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BSc in Biology

Studies of the Regulation of the MYC Oncoprotein by the VHL Tumor Suppressor Protein

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To my cherished family and friends, whose unwavering belief in me has been a source of inspiration ♥. I dedicate this to those who are battling cancer, with a special remembrance for my beloved grandpa, Opi Heini, who lost his battle to this devastating illness.

An meine geliebte Familie und meine Freunde, die immer an mich geglaubt haben und mich inspiriert haben ♥. Ich widme dies denjenigen, die gegen den Krebs kämpfen, mit einer besonderen Erinnerung an meinen geliebten Großvater, Opi Heini, der seinen Kampf gegen diese verheerende Krankheit verloren hat.

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To my boyfriend, Kons, your unwavering support, humor, motivation, and belief in me have been my constant pillars of strength. I cannot thank you enough for everything.

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ABSTRACT

This study explored the interplay between MYC, VHL and SKP2. The *MYC* oncogene is dysregulated in most human cancers, the VHL tumor suppressor is lost in many kidney cancers and SKP2 is often overexpressed in cancer. Both VHL and SKP2 are E3 ubiquitin ligase components. Previous findings suggest that MYC interacts with both SKP2 and VHL in cancer cells, but the biological roles of these interactions are still unclear.

Here, we hypothesized that SKP2 and VHL might compete for MYC binding. By transient overexpression in cells followed by co-immunoprecipitation, I confirmed MYC interaction with VHL and SKP2. However, co-transfections of MYC, VHL, and SKP2 showed that SKP2 rather than competing seemed to favor formation of the MYC-VHL complex, and also interacted with VHL, suggesting possible formation of a ternary complex.

Additionally, I utilized site-directed mutagenesis to generate single and double mutations of amino acids implicated in MYC-VHL interactions based on NMR data. So far, none of these fully disrupted the interaction, but ongoing work hopefully will provide mechanistic insights into the interaction.

This research has advanced our understanding of the interplay between MYC, VHL and SKP2, and will hopefully contribute to new therapeutic possibilities for cancer treatment in the future.

RESUMO

Este estudo explorou a interação entre MYC, VHL e SKP2. O oncogene MYC é frequentemente desregulado na maioria dos cânceros humanos, o supressor de tumor VHL está ausente em muitos cânceros renais e o SKP2 é frequentemente superexpresso em cânceros. Tanto o VHL quanto o SKP2 são componentes da E3 ubiquitin ligase. Estudos anteriores sugerem que o MYC interage com ambos SKP2 e VHL em células cancerígenas, mas as funções biológicas dessas interações ainda são incertas.

Neste estudo, hipotetizamos que SKP2 e VHL poderiam competir pela ligação ao MYC. Por superexpressão transitente em células seguida de co-imunoprecipitação, confirmei a interação do MYC com VHL e SKP2. No entanto, co-transfeções de MYC, VHL e SKP2 mostraram que SKP2, em vez de competir, parecia favorecer a formação do complexo MYC-VHL e também interagiu com VHL, sugerindo a possível formação de um complexo ternário.

Além disso, utilizei mutagênese dirigida para gerar mutações únicas e duplas de aminoácidos envolvidos nas interações MYC-VHL com base em dados de RMN. Até o momento, nenhuma delas interrompeu completamente a interação, mas o trabalho em curso, esperançosamente, fornecerá conhecimentos dos mecanismos de interação.

Esta pesquisa avançou o nosso conhecimento da interação entre MYC, VHL e SKP2 e, esperançosamente, contribuirá para novas possibilidades terapêuticas para o tratamento do câncer no futuro.

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INTRODUCTION

1.1 Cancer

Cancer is a complex and often deadly disease caused by the abnormal growth and spread of cells that can develop in any part of the body. The incidence of cancer is increasing globally, making it one of the leading causes of death worldwide (World Health Organization, 2022). Cancers are traditionally classified based on the originating organ or tissue, but more recently, they are also categorized based on molecular characteristics of cancer cells (Kriegshoff-Henning et al., 2017)

Cancer development arises from genetic, epigenetic, and environmental factors that bring about crucial changes in cellular physiology, driving malignant growth (Hanahan & Weinberg, 2000). These changes, known as cancer hallmarks, provide fundamental insights into tumor complexity. These hallmarks, shown In Figure 1, include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis (blood vessel formation), and promoting invasion and metastasis. In 2011, two emerging hallmarks were recognized and have since been validated as core components: deregulating cellular metabolism and avoiding immune destruction (Hanahan & Weinberg, 2011). Additionally, two enabling characteristics that play roles in activating these hallmarks were identified: genome instability and mutation, as well as tumor-promoting inflammation. In 2022, the framework was further expanded to include two additional emerging hallmarks: "unlocking phenotypic plasticity" and "senescent cells." Additionally, two more enabling characteristics were introduced: "nonmutational epigenetic reprogramming" and "polymorphic microbiomes" (Hanahan, 2022). The development of new cancer therapies will increasingly benefit from the broad relevance of these concepts. Importantly, tumors consist not only of cancer cells but also a diverse range of cells in the tumor microenvironment (Hanahan, 2022).

