIF1α protein degradation\textsuperscript{257,258}. PX-478 and YC-1, agents with an unknown mechanism of action have also been shown to decrease HIF1α protein levels\textsuperscript{259,260}. DNA-binding molecule echinomycin interrupts the DNA binding of HIF1α, while HIF1α-mediated transcription is reduced by the p300 inhibitor chetomin, the proteasome inhibitor bortezomib and the antifungal agent amphotericin B\textsuperscript{261-264}.

Despite the growing number of HIF1α inhibitors, none of the above agents target HIF1α directly and all possess additional functions other than blocking HIF1α. Moreover, the inhibitory effect of these agents on HIF2α is largely unknown, despite the previously outlined evidence supporting an important role of HIF2α in tumourigenesis.

\textbf{1.5.4 Treatment options for CCRCC}

CCRCC is the most common form of sporadic kidney cancer and the major cause of morbidity and mortality in VHL patients\textsuperscript{1,265}. The most effective treatment option for localized CCRCC is surgery by radical or partial nephrectomy, which is associated with high 5-year survival rates in patients with sporadic disease\textsuperscript{265}. However, due to the lack of standard screening, up to one-third of patients have metastatic disease at diagnosis, which reduces their 5-year survival rate to less than 10%\textsuperscript{266,267}. CCRCC is notoriously resistant to conventional chemotherapy and radiotherapy, but can evoke an immune response that infrequently results in spontaneous remissions, such that the standard of care for metastatic CCRCC has been immunotherapy with interleukin-2 (IL-2) or interferon α (IFNα) cytokines\textsuperscript{268}. In 1992, high-dose IL-2 was granted FDA approval based on its ability to produce durable complete responses in 5% of patients with metastatic CCRCC. However, IL-2 treatment is highly toxic with life-threatening cardiac and pulmonary adverse events that can result in treatment-related deaths. Thus, patients need to have excellent performance status and adequate organ function to be eligible for this treatment, which is only performed at specialized centres\textsuperscript{268}. Studies aimed at identifying predictors of treatment-response are currently underway.
Functional inactivation of VHL is frequently observed in sporadic CCRCC\(^1\). Based on our recent understanding of VHL-HIF oxygen sensing, targeted therapies against cellular proteins involved in this pathway have been developed, and a number of these have been approved for treatment of metastatic CCRCC in the last four years (Fig. 1.5). The HIF-mediated angiogenic switch in CCRCC has been targeted by anti-angiogenic agents sorafenib (Nexavar), sunitinib malate (Sutent) and bevacizumab (Avastin)\(^{267,269}\). While bevacizumab is a humanized monoclonal antibody against VEGF, sunitinib and sorafenib are both receptor tyrosine kinase inhibitors acting on multiple receptors involved in angiogenesis, such as VEGFR2 and PDGFR\(\beta\)\(^{267,269}\) (Fig. 1.5). First-line treatment of metastatic CCRCC with sunitinib, or bevacizumab and IFN\(\alpha\), or second-line therapy with sorafenib, significantly prolonged progression-free survival of patients in large international phase III trials\(^{270-272}\). Similarly, phase III trials with mTOR inhibitors temsirolimus (CCI-779) and everolimus (RAD-001) (Fig. 1.5) also resulted in increased progression-free survival in patients with metastatic CCRCC\(^{273,274}\). In these trials, temsirolimus was administered as a first-line treatment, while everolimus served as a second-line therapy upon disease relapse on sorafenib or sunitinib.

Even though all of the above agents significantly augmented progression-free survival in metastatic CCRCC patients, only temsirolimus also increased overall patient survival\(^{270-274}\). Hoping to prolong overall survival, studies are now underway testing different combinations of these agents, as well as their use in the adjuvant therapy setting\(^{267}\). While the anti-angiogenic agents target a major cellular response initiated by HIF, there are other important HIF-regulated functions contributing to disease progression, including anaerobic metabolism, tumour cell growth and survival, unlikely to be affected by these drugs. Thus, targeting HIF\(\alpha\) through inhibition of mTOR-mediated HIF\(\alpha\) translation may seem a better approach. However, it has been shown that mTOR-mediated translation decreases under hypoxia as an energy-conserving measure\(^{275}\). Under these circumstances, HIF\(\alpha\) mRNA translation is ensured through other mechanisms, possibly IRES-dependent. Given the invariable existence of hypoxic regions in CCRCC, mTOR inhibitors would be rendered ineffectual in these regions. Thus, new strategies to inactivate HIF\(\alpha\) directly and constitutively would represent a major conceptual advancement in anti-CCRCC and other anti-cancer therapeutics.
Figure 1.5. New targeted therapies for the treatment of CCRCC. Schematic illustrating the mechanisms of action of temsirolimus, everolimus, bevacizumab, sunitinib and sorafenib with respect to inhibition of HIF and its target proteins.
1.6 HIF-independent VHL functions

1.6.1 ECM regulation

In addition to its well-characterized role in the negative regulation of HIFα, other tumour-suppressor functions have also been attributed to VHL. Early experiments established a direct interaction between VHL and fibronectin, an extracellular glycoprotein which interacts with integrins to bridge cells to the structural proteins of the extracellular matrix (ECM)\(^\text{276}\). Biochemical fractionation and immunofluorescence studies showed VHL and fibronectin to co-localize in the membrane compartment, specifically in the ER/Golgi\(^\text{276}\). VHL binding to fibronectin is important for deposition of an extracellular fibronectin matrix, as CCRCC cells devoid of VHL do not deposit a proper fibronectin matrix, a defect which can be corrected by re-introducing VHL\(^\text{276}\). Except for VHL mutants solely giving rise to polycythemia (further discussed in chapter 4), all VHL-disease mutants characterized to date are defective for fibronectin binding and/or fibronectin matrix assembly\(^\text{277,278}\). Notably, disruption of VHL engagement to fibronectin promotes tumour formation in CCRCC xenografts\(^\text{279,280}\). Both phosphorylation of the VHL N-terminus and VHL NEDD8-modification have been shown to be important for fibronectin matrix deposition\(^\text{279,280}\). Interestingly, NEDD8 conjugation to VHL acts as a molecular switch, preventing ECV complex formation and allowing engagement to fibronectin\(^\text{281}\).

Recently, VHL has also been implicated in collagen IV matrix assembly through direct interaction with α-chains of collagen IV\(^\text{282,283}\). Collagen IV, similar to fibronectin, is a component of the ECM. Interestingly, VHL binding to collagen IV was hydroxylation-dependent\(^\text{282,283}\). While VHL bound fibrillar collagen chains, it did not associate with the folded collagen triple helix, suggesting VHL to have a possible role in collagen processing\(^\text{282}\). Similar to fibronectin, a wide range of VHL tumour-associated mutations, including VHL mutants intact for HIFα degradation, were defective in collagen binding, supporting a HIF-independent tumour suppressor role for VHL\(^\text{282,283}\).

1.6.2 The primary cilium and microtubule stability

Renal cysts are a common feature of VHL disease and are considered preneoplastic precursor lesions of CCRCC. In addition, many different hereditary conditions give rise to renal cysts,
which are associated with altered structure or function of the primary cilium, a hair-like extracellular appendage, which allows cells to respond to flow by transmitting calcium-mediated intracellular signals upon mechanical bending. In renal epithelial cells, these calcium signals are thought to regulate proliferation and cytoarchitecture in response to urine flow. A number of recent studies indicate that loss of VHL leads to loss of the primary cilium in CCRCC cells and that re-introduction of wild-type VHL, but not VHL-disease mutants, can rescue cilia formation. The renal primary cilium emerges from the basal body, a microtubule-based structure, consists of nine microtubule doublets forming the axoneme, and is surrounded by a lipid bilayer membrane which is continuous with the plasma membrane. Notably, VHL directly localizes to the primary cilium and has also been shown to interact with microtubules. The precise mechanism for VHL-mediated regulation of the primary cilium needs to be further defined, but correlates with its ability to maintain microtubule stability, possibly through engagement of kinesin. VHL has also been shown to be essential for the oriented growth of microtubules towards the cell periphery, a prerequisite step for cilia formation.

Interestingly, a recent report showed VHL and glycogen synthase kinase 3β (GSK3β) to have redundant roles in the maintenance of the primary cilium, with GSK3β capable of inactivating VHL by phosphorylation. However, following activation of the PI3K-Akt pathway, GSK3β is itself inactivated, and microtubule stability and cilium maintenance falls to VHL. Notably, in renal cysts of VHL patients, both VHL and GSK3β are inactivated, leading to cilia loss and cyst formation. In agreement, loss of both VHL and PTEN, an antagonist of the PI3K pathway, promotes renal and genital tract cyst formation.

### 1.6.3 Apoptosis

Several studies have reported increased NF-κB activity upon VHL loss in CCRCC cells, which correlates with the observed resistance of these cells to TNF-α-mediated apoptosis. Recently, VHL was found to sensitize CCRCC cells to TNF-α induced apoptosis by directly engaging with casein kinase 2 (CK2) and promoting the inhibitory phosphorylation of the NF-κB agonist Card9. Lack of Card9 phosphorylation, as observed in VHL-/- cells, results in increased NF-κB activity and resistance to apoptosis. shRNA-mediated knockdown of Card9 in VHL-/- CCRCC cells restored sensitivity to TNF-α and retarded tumour growth in an...
orthotopic xenograft assay. Contrary to previous reports, Yang et al. also showed that NF-κB regulation is HIF-independent, as HIF2α or ARNT knockdown did not affect NF-κB activity.

A subset of germline VHL mutations are known to cause phaeochromocytoma, which is an intra-adrenal paraganglioma, derived from sympathetic neuronal precursor cells. During normal development, many of these neuronal precursors undergo c-Jun-mediated apoptosis, as they compete for nerve growth factor (NGF). VHL has recently been shown to negatively regulate the c-Jun antagonist JunB, through its ubiquitin-mediated degradation of active, atypical protein kinase C (aPKC). Interestingly, VHL mutations linked to phaeochromocytoma development were defective for negative regulation of aPKC, and thus JunB. This resulted in increased survival, as increased JunB activity protected phaeochromocytoma cells from apoptosis upon NGF withdrawal.

1.7 Mouse models of VHL function

1.7.1 Germline and tissue-specific VHL inactivation

Germline inactivation of VHL is embryonically lethal due to severe placental hemorrhage. While heterozygous VHL(+/−) mice are viable and VHL(−/−) embryos and placentas appear phenotypically normal until embryonic day (E) E9.5-10.5, VHL(−/−) embryos succumb by day E10.5-12.5 due to abnormal placental vascularization. This phenotype is likely mediated by inappropriate HIF activation, since PHD2 knockout mice, which naturally display significant global increases in HIFα protein levels, also exhibit severe placental defects and die during midgestation. However, the pathologies of VHL and PHD2 null mice are not identical, pointing to HIF-independent functions of either/both proteins in placental development.

Mice heterozygous for the germline loss of one VHL allele develop cavernous liver haemangiomas, likely due to local inactivation of the remaining wild-type VHL allele, as predicted by Knudson’s two-hit model. Liver haemangiomas are a rare manifestation of VHL disease in humans and seem to be affected by polymorphic differences in modifier genes, since the incidence of this phenotype greatly varies between different mouse strains. The liver haemangiomas found in VHL(+/−) heterozygotes are phenocopied by conditional inactivation of


*VHL* in hepatocytes, using hepatocyte-specific Albumin-Cre and phosphoenolpyruvate carboxykinase (PEPCK)-Cre transgenes. This points to hepatocytes as the cells of origin of the liver haemangiomas, as opposed to other liver cell types, such as endothelial cells, and parallels human VHL-associated haemangioblastoma, which arises from stromal cells, not endothelial cells. Interestingly, hepatocyte-specific *ARNT* inactivation abrogated the hepatic lesions observed in conditional *VHL(-/-)* mice, but similar *HIF1α* inactivation had no effect, implicating another ARNT-binding partner, such as HIF2α in liver pathology. In agreement, hepatocyte-specific overexpression of non-degradable HIF2α was sufficient to induce the liver pathology observed after VHL loss.

Intriguingly, characteristic VHL-disease tumours such as CCRCC and CNS and retinal haemangioblastomas were not observed in the germline heterozygous VHL mice. However, upon conditional inactivation of both VHL alleles in the renal cortex, approximately 20% of mice developed renal cysts, a precursor lesion to CCRCC. Notably, CCRCC itself was not observed. Renal cyst development was dependent upon intact HIF signalling, again implicating HIF2α activity, as conditional kidney VHL/HIF1α double knockout mice did not develop cysts, but VHL/ARNT inactivation resulted in cyst formation. The absence of CCRCC formation in these mice may point to additional genetic events required downstream of VHL inactivation for renal tumorigenesis.

### 1.7.2 Mouse model of Chuvash polycythemia

In an effort to investigate the mechanism underlying Chuvash polycythemia, Hickey *et al.* recently created a mouse model for the disease by generating mice homozygous for the R200W mutation (R166W in the mouse). (Homozygous mutant mice are referred to as *VHL*R/R, or R200W/R200W in this text). As expected, *VHL*R/R mice developed polycythemia with hematocrits rising to nearly 55%, a value similar to that observed in CP patients. Interestingly, low HIF2α protein levels were detectable even under normoxia in *VHL*R/R ES cells. In accordance, serum levels of HIF2α-regulated genes, such as *EPO* and *VEGF* were elevated in *VHL*R/R mice and *EPO* mRNA was increased in the kidneys, the physiological site of EPO production. The R200W mutation was found to stimulate extramedullary haematopoiesis in the spleen of *VHL*R/R mice. Compared to WT and *VHL*R/+ littermates, *VHL*R/R spleens were darker in colour and contained many clusters of erythroid precursors and interestingly, also contained an
abundance of clustered megakaryocytes. The stress response to acute hemolytic anaemia induced by phenylhydrazine (PHZ) treatment was also significantly prolonged in VHL\textsuperscript{R/R} mice, which were unable to stop erythroid expansion upon reaching WT hematocrit values. Instead, mutant mice continued erythropoiesis until reaching hematocrits of polycythemic levels observed prior to PHZ treatment. In addition, when plated in methylcellulose containing various cytokines, VHL\textsuperscript{R/R} splenocytes gave rise to 3.5-fold more erythroid colonies compared with WT splenocytes, as well as 3-fold more CFU-G (CFU-granulocyte) and CFU-GEMM (CFU-granulocyte-erythroid-macrophage-megakaryocyte) colonies. Notably, splenocytes from VHL\textsuperscript{R/R} mice were also hypersensitive to EPO, giving rise to more burst-forming-erythroid (BFU-E) erythroid precursors than WT at each EPO concentration tested. In summary, the polycythemic VHL\textsuperscript{R/R} mice faithfully recapitulate the human CP condition, as they contain erythroid precursors that are hypersensitive to EPO as well as high serum EPO levels, prominent features of primary and secondary polycythemia, respectively.

1.8 Molecular characterization of the VHL ubiquitin ligase

The best-characterized function of VHL is as the substrate-recognition component of the ECV E3 ubiquitin ligase, targeting HIF\textalpha for degradation under normal oxygen tension. However, the precise regulation of this VHL ubiquitin ligase is still unknown. Recently, covalent modification of the ECV scaffolding component Cul2 by the ubiquitin-like molecule NEDD8 has been shown to facilitate ECV-mediated HIF\textalpha ubiquitination\textsuperscript{70}. Hence, to better understand the sequence of events leading to HIF\textalpha degradation, the mechanism by which Cul2 neddylation enhances ECV ubiquitin ligase activity is investigated in this thesis. Furthermore, HIF\textalpha is highly expressed in a large number of solid tumours and is associated with tumour aggressiveness and resistance to treatment, rendering it a valid therapeutic target for anti-cancer therapy\textsuperscript{10}. Thus, work contained in this thesis applies the current understanding of the VHL ubiquitin ligase to bioengineer a VHL protein capable of constitutively targeting HIF1\textalpha and HIF2\textalpha for ubiquitin-mediated degradation. Subsequently, the effect of this recombinant VHL on tumour progression is tested.

Two VHL mutations, VHL(R200W) and VHL(H191D), have recently been shown to cause Chuvash polycythemia, characterized by hypersensitive erythroid progenitors to EPO and
elevated serum EPO levels\textsuperscript{13,32}. The current model for this disease proposes an insufficient negative regulation of HIF\textalpha by the VHL(R200W) ubiquitin ligase, leading to mild HIF\textalpha stabilization under normoxia and thus increased production of the HIF-target gene EPO\textsuperscript{13}. However, an increased serum EPO concentration due to inappropriate HIF activity only explains the secondary polycythemic features of CP. A mechanism addressing the erythroid progenitor hypersensitivity to EPO is still outstanding, and is therefore investigated in this thesis.
Chapter 2
Role of NEDD8 modification of Cul2 in the sequential activation of the ECV complex

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2.1. Introduction and Rationale

VHL is the substrate-recognition component of an SCF (Skp1/Cdc53 or Cul1/F-box protein)-like E3 ubiquitin ligase complex (ECV) composed of elongin C, elongin B, Cul2, and a RING-finger protein, Rbx1, that targets HIF\(\alpha\) subunits for ubiquitin-mediated degradation selectively under normal oxygen tension. The E3 function of both SCF and ECV is dependent on the recruitment of their individual E2 ubiquitin-conjugating enzymes, Cdc34 and UbcH5a, respectively\(^7,303\).

Cullins are the scaffold components of SCF and ECV ubiquitin ligases and are covalently modified by the ubiquitin-like molecule NEDD8 in a manner analogous to ubiquitin attachment, involving the NEDD8-activating APP-BP1/Uba3 heterodimeric enzyme (E1; NAE) and a NEDD8-conjugating enzyme UbcH12 (E2; NCE)\(^76,80,81\) (Fig. 1.2). Importantly, the overall E3 ubiquitin ligase activity of a number of yeast and mammalian SCF complexes is enhanced by Cullin neddylation. Recently, the NEDD8 modification of Cul2 on Lysine 689 was shown to enhance the E3 activity of the SCF-like ECV complex \textit{in vivo}\(^70\).