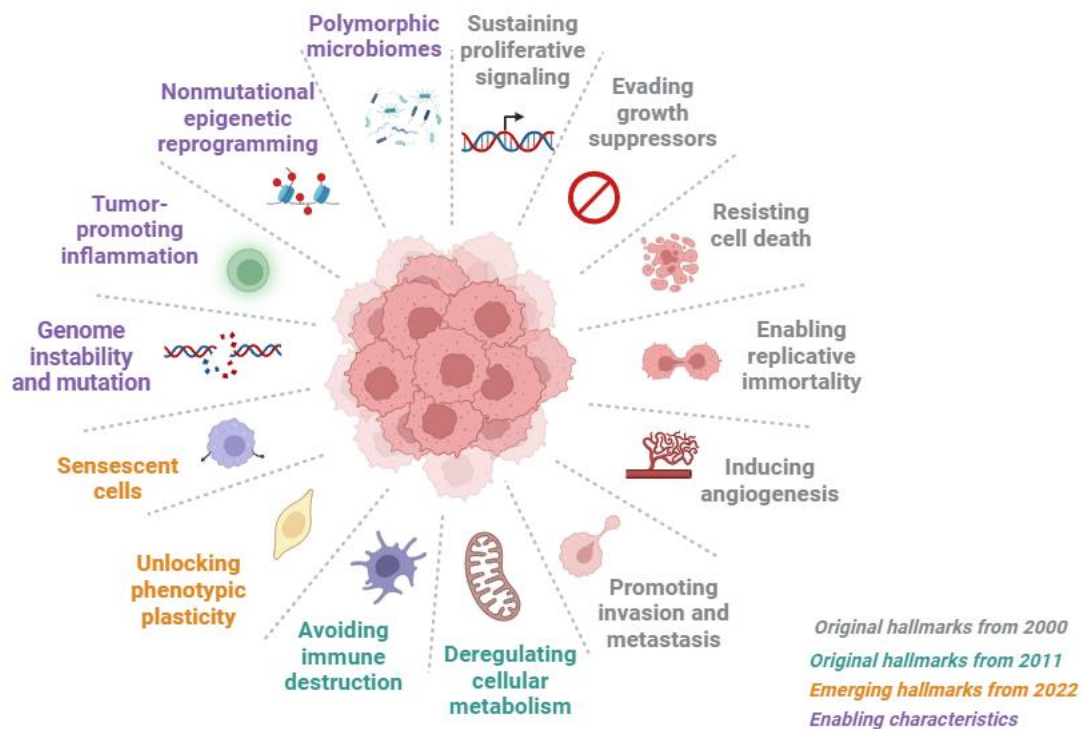


Figure 1: Hallmarks of Cancer: new dimensions. The different hallmarks of cancer described during the last two decades

Evolving landscape of cancer hallmarks over two decades, featuring the hallmarks identified in the years 2000, 2011, and 2022. Additionally, enabling characteristics that contribute to cancer progression are highlighted.

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In 2020, there were roughly 19.3 million new cases of cancer and nearly 10 million deaths caused by cancer worldwide. The likelihood of experiencing cancer at some point in life is approximately 20%, accompanied by an associated risk of about 10% for cancer-related mortality (Ferlay et al., 2021). Notably, cancer ranks as a major global public health concern and a leading cause of death worldwide. The worldwide cancer burden is projected to rise significantly to 28.4 million cases by 2040, with the most substantial increase observed in transitioning countries (Siegel et al., 2022; Sung et al., 2021).

Despite vast amounts of research, the root causes and contributing factors of cancer are not fully understood. Traditional treatment methods, including surgery, chemotherapy, radiotherapy, and immunotherapy, often fail due to drug resistance, intra-tumor heterogeneity, and a lack of personalized treatment options. Researchers must focus on overcoming these challenges, analyzing existing gaps in knowledge, and continuously assessing new developments to create better and more comprehensive treatment plans that may one day lead to a cure (Upadhyay, 2021).

Cancer is a genetic disease triggered by gene alterations that influence cell function, especially growth and division. These changes arise from cell division errors, exposure to harmful substances, or inherited genetic mutations (National Cancer Institute, 2021). Cancer results from the accumulation of mutations, driving uncontrolled growth, invasion, and metastasis (Morales et al., 2002; Wei Dai, 2014). It primarily stems from somatic mutations, requiring multiple mutations for full development (Vogelstein & Kinzler, 1993). Understanding the genetic basis of cancer is crucial for effective treatments and prevention.