In an effort to define the mechanisms regulating SCF function, the core Cullin/Rbx1 complex was shown to be required for Cdc34 recruitment by the yeast SCF\(^56,304\). Wu and colleagues subsequently demonstrated that the neddylated-Cul1/Rbx1 complex was significantly better at supporting Cdc34-mediated assembly of polyubiquitin chains than unneddylated-Cul1/Rbx1\(^305\). In support, NEDD8 modification of Cul1 was shown to directly enhance the binding of ubiquitin-conjugated E2 Ubc4 to SCF\(^8\text{TrCP}\)\(^97\). Although these aforementioned studies provide several models that explain, in part, Cul1 neddylation-mediated regulation of SCF activity, the
mechanism by which Cul2 neddylation promotes ECV ubiquitin ligase function remains entirely unknown and is the subject of our investigation.

2.2. Materials and Methods

Unless otherwise indicated, all experiments were performed in triplicate.

2.2.1. Cells
The HEK293 human embryonic kidney cell line (American Type Culture Collection, Rockville, MD) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Oakville, Canada) at 37°C in a humidified atmosphere with 5% CO₂.

2.2.2. Antibodies
Anti-HA (Y11), anti-myc polyclonal and anti-His monoclonal antibodies were obtained from Santa Cruz (Santa Cruz, CA). Anti-HA (12CA5), anti-HIF1α monoclonal and anti-Cul2 polyclonal antibodies were obtained from Boehringer (Mannheim, Germany), Novus Biologicals (Littleton, CO), and Zymed (San Francisco, CA), respectively. MG132 proteasome inhibitor was obtained from Boston Biochem (Cambridge, MA).

2.2.3. Plasmids
The generation of mammalian expression plasmids pRC-CMV-HA-Cul2(WT), pRC-CMV-HA-Cul2(K689R) was described previously, and pcDNA3.1-HA-Cul2ΔC(1-600) was kindly provided by Dr. William G. Kaelin. pcDNA3.1-myc-Rbx1 was generously provided by Dr. James DeCaprio. pRC-CMV-HA-VHL(WT) and pcDNA3-HA-HIF1α were previously described. Purified His-UbcH5a was obtained from Boston Biochem (Cambridge, MA).

2.2.4. Immunoprecipitation and immunoblotting
Cells were lysed in EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40), supplemented with protease and phosphatase inhibitors (Roche, Laval, Canada). Cell lysates were
immunoprecipitated with the indicated antibodies in the presence of protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ). Bound proteins were washed five times with NETN buffer (20 mM Tris pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% NP-40), eluted by boiling in sodium dodecyl sulfate (SDS)-containing sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). For western blotting, resolved proteins were transferred to PVDF membrane in 1x transfer buffer (1.3M Tris, 170 mM glycine, 0.1% SDS, 20% Methanol) for 4-5 hrs at 450-500 mA. The membrane was subsequently blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 4% skim milk powder for 1 hr and incubated for 16 hrs with primary antibody diluted in TBST. Five washes with TBST were followed by 1 hr incubation with secondary antibody (goat anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase or alkaline phosphatase, Pierce Chemical, Rockford, IL, USA) diluted 1:7500 in TBST containing 2% skim milk. The membrane was washed again five times in TBST and developed using film and ECL reagents (Lumi-light, Roche, Laval, Canada), or directly in alkaline phosphatase developing solution (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 0.04% NBT and 0.017% BCIP).

2.2.5. In vitro binding assays

*In vitro* translation in the presence of [³⁵S]-methionine was performed using TNT reticulocyte *in vitro* transcription/translation system (Promega, Madison, WI) according to the manufacturer’s instructions. Indicated translation products were mixed at 37°C for 60 min. Reaction mixtures were then incubated with the indicated antibodies in the presence of protein A-Sepharose beads in 700 µl of EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% NP-40) at 4°C for 1hr. After five washes with NETN buffer (20 mM Tris [pH 8.0], 120 mM NaCl, 1 mM EDTA, 0.5% NP-40), the bound proteins were resolved on SDS-PAGE and visualized by autoradiography.

2.2.6. In vitro neddylation assay

*In vitro* neddylation assay was performed by incubating ³⁵S-labelled Cul2 *in vitro* translates with 1 µg each of NAE, UbcH12 and NEDD8, and 1 µl 10 mM ATP. NAE, UbcH12 and NEDD8 were obtained from Boston Biochem (Cambridge, MA). Reactions were incubated at 37°C for 60 min and subsequently incubated with the indicated antibodies in the presence of protein A-Sepharose beads in 700 µl of EBC buffer at 4°C for 1hr. After five washes with NETN buffer, the bound proteins were resolved on SDS-PAGE and visualized by autoradiography.
2.2.7. Hypoxia treatment of cells
Cells were maintained at 1% O\textsubscript{2} in a ThermoForma (Marietta, OH) hypoxia chamber. Cell lysates were prepared in the chamber in hypoxic environment prior to further experimentation.

2.3. Results and Discussion

2.3.1. Neddylation status of Cul2 does not influence binding to Rbx1

Rbx1 has been shown to be involved in the recruitment of E2 ubiquitin-conjugating enzymes\textsuperscript{56,304}. Binding of Rbx1 has been mapped outside of the Cul1 neddylation site\textsuperscript{96}. However, the Rbx1 binding region on Cul2 has not been established and it is unclear whether neddylation of Cul2 would influence binding to Rbx1 via promoting subtle conformational changes to Cul2. Thus, we asked whether the neddylation of Cul2 influenced the engagement of Rbx1 as a potential mechanism that promotes activity of the ECV. \textsuperscript{35}S-labelled \textit{in vitro} translated HA-Cul2(WT) or the neddylation-defective HA-Cul2(K689R) mutant\textsuperscript{70} were mixed with \textsuperscript{35}S-labelled \textit{in vitro} translated myc-Rbx1, immunoprecipitated with an anti-HA antibody, resolved on SDS-PAGE, and visualized by autoradiography (Fig. 2.1A). As previously demonstrated\textsuperscript{70}, HA-Cul2(WT) migrated as a doublet, where the slower migrating protein represents Cul2 covalently linked to NEDD8. As expected and previously shown\textsuperscript{70}, HA-Cul2(K689R), failed to be neddylated and therefore migrated as a single band. However, Rbx1 co-precipitated with both HA-Cul2(WT) and HA-Cul2(K689R) (Fig. 2.1A). An analogous experiment was performed \textit{in vivo}, where human embryonic kidney HEK293 cells were transiently transfected with plasmids encoding HA-Cul2(WT) or HA-Cul2(K689R) with or without a plasmid encoding myc-Rbx1. Cells were lysed, immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and immunoblotted with an anti-HA (upper panel) or anti-myc (lower panel) antibody (Fig. 2.1B). Consistent with the \textit{in vitro} data, similar levels of myc-Rbx1 co-precipitated with either neddylatable HA-Cul2(WT) or un-neddylatable HA-Cul2(K689R), suggesting that the neddylation status/capability of Cul2 does not influence its interaction with Rbx1 (Fig. 2.1B).
Figure 2.1. Cul2 neddylation status does not influence association with Rbx1. (A) ^35S-labelled in vitro translated HA-Cul2(WT) or neddylation-defective HA-Cul2(K689R) mutant were, where indicated, mixed with ^35S-labelled in vitro translated myc-Rbx1 and immunoprecipitated with the indicated antibodies. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. (B) HEK293A cells were transiently transfected with mammalian expression plasmids encoding myc-Rbx1, HA-Cul2(WT), or HA-Cul2(K689R) alone or in combination. Cells were lysed and immunoprecipitated with the indicated antibodies. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Cul2 (upper panel) and anti-myc (lower panel) antibodies. IP: immunoprecipitation.
2.3.2. Rbx1 acts as an E3 NEDD8 ligase to promote the neddylation of Cul2

Interestingly, the ectopic expression of Rbx1 promoted the accumulation, albeit modest, of NEDD8-modified Cul2 (Fig. 2.1B, compare lanes 3 and 4). We asked whether Rbx1 promoted Cul2 neddylation by performing an \textit{in vitro} Cul2 neddylation assay, as previously described\textsuperscript{70}. \textit{In vitro} translated HA-Cul2(WT) in the absence of a neddylation reaction produced minimal modification of Cul2 by NEDD8 (Fig. 2.2A, lane 1), whereas in the presence of a neddylation reaction (containing purified NAE, UbcH12, NEDD8 and ATP; see Materials and Methods), a greater fraction of HA-Cul2 became neddylated. Addition of exogenous \textit{in vitro} translated Rbx1 to this reaction significantly shifted the status of Cul2 towards NEDD8-modified Cul2 (Fig. 2.2A, lane 3). The slight modification of Cul2 observed in the absence of exogenous Rbx1 was likely due to the presence of endogenous Rbx1 in the rabbit reticulocyte lysate used in the \textit{in vitro} translation reaction (Fig. 2.2A, lane 1). In a complementary experiment, we made use of the Cul2\textDeltaC mutant, which is a C-terminal Cul2 truncation mutant (1-600) capable of binding Rbx1, but lacking the NEDD8-conjugation site (Fig. 2.2B). Incubation of co-\textit{in vitro} translated HA-Cul2/myc-Rbx1 with increasing amounts of Cul2\textDeltaC prior to the neddylation reaction resulted in the attenuation of Cul2 neddylation (Fig. 2.2C, left panel). In addition, incubation of \textit{in vitro} translated myc-Rbx1 with increasing amounts of Cul2\textDeltaC prior to the \textit{in vitro} Cul2 neddylation reaction resulted in a similar diminution of Cul2 neddylation (Fig. 2.2C, right panel). These results, taken together, suggest that Rbx1 functions as an E3 NEDD8 ligase to direct the specific targeting of Cul2 for neddylation in concert with NAE and NCE/UbcH12.
Figure 2.2. Rbx1 promotes Cul2 neddylation. (A) HA-Cul2(WT) was in vitro translated alone (lanes 1 and 2) or co-translated with myc-Rbx1 (lane 3) in the presence of $^{35}$S-Met and subjected to a neddylation reaction where indicated. Reaction mixtures were subsequently immunoprecipitated with anti-HA antibody, resolved on SDS-PAGE and visualized by autoradiography. (B) Myc-Rbx1 in combination with HA-Cul2ΔC were in vitro translated in the presence of $^{35}$S-Met and immunoprecipitated with the indicated antibodies. Bound proteins were resolved on SDS-PAGE and visualized by autoradiography. (C) HA-Cul2(WT) was co-in vitro translated with myc-Rbx1 in the presence of $^{35}$S-Met and incubated with increasing amounts of unlabelled in vitro translated HA-Cul2ΔC prior to performing a neddylation reaction (left panel). HA-Cul2(WT) and myc-Rbx1 were in vitro translated independently in the presence of $^{35}$S-Met. Myc-Rbx1 was subsequently incubated with increasing amounts of unlabelled in vitro translated HA-Cul2ΔC prior to adding radiolabelled in vitro translated HA-Cul2(WT) and performing a neddylation reaction (right panel). All reaction mixtures were immunoprecipitated with anti-HA antibody, resolved on SDS-PAGE and visualized by autoradiography. IP: immunoprecipitation, AR: autoradiography, NEDD8 RxN: neddylation reaction.
2.3.3. UbcH5a preferentially engages Cul2 modified by NEDD8

Polyubiquitination of cellular proteins requires the coordinated action of an E3 with E2. The physiological E2 for ECV is yet unknown. However, both Cdc34 and UbcH5a have been shown to effectively support ECV-dependent ubiquitination of HIFα \textit{in vitro} with UbcH5a displaying greater activity\textsuperscript{7,53,303}. We asked whether neddylation of Cul2 affected its association with UbcH5a as a potential mechanism of regulating E3 function of the ECV. HA-Cul2(WT) and the neddylation-defective HA-Cul2(K689R) mutant were \textsuperscript{35}S-\textit{in vitro} translated in the presence or absence of neddylation conditions and incubated with purified His-UbcH5a prior to immunoprecipitation with anti-HA antibody. Bound proteins were resolved on SDS-PAGE and visualized by autoradiography and by immunoblotting with anti-His antibody (Fig. 2.3A). Both HA-Cul2(WT) and the HA-Cul2(K689R) mutant co-precipitated equal amounts of His-UbcH5a in the absence of neddylation reactions. Similar results were obtained \textit{in vivo} from transfected HEK293 cells, in which HA-Cul2(WT) and HA-Cul2(K689R) immunoprecipitated equal amounts of co-transfected FLAG-UbcH5a (data not shown). However, an \textit{in vitro} neddylation reaction resulted in a dramatic increase of the slower migrating neddylated Cul2 and, importantly, of the Cul2-associated UbcH5a. This was not due to the neddylation reaction itself, since the amount of His-UbcH5a associating with the neddylation-defective Cul2(K689R) was comparable irrespective of the neddylation conditions (Fig. 2.3B). These results suggest that the promotion of Cul2 neddylation increases UbcH5a engagement. It should be mentioned that the detection of endogenous E2s in complex with E3s is notoriously difficult, and we have to-date been unsuccessful in establishing the interaction of UbcH5a (or Cdc34) with Cul2(WT or K689R) in the absence of over-expression.
Figure 2.3. UbcH5a preferentially binds NEDD8-modified Cul2. (A) $^{35}$S-labelled in vitro translated HA-Cul2(WT) or HA-Cul2(K689R) were mixed with purified His-UbcH5a, subjected to a neddylation reaction where indicated, and immunoprecipitated with anti-His or anti-HA antibodies. Bound proteins were resolved by SDS-PAGE; $^{35}$S-Cul2 was visualized by autoradiography and Cul2-bound UbcH5a was visualized by performing anti-His immunoblot. (B) Performed as in A with HA-Cul2(K689R) and His-UbcH5a in the absence (-) or presence (+) of a neddylation reaction. IP: immunoprecipitation, IB: immunoblot, NEDD8 RxN: neddylation reaction.
2.3.4. HIF1α-bound ECV preferentially contains neddylated Cul2

An active E3 of the SCF family is invariably associated with its respective E2 and bound by the F-box protein-specific substrate. A prediction would be that an ECV complex which has recruited HIFα for ubiquitination would be associated with the neddylated Cul2 that has engaged UbcH5a. To test this hypothesis, HEK293 cells were transiently transfected with plasmids encoding HA-VHL in combination with HIF1α and treated with proteasome inhibitor MG132 prior to lysis. Lysates were immunoprecipitated with either anti-HA or anti-HIF1α antibody, resolved by SDS-PAGE and immunoblotted with anti-HIF1α, anti-Cul2 or anti-HA antibodies (Fig. 2.4A). As expected and previously observed\(^4\), VHL co-precipitated both the neddylated and unnedylated Cul2, but primarily the latter form (Fig. 2.4A, lane 1). HIF1α co-precipitated VHL as expected, but strikingly and in contrast, preferentially co-precipitated NEDD8-modified Cul2 (Fig. 2.4A, lane 2). In a complementary experiment, HEK293 cells were treated with or without MG132 and immunoprecipitated for endogenous HIF1α (Fig. 2.4B). Proteasomal inhibition, as expected, resulted in the accumulation of HIF1α, which, consistent with the overexpression data, preferentially co-precipitated neddylated Cul2 (Fig. 2.4B, lane 2). These results suggest that ECV recognition of HIF1α via VHL is temporally coordinated with the NEDD8-modification of Cul2, resulting in the recruitment of UbcH5a and thereby triggering the ubiquitination of HIF1α. Notably, we observed a slight but nevertheless significant accumulation of total neddylated Cul2 in the presence of proteasome inhibitor, suggesting that the NEDD8-conjugated form of Cul2 is also unstable and subjected to degradation via the proteasome (Fig. 2.4B, compare lanes 3 and 4). In support of this notion, neddylated Cul1 and Cul3 were recently shown to be targeted for proteasome-mediated destruction\(^8,5\). However, this accumulation of neddylated Cul2 in the presence of a proteasomal inhibitor does not account for the Cul2 profile associated with HIF1α (Fig. 2.4B, compare lane 2 in middle panel and lane 4 in lower panel).
**Figure 2.4. ECV engaged to HIF1α contains preferentially neddylated Cul2.** (A) HEK293A cells were transiently transfected with a mammalian expression plasmid encoding HA-VHL alone or in combination with a plasmid encoding HIF1α. Cells were treated with the proteasome inhibitor MG132 for 4 hrs, lysed and immunoprecipitated with the indicated antibodies. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-HIF1α (upper panel), anti-Cul2 (middle panel) and anti-HA (lower panel) antibodies. (B) HEK293A cells were treated with MG132 for 4 hrs where indicated, lysed and immunoprecipitated with anti-HIF1α antibody (lanes 1, 2). Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-HIF1α (upper panel) and anti-Cul2 (middle panel) antibodies. In parallel, 20µg of whole cell extract (lanes 3, 4) collected prior to the anti-HIF1α immunoprecipitation experiment was resolved by SDS-PAGE and immunoblotted with anti-Cul2 antibody (lower panel). IP: immunoprecipitation, IB: immunoblot, WCE: whole cell extract.
2.3.5. HIF1α binding to ECV triggers Cul2 neddylation

We next asked whether the binding of substrate HIFα by the substrate-recruiting protein VHL determined Cul2 neddylation. HEK293 cells were transfected with plasmids encoding HA-full-length VHL (1-213) or HA-α domain (155-213) of VHL. The latter is incapable of binding HIFα due to lack of the substrate-recognition β domain. Cells were lysed, immunoprecipitated against HA, resolved by SDS-PAGE, and immunoblotted with anti-Cul2 and anti-HA antibodies (Fig. 2.5A). While the full-length VHL co-precipitated both neddylated and unneddylated Cul2, the α domain of VHL exclusively bound unmodified Cul2 (Fig. 2.5A), suggesting that the VHL engagement of substrate is critical for subsequent neddylation of Cul2. In a complementary experiment, we asked whether an increase in available substrate enhanced the neddylation of Cul2. HEK293 cells were transiently transfected with plasmids encoding HIF1α and T7-VHL and incubated with proteasome inhibitor for 4 hrs prior to lysis. Cells were lysed, immunoprecipitated with anti-T7 antibody, bound proteins resolved by SDS-PAGE and immunoblotted with anti-HIF1α, anti-Cul2 and anti-T7 antibodies. While T7-VHL immunoprecipitated mainly unnedylated Cul2 as previously observed, the ectopic expression of HIF1α resulted in a dramatic increase of neddylated Cul2 (Fig. 2.5B). This increase in neddylated Cul2 was dose-dependent on the amount of available HIF1α (Fig. 2.5C). Notably, ectopic expression of HIF1α had negligible effect on the general profile of Cul2, suggesting that ECV is not the sole occupier of Cul2. In support of this notion, Kamura et al.307 identified several elonginBC/Cul2 complexes associated with other potential substrate-recruiting proteins with ‘loose’ F-box motifs. Together, these results demonstrate that HIF1α is not merely recruited to the ECV for ubiquitination, but that it plays an active role in its own demise by triggering the neddylation-dependent activation of the ECV. Furthermore, the ability of VHL to bind HIFα was not affected by the NEDD8 status of Cul2 (data not shown), precluding the possibility that neddylation of Cul2 promotes ECV engagement of HIFα.
The best determinant of VHL engagement of HIFα is oxygen tension. Therefore, we utilized hypoxia to abrogate VHL association with HIFα, and then asked whether substrate disengagement from VHL affected Cul2 neddylation. HEK293 cells were transiently transfected with plasmids encoding HA-VHL and maintained under normoxia (21% O₂) or hypoxia (1% O₂). Cells were lysed, immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE and immunoblotted with anti-HA antibodies. Under hypoxia, where VHL fails to recognize HIFα, there was a striking absence of the neddylated Cul2, while under normoxia, the neddylated Cul2 was found in a complex with VHL (Fig. 2.5D). This result further confirms our notion that the binding of substrate HIFα by the substrate-recruiting protein VHL determined Cul2 neddylation. It also suggests that the ECV complex is maintained at an energy-conserving ‘OFF’ position when no substrate is recruited for the ubiquitin-mediated destruction.

In summary, we propose (see Fig. 2.6) that oxygen-dependent HIF1α engagement to ECV is critical in the promotion of Rbx1-mediated neddylation of Cul2. Indeed, an ECV complex containing a mutant VHL lacking substrate-recognition domain is exclusively associated with unneddylated Cul2. Neddylation of Cul2 then preferentially recruits UbcH5a, triggering the ubiquitination of VHL-bound HIFα. Thus, these events establish a central role for the
neddylation of Cul2 in a previously unrecognized temporally coordinated activation of the ECV with the recruitment of its substrate HIFα. How precisely HIFα binding to VHL leads to the neddylation of Cul2 in the context of ECV remains an outstanding question yet to be resolved.
Figure 2.6. Sequential activation of ECV. See text for details. N8: NEDD8, NE1 or NAE: NEDD8-activating enzyme, NE2 or NCE: NEDD8-conjugating enzyme, Ub: Ubiquitin, UE1 or UAE: Ubiquitin-activating enzyme, UE2 or UCE: Ubiquitin-conjugating enzyme, UE3: Ubiquitin ligase, eB: Elongin B, eC: Elongin C, red box within HIFα: prolyl-hydroxylated ODD.
Chapter 3
Oxygen-independent degradation of HIFα via bioengineered VHL tumour suppressor complex

This chapter is now published:

3.1. Introduction and Rationale

Tumour growth invariably outstrips its blood supply as the diffusion capacity of oxygen from the nearest blood vessel is surpassed, leading to regions of hypoxia within the tumour mass. In addition, irregular blood flow in the commonly malfolded tumour vasculature can also cause cells adjacent to tumour blood vessels to experience hypoxia\(^3\). In these hypoxic regions, HIFα is not prolyl-hydroxylated and thus escapes VHL recognition and ECV-mediated polyubiquitination and degradation\(^1\). Furthermore, cancer-causing mutations on a growing list of oncogenes and tumour suppressor genes have been identified to enhance the expression of HIFα by increasing its rate of synthesis\(^10\). Moreover, tumour cells including CCRCC devoid of functional VHL have high levels of HIFα irrespective of oxygen tension\(^1\). In these scenarios, HIFα is able to engage to ARNT to form the active transcription factor HIF, triggering the transcription of a large number of genes that promote various adaptive cellular responses ranging from anaerobic metabolism, erythropoiesis, angiogenesis, invasion to cell survival\(^10\). HIF-induced genes are known to drive oncogenesis and as a result, HIF1α and HIF2α overexpression is frequently associated with increased phenotypic aggressiveness and poor prognosis in numerous tumour types including brain, breast, lung, colon, skin, prostate, and kidney cancers\(^10\). These observations implicate both HIF1α and HIF2α as compelling targets for anti-cancer therapy.
Currently, a number of compounds that inhibit HIF1α activity are in clinical development or have been approved by the FDA. However, none of these compounds directly targets HIF1α and each drug has multiple functions other than blocking HIF1α. Moreover, the inhibitory effect of these agents on HIF2α is largely unknown, despite increasing evidence supporting an important role of HIF2α in tumourigenesis, especially in CCRCC. We therefore sought to directly and constitutively target both HIF1α and HIF2α for degradation. Here, we describe the bioengineering of VHL that removes the oxygen constraint in the recognition of HIFα while preserving its E3 enzymatic activity, allowing HIFα degradation irrespective of oxygen tension.

3.2. Materials and Methods

Unless otherwise indicated, all experiments were performed in triplicate.

3.2.1. Cells

786-O (VHL-/-; HIF1α-/-) CCRCC cells and HEK293 embryonic kidney cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) at 37°C in a humidified 5% CO₂ atmosphere. 786-O cells stably expressing DsRed2 (786-dsRed) were generated by nucleofecting (Amaxa, Gaithersburg, MD, USA) the mammalian expression plasmid encoding DsRed2 (Clontech, Palo Alto, CA, USA). Clones were selected with 500 µg/ml G418 (Sigma-Aldrich, Oakville, ON, Canada) and red fluorescence was confirmed using a fluorescence microscope (Nikon Eclipse TE200). Similarly, 786-HRE-Luc cells were generated by nucleofecting 5xHRE-Luciferase mammalian expression plasmid (kindly provided by Dr. Richard P. Hill) in combination with an empty mammalian expression plasmid pcDNA3.1 encoding the neomycin resistance gene. Positive clones were selected with 500 µg/ml G418 and luciferase expression was confirmed by luciferase assay.
3.2.2. Antibodies

Monoclonal anti-T7, anti-HA (12CA5), anti-α-tubulin, anti-vinculin, anti-GFP and anti-AhR antibodies were obtained from Novagen (Madison, WI, USA), Boehringer Ingelheim (Laval, QC, Canada), Sigma-Aldrich (Oakville, ON, Canada), Millipore (Billerica, MA, USA), Covalence (Berkeley, CA, USA), and Abcam (Cambridge, MA, USA) respectively. Monoclonal anti-HIF1α and polyclonal anti-HIF2α were obtained from Novus Biologicals (Littleton, CO, USA). Anti-Cul2, anti-Elongin B and anti-GLUT1 polyclonal antibodies were obtained from Invitrogen (Burlington, ON, Canada), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Alpha Diagnostics International (San Antonio, TX, USA), respectively. MG132 proteasome inhibitor was obtained from Boston Biochem (Cambridge, MA, USA).

3.2.3. Plasmids

The ARNT truncation plasmids T7-bHPAS (residues 90-419), T7-HPAS (103-419), T7-bHPAC (90-467), and T7-HPAC (103-467) were generated using the following primers introducing 5’ BamHI and 3’ XbaI sites. ARNT basic region forward primer: 5’-GCGCGGATCCATGGCCAGGGAAAATCAC-3’, ARNT HLH forward primer: 5’-GCGCGGATCCATGAACAAGATGACAGCC-3’, ARNT PAS B reverse primer: 5’-GCGCTCTAGATCATTTTAATTTCACTAC-3’, ARNT PAC reverse primer: 5’-GCGCTCTAGATCAAGAGTTCTTCACATTG-3’. The PCR products were digested with BamHI and XbaI and ligated into pcDNA3-T7 cut with these restriction enzymes. T7-HPACV, T7-HPACGV, T7-bHPACV, and T7-bHPACGV (VHL α domain residues 151-194) were generated using the above-mentioned forward primers, a reverse PAC primer introducing a 3’ XbaI site without a Stop codon: 5’-GCGCTCTAGAAGAGTTCTTCACATTG-3’, and the appropriate combination of the following VHL primers, introducing a 5’XbaI site and a 3’ApaI site. VHL forward primer: 5’-GCGCTCTAGAATCACACTGCCAGTGATATAC-3’, VHL forward primer including the Gly(6) linker: 5’-GCGCTCTAGAGGTGGTGGTTGTTGTTGTTGTTGATATACACTGCCAGTGATAC-3’ and VHL reverse primer: 5’-GCGCGGCGCCCTTCACACATTGCTGGTGTTGTC-3’. The corresponding PCR products were ligated into pcDNA3-T7 cut with BamHI and ApaI by a three-way ligation. All plasmids were confirmed by DNA sequencing. pcDNA3-HA-HIF1α, pcDNA3-HA-HIF1α(P564A) pcDNA3-T7-VHL, pcDNA3.1-(HRE)₃-Luc expression plasmids were described
previously. AhR was a kind gift from Dr. Christopher A. Bradfield and pGL3-5x-HRE-Luc was kindly provided by Dr. Richard P. Hill. A plasmid encoding DsRed2 was obtained from Clontech (Palo Alto, CA, USA).

3.2.4. Immunoprecipitation and immunoblotting
Immunoprecipitation and immunoblotting were performed as described in chapter 2.2.4.

3.2.5. In vitro binding assays
In vitro binding assays were performed described in chapter 2.2.5.

3.2.6. Hypoxia treatment of cells
Cells were treated as described in chapter 2.2.7.

3.2.7. Luciferase assays
The dual-luciferase assay was performed on HEK293 cells transfected with a total of 2.5 µg expression plasmids using Escort (Sigma-Aldrich, Oakville, ON, Canada). (HRE)$_5$-Luc (0.9 µg) was used to measure HRE-mediated transcription and 0.1 µg of pRL-SV40 renilla luciferase plasmid (Promega, Madison, WI, USA) was used as a transfection control. Empty pcDNA3.1 plasmid (Invitrogen, Burlington, ON, Canada) was used to maintain a constant final amount of transfected DNA. Where indicated, T7-VHL, T7-HPACV and T7-HPACGV were co-transfected with the luciferase plasmids. Transfections were performed in triplicate and luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) and Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase relative light units (RLUs) were normalized against Renilla luciferase RLUs. The stable 786-HRE-Luc cells infected with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV for 48 hours were lysed and 4 µg of total cell lysates were used to measure luciferase activity. The LARII substrate (Promega, Madison, WI, USA) was used to obtain RLU readings using the Lumat LB9507 luminometer.
3.2.8. Infection of 786-O or 786-HRE-Luc cells with recombinant adenovirus

Ad-EGFP, Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV were generated by the Viral Vector Core Facility (Neuroscience Research Institute, University of Ottawa, Ottawa, ON, Canada). 786-O or 786-HRE-Luc cells were infected at 70% confluency with a final multiplicity of infection (MOI) of 300. Cells were lysed 48 hours after infection for further experimentation.

3.2.9. Quantitative real-time PCR

Quantitative real-time PCR was performed and results were interpreted as previously described\textsuperscript{195}. The following primer sets were used: GLUT-1 5’-CACCACCTCACTCGGTACTT-3’ and 5’-CAAGCATTAAAACCATGTCTTCTA-3’, VEGF 5’-CTCTCTCCCTCATCGGTGACA-3’ and 5’-GGAGGGCAGAGCTGAGTGTTAG-3’, BNIP3L 5’-CTGCACAAACTTGCACATTG-3’ and 5’-TAATTTCCACAACGGGTCA-3’, and TCF8 5’-CCTAGTAAGCACAATTAGGATTAAC-3’ and 5’-GGAACAAATTGGCACAAAATGTT-3’. Values were normalized to the expression of TCF8 mRNA, expressed relatively to Ad-EGFP-infected samples (arbitrarily set to 1.0), and represented as the mean value ± standard deviation of three independent experiments performed in triplicate.

3.2.10. Mouse dorsal skin-fold window chamber assay

All animal experiments were performed in accordance with the institutional animal care guidelines (University Health Network, ON, Canada). SCID mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (80 and 5 mg/Kg, respectively). Dorsal window chambers were installed as described previously\textsuperscript{309}. Briefly, a circular incision of 1 cm diameter was made in the dorsal skin and the titanium chamber was surgically implanted. 7x10\textsuperscript{5} 786-dsRed tumour cells were implanted in the dermis of the mouse right dorsal side using an 18G syringe. A circular glass coverslip was positioned over the incision allowing the visualization of the tumour and longitudinal monitoring of associated vasculature. Adenoviral intratumoural injections with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV were performed using 2x10\textsuperscript{8} infectious units (ifu).
3.2.11. *In vivo* imaging

Fluorescence and white-light imaging were performed using a MZ FLIII, Leica stereomicroscope. Tumours, as identified by red fluorescence and white-light stereomicroscope images, were also imaged non-invasively using optical coherence tomography (OCT). A 36 kHz 1.3 µm swept source OCT system was used to probe tissue microstructure and microvasculature with approximately 10 µm resolution. 3-dimensional image stacks over a 5 mm x 5 mm x 2 mm volume of the window chamber were acquired with 1600 x 2000 x 512 pixels. Image stacks were processed to produce 2-dimensional projection maps of microvasculature within the volume using the interframe speckle variance algorithm as previously described. Low pass Gaussian filter was used for motion artefact removal and all images were normalized to the same false colour map. This technique allows for blood vessels with diameters as small as 25 µm to be visualized without the use of any exogenous contrast agents during longitudinal studies.

3.2.12. Immunohistochemical staining

Sample preparation and immunohistochemical staining were performed as previously described. Briefly, window chamber tissues were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin and 5-µm sections were cut and placed on coated slides for light microscopy. Standard hematoxylin and eosin staining was performed on the samples. Immunohistochemical staining for GFP was performed manually using a standard avidin-biotin-peroxidase complex method. Sections were incubated overnight in a humidified chamber with unlabeled mouse anti-GFP antibody at a 1:5,000 dilution, following microwave pretreatment for antigen retrieval. The sections were then incubated with a biotinylated secondary antibody (horse anti-mouse immunoglobulin G at a dilution of 1:200) and the avidin-peroxidase complex. The color reaction was visualized using diaminobenzidine as the chromogen. The tissue was then lightly counterstained with hematoxylin.
3.3. Results and Discussion

Heterodimerization of HIFα with ARNT occurs through the bHLH-PAS domains, which are unaffected by oxygen tension. Thus, in contrast to VHL binding, prolyl-hydroxylation of HIFα is not required for binding ARNT. We therefore sought to generate a VHL-ARNT chimera containing the minimal region of ARNT required for binding HIFα fused to the α domain of VHL known to bind elongin C, which bridges VHL to the rest of the ECV complex (Fig. 3.1A). A prediction is that VHL-ARNT would bind HIFα irrespective of oxygen to promote its degradation.

3.3.1. VHL-ARNT fusion proteins bind HIFα and form an ECV complex

The bHLH, PAS A and PAS B domains of ARNT are required for dimerization with HIF1α and HIF2α. C-terminal to the PAS B domain is the PAS C-terminal domain (PAC) that is less well described, but has been proposed to play a similar role in heterodimerization. To define the minimal region of ARNT required for strong dimerization with HIFα, we generated T7-tagged truncation plasmids encoding the following domains: HLH, PAS A, PAS B (T7-HPAS, residues 103-419); bHLH, PAS A, PAS B (T7-bHPAS, residues 90-419); HLH, PAS A, PAS B, PAC (T7-HPAC, residues 103-467); and bHLH, PAS A, PAS B, PAC (T7-bHPAC, residues 90-467) (Fig. 3.1B). An in vitro binding assay was performed with 35S-labelled in vitro translated HA-HIF1α mixed with 35S-labelled in vitro translated T7-tagged ARNT truncation mutants. The reaction mixtures were immunoprecipitated with an anti-T7 antibody, resolved by SDS-PAGE and visualized by autoradiography (Fig. 3.1C). The ARNT truncation mutants containing the PAC domain showed increased binding to HIF1α (Fig. 3.1C, compare lanes 5 and 8 with lanes 11 and 14). Based on these findings, T7-HPAC and T7-bHPAC were used to generate the VHL-ARNT chimera.

VHL-ARNT chimeras were generated by fusing the VHL α domain (residues 151-194) C-terminal to T7-HPAC and T7-bHPAC with or without a six Glycine flexible linker between the two heterologous protein fragments, giving rise to the following constructs: T7-HPACV, T7-HPACGV, T7-bHPACV, and T7-bHPACGV (Fig. 3.1D). We next tested the ability of the fusion proteins to bind HIF1α by performing an analogous in vitro binding assay (Fig. 3.1E). The
addition of VHL α domain did not diminish the ability of ARNT truncation mutants to bind HIF1α, and T7-HPACV and T7-HPACGV chimeras displayed stronger interaction with HIF1α than T7-bHPACV or T7-bHPACGV containing the basic DNA binding sequences (Fig. 3.1E, compare lanes 9 and 12 with lanes 18 and 21).
Figure 3.1. VHL-ARNT fusion protein binds HIF1α in vitro. (A) Schematic diagram of a model depicting VHL-ARNT binding HIFα independent of its prolyl-hydroxylation status and promoting HIFα polyubiquitylation via ECV. See text for details. B/C: Elongins BC; bHP: basic Helix-Loop-Helix and PAS; HRE: hypoxia-responsive element; Ub: ubiquitin. (B) Schematic diagram of the various T7-tagged ARNT truncation mutants generated for defining minimal regions required for binding HIF1α. FL ARNT: full-length ARNT; b: basic; HLH: Helix-Loop-Helix; PAS: Per-ARNT-Sim; PAC: PAS-associated C-terminal domain. (C) 35S-labelled in vitro translated HA-HIF1α was mixed with the indicated 35S-labelled in vitro translated T7-ARNT truncation mutants. Sample mixtures were immunoprecipitated with anti-T7 antibody, resolved by SDS-PAGE and visualized by autoradiography. The image shown was generated from one autoradiograph. (D) Schematic diagram of the VHLα domain fused to the indicated ARNT truncation mutants with or without the flexible Gly₆ linker. (E) 35S-labelled in vitro translated HA-HIF1α was mixed with the indicated 35S-labelled in vitro translated T7-tagged VHL-ARNT fusion proteins. Sample mixtures were immunoprecipitated with anti-T7 or anti-HA antibodies. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. The image shown was generated from one autoradiograph. IP: immunoprecipitation.
We asked whether the VHL-ARNT fusion proteins bound HIF1α in vivo. Human embryonic kidney cells (HEK293) were transiently co-transfected with the mammalian expression plasmids encoding HA-HIF1α and empty plasmid (MOCK) or T7-HPACV, T7-HPACGV, T7-bHPACV or T7-bHPACGV. Cells were treated with the proteasome inhibitor MG132 to stabilize the oxygen-labile HIF1α. Cells were then lysed, immunoprecipitated with an anti-HA antibody, bound proteins resolved by SDS-PAGE, and immunoblotted with anti-HA and anti-T7 antibodies. All VHL-ARNT chimeras co-precipitated HA-HIF1α (Fig. 3.2A). Moreover, VHL-ARNT chimeras likewise bound HA-HIF2α (data not shown). We next asked whether the VHL-ARNT chimeras formed an ECV complex. HEK293 cells were transiently transfected with an empty plasmid, T7-VHL, T7-bHPAC, T7-HPACV, T7-HPACGV, T7-bHPACV or T7-bHPACGV. Cells were then lysed, immunoprecipitated with anti-T7 antibody, bound proteins resolved by SDS-PAGE, and immunoblotted with anti-Cul2 and anti-T7 antibodies (Fig. 3.2B). T7-VHL served as a positive control showing co-precipitation of the scaffold component Cul2 (Fig. 3.2B, lane 3), while the ARNT truncation mutant T7-bHPAC lacking the VHL α domain served as a negative control showing a failure in co-precipitating Cul2 (Fig. 3.2B, lane 4). VHL-ARNT chimeras when normalized for expression exhibited Cul2 binding with efficiency comparable to that of wild-type VHL, indicating their ability to form an ECV complex.
Figure 3.2. VHL-ARNT fusion proteins bind HIF1α and form ECV complexes *in vivo*. (A) HEK293A cells transfected with the indicated plasmids were treated with the proteasome inhibitor MG132, lysed, immunoprecipitated with anti-HA antibody and visualized by immunoblotting. (B) HEK293A cells transfected with indicated plasmids were lysed, immunoprecipitated with anti-T7 antibody, resolved by SDS-PAGE, and immunoblotted with anti-Cul2 and anti-T7 antibodies. WCE: whole cell extract; IP: immunoprecipitation; IB: immunoblot; IgG-H: immunoglobulin heavy chain.
3.3.2. VHL-ARNT chimeras promote HIF$\alpha$ degradation and inhibit HRE-mediated transcription under hypoxia

We asked whether T7-HPACV or T7-HPACGV could degrade HIF1$\alpha$ under hypoxia. HEK293 cells were transiently co-transfected with plasmids encoding HA-HIF1$\alpha$ and empty plasmid or T7-VHL, T7-HPACV or T7-HPACGV. Prior to lysis, cells were treated with or without proteasome inhibitor MG132 and maintained under hypoxia (1% oxygen). Cells were lysed and equal amounts of the whole cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-HIF1$\alpha$ antibody (Fig. 3.3A). As expected, co-transfection of T7-VHL had negligible effect on HIF1$\alpha$ protein levels, evidenced by the similar amounts of HIF1$\alpha$ detected with or without MG132 (Fig. 3.3A, compare lanes 3 and 4). Notably, the endogenous VHL in HEK293 cells had likewise no discernable effect on HA-HIF1$\alpha$ expression under hypoxia (Fig. 3.3A, lane 2). In contrast, the expression of either T7-HPACV or T7-HPACGV caused dramatic attenuation of HIF1$\alpha$ levels in the absence of MG132 (Fig. 3.3A, lanes 6 and 8). These results indicate that both T7-HPACV and T7-HPACGV effectively promote HIF1$\alpha$ for proteasome-dependent degradation under hypoxia. Consistent with this notion, non-hydroxylated HIF1$\alpha$(P564A)$^{11,12}$, which has been shown to be stable in the presence of wild-type VHL under normoxia, was effectively degraded by T7-HPACGV in a proteasome-dependent manner (Fig. 3.3B).

To assess the binding of VHL-ARNT chimeras to HIF1$\alpha$ under hypoxia, an analogous experiment in HEK293 cells was performed with anti-HA and anti-T7 immunoprecipitations of the whole cell lysates. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-HIF1$\alpha$ and anti-T7 antibodies (Fig. 3.3C). As expected, T7-VHL failed to co-precipitate HA-HIF1$\alpha$ under hypoxia even in the presence of MG132 (Fig. 3.3C, lane 3). In contrast, both T7-HPACV and T7-HPACGV co-precipitated HA-HIF1$\alpha$ in the presence of MG132 (Fig. 3.3C, lanes 5 and 7). These results strongly suggest that VHL-ARNT chimeras bind HIF1$\alpha$ under hypoxia to promote its proteasome-dependent degradation. Aryl Hydrocarbon Receptor (AhR) is another well-characterized ARNT binding partner ubiquitously expressed and involved in endo- and xeno-biotic metabolism$^{313}$. AhR as expected bound ARNT, but failed to bind HPACGV in an in vitro binding assay (Fig. 3.4A and B). In accord, HPACGV failed to downregulate the expression of AhR in vitro or in vivo (Fig. 3.4C and D). Thus, HPACGV, which was generated
and optimized for binding and degrading HIFα, does not interact with arguably the next best-characterized ARNT-binding partner AhR.
Figure 3.3. HPACV and HPACGV bind and degrade HIF1α independent of its prolyl-hydroxylation status. (A)/(B)/(C) HEK293 cells transfected with the indicated plasmids were treated with (+) or without (-) MG132 for 4 hours. (A) Cells were maintained at 1% oxygen, lysed and whole cell extracts resolved by SDS-PAGE were immunoblotted with the indicated antibodies. (B) Equal amounts of protein, as indicated by anti-Vinculin immunoblot, were immunoprecipitated with anti-HA or anti-T7 antibodies. Bound proteins were resolved by SDS-PAGE and immunoblotted with indicated antibodies. (C) Cells were treated as in (A), immunoprecipitated with anti-HA or anti-T7 antibodies and bound proteins were immunoblotted with anti-HIF1α or anti-T7 antibodies. IP: immunoprecipitation, IB: immunoblot.
The HIF transcription factor, which is a heterodimer of HIFα and ARNT, engages HREs in the promoters of a myriad of hypoxia-inducible genes to initiate their transcription. We sought to determine the effect of T7-HPACV and T7-HPACGV on HRE-driven transcription by performing a dual-luciferase assay using a firefly luciferase reporter driven by five contiguous HRE elements from the phosphoglycerate kinase-1 promoter (Fig. 3.5). HEK293 cells were transiently co-transfected with plasmids encoding (HRE)$_5$-Luc and T7-VHL, T7-HPACCV or T7-HPACGV. Cells were maintained at either normoxia (21% oxygen) or hypoxia (1% oxygen) for 16 hours prior to lysis. The transactivation activity from the HRE promoter was markedly higher under hypoxia than normoxia, as expected. Also, the ectopic expression of T7-VHL did not influence HRE-driven transactivation under hypoxia, since VHL is ineffective in targeting HIFα for destruction under hypoxia. In contrast, T7-HPACV and T7-HPACGV significantly reduced the transactivation from the HRE promoter under hypoxia (Fig. 3.5), indicating a marked loss of endogenous HIF function. Notably, T7-HPACGV was reproducibly more potent in attenuating HIF-mediated transcription than T7-HPACV (Fig. 3.5), and thus the T7-HPACGV chimera was selected for subsequent experimentation. Furthermore, HPAC in the absence of VHL diminished HIF-driven transcription under hypoxia via forming an inactive transcriptional complex, while HPACGV in comparison dramatically attenuated HIF-driven transcription (Fig. 3.6), suggesting that the E3 enzymatic activity to rapidly degrade HIFα upon binding underlies the potency of HPACGV.

Figure 3.4. HPACGV does not bind or degrade AhR. (A) $^{35}$S-labeled in vitro translated HPACGV was mixed with $^{35}$S-labeled in vitro translated AhR. Sample mixtures were immunoprecipitated with anti-AhR antibody, resolved by SDS-PAGE and visualized by autoradiography. (B) $^{35}$S-labeled in vitro translated ARNT was mixed with cold in vitro translated AhR. Sample mixtures were immunoprecipitated with anti-AhR antibody. (C) HEK293 cells transfected with empty plasmid (MOCK) or plasmid encoding HPACGV were lysed and equal amounts of whole cell lysates were mixed with $^{35}$S-labeled in vitro translated AhR. Sample mixtures were resolved by SDS-PAGE and visualized by autoradiography. (D) HEK293 cells transiently transfected with the indicated plasmids were lysed and equal amounts of protein, as indicated by anti-Vinculin immunoblot, were immunoprecipitated with anti-AhR or anti-T7 antibodies. Bound proteins were resolved by SDS-PAGE and immunoblotted with indicated antibodies. AR: autoradiography, IP: immunoprecipitation, IB: immunoblot.
Figure 3.5. HPACV and HPACGV decrease HRE-mediated transcription under hypoxia. Dual-luciferase assay was performed in HEK293 cells transfected with (HRE)$_5$-Luc in combination with indicated plasmids. SV40-driven renilla luciferase was transfected as internal control. Cells were maintained at 21% or 1% oxygen for 16 hours prior to lysis and dual-luciferase assay performed. Experiments and transfections were performed in triplicates with one representative experiment shown. Error bars represent standard deviations. RLU: relative light units.
Figure 3.6. HPACGV is more potent than HPAC at inhibiting HIF-mediated transcriptional activity. (A) Dual-luciferase assay was performed in HEK293 cells transiently transfected with (HRE)$_5$-Luc in combination with plasmids encoding T7-HPAC or T7-HPACGV. SV40-driven renilla luciferase was transfected as an internal control. Cells were maintained at 1% oxygen for 16 hours prior to analysis. Experiments and transfections were performed in triplicates with one representative experiment shown. Error bars represent standard deviations. (B) Expression levels of transfected T7-HPAC and T7-HPACGV in (A) were determined via Western blot analysis. Asterisk represents proteins non-specifically recognized by T7 antibody. RLU: relative light units, IB: immunoblot.
3.3.3. Adenoviral delivery of T7-HPACGV attenuates HIFα and HIF-target gene expression independent of oxygen tension

A cardinal feature of CCRCC is the overexpression of hypoxia-inducible genes. This is principally due to the inactivation of VHL that is observed in the vast majority of CCRCC. Interestingly, CCRCC harbouring wild-type VHL also displays strong hypoxic signatures and several lines of evidence suggest the stabilization of HIF2α to be a critical oncogenic event in the pathogenesis of CCRCC\textsuperscript{223,225}. We generated recombinant adenoviruses expressing enhanced green fluorescence protein (EGFP) alone or in combination with T7-VHL (Ad-EGFP-T7-VHL) or T7-HPACGV (Ad-EGFP-T7-HPACGV) and tested their ability to form an ECV complex in the CCRCC cell line 786-O (VHL\textsuperscript{−/−}; HIF1α\textsuperscript{−/−}), a widely used cell system for CCRCC with constitutive activation of HIF2α. Upon high-efficiency infection of 786-O cells with the indicated adenoviruses (>95%, as determined by EGFP fluorescence; data not shown), cells were lysed and immunoprecipitated with anti-T7 antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-T7, anti-Cul2 and anti-Elongin B antibodies (Fig. 3.7A). Both Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, co-precipitated Cul2 and Elongin B to comparable levels, suggesting equal ability of ECV complex formation (Fig. 3.7A).

We next determined the effect of Ad-EGFP-T7-HPACGV on HIF2α protein levels under normoxia and hypoxia. 786-O cells were uninfected (MOCK) or infected with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV and maintained at 21% or 1% oxygen for 48 hours. Cells were then lysed, resolved by SDS-PAGE and immunoblotted with anti-HIF2α and anti-T7 antibodies (Fig. 3.7B). Cells infected with Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, showed near-complete loss of HIF2α expression under normoxia (Fig. 3.7B, left panel). Importantly, while HIF2α protein level was unaffected by Ad-EGFP-T7-VHL or Ad-EGFP infection, Ad-EGFP-T7-HPACGV dramatically attenuated the expression of HIF2α under hypoxia (Fig. 3.7B, right panel). These results demonstrate that, unlike Ad-EGFP-T7-VHL, which is only capable of degrading HIF2α under normoxia, Ad-EGFP-T7-HPACGV degrades HIF2α irrespective of oxygen tension.

We next generated 786-O cells stably expressing a firefly luciferase reporter gene driven by five HRE elements from the VEGF promoter (786-HRE-Luc). Infection of 786-HRE-Luc cells with
Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, significantly decreased HIF-dependent HRE-driven luciferase activity under normoxia (Fig. 3.7C). Under hypoxia, only Ad-EGFP-T7-HPACGV infected cells showed marked loss of luciferase activity (Fig. 3.7C). Intriguingly, Ad-EGFP-T7-HPACGV had noticeably greater effect on the attenuation of HIF transactivation activity than Ad-EGFP-T7-VHL, even under normoxia. This is likely a reflection of the restricted binding of VHL to prolyl-hydroxylated HIFα, which is critically dependent on the Fe^{2+}, 2-oxoglutarate and oxygen-dependent activity of PHD enzymes, versus the unrestricted capacity of VHL-ARNT fusion protein to bind any and all unmodified or modified HIFα.

We next assessed the endogenous HIF transcriptional activity in 786-O cells infected with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV. Consistent with the aforementioned biochemical analyses, Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, significantly decreased the protein level of GLUT1 under normoxia (Fig. 3.7D, left panel). Under hypoxia, only Ad-EGFP-T7-HPACGV resulted in the marked reduction in GLUT1 (Fig. 3.7D, right panel). Moreover, HIF-target gene transcripts such as GLUT1, VEGF and BNIP3L were reduced upon Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV infection under normoxia (Fig. 3.7E). In contrast, only Ad-EGFP-T7-HPACGV was effective at decreasing GLUT1, VEGF and BNIP3L mRNA levels under hypoxia (Fig. 3.7E).
E

Fold change - GLUT1

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3.3.4. Treatment of CCRCC xenografts with Adeno-EGFP-T7-HPACGV results in decreased angiogenesis and significant tumour necrosis

Optical coherence tomography (OCT) is a non-invasive near infrared imaging technique that provides depth-resolved microstructural information in biological tissue at near histology resolution (1 to 10 μm)\(^{310}\). Depending on tissue optical properties, OCT imaging depth ranges from 2 mm in highly scattering samples such as solid tumors to > 20 mm in the eye. Recent advances in spectral domain OCT made 3-dimensional \textit{in vivo} imaging feasible. We developed a method of mapping normal microvasculature with high sensitivity and tracking photodynamic therapy-induced microvascular changes within the window chamber using an interframe speckle variance technique\(^{311}\). We apply, for the first time, the speckle variance technique to monitor tumour angiogenesis in response to the various recombinant adenoviral treatments within the skin-fold window chamber.

On day 2, 3 and 8 post-implantation of 786-O cells stably expressing red fluorescent protein DsRed2 (786-dsRed) in the dorsal skin-fold window chambers on SCID mice (Fig. 3.8), intratumoural injections of Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV were administered at 2x10\(^8\) ifu. The adenovirus infections were monitored by the area and intensity of EGFP expression as measured by green fluorescence imaging, and tumour xenografts were visualized using red fluorescence microscopy, while angiogenesis was assessed using white-
light microscopy and speckle variance-OCT (Fig. 3.9). Adenoviruses infected the tumours with high efficiency and importantly, there was a complete lack of EGFP signal in the normal mouse tissues surrounding the xenografts. This allowed the observed effects to be directly attributed to specific adenoviral infection of the tumour (Fig. 3.9, second rows). Ad-EGFP treatment had negligible effect on the growth of tumours, which showed extensive angiogenesis within the tumour mass (Fig. 3.9A). Ad-EGFP-T7-VHL treatment had a modest negative effect on tumour growth with some angiogenesis occurring within the tumour as compared to the Ad-EGFP treated group (Fig. 3.9B). In contrast, Ad-EGFP-T7-HPACGV treatment dramatically inhibited tumour angiogenesis and showed significant tumour regression, especially in the central core region by day 8 post-implantation and most noticeably by day 10, the final day of the assay (Fig. 3.9C).

**Figure 3.8. Dorsal skin-fold window chamber setup.** The titanium window chamber was surgically implanted into the dorsal skin of SCID mice. 786-dsRed cells were injected into the dorsal dermis. Visualization of the tumour and associated vasculature was facilitated by a circular glass coverslip positioned over the incision.
To further examine the regression in Ad-EGFP-T7-HPACGV-infected tumours, we performed analogous experiments in which the tumours were resected 7 days post-implantation, corresponding to 4 days post-first adenoviral treatment, for immunohistochemical analysis (Fig. 3.10). Immediately prior to sacrificing the mice, fluorescent, white-light and svOCT images were collected for analysis. Consistently, Ad-EGFP-infected tumours were highly angiogenic, while Ad-EGFP-T7-HPACGV-infected tumours exhibited markedly lower levels of neovascularization in the tumour core (Fig. 3.10A). Green fluorescence microscopy and anti-GFP immunohistochemical analysis of the resected tumours revealed positive GFP expression throughout Ad-EGFP-infected specimens (Fig. 3.10A). However, while green fluorescence microscopy showed similar GFP expression in the Ad-EGFP-T7-HPACGV-infected tumours, anti-GFP immunohistochemical staining from numerous Z-stacked sections of the tumour revealed a striking absence of GFP staining in the tumour core (Fig. 3.10A). Consistent with this observation, hematoxylin and eosin (H&E) staining showed viable tumour cells throughout the Ad-EGFP-treated tumour mass, while Ad-EGFP-T7-HPACGV-treated tumours displayed an interface of viable to necrotic tumour cells in which the tumour periphery contained mostly viable cells with admixed early necrotic changes at the viable/necrotic interface to a largely necrotic tumour core with infiltrating inflammatory cells (Fig. 3.10A and B). These results show that adenovirus-mediated expression of VHL-ARNT fusion protein dramatically attenuates tumour angiogenesis and growth in a mouse xenograft model.
A

786-dsRed

Ad-EGFP

Ad-EGFP-T7-HPACGV

White Light

svOCT

Anti-GFP

H&E
Adenoviral delivery of T7-HPACGV causes tumour cell death by necrosis. (A) Analogue experiments were performed as in Fig. 3.9 using Ad-EGFP (left panel) and Ad-EGFP-T7-HPACGV (right panel). Images were taken from day 7 post-implantation, corresponding to 4 days post-first adenoviral treatment. Tumours were visualized by red fluorescence microscopy and positivity of adenoviral infection was monitored by green fluorescence microscopy. Tumour angiogenesis was visualized by white-light microscopy and speckle variance-optical coherence tomography (svOCT). Tumours were then resected and H&E and anti-GFP immunohistochemistry were performed. Dashed line: viable/necrotic interface; V: viable cells; N: necrotic cells; N+I: necrotic and inflammatory cells. (B) Higher magnifications of the H&E images from (A).
In summary, we have re-engineered the ECV E3 ligase containing the VHL-ARNT chimeric F-box component to remove the oxygen constraint in the recruitment of HIFα, promoting its rapid degradation under any oxygen tension. The expression of the VHL-ARNT fusion protein resulted in the degradation of both HIF1α and HIF2α under normoxia and hypoxia. This led to decreased HIF transcriptional activity and reduced expression of HIF-target genes. Ultimately, the HPACGV VHL-ARNT chimera was able to suppress the growth of CCRCC xenografts by inhibiting tumour angiogenesis and causing tumour necrosis. While we have presented evidence of the feasibility in promoting human CCRCC xenograft regression and cell death using the bioengineered VHL-ARNT chimera in an animal model system, it remains an outstanding question whether such a treatment would lead to the growth suppression of other tumour types exhibiting strong hypoxic signatures with overexpression of HIFα.
Chapter 4
VHL and SOCS1 cooperate to degrade JAK2: implications for polycythemia

This chapter has not yet been published:
Russell, RC*, Sufan, RI*, Roche, O, Richmond TD, Barber, DL, Irwin, MS, and Ohh, M. VHL and SOCS1 cooperate to degrade JAK2: implications for polycythemia.
* Both authors contributed equally to this work.

4.1. Introduction

The 598C→T (R200W) homozygous VHL mutation was recently shown to cause a congenital autosomal recessive form of polycythemia, Chuvash Polycythemia (CP), endemic to the Chuvash Autonomous Republic of the Russian Federation\textsuperscript{13}. Since the discovery of the R200W mutation, R200W and other VHL mutations leading to CP, such as H191D, have been identified in patients of various ethnicities\textsuperscript{2,32}. Chuvash polycythemia is unique in that it has features of both primary and secondary polycythemia, as CP erythroid progenitors are hypersensitive to EPO stimulation, and serum EPO levels are high in most CP patients\textsuperscript{13} (Table 1). Primary polycythemia, the most common form of which is polycythemia vera (PV), is defined as excessive erythrocytosis arising from an intrinsic defect in erythroid progenitors rendering them hypersensitive to or independent of EPO stimulation\textsuperscript{33}. Secondary polycythemia is defined as excessive erythrocytosis arising from increased production of EPO\textsuperscript{33}. For example, perturbation of the oxygen-sensing pathway due to mutations in PHD2 and HIF2α has been identified in individuals with congenital secondary polycythemia\textsuperscript{314-317}. Polycythemia can also develop secondary to increased EPO production by some renal tumors or in mice with constitutive expression of HIF2α\textsuperscript{186,187}.

4.1.1. Erythropoiesis and EPOR signalling

Erythropoiesis is defined as the process by which primitive committed erythroid progenitor cells proliferate and differentiate into mature, non-nucleated red cells\textsuperscript{318}. Erythropoietin, EPO, a 34
kDa glycoprotein hormone produced by peritubular interstitial cells in the cortex/outer medulla of the kidney, is the crucial regulator of red-blood cell production\textsuperscript{318,319}. EPO signals through the erythropoietin receptor (EPOR), which is first expressed on the earliest committed erythroid progenitor cell, the burst-forming unit erythroid (BFU-E)\textsuperscript{320-322}. For BFU-Es, which are largely dormant, but have a high proliferative capacity, EPO acts both as a mitogen and survival factor\textsuperscript{322}. The lineal descendant of BFU-E is the colony-forming unit-erythroid (CFU-E), which has limited proliferative potential and is incapable of self-renewal\textsuperscript{320-322}. In contrast to BFU-Es, CFU-Es have an absolute requirement for EPO, which acts primarily as a survival factor in these cells. Indeed, in mice unable to express EPO or EPOR due to engineered null mutations in either gene, erythropoiesis is arrested at the CFU-E stage\textsuperscript{323}. This indicates that although EPO-mediated EPOR signalling is not required for erythroid lineage commitment, it is essential for the proliferation and survival of CFU-E progenitors and their irreversible terminal differentiation. CFU-E cells give rise to proerythroblasts, which are the first morphologically recognizable erythroid cells in the bone marrow\textsuperscript{322}. As proerythroblasts mature through various erythroblast stages into the reticulocyte, EPO requirement of these cells diminishes\textsuperscript{324}.

Originally described in 1989, the homodimeric EPOR is a member of the cytokine receptor superfamily\textsuperscript{325}. It contains a ligand-binding extracellular domain that engages to EPO, a single transmembrane domain and an intracellular domain associated with Janus kinase 2 (JAK2), which provides the kinase activity for the receptor\textsuperscript{325,326} (Fig. 4.1). EPOR exists in an unliganded, dimeric state, pre-associated with two JAK2 molecules, one per receptor monomer\textsuperscript{327}. Upon EPO ligand binding to the receptor, EPOR undergoes a conformational change that brings the JAK2 proteins into close proximity, leading to JAK2 activation by autophosphorylation\textsuperscript{327} (Fig. 4.1). Active JAK2 phosphorylates EPOR on several cytoplasmic tyrosine residues, which act as docking sites for Src-homology 2 (SH2) domain-containing proteins, allowing downstream signal transduction\textsuperscript{318}. Signals emanating from the EPOR converge to induce proliferation and inhibit apoptosis of erythroid progenitors\textsuperscript{318}. The critical role of JAK2 in erythropoiesis has been established by JAK2 knockout mice, which, similarly to EPO-/- and EPOR-/- mice, are severely anemic and die around E10-12 due to an absence of definitive erythropoiesis\textsuperscript{323,328,329}. A critical transcription factor that associates with the phosphorylated EPOR is signal transducer and activator of transcription 5a/b (STAT5a/b, commonly referred to as STAT5). Upon EPOR binding, STAT5 is phosphorylated, which allows its dimerization, nuclear translocation and
transcriptional activation of genes implicated in proliferation and survival\textsuperscript{318} (Fig. 4.1). \textit{STAT5}\textsubscript{a}\textsuperscript{−/−} \textit{5b}\textsuperscript{−/−} embryos are severely anaemic and display increased erythroid progenitor apoptosis, which may be explained by STAT5-mediated transactivation of the antiapoptotic Bcl-x\textsubscript{L} in erythroid progenitors\textsuperscript{330,331}. In agreement, conditional knockout of Bcl-x\textsubscript{L} in the haematopoietic system results in severe anaemia, while overexpression of constitutively active STAT5 or Bcl-x\textsubscript{L} in erythroid progenitors drives erythropoiesis independently of EPO stimulation\textsuperscript{332-334}.

Upon EPO stimulation, STAT5 is also known to initiate a negative feedback loop that effectively inhibits EPOR signalling. This involves members of the suppressor of cytokine signalling (SOCS) family SOCS1 (also known as JAB, SSI), SOCS3 (also known as CIS3) and cytokine-inducible SH2-containing protein (CIS)\textsuperscript{335-337}. The SOCS family consists of eight members, CIS and SOCS1-7, which are induced in response to a broad range of cytokines and act to repress cytokine signalling\textsuperscript{337,338}. All SOCS family members contain a variable N-terminal region, an SH2 domain, and a conserved C-terminal SOCS box\textsuperscript{338}. Specifically, CIS, SOCS1 and SOCS3 are induced following EPO stimulation and both CIS and SOCS3 have been shown to directly bind to the EPOR and inhibit signal transduction, possibly by competing with STAT5 binding to the EPOR\textsuperscript{335,337,339-341}. In addition, SOCS3 and SOCS1 can also engage phosphorylated JAK2 (pJAK2) and inhibit its kinase activity\textsuperscript{341,342}. Similarly to VHL, SOCS1 forms an E3 ubiquitin ligase termed ECS, with Elongins BC and Cul2/5\textsuperscript{343-345} (Fig. 4.1). The SOCS box of SOCS1 acts similarly to the VHL \textalpha domain, in that it nucleates the ECS by engaging with Elongins BC\textsuperscript{343-345}. Interestingly, SOCS1 has been shown to specifically target pJAK2 for ubiquitin-mediated degradation\textsuperscript{346} (Fig. 4.1). In addition, BFU-E and CFU-E cells from the fetal livers of \textit{SOCS1}\textsubscript{−/−} mice were shown to be hyper-responsive to EPO, thus implicating ubiquitination of EPOR-associated pJAK2 as a viable mechanism for signal attenuation\textsuperscript{347}. 
Figure 4.1. JAK2-mediated EPOR signalling and negative feedback loop. Upon EPO stimulation, JAK2 is activated and phosphorylates the EPOR cytoplasmic tail and STAT5 transcription factors. STAT5 phosphorylation allows dimerization, nuclear translocation and transcription of STAT5-responsive genes promoting survival and proliferation of erythroid progenitors. As part of the negative feedback loop, SOCS1 forms an E3 ubiquitin ligase with Elongin BC, Cul2/5 and Rbx1 which targets phosphorylated JAK2 for ubiquitin-mediated degradation, thus terminating EPO-mediated signal transduction. Ub: ubiquitin; EloBC: Elongin BC; P: phosphoryl group.
4.1.2. Polycythemia vera and the JAK2(V617F) mutation

Recently, gain-of-function mutations in JAK2, predominated by JAK2(V617F), have been identified in the vast majority of polycythemia vera (PV) patients. PV, the most common form of primary polycythemia, is characterized by erythroid progenitors that are hypersensitive to, or independent of EPO for survival. As such, EPO-independent erythroid colonies are found in the majority of PV patients. The significance of the JAK2(V617F) mutation in PV has been demonstrated by its ability to induce a PV phenotype in mouse bone marrow transplantation assays and to promote cytokine-independent signalling in cytokine-dependent cell lines.

The V617F mutation is located in the JH2 pseudo-kinase domain of JAK2, which is known to be involved in the auto-inhibition of JAK2 tyrosine kinase activity, most likely through blocking the activation loop of the JH1 kinase domain. JAK2(V617F) thus leads to constitutive phosphorylation of JH1 Y1007, which is required for JAK2 activation. Consequently, the JAK2(V617F) mutant has been shown to activate STAT5-mediated transcription in the absence of EPO, and high STAT5 phosphorylation has also been detected in bone marrow biopsies of PV patients. The antiapoptotic STAT5-target gene Bcl-xL was also shown to be constitutively expressed in PV erythroid progenitors, leading to EPO-independent erythroid survival, thus contributing to the substantial erythrocytosis observed in this disease. These lines of evidence suggest that constitutive activation of JAK2-STAT5 signalling is a major causative determinant of PV pathogenesis, and that JAK2(V617F)-negative patients might harbor mutations in other genes perhaps encoding proteins yet-to-be-identified in the JAK2-STAT5 pathway.

4.1.3. Current model for Chuvash polycythemia and rationale

Chuvash polycythemia patients frequently present with high serum EPO levels and invariably contain BFU-E erythroid progenitors which are hypersensitive to EPO. Recently, Ang et al. have proposed a defect in the ability of CP-VHL to maintain proper oxygen homeostasis, due to dysregulation of the HIF pathway, as the principal mechanism of CP pathogenesis. CP-VHL(R200W) was shown to have a decreased capacity for binding HIFα, resulting in mild HIFα stabilization and elevated levels of EPO. Similar results were obtained by Hickey et al., who showed mild HIF2α stabilization under normoxia in the R200W/R200W mouse model, leading to increased EPO production. However, while these findings explain the secondary
polycythemic features of CP, HIF has not been associated with hypersensitivity of erythroid progenitors to EPO and thus, the molecular mechanism underlying the primary polycythemic features of CP remains unknown. In addition, it is currently unclear why polycythemia is rarely observed in VHL disease patients, even though mutations promoting HIFα stabilization are common in this population. Based on the fact that constitutively active PV JAK2(V617F) can lead to EPO hypersensitivity of erythroid progenitors, we investigated a possible link between VHL and JAK2 for CP, proposing that CP-VHL causes erythrocytosis by augmenting JAK2 signalling.

4.2. Materials and Methods

Unless otherwise indicated, all experiments were performed in triplicate.

4.2.1. Cells

786-O CCRCC and HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Milwaukee, WI, USA) at 37°C in a humidified 5% CO₂ atmosphere. 786-O subclones ectopically expressing HA-VHL(WT), HA-VHL(C162F) or empty plasmid were previously described. 786-O subclones ectopically expressing HA-VHL(R200W) and HA-VHL(H191D) were generated as previously described. Ba/F3 pro-B cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% FBS and 0.5 U/ml recombinant human EPO (Janssen Ortho, Toronto, ON, Canada).

4.2.2. Antibodies

Rabbit antibodies against JAK2, VHL, pJAK2, and pSTAT5 were obtained from Cell Signalling Technologies (Danvers, MA, USA). Polyclonal antibodies against ubiquitin, Elongin B and HIF2α antibodies were obtained from DAKO Canada (Mississauga, ON, Canada), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Monoclonal antibodies against HA (12CA5), T7 and VHL(IG32) were obtained from Boehringer Ingelheim (Laval, QC, Canada), Novagen (Madison, WI, USA) and BD
Biosciences (Mississauga, ON, Canada), respectively. Monoclonal anti-α-tubulin antibody was obtained from Abcam (Cambridge, MA, USA). Polyclonal anti-Cul2 and anti-SOCS1 antibodies were obtained from Invitrogen (Burlington, ON, Canada) and Novus Biologicals (Littleton, CO, USA), respectively. MG132 proteasome inhibitor was obtained from Boston Biochem (Cambridge, MA, USA).

4.2.3. Plasmids
Plasmid encoding HA-SOCS1 was generously provided by Dr. Robert Rottapel (Ontario Cancer Institute, Toronto, ON, Canada). T7-VHL and HA-VHL(WT, R64P, V74G, Y98H, S111H, Y112H, Y112N, F119S, L128F, L158S, K159E, C162F, L188V) were previously described47,278,280,362. HA-VHL(R200W, H191D) were generated using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and mutations verified by direct DNA sequencing.

4.2.4. Immunoprecipitation and immunoblotting
Immunoprecipitation and immunoblotting were performed as described in chapter 2.2.4.

4.2.5. Metabolic labeling
Metabolic labeling was performed as described previously276. In brief, 786-O cells were maintained in methionine-free Dulbecco's modified Eagle's medium for 45 min then supplemented with \(^{35}\)S-methionine (100 µCi/ml of medium; Amersham Biosciences, Buckinghamshire, United Kingdom) and 2% dialyzed fetal bovine serum for 3 h at 37 °C in a humidified 5% CO₂ atmosphere.

4.2.6. In vitro ubiquitination assay
T7-JAK2 and T7-pJAK2 were purified on Protein-A agarose beads (Waltham, MA, USA) with anti-T7 antibody from HEK293 cells transfected with T7-JAK2 and EPOR stimulated with or without EPO. Ubiquitination reaction was then performed as described previously on JAK2 or pJAK2 bound on beads. Briefly, JAK2/pJAK2 bound on beads were incubated in the presence of S100 extracts (100–200 µg) supplemented with 8 µg/µl ubiquitin (Sigma-Aldrich, Oakville, Canada), 100 ng/µl ubiquitin aldehyde (BostonBiochem, Cambridge, Massachusetts) and
energy-regenerating system (20 mM Tris pH 7.4, 2 mM ATP, 5 mM MgCl₂, 40 mM creatine phosphate and 0.5 µg/µl creatine kinase) in a reaction volume of 20–30 µl for 1–2 h at 30 °C. S100 extracts from 786-O VHL(-/-) subclones stably transfected with empty vector or VHL mammalian expression plasmid were prepared as previously described. In brief, cells were resuspended and incubated in ice-cold hypotonic buffer (20 mM Tris pH 7.4, 5 mM MgCl₂, 8 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethyl sulphonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 0.1 mM PABA and 10 µg/ml aprotinin) for 15 min. Cells were subjected to three freeze/thaw cycles and pelleted by centrifugation at 14,000g for 5 min. The resulting supernatant was ultracentrifuged at 100,000g for 4 h. The supernatant (S100 fraction) was aliquoted and stored at −80 °C.

4.2.7. Generation of phenylhydrazine-primed splenic erythroblasts
Mice were injected intraperitoneally with 50 mg/kg phenylhydrazine hydrochloride (Sigma-Aldrich, Oakville, ON, Canada) in PBS on days 1 and 2, as previously described. Mice were sacrificed and spleens removed on day 4 under sterile conditions. Single-cell suspensions for further analysis were generated using a 70-µm cell strainer.

4.2.8. Cytokine deprivation and stimulation of murine splenic erythroblasts
Cells were washed twice in PBS, starved in α-MEM supplemented with 2% FCS for 4 hrs at 37°C, and then stimulated with various concentrations of EPO for 15 min at 37°C. Cells were pelleted at 6000 rpm for 1 min and lysed in 1% Triton X-100 lysis buffer supplemented with phosphatase inhibitors (Roche, Laval, Canada) supplemented with 20mM Na₃P₂O₇, 10mM NaF and 1mM Na₃VO₄.

4.3. Results and Discussion

4.3.1. CP-VHL mutants have reduced capacity to form an ECV
CP-VHL(R200W and H191D) mutants showed diminished association with Elongins BC and Cul2, the core components of ECV, when expressed in human embryonic kidney epithelial cells HEK293 or CCRCC 786-O(VHL-/-) cells (Fig. 4.2A and B). Tumor-associated VHL(C162F)
mutant, which is known to be defective in forming an ECV complex, served as control. Thus, in addition to the previously reported defect in HIFα binding, CP-VHL mutants are compromised in ECV assembly, which is also likely to contribute to HIFα stabilization.

Furthermore, all tumour-associated VHL mutants tested-to-date have invariably shown a failure in binding to fibronectin (FN) and formation of FN fibrillar array in the extracellular space. In contrast, CP-VHL mutants, but as expected not VHL(C162F), showed intact interaction with FN and robust extracellular FN matrix deposition (Fig. 4.2C and data not shown). Thus, VHL(R200W) and VHL(H191D) are the first naturally occurring VHL mutants exhibiting proper FN matrix deposition, which is consistent with the absence of cancer predisposition in individuals with CP.
Figure 4.2. CP-VHL exhibit decreased binding to ECV components but retain fibronectin matrix deposition. (A) HEK293 cells transfected with the indicated plasmids were lysed, immunoprecipitated with anti-HA antibody and immunoblotted with 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) 786-O subclones stably expressing the indicated HA-VHL were grown on glass coverslips and immunostained for fibronectin (red) and visualized by fluorescent microscopy. DAPI (blue) staining indicates nuclei. IP: immunoprecipitation; IB: immunoblot; AR: autoradiography.
4.3.2. VHL binds JAK2 in a proteasome-sensitive manner

In addition to reduced Cul2 binding, $^{35}$S-metabolic labeling of 786-O cells stably expressing VHL(R200W or H191D) revealed an associated protein of 120 kDa in the absence of proteasome inhibitor (Fig. 4.3A). JAK2 is approximately 120 kDa and aberrant JAK2-STAT5 signalling has been reported to cause hypersensitivity to EPO in BFU-E cells of PV patients. Similarly, BFU-E cells of CP patients are also hypersensitive to EPO and thus, we asked whether VHL interacts with JAK2. HEK293 cells transfected with plasmids encoding HA-VHL(WT) and T7-JAK2 were treated with or without proteasome inhibitor MG132 and immunoprecipitated with anti-VHL antibody. HA-VHL(WT) co-precipitated JAK2 preferentially in the presence of MG132 (Fig. 4.3B). HA-VHL(R200W) and HA-VHL(H191D) showed increased association with JAK2 in comparison to VHL(WT) in the absence of MG132 (Fig. 4.3C). These results identify JAK2 as a novel substrate of VHL and suggest that CP-VHL mutants have a diminished capacity to promote proteasome-dependent degradation of JAK2.
4.3.3. VHL promotes ubiquitin-mediated degradation of pJAK2

The level of total JAK2 remained unaffected by ectopic expression of VHL (Fig. 4.3B and C, bottom panels), which suggests that VHL promotes diminution of a select population of JAK2 upon engagement. We asked whether VHL promoted degradation of activated JAK2, which is defined by Y1007 phosphorylation. Introduction of HA-VHL(WT) in HEK293 cells resulted in a dramatic loss of phosphorylated JAK2 (pJAK2) (Fig. 4.4A). We next asked whether the loss of pJAK2 was due to VHL-mediated ubiquitination of pJAK2. HEK293 cells were co-transfected with plasmids encoding T7-JAK2 and EPOR and stimulated with EPO (20U/ml) for 15 min to generate robust levels of pJAK2 (Fig. 4.4B, left panel), which was subsequently isolated via anti-T7 immunoprecipitation. The enriched T7-pJAK2 was then subjected to an in vitro ubiquitination reaction using S100 extracts devoid of or reconstituted with VHL(WT) (Fig. 4.4B, right panel). While the total JAK2 levels were unaffected, the level of pJAK2 decreased dramatically in the presence of VHL(WT), which was accompanied by the appearance of pJAK2 polyubiquitination (Fig. 4.4B, lane 4). Notably, the low level of VHL-dependent ubiquitination observed in the absence of EPO is likely due to limited spontaneous JAK2 autophosphorylation, commonly observed upon ectopic JAK2 expression (Fig. 4.4B, right panel, lane 3). These results demonstrate that VHL promotes pJAK2 ubiquitination.

We next investigated the effect of CP-VHL mutants on pJAK2 stability and observed that while VHL(WT) co-precipitated negligible levels of pJAK2 in the absence of MG132, both CP-VHL mutants R200W and H191D co-precipitated higher levels of pJAK2, supporting the notion that CP-VHL mutants have a diminished capacity to promote pJAK2 degradation (Fig. 4.4C).

VHL patients rarely develop polycythemia despite harboring VHL mutations that abolish HIFα degradation. Thus, tumour-causing VHL mutants incapable of binding or ubiquitinating HIFα
are predicted to retain the ability to promote ubiquitin-mediated destruction of pJAK2. A panel of VHL substitution mutants spanning the open reading frame were tested for their ability to degrade pJAK2. Consistent with our prediction, expression of VHL mutants, with the exception of F119S and L128F (discussed below), resulted in negligible levels of pJAK2 in the absence of MG132 (Fig. 4.4D). In addition, a panel of tumour-causing VHL mutants retained binding to JAK2 in the presence of MG132 (Fig. 4.4E). Notably, well established α domain VHL mutants C162F and L158S, which cannot form an ECV\textsuperscript{47,363}, decreased pJAK2 levels comparable to that of VHL(WT). These results infer that a novel, ECV-independent, mechanism is responsible for VHL-mediated pJAK2 degradation.
4.3.4. VHL binds and requires SOCS1 to promote pJAK2 degradation

The F-box protein SOCS1 is the principal negative regulator of pJAK2 via ubiquitin-mediated degradation. VHL, as well as other F-box proteins that confer substrate specificity, have been shown to homodimerize\(^{364-368}\). Moreover, homodimerization of entire E3 enzymes such as the SCF (Skp1/Cdc53/F-box protein) has been shown to increase the efficiency of ubiquitination by improving spatial orientation of substrate to active site\(^{368}\). We asked whether SOCS1 interacts with VHL to promote ECV-independent degradation of pJAK2. T7-VHL co-precipitated HA-SOCS1 when ectopically expressed in HEK293 cells (Fig. 4.5A, left panel), and similar results were obtained by reciprocal immunoprecipitation (Fig. 4.5A, right panel). We then asked whether VHL/SOCS1 interaction occurred under physiologic conditions. BaF3 cells stably expressing EPOR were treated with or without MG132. Cell lysates were immunoprecipitated with anti-VHL or isotype-matched control antibody and bound proteins were visualized by Western blot analysis, which showed endogenous VHL co-precipitating SOCS1 in the presence of MG132 (Fig. 4.5B). Notably, VHL/SOCS1 interaction was significantly reduced in the absence of MG132, suggesting perhaps that the complex is sensitive to proteasomal degradation.
Figure 4.5. VHL engages SOCS1. (A) HEK293 cells transfected with the indicated plasmids were lysed, immunoprecipitated with either anti-T7 (left panels) or anti-HA (right panels) antibody, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. (B) BaF3 cells stably expressing EPOR were stimulated with EPO in the presence (+) or absence (-) of MG132. Cells were lysed and immunoprecipitated with anti-VHL or isotype-matched control antibody and immunoblotted with the indicated antibodies. IP: immunoprecipitation; IB: immunoblot; WCE: whole cell extract. Asterisk denotes non-specific protein bands.
VHL(F119S) and VHL(L128F) mutants are capable of forming an intact ECV and targeting HIFα for degradation (Fig. 4.6A and B), but fail to promote pJAK2 degradation (Fig. 4.6C and see Fig. 4.4D) thus supporting again the notion that the defect in pJAK2 regulation is independent of ECV. One possibility is that the failure in pJAK2 degradation is due to a defect in F119S and L128F to engage SOCS1. As predicted, unlike VHL(WT), both F119S and L128F mutants were severely compromised in binding SOCS1 (Fig. 4.6D), underscoring the potential requirement of SOCS1, rather than ECV complex formation, in the degradation of pJAK2. Notably, F119S and L128F mutants retained the ability to bind JAK2 (data not shown).

We asked whether the ability of SOCS1 to recruit the E3 ubiquitin ligase components was required for VHL-dependent pJAK2 degradation. Analogous to the α domain of VHL, the SOCS-box of SOCS1 facilitates the recruitment of the various ECS components including Cul5 or Cul2, Elongins BC and Rbx1. 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 SOCS1ΔSOCS-box mutant abrogated pJAK2 degradation (Fig. 4.6E). These results suggest that the SOCS-box is required for enzymatic activity of the VHL/SOCS1 heterodimer towards the degradation of pJAK2.
4.3.5. CP-VHL/SOCS1 association inhibits pJAK2 binding and degradation

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 VHL(H191D) mutants showed a dramatic increase in SOCS1 binding in comparison to their wild-type VHL counterpart (Fig. 4.7A), 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. HEK293 cells transfected with plasmids encoding EPOR, T7-JAK2 and HA-SOCS1 in combination with plasmids encoding HA-VHL(WT or R200W or H191D) were stimulated with EPO in the presence of MG132 to minimize the degradation of pJAK2. pJAK2 co-precipitated significantly lower levels of CP-VHL mutants in comparison to VHL(WT), suggesting that the abnormal association between CP-VHL and SOCS1 hinders pJAK2 substrate binding (Fig. 4.7B). 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 beads were washed and equally distributed into 4 reaction tubes, as confirmed by comparable levels of IgG\textsubscript{L} (Fig. 4.7C, bottom panel), and mixed with HEK293 cell lysates expressing empty plasmid (MOCK), HA-VHL(WT), HA-VHL(R200W) or HA-VHL(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. 4.7C). These results collectively suggest that the CP-VHL/SOCS1 heterocomplex is defective in promoting pJAK2 degradation.
4.3.6. pJAK2 and pSTAT5 are elevated in CP-mice

Erythroid progenitors from PV patients are hypersensitive to EPO due to JAK2 activating mutations associated with increased levels of phosphorylated JAK2 and STAT5\textsuperscript{560}. Erythroid progenitors from CP patients or R200W/R200W mice have likewise been shown to be hypersensitive to EPO\textsuperscript{13,34}. Single cell suspensions enriched with erythroid progenitors were generated from spleens of phenylhydrazine (PHZ)-treated R200W/R200W or WT mice and residual cytokines were removed by washes in cytokine-free media. Cells were cytokine starved for 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 noticeably higher in R200W/R200W compared to the WT erythroid progenitor-enriched cell lysates (Fig. 4.7D, compare lanes 3 and 4 against 7 and 8). Densitometry performed on unsaturated exposures of the immunoblots validated the observed trend (data not shown). These results demonstrate that homozygous inheritance of the CP-causing R200W mutation increases JAK2-STAT5 signalling pathway \textit{in vivo}. 
Mapping of VHL disease-causing mutations on VHL/Elongin B/Elongin C (VBC) crystal structure engaged with HIF1α peptide has revealed two major domains, α and β, required for Elongin C and HIF1α binding, respectively. VHL mutations that disrupt (F119S and L128F) or enhance (R200W and H191D) SOCS1 binding interestingly clustered to a unique region of VHL, revealing a likely interface required for the engagement of SOCS1, which we have termed the ‘SOCS groove’ (Fig. 4.8A). Notably, the SOCS groove does not overlap with the Elongin C or HIF1α binding interface. This is consistent with the observed autonomy of HIF- and JAK2-associated functions of VHL clearly revealed by specific mutants F119S and L128F, which retain the ability to degrade HIFα but fail to degrade pJAK2 despite their ability to form an ECV. Conversely, C162F retains the ability to degrade pJAK2 despite its inability to form an ECV or degrade HIFα. Thus, mutations within the groove may alter VHL’s affinity for SOCS1 positively or negatively via steric conformational change.

We propose the following model of CP (Fig. 4.8B). In normal individuals, VHL forms a proper ECV complex and negatively regulates HIFα via the ubiquitin-proteasome pathway. In contrast, CP-associated VHL mutations (e.g., R200W) attenuate HIFα binding and ECV complex formation, causing the reported mild stabilization of HIFα, which leads to overproduction of HIF-target EPO in the kidney and secondary polycythemia. In normal individuals, VHL also binds SOCS1 through its SOCS groove, and together the complex recognizes pJAK2 for...
ubiquitin-mediated degradation, thus negatively regulating JAK2-mediated signalling. The R200W VHL mutation in CP patients causes a conformational change within the SOCS groove, leading to an inordinately tight CP-VHL/SOCS1 association, thereby blocking pJAK2 binding and degradation. Resulting pJAK2 stabilization promotes hyperactivation of the JAK2-STAT5 pathway in erythroid progenitors, causing hypersensitivity to EPO and primary polycythemia.

The present findings also provide molecular explanations to several mysteries and paradoxes in VHL and CP fields. For example, it has been unclear why polycythemia is rarely observed in VHL patients, despite the fact that mutations promoting HIF\(\alpha\) stabilization are common in this population\(^{34,277,278}\). We show here that most tumour-causing VHL mutants, including those that have lost the ability to degrade HIF\(\alpha\), retain the ability to negatively regulate pJAK2 downstream of EPO signalling, which likely explains the rarity of polycythemia among VHL patients (see Fig. 4.8B). Furthermore, R200W/WT heterozygous mice, which do not show detectable HIF\(\alpha\) accumulation or EPO overproduction, have BFU-E cells that are modestly hypersensitive to EPO \textit{ex vivo}\(^{34}\). In addition, even though the average serum EPO levels are higher in CP patients compared to their unaffected relatives, there is overlap in the range of serum EPO between the two groups\(^{35}\) (see Table 1). The fact that not all CP patients have abnormally high serum EPO levels argues against the current model, which presents high EPO levels due to HIF dysregulation as the sole explanation for the excessive erythropoiesis observed in CP.
Figure 4.8. The ‘SOCS groove’ and the revised molecular model of CP. (A) Mutations (red) that influence SOCS1 binding are indicated on the VHL/Elongin B/Elongin C (VBC) crystal structure bound to HIF1α peptide, which cluster within the ‘SOCS groove’. Analyzed using DeepView/Swiss-PdbViewer v4.0. (B) Molecular model of CP. See text for details. OH: hydroxyl group, P: phosphoryl group.
Chapter 5
The VHL ubiquitin ligase: Summary of our current knowledge and future directions

5.1. Functions of the VHL ubiquitin ligase

The VHL tumour suppressor gene is inactivated in the majority of sporadic CCRCC and haemangioblastomas. Germline mutations resulting in functional inactivation of one VHL allele are associated with VHL disease, a hereditary cancer syndrome which leads to tumour formation in multiple organs upon acquisition of mutations inactivating the remaining wild-type VHL allele in the respective tissues. Additionally, certain VHL mutations have been shown to cause a congenital form of polycythemia, Chuvash polycythemia, a disorder characterized by excessive production of erythrocytes. Although a number of functions have been attributed to VHL, its best-characterized role is as the substrate-recognition component of an E3 ubiquitin ligase, the ECV, which is composed of VHL, Elongins BC, Cul2 and Rbx. ECV is part of the cullin-RING-ligase (CRL) group of ubiquitin ligases, which have the cullin scaffolding component and the Rbx RING finger protein in common. Substrate-recognition components engage to CRL complexes via F-box or SOCS-box domains, which are conserved in over 70 mammalian proteins. The best-characterized substrate of the ECV is the α subunit of the heterodimeric transcription factor HIF, a key regulator of oxygen homeostasis. HIFα is hydroxylated on proline residues under normal oxygen tension, which leads to its recognition by VHL and subsequent ECV-mediated polyubiquitination and destruction by the 26S proteasome. Under low oxygen tension, or hypoxia, HIFα is not prolyl-hydroxylated and thus escapes VHL recognition and subsequent degradation, which allows it to bind its dimerization partner ARNT, forming the active transcription factor HIF. A myriad of genes allowing cellular adaptation to reduced oxygen tension are transactivated by HIF, which permit tissue survival until normal oxygen levels resume. However, HIF is frequently overexpressed in a large number of cancers and is associated with tumour aggressiveness, as HIF-target gene expression promotes tumour growth, survival, angiogenesis, anaerobic metabolism, invasion, metastasis and resistance to radiation- and chemotherapy.
Work performed in this thesis further characterized the VHL ubiquitin ligase by describing the intricate regulation of the ECV. Furthermore, existing knowledge about the molecular VHL-HIF oxygen-sensing pathway was utilized to genetically engineer a unique form of VHL, capable of constitutively degrading HIFα through the ECV, ultimately leading to suppression of CCRCC tumour growth in a mouse xenograft model. In addition, a new VHL ubiquitin ligase composed of the two F-box proteins VHL and SOCS1 was characterized and shown to be essential for the negative regulation of erythropoiesis. The malfunction of this novel E3 ligase explains the excessive erythrocytosis observed in Chuvash polycythemia patients. Knowledge obtained from this thesis can be directly applied towards the development of better therapeutic options for polycythemica patients or patients with HIF-overexpressing tumours.

5.2. Regulation of ECV activity

VHL recognizes HIFα that has undergone prolyl-hydroxylation in the presence of oxygen11,12. The recruited HIFα is then subjected to ubiquitination via the ECV complex, subsequently leading to 26S proteasome-mediated destruction5,7,8. Recent evidence has shown that NEDD8-modification of Cul2, the scaffolding component of the ECV, dramatically increases the E3 ubiquitin ligase activity of the ECV70. However, the molecular mechanism underlying this process remained unresolved. Here, we show that NEDD8 modification of Cul2 requires Rbx1, which dramatically enhances binding to UbcH5a. ECV that has engaged HIF1α contains preferentially NEDD8-modified Cul2, while ECV consisting of mutant VHL incapable of binding HIFα exclusively associates with unmodified Cul2. In addition, increased HIF1α association with VHL results in a corresponding increase of Cul2 neddylation in the context of ECV. These results suggest that the oxygen-dependent recognition/binding of HIFα via VHL triggers Rbx1-mediated neddylation of Cul2, which promotes the engagement of UbcH5a to the ECV complex. We thereby establish for the first time a central role for the neddylation of Cul2 in the temporally coordinated activation of the ECV with the recruitment of its substrate HIFα. It remains to be investigated how HIFα triggers Cul2 neddylation upon engagement to the ECV. We have preliminary data (discussed in detail in the Appendix) showing that HIF1α does not
promote Rbx1 association with the ECV, but that it can bind UbcH12, thus likely recruiting it to the ECV, where it can act in conjunction with Rbx1 to neddylate Cul2.

Cullins are scaffolding components of the multisubunit CRL ubiquitin ligases and are the best-characterized substrates of NEDD8/Rub1-conjugating enzymes. Interestingly, Rbx1 has been implicated in both substrate ubiquitination and cullin neddylation. Rbx1 has been shown to recruit charged ubiquitin-conjugating enzymes to the ligase complex and to catalyze substrate ubiquitination. The RING domain itself serves as a scaffold to direct the transfer of ubiquitin from the E2 to the substrate. Using a baculovirus protein expression system in Sf-9 insect cells, Kamura and colleagues have shown that conjugation of the NEDD8-orthologue Rub1 to Cdc53 depended on Rbx1. Similarly, co-expression of Cul1 or Cul2 with Rbx1 in insect cells resulted in increased neddylation of Cul1/2. Morimoto and colleagues recently showed that Rbx1 binds to the E2 NEDD8-conjugating enzyme UbcH12, thus providing the mechanism responsible for the Rbx1-mediated neddylation of Cul1. Together with our data showing Rbx1 to be necessary for Cul2 neddylation, Rbx1 can be proposed as a NEDD8 E3 ligase. Since our study was published, a number of reports have also implicated a novel protein conserved from yeast to humans, Dcn1 (defective in cullin neddylation 1), in cullin NEDD8 modification. Specifically, Dcn1 was found to interact directly with NEDD8, yeast Cdc53 and mammalian Cul1, and to stimulate the neddylation reaction in vitro and in vivo in conditions of limited Ubc12. Similar to Rbx1, Dcn1 also binds to Ubc12 and thus fulfills criteria of a NEDD8 E3 ligase. Interestingly, Rbx1 and Dcn1 associate and thus may cooperate to promote cullin neddylation. Further experimentation is required to elucidate the mechanism by which Dcn1 promotes cullin NEDD8 modification and to determine if it has a role in Cul2 neddylation, specifically in the context of ECV.

In an effort to understand how increased CRL activity is induced by cullin neddylation, Kawakami et al. showed that promoting neddylation in an in vitro system increased the recruitment of the E2 UbcH4 to the SCFβTrCP1 bound to phosphorylated IκBα. Additionally, overexpressed UbcH4 in HEK293 cells precipitated exclusively NEDD8-modified Cul1. We have shown that neddylated Cul2 preferentially engages UbcH5a. In support of these results, nuclear magnetic resonance and mutational analysis of the NEDD8/UbcH4 complex revealed that NEDD8 directly binds UbcH4. A very recent enzymatic study suggests that in addition to stimulating ubiquitin-charged E2 (E2-Ub) recruitment, cullin neddylation significantly stimulates...
ubiquitin transfer from SCF-bound E2-Ub to the substrate acceptor lysine\textsuperscript{375}. NEDD8 conjugation of Cul1 increased the rate of ubiquitination of both p27 and β-catenin SCF substrates by stimulating transfer of the first ubiquitin and also promoting ubiquitin chain elongation. These results are in agreement with very recent crystallographic data, showing that NEDD8-modification of Cul1 and Cul5 induces a substantial conformational change in the cullins which frees the Rbx1 RING domain from being embedded in the cullin C-terminal region\textsuperscript{371}. This allows the flexibly-linked RING domain to adopt multiple orientations important for substrate polyubiquitination, while the Rbx1 N-terminal region is anchored in the cullin C-terminal domain. Thus, upon NEDD8-modification of cullins, Rbx1 and the associated ubiquitin-charged E2 can be positioned closely to the substrate acceptor lysine, promoting ubiquitination. Due to its flexibility, the Rbx1/E2 can adopt various spatial orientations to accommodate a growing polyubiquitin chain. These observations suggest that, rather than NEDD8 serving as a docking site for the E2, the increased E2 association observed upon cullin neddylation may be due to the release of the E2-interacting Rbx1 RING domain.

In light of the well-established oncogenic role of HIF in tumour progression, the importance of an intact neddylation pathway to promote HIFα degradation should not be underestimated. As such, inhibition of Cul2 neddylation through incubation at non-permissive temperature of the ts41 CHO cell line containing a temperature-sensitive mutation in the NAE, led to HIFα stabilization under normal oxygen tension\textsuperscript{70}. As previously described, HIFα is overexpressed in a number of tumours containing wild-type VHL. One possible mechanism explaining this phenomenon is inefficient HIFα degradation by the ECV. It thus remains to be investigated if such tumours harbor mutations interfering with Cul2 neddylation. Cul2 is located on chromosome 10p11.1-11.2, a region frequently affected by deletions or loss of heterozygosity in tumours known to overexpress HIF, such as gliomas and prostate carcinomas\textsuperscript{376-380}. In addition to the potential loss of Cul2 or a mutation rendering Cul2 unable to form an ECV complex in these tumours, it is formally possible that mutations in Cul2 may be found that impinge on its neddylation. Such mutations could affect the attachment of the NEDD8 molecule itself, or could interfere with Rbx1 engagement or Rbx1 RING release upon neddylation.
5.3. Bioengineered VHL constitutively degrades HIFα

Accumulation of HIF1/2α due to tumour hypoxia promotes progression and aggressiveness of cancer, and is associated with resistance to conventional anti-cancer therapies\textsuperscript{10}. In addition, tumour-causing mutations on a growing list of oncogenes and tumour suppressor genes have been identified to enhance the expression of HIFα, underscoring the significance of HIF in oncogenesis\textsuperscript{10}. Perhaps the most direct and convincing association between tumour-causing mutations and HIF activation is VHL, as it triggers the polyubiquitination of prolyl-hydroxylated HIFα, leading to immediate HIFα destruction via the 26S proteasome. Moreover, CCRCC that frequently harbours VHL inactivating mutations or displays loss of VHL, overexpresses HIF2α and is one of the most resistant tumours to radiation or chemotherapies\textsuperscript{265}. Thus, the molecular understanding of the VHL-HIF oxygen-sensing pathway has been invaluable for conceptualizing new and targeted anti-cancer therapeutics.

Surgery by radical or partial nephrectomy is the most effective treatment option for localized CCRCC. However, in one-third of patients tumours recur post-operatively as distant metastases, and only 4-6\% of these tumours respond to chemotherapy\textsuperscript{265}. The standard non-surgical treatment for advanced CCRCC has been the administration of IL-2 or IFNα. However, the response rates were generally low with considerable toxicity. Recently, a number of anti-angiogenic agents and mTOR inhibitors have received regulatory approval for treatment of advanced CCRCC\textsuperscript{267}. While anti-angiogenic agents prevent the HIF-induced angiogenic switch, the anti-tumour effects of mTOR inhibitors are presumed to be through inhibiting cap-dependent translation of HIF1α, although their activity in hypoxic tumour regions is unknown. However, despite prolonging progression-free survival, a major hurdle for most of these agents is to increase overall patient survival. As the oncogenic effect of HIF activity in tumours gained more appreciation, a number of additional agents have been shown to reduce the expression of HIF1α\textsuperscript{247}. However, none of the available compounds directly targets HIF1α, with each drug having additional functions other than blocking HIF1α. Additionally, despite the established oncogenic role of HIF2α in CCRCC, no agents have been shown to inhibit HIF2α activity. Thus, new strategies to inactivate HIF1α, as well as HIF2α, directly and constitutively would represent a major conceptual advancement in anti-cancer therapeutics. Here, we demonstrate that a bioengineered VHL, VHL-ARNT, comprised of the VHL α domain fused to the ARNT
HLH/PAC dimerization region, can engage and degrade HIF\(1\alpha\) and HIF\(2\alpha\) irrespective of oxygen tension, relieving the necessity of HIF\(\alpha\) prolyl-hydroxylation for degradation. We further show that adenovirus-mediated delivery of the bioengineered VHL dramatically inhibits angiogenesis and regresses CCRCC xenografts in vivo. This is the first report illustrating the feasibility of a VHL ubiquitin ligase designed to remove the oxygen constraint as an alternative mode to directly and constitutively target HIF\(\alpha\) for destruction for rational anti-cancer therapy.

The strategy of employing chimeric F-box proteins for targeted proteolysis of cellular proteins has been described in the context of the SCF (Skp1/Cull1/F-box protein) E3 ligase\(^{381}\). All generated chimeras used either full length or the F-box of \(\beta\)TrCP, the substrate-recognition component of the SCF\(^{\beta\text{TrCP}}\) complex. Typically, the SCF\(^{\beta\text{TrCP}}\) complex targets phosphorylated \(\beta\)-catenin for ubiquitin-mediated degradation. To circumvent the need for \(\beta\)-catenin phosphorylation, \(\beta\)TrCP was fused to the \(\beta\)-catenin-binding domain of E-cadherin, APC or Tcf4, which resulted in constitutive degradation of cytosolic \(\beta\)-catenin\(^{382-384}\). Additionally, substrates not normally recognized by the SCF\(^{\beta\text{TrCP}}\) complex, such as Rb, c-myc and the cyclin A/Cdk2 complex were targeted for degradation by fusing \(\beta\)TrCP to specific regions of proteins that interact with the respective targets\(^{385-387}\). As such, regions of the HPV oncoprotein E7 and Max were used to respectively tether Rb and c-myc to the SCF for degradation, while a short peptide containing a cyclin A/Cdk2 binding motif normally found in p21-like cdk inhibitors was used to cause degradation of the cyclin A/Cdk2 complex\(^{385-387}\). In light of these studies, replacement of the substrate-binding interface of any F-box protein with the HIF\(\alpha\)-binding region of ARNT (i.e., HPAC) may have been sufficient to promote oxygen-independent degradation of HIF\(\alpha\).

However, as discussed above, we have shown that HIF\(\alpha\) triggers its own degradation via initiating the conjugation of NEDD8 onto Cul2, which enhances the E3 activity of the ECV. Although the precise mechanism(s) governing substrate-dependent triggering of E3 function remains incompletely understood, it suggests a molecular interplay between substrates and their specific E3s, arguing for a more efficient degradation of intrinsic E3 substrates.

As outlined in chapter 1.4, HIF expression also promotes an invasive and metastatic tumor phenotype by inducing protease activity via upregulating MMP-2 and uPAR, promoting epithelial to mesenchymal transition through loss of E-cadherin and enhancing directional migration of tumor cells through CXCR4 expression\(^{188,189,191,192,194-196}\). Thus, the effect of the
potent HIF-inhibitor VHL-ARNT on CCRCC metastatic potential should be addressed. This could be achieved by implanting CCRCC cells with known metastatic potential subcutaneously into flanks of immunocompromised mice, or by generating a murine orthotopic model of metastatic CCRCC. Stable transfection with the red-fluorescent protein dsRed2 prior to implantation would facilitate visualization of the primary tumour and its metastases. The effect of repeated intratumoural injections with either Ad-EGFP or Ad-EGFP-T7-HPACGV on tumour metastasis would be assessed after sacrificing the mice, by imaging the dissected animals for red fluorescence. In particular, common CCRCC metastatic sites, such as regional lymph nodes, adrenal glands, liver and lungs should be carefully examined. Based on the positive effect of HIF on tumour metastatic potential, we hypothesize that VHL-ARNT-mediated HIFα degradation decreases tumour metastases.

High HIF expression in tumours has also been associated with resistance to chemotherapy, which can be in part attributed to HIF-induced expression of the P-gp/MDR-1 drug efflux pump and hTERT telomerase reverse transcriptase, as well as the implication of HIF in upregulation of double-strand break repair enzymes199-203. In particular, CCRCC is characterized by very low response rates to frequently used chemotherapeutic agents, such as gemcitabine, fluorouracil, capecitabine and vinblastine267. Therefore, a possible synergistic effect of VHL-ARNT tumour treatment with chemotherapy could be investigated, as HIF inhibition may sensitize cells to chemotherapy-induced cell death. The above-mentioned heterotopic or orthotopic CCRCC xenograft tumour models could be used to test this hypothesis. Intratumoural injection with Ad-EGFP or Ad-EGFP-T7-HPACGV could be performed in tandem with administration of chemotherapeutic agents. The effect on primary tumour size and metastasis could be assessed by fluorescence microscopy and compared between Ad-EGFP, Ad-EGFP plus chemotherapy and Ad-EGFP-T7-HPACGV, Ad-EGFP-T7-HPACGV plus chemotherapy treatment groups. Additionally, cell death within the primary tumours could be assessed by performing H&E stains.

In addition to CCRCC, overexpression of HIFα has been associated with tumour aggressiveness in a large number of solid tumours, including brain, breast, lung, colon, skin and prostate cancer10. Therefore, cell lines derived from these tumours could serve as mouse xenograft models, as described above, to test the efficacy of VHL-ARNT treatment on tumour angiogenesis, growth, metastasis and resistance to chemotherapy.
5.4. VHL and SOCS1: two F-box proteins cooperate to control erythropoiesis

Polycythemia is a condition characterized by excessive erythrocytosis, resulting in high hematocrit. Recently, VHL homozygous germline mutations, VHL(R200W) and VHL(H191D) have been identified in patients with a unique form of polycythemia, Chuvash polycythemia (CP)\textsuperscript{13,32}. CP patients have erythroid progenitors that are hypersensitive to EPO stimulation, and many patients also present with high serum EPO levels, features of primary and secondary polycythemia, respectively\textsuperscript{13}. The current model proposes CP-VHL to be defective in maintaining proper oxygen homeostasis, by allowing inappropriate HIF\(\alpha\) stabilization and thus leading to high expression of the HIF-target gene EPO\textsuperscript{13}. However, the primary polycythemic features of CP remain unexplained. In parallel, activating JAK2 mutations leading to excessive erythrocytosis due to aberrant JAK2-mediated signalling in the context of the EPOR were recently identified in the vast majority of individuals with polycythemia vera, the most common form of primary polycythemia\textsuperscript{348-352}. Here we reveal a novel genetic link between the seemingly distinct genes VHL and JAK2 in the development of polycythemia. Specifically, we show that the wild-type VHL and tumour-causing VHL mutants form a complex with SOCS1 to target phosphorylated JAK2 for ubiquitin-mediated destruction. VHL and other F-box proteins have been previously shown to homodimerize\textsuperscript{364-368}, however, this is the first evidence of two different F-box proteins forming a heterodimer with unique substrate-recognition specificity. It is formally possible that other substrate recognition components of E3 ubiquitin ligases also form heterodimers, and that the resulting combinatorial diversity leads to a significant increase in the number of substrates recognized and targeted for ubiquitination. We further show that CP-VHL(R200W and H191D) mutants form a defective heterodimer with SOCS1, severely compromising JAK2 degradation and consequently enhancing the JAK2-STAT5 signalling pathway. These findings provide the mechanism underlying the primary polycythemic features of CP and introduce VHL as a novel regulator of JAK2.

As mentioned previously, the SOCS family of proteins consists of eight family members, CIS and SOCS1-7\textsuperscript{337,338}. Based on our findings showing VHL interaction with SOCS1, the question arises whether VHL also interacts with other SOCS proteins. Co-immunoprecipitation experiments performed on cells transiently transfected with mammalian expression plasmids
encoding VHL and the respective SOCS proteins would clarify this question. In case new interactions are found, these could be confirmed by showing binding endogenously. In addition to SOCS1, SOCS3 has also been shown to interact with pJAK2 and to serve as a negative regulator of EPO-induced JAK2 signalling. Therefore, the significance of SOCS3 in the context of VHL-mediated ubiquitination of pJAK2 could be elucidated and compared to SOCS1. If SOCS3, similarly to SOCS1, forms a heterodimer with VHL capable of targeting pJAK2 for ubiquitin-mediated degradation, both SOCS3-binding of the VHL(R200W) and VHL(H191D) CP mutants and pJAK2 degradation by the CP-VHL/SOCS3 complex should be investigated.

The unique combination of primary and secondary polycythemic features found in Chuvash polycythemia prompts investigation of the relative contribution of each feature to disease manifestation. This question could be addressed by generating chimeric mouse models using WT and R200W/R200W CP mice. While human erythropoiesis occurs almost exclusively in the bone marrow, the spleen is a frequent site of extramedullary haematopoiesis in the mouse. Notably, in the R200W/R200W CP mouse model, the increase in erythroid precursors is mainly observed in the spleen. Therefore, to assess the effect of the VHL(R200W) mutation exclusively in haematopoietic progenitor cells, bone marrow cells and splenocytes from the R200W/R200W mice could be transplanted into lethally irradiated WT syngeneic recipient mice. These chimeric mice are predicted to have normal serum EPO levels, since their kidneys, the primary site of EPO production, will contain VHL(WT). Based on our findings showing VHL(R200W) to be defective in negative pJAK2 regulation, we predict these mice to have erythroid progenitors which are hypersensitive to EPO, and to thus develop polycythemia despite their normal serum EPO levels. This would further distinguish our model from the current model, which relies only on high serum EPO concentration to explain the erythrocytosis observed in CP. The degree of erythrocytosis observed in the chimeric mice compared to WT and R200W/R200W mice will also indicate the relative contribution of primary polycythemia to the CP disease phenotype. To determine the relative contribution of secondary polycythemia to CP, a complementary experiment could be performed, in which bone marrow cells and splenocytes from VHL(WT) mice would be transplanted into R200W/R200W lethally irradiated syngeneic recipients. As the kidneys of the chimeric mice will contain VHL(R200W), which has been shown to allow inappropriate low-level HIFα stabilization under normoxia, these mice are predicted to have elevated serum EPO levels. Since most, but not all CP patients have high serum EPO levels, it
would be very interesting to determine the effect of EPO on erythropoiesis in these chimeric mice. The comparison of hematocrit, hemoglobin levels, RBC counts and numbers of erythroid progenitors of the chimeric mice to WT or R200W/R200W mice will reveal the answer. Together, these experiments will help to further distinguish the relative contribution of aberrant JAK2 signalling from the effect of high EPO levels on erythroid progenitors in CP.

The PV-associated JAK2(V617F) mutation causes uncontrolled expansion of RBCs, but also gives rise to pleomorphic and clustered megakaryocytes hypersensitive to thrombopoietin (TPO), which, similarly to EPO, signals through JAK2. Abnormal megakaryocyte function is thought to be critical in thrombotic complications frequently observed in PV patients. Strikingly, R200W/R200W mice exhibit increased number of megakaryocytes that cluster and CP patients, like PV patients, often present with thrombotic complications. In contrast, secondary polycythemia associated with elevated EPO does not give rise to megakaryocytic defects; an observation supported in mice with constitutive overexpression of EPO that do not develop thrombotic complications despite an inordinately high hematocrit. These observations suggest that the hyperactive JAK2-mediated signalling, but not the increased EPO production due to a mild defect in HIF regulation, is the principal mechanism underlying thrombotic complications observed in CP patients. Haematopoietic cell lineages other than the erythroid lineage have not been found to be increased in CP patients, but the number of megakaryocytes has not been explicitly investigated. The significant increase in megakaryocytes found in the R200W/R200W CP mouse model, however, warrants the detailed investigation of megakaryocyte status of CP patients. The hypersensitivity to TPO observed in PV and the increased numbers of megakaryocytes observed in both PV and the CP mouse model, may point to a novel role of VHL in thrombopoiesis through the negative regulation of JAK2-mediated signalling downstream of the TPO receptor (TPOR). Such a hypothesis could be tested by determining if the R200W/R200W megakaryocyte progenitors are hypersensitive to TPO, similar to PV megakaryocyte progenitors. In addition, the effects of transient knock-down of VHL in megakaryocyte cell lines with respect to clearing of pJAK2 should be investigated.

Considerable overlap in clinical and laboratory features led to the grouping of PV, essential thrombocythemia (ET) and primary myelofibrosis (PMF) as myeloproliferative neoplasms (MPNs). These conditions affect the erythroid, megakaryocyte and granulocyte lineages, respectively, which are hypersensitive to EPO, TPO or G-CSF cytokines. Interestingly, while
the JAK2(V617F) mutation is prevalent in 95% of PV patients, it has also been found in 50% of ET and PMF patients. In addition to signalling from the EPO and TPO receptors, JAK2 is also involved in G-CSFR-mediated downstream signal transduction. Thus, cytokine hypersensitivity of these conditions can be likely explained by aberrant JAK2 signalling. Based on our model of VHL-mediated negative regulation of JAK2 signalling, the VHL status in JAK2(V617F)-negative MPN patients should be investigated. Likewise, patients with a congenital form of polycythemia, named primary familial and congenital polycythemia (PFCP) display increased red-cell mass and have erythroid progenitors that are hypersensitive to EPO. Recently, mutations in the EPOR have been identified in 12% of PFCP patients. Sequencing of the VHL locus in the remaining 88% of patients may reveal possible VHL mutations incapable of negative JAK2 regulation.

The discovery of JAK2 mutations in PV patients has certainly expedited the clinical trials of JAK2 inhibitors in the management of PV and MPN. Several selective JAK2 inhibitors have been tested in preclinical models of these diseases and three have moved into first-in-human Phase 1 clinical trials. The results of these and following trials are highly anticipated, however, careful dosing is likely required to only inhibit constitutively active JAK2 signalling in affected myeloid progenitor cells and not to interfere with normal erythropoiesis and other JAK2-mediated normal cellular functions. Nevertheless, targeted therapies for PV and MPN patients would constitute a major advantage to the current treatment methods, which are mainly aimed at managing the symptoms, rather than treating the cause of the disease. However, despite the clinical features shared between PV and CP, including hypersensitivity to erythropoietin and megakaryocytic defects associated with thrombotic complications, JAK2 inhibitors have not yet been considered for CP. Thus, the present findings linking VHL to the JAK2-STAT5 pathway provide a biochemical rationale for JAK2-targeted therapies in CP.

5.5. Potential therapeutic implications

The VHL-ARNT chimera described in this thesis led to constitutive HIFα degradation and resulted in decreased CCRCC tumour angiogenesis and significant tumour cell death in our murine window chamber CCRCC xenograft model. Based on these promising results, a series of
preclinical studies could be performed, aimed at developing VHL-ARNT as a potential treatment for patients with CCRCC. To better mimic the natural tumour microenvironment, a murine orthotopic model of CCRCC could be generated and used to assess treatment efficacy in large numbers of animals. Furthermore, pharmacokinetics, pharmacodynamics and the drug safety profile of VHL-ARNT should be established. If these studies succeed, clinical development for VHL-ARNT to treat CCRCC patients could be envisioned. Despite the recent emergence of targeted therapies for advanced CCRCC, there is still an unmet medical need for treatment of this disease, as only therapy with the mTOR inhibitor temsirolimus increased overall survival by 3.6 months in patients with poor risk characteristics. The mechanism of action of VHL-ARNT has a theoretical advantage over temsirolimus, as VHL-ARNT constitutively degrades HIF1α and HIF2α under normoxia and hypoxia, while the effect of temsirolimus under hypoxia and against HIF2α is unknown. Thus, Phase I studies to determine drug safety and dose range in CCRCC patients could be conducted. If VHL-ARNT is found to be safe, Phase II studies aimed at determining its efficacy could be undergone. Should CCRCC tumours respond well to VHL-ARNT treatment, Phase III studies in large numbers of patients, comparing VHL-ARNT treatment safety and efficacy to CCRCC standard of care could be performed. To potentially obtain regulatory approval, better patient progression-free survival and preferably also better overall survival should be demonstrated in these studies. In parallel, as described above, preclinical proof-of-concept studies with VHL-ARNT could also be performed in other solid tumour types for which high HIFα expression has been associated with tumour progression. If the tumours respond to VHL-ARNT treatment, similar drug development plans for the respective tumour types could be set up.

Over 70% of Chuvash polycythemia patients die before the age of 65, mainly due to cerebral vascular events and peripheral thrombosis. Phlebotomy and aspirin are common treatment methods for CP patients, however, no studies have been conducted to validate their efficacy, and their effect on improving patient long-term survival is unknown. Since the discovery of the JAK2(V617F) mutation as the cause of polycythemia vera, three small-molecule JAK2 inhibitors, INCB018424, XL019 and TG101348 have entered Phase I studies for primary myelofibrosis, a myeloproliferative neoplasm associated with aberrant JAK2 signalling. If these studies prove successful, PV patients are next in line for treatment with these inhibitors. Importantly, based on our results showing a defect in the VHL-mediated negative regulation of
JAK2 signalling in CP, proof-of-concept preclinical trials with JAK2 inhibitors ought to be performed in CP. The R200W/R200W CP mouse model could be used to study the effect of JAK2 inhibitors on erythrocytosis. Should these agents indeed decrease erythropoiesis to normal levels, preclinical studies aimed at characterizing pharmacokinetics, pharmacodynamics and drug safety profile of the active compounds could be performed in the CP mouse model. After careful dosing, if the drug is well-tolerated and proves efficacious specifically against hyperactive JAK2 signalling in the affected myeloid precursors cells, clinical development with phase I, II and III trials could be envisioned to benefit CP patients.

Even though commonly overlooked in the CP literature, not all CP patients present with high serum EPO levels. Indeed, there is overlap in the range of serum EPO concentrations measured in CP patients and their unaffected relatives (see Table 1). However, all CP patients have high erythrocytosis, the very definition of polycythemia. Thus, the contribution of EPO to CP erythrocytosis is an outstanding question. As described above, generating chimeric mice by injecting VHL(WT) bone marrow cells and splenocytes into lethally irradiated R200W/R200W recipients, would help answer this question. Based on the varying EPO levels of different CP patients, a small or negligible effect of EPO on CP erythrocytosis could be postulated, hypothesizing that the defect in negative JAK2 regulation accounts for the polycythemic features of these patients. If this is indeed the case, bone marrow transplantation could be envisioned as a possible therapeutic option for CP patients. While treatment with a JAK2 inhibitor could likely alleviate polycythemia, CP patients would have to be continuously treated with this agent for the rest of their life. However, if bone marrow transplantation proves to be a sound therapeutic option, this would represent a cure for the disease, an extraordinary advantage compared to any other possible treatment.

In summary, building upon the knowledge presented in this thesis, possible therapeutics could be developed for CCRCC patients and other cancer patients whose tumours overexpress HIFα, and for Chuvash polycythemia patients, for whom no validated treatment option exists.
Appendix
Investigation of HIFα-induced Cul2 NEDD8-modification

A.1. Introduction and Rationale

As shown in chapter 2, we found HIF1α to induce its own degradation by initiating the NEDD8 modification of Cul2. Rbx1 mediates Cul2 neddylation, which in turn facilitates recruitment of the ubiquitin-conjugating enzyme UbcH5, ultimately leading to HIF1α ubiquitination and proteasomal degradation. A recent report implied Cullin neddylation of Cul1, Cul2, Cul3 and Cul4A to be driven by E3 substrate engagement by showing that association of these Cullins with the rest of the E3 components was required for Cullin NEDD8 modification\textsuperscript{399}. Furthermore, the substrate-recognition domain of the SCF F-box protein Skp2 was required for Cul1 neddylation, directly linking substrate binding to the SCF to Cul1 neddylation\textsuperscript{399}. Confirming our results, this report also demonstrates that HIF1α induces Cul2 NEDD8 modification. However, the mechanism by which HIF1α leads to Cul2 neddylation remains largely unknown and forms the subject of our investigation.

A.2. Materials and Methods

Unless otherwise indicated, all experiments were performed in triplicate.

A.2.1. Cells

RCC4 CCRCC and HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Milwaukee, WI, USA) at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. RCC4 VHL(-/-) subclones ectopically expressing HA-VHL(WT) were previously described\textsuperscript{8}.
A.2.2. Antibodies

Relevant antibodies were described in chapter 2.2.2. Polyclonal anti-Rbx1 and anti-UbcH12 antibodies were obtained from Sigma-Aldrich (Oakville, Canada) and Boston Biochem (Cambridge, MA, USA), respectively. Monoclonal anti-VHL (IG32) and anti-GST antibodies were obtained from BD Biosciences (Mississauga, ON, Canada) and Millipore (Billerica, MA, USA), respectively. Purified GST-UbcH12 was obtained from Boston Biochem (Cambridge, MA, USA).

A.2.3. Plasmids

Relevant mammalian expression plasmids were described in chapter 2.2.3. HA-HIF1α(P564A), HA-HIF1α(ODD) containing amino acids 530-652 of HIF1α, HA-HIF1α(∆ODD) lacking residues 530-652, HA-HIF2α and HA-HIF3α were previously described.

A.2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described in chapter 2.2.4.

A.2.5. In vitro binding assays

*In vitro* translation of wild-type and mutant HIF1α was performed as described in chapter 2.2.5. 0.5µg of GST-UbcH12 was incubated with indicated HIF1α translation products and with anti-GST antibody in the presence of protein A-Sepharose beads in 700 µl of EBC buffer at 4°C for 2 hrs. After five washes with NETN buffer, the bound proteins were resolved on SDS-PAGE and visualized by autoradiography and immunoblotting with anti-UbcH12 antibody.

A.3. Results and Discussion

A.3.1. HIF1α, HIF2α, HIF3α binding to ECV triggers Cul2 neddylation

There are three HIFα genes in humans, HIF1α, HIF2α and HIF3α (referring to HIF3α1, the full length isoform), and all are known to engage to the ECV. We therefore asked whether HIF2α and HIF3α were also able to trigger Cul2 NEDD8 modification in a similar manner to HIF1α.
HEK293 cells were transiently transfected with mammalian expression plasmids encoding empty vector (mock), T7-VHL, or T7-VHL in conjunction with HA-HIF1α, HA-HIF2α or HA-HIF3α. Cells were incubated with the proteasome inhibitor MG132 for 4 hrs prior to lysis. Upon lysis, cells were immunoprecipitated with anti-T7 antibody, bound proteins were resolved by SDS-PAGE and immunoblotted with anti-HA, anti-Cul2 and anti-T7 antibodies. While T7-VHL immunoprecipitated mainly unnedylated Cul2, the ectopic expression of all HA-HIF1α, HA-HIF2α and HA-HIF3α resulted in a dramatic increase of neddylated Cul2 (Fig. A.1). The extent of the induction of Cul2 neddylation was comparable across the different HIFαs, suggesting that Cul2 NEDD8 modification upon substrate engagement is not limited to HIF1α.

Figure A.1. HIF1α, HIF2α and HIF3α engagement to VHL triggers Cul2 NEDD8 modification. HEK293 cells were transiently transfected with the indicated mammalian expression plasmids and treated with the proteasome inhibitor MG132 4 hrs prior to lysis. Cells were lysed, immunoprecipitated with anti-T7 antibody, resolved by SDS-PAGE and visualized by immunoblotting with the indicated antibodies. IP: immunoprecipitation, IB: immunoblot, WCE: whole cell extract.
A.3.2. HIF1α does not promote Cul2 neddylation by recruiting Rbx1

Accumulating evidence suggests that Rbx1 acts as an E3 NEDD8 ligase for Cullins. We have previously shown that Rbx1 is required for Cul2 modification by NEDD8. Based on this finding, we asked whether HIF1α led to Cul2 neddylation by promoting Rbx1 recruitment to the ECV.

To address if HIF1α directly engaged with Rbx1, we transiently transfected HEK293 cells with HA-HIF1α or the HA-HIF1α(P564A) mutant, which does not interact with VHL due to lack of prolyl-hydroxylation. Cells were treated with proteasome inhibitor 4 hrs prior to lysis, lysed, immunoprecipitated with anti-HA antibody and bound proteins were resolved by SDS-PAGE (Fig. A.2A). Immunoblotting with anti-HA and anti-Rbx1 antibodies revealed association of wild-type HA-HIF1α, but not of the HA-HIF1α(P564A) mutant with endogenous Rbx1, arguing against a direct association of HIF1α with Rbx1 (Fig. A.2A). In a complementary experiment, endogenous HIF1α was immunoprecipitated from the VHL(-/-) CCRCC cell line RCC4. In the presence of proteasomal inhibition, HIF1α association with Rbx1 and ECV components Cul2 and VHL was only observed in RCC4 cells stably transfected with VHL (RCC4-VHL) (Fig. A.2B). In contrast, in RCC4 cells stably transfected with empty plasmid (RCC4-Mock), HIF1α did not co-immunoprecipitate Rbx1, despite similar total levels of Rbx1 in the whole cell extract (Fig. A.2B). Together, these results illustrate the need for HIF1α to be engaged with VHL in order for association with Rbx1 to be observed. Nevertheless, it is conceivable that engagement of HIF1α to VHL leads to an ECV conformation that favours Rbx1 association and thus leads to Cullin neddylation. To test this notion, HEK293 cells were transiently transfected with empty plasmid, T7-VHL, or T7-VHL in conjunction with HA-HIF1α or HA-HIF2α, and incubated with the proteasome inhibitor MG132 for 4 hrs prior to lysis. Cells were lysed, immunoprecipitated with anti-T7 antibody, and western blotting was performed with anti-HA, anti-Cul2, anti-T7 and anti-Rbx1 antibodies on SDS-PAGE-resolved proteins (Fig. A.2C). While overexpression of HIF1α and HIF2α led to increased levels of Cul2-NEDD8 as previously observed, it did not lead to increased Rbx1 association (Fig. A.2C, compare lanes 2-4). Notably, Rbx1 was also associated with the rest of the ECV in the absence of ectopic substrate expression, suggesting that Rbx1 is a default component of the ECV complex and is not recruited only upon substrate engagement. In summary, these results indicate that HIFα-induced Cul2 neddylation occurs through a mechanism not involving Rbx1 recruitment.
A.3.3. HIF1α directly engages UbcH12

UbcH12 is the relevant E2 for Cul2 neddylation, as a dominant-negative UbcH12 mutant was shown to abrogate NEDD8 conjugation to Cul2. In order to investigate whether HIF1α engagement to ECV triggers Cul2 neddylation by recruiting UbcH12, we performed an in vitro binding assay with purified GST-tagged UbcH12 and 35S-labelled, in vitro translated HIF1α(WT, P564A, ΔODD, ODD). Interestingly, immunoprecipitation with anti-GST antibody revealed association of HIF1α with UbcH12 (Fig. A.3). This association was not dependent on HIF1α engagement to VHL in the reticulocyte lysate, as both HIF1α(P564A) and the HIF1α mutant lacking the oxygen-dependent degradation domain, HIF1α(ΔODD), engaged with GST-UbcH12 (Fig. A.3, compare lanes 6, 8, 10). In contrast, HIF1α only containing the ODD did not associate with UbcH12. These preliminary results show for the first time a substrate engaging with a NEDD8-conjugating enzyme and indicate a mechanism responsible for the HIFα-induced Cul2 neddylation.

Figure A.2. HIFα does not recruit Rbx1 to ECV. (A) HEK293 cells were transiently transfected with the indicated mammalian expression plasmids and treated with MG132 4 hrs prior to lysis. Cells were lysed, immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE and visualized by immunoblotting with the indicated antibodies. (B) RCC4 subclones were immunoprecipitated for endogenous HIF1α and immunoblotted with indicated antibodies. (C) HEK293 cells were treated as in (A), immunoprecipitated with anti-T7 antibody and immunoblotted with indicated antibodies. IP: immunoprecipitation, IB: immunoblot, WCE: whole cell extract. Asterisk denotes non-specific protein bands.
Figure A.3. HIF1α binds UbcH12. HIF1α(WT, P564A, ΔODD, ODD) were \textit{in vitro} translated in the presence of $^{35}$S-methionine and mixed with purified GST-UbcH12, where indicated. Sample mixtures were immunoprecipitated with anti-GST antibody, resolved by SDS-PAGE and visualized by autoradiography and immunoblotting with anti-UbcH12 antibody. IP: immunoprecipitation, IB: immunoblot, AR: autoradiography.
In summary, we show that all three HIFαs, HIF1α, HIF2α and the full-length HIF3α, potently induce Cul2 neddylation upon engaging to VHL. This NEDD8-modification of Cul2 is not achieved by HIFα-induced recruitment of the NEDD8 E3 ligase Rbx1 to the ECV complex; in contrast, Rbx1 is associated with the ECV irrespective of substrate engagement. Notably, we found HIF1α to directly bind to UbcH12, the NEDD8 conjugating enzyme involved in the Cul2 neddylation reaction. Therefore, we propose that HIF1α recruits UbcH12 to the ECV and thus induces Cul2 neddylation. Whether HIF1α engagement to the ECV indeed results in increased associated UbcH12 levels has to be determined. This could be addressed by performing an experiment similar to the one shown in Fig.A.2.C. Specifically, endogenous UbcH12 levels associated with transiently transfected VHL could be compared to VHL-associated UbcH12 levels upon HIF1α co-expression. As HIF2α and HIF3α also lead to Cul2 neddylation upon ECV-engagement, their effect on amounts of ECV-associated UbcH12 could also be assessed. In addition, HIF1α mutants not able to engage to UbcH12, such as HIF1α(ODD) should be tested for their ability to induce Cul2 neddylation, compared to full-length HIF1α. An experiment similar to the one illustrated in Fig.A.1. could be performed, comparing the neddylation profile of VHL-associated Cul2 upon co-expression of HIF1α, or HIF1α(ODD).
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