1.2 Oncogenes and tumor suppressor genes

Cancer results from uncontrolled cell division and growth due to genetic changes in genes that encode growth-regulating proteins. These cancer-related genes are divided into two groups: Proto-oncogenes code for proteins that facilitate cellular growth and, when activated by mutations, can transform normal cells into cancerous ones. Tumor suppressor genes (TSGs) encode proteins that play crucial roles in DNA damage repair, inhibition of cell division, induction of apoptosis, and suppression of metastasis. Loss of TSG function can lead to the onset and progression of cancer (Chow, 2010; L. H. Wang et al., 2019). It typically involves mutations reducing tumor suppressor function and enhancing proto-oncogene activity, leading to heterogeneous tumor cells that resist normal death signals. These cells exhibit uncontrolled growth and invasive potential due to genetic and epigenetic shifts (Chial, 2008b).

1.2.1 Oncogenes

Oncogenes are genes that code for proteins that, when they are deregulated, can transform normal cells into cancerous ones. Those proteins are often involved in stimulating proliferation, cell signaling, inhibition of differentiation and blocking cell senescence and apoptosis induction. Proto-oncogenes are the normal versions of these genes, but mutations can permanently activate them, leading to the formation of oncogenes. Chromosomal translocations and gene amplification are two other ways that proto-oncogenes can become oncogenes (Chow, 2010).

One example of a prototypic oncogene is *MYC*. *MYC* is a potent driver of cell proliferation, activated by multiple signal transduction pathways. Deregulated *MYC* is frequently involved in human cancers (Shortt & Johnstone, 2012).

Mutations in proto-oncogenes are typically dominant. Cancer cells may rely on certain oncogenic mutations more heavily than others for their growth, proliferation, and survival. This concept is called oncogene addiction. By targeting the activity of specific oncogenes, cancer cells can in many cases be effectively eliminated. The theory of oncogene addiction is

supported by the success of imatinib in treating chronic myelogenous leukemia, but drug resistance remains a concern. It is necessary to continue to develop approaches for targeting oncogene activity in the ongoing war on cancer (Chial, 2008a).

1.2.2 Tumor Suppressor Genes

Tumor suppressor genes (TSGs) are genes that regulate several cellular functions and act to prevent the development of tumors (Joyce et al., 2022). They function to inhibit cell growth, division, and proliferation and stimulate cell death to keep cells in proper balance. Additionally, certain tumor suppressor genes also contribute to the prevention of cancer by participating in DNA repair mechanisms, which serve to prevent the accumulation of mutations in cancer-related genes (Chial, 2008b).

Loss of tumor suppressor gene function, which can occur through loss of function mutations, gene deletion and epigenetic silencing, can lead to dysregulated cell growth, a well-known mechanism for the development of cancers. There are many types of cancers associated with loss of function mutations in tumor suppressor genes, and even familial cancer syndromes associated with the loss of function germline mutations of specific tumor suppressor genes (Joyce et al., 2022).

Tumor suppressor genes can be broadly classified into five types based on their function: genes that encode intracellular proteins that control cell cycle progression, genes that encode receptors or signal transducers that inhibit cell proliferation, genes that encode checkpoint-control proteins that trigger cell cycle arrest in response to DNA damage or chromosomal defects, genes that encode proteins that induce apoptosis, and genes that encode proteins involved in repairing mistakes in DNA (L. H. Wang et al., 2019).

There are currently 73 known tumor suppressor genes that may contribute to cancer development, with the possibility of discovering more in the future (Healthline, 2022).

The central tumor suppressor gene that holds importance for this thesis is the von Hippel-Lindau gene (*VHL*). It encodes the substrate-binding subunit of the ubiquitin ligase complex VCB that degrades hypoxia-inducible factors (HIFs), which regulate gene expression in response to low oxygen. Mutations raise cancer risk, including renal cell carcinoma (Joyce et al., 2022).

Tumor suppressor genes are frequently mutated in cancer. These mutations are typically recessive, requiring both gene copies to be altered for malignancy - a concept called the "two-hit" hypothesis. The initial hit can be a somatic mutation, and the second often involves loss of heterozygosity, deactivating the remaining functional gene copy. Most TSGs discovered to date follow this model, with both alleles being deleted, mutated, or epigenetically silenced in cancer (Chial, 2008b; Joyce et al., 2022). Also aberrant transcription factor (TF) regulation and mislocalization of TSGs can interfere with their normal function and lead to abnormal cell

growth and division. That's why a revised 'multiple-hit' model is proposed, which includes non-genetic/epigenetic events such as transcriptional regulation, proteasome degradation, or abnormal nucleocytoplasmic shuttling. This could suggest novel targets for anti-cancer therapy (L. H. Wang et al., 2019).

1.3 MYC

MYC was first identified as a viral oncogene, known as v-*MYC*, carried by some avian retroviruses that cause **myelocytomatosis**, a type of leukemia. The cellular homolog (*MYC* or c-*MYC*) was subsequently discovered in chicken cells, and later, the human homolog of the *MYC* gene was identified in Burkitt lymphoma. It was found to be altered and over-expressed in various other types of cancer, making it an important area of research in cancer biology for several decades (Dalla-Favera et al., 1982; Lee & Reddy, 1999; Sheiness & Michael Bishop, 1979; Vennstrom et al., 1982).

1.3.1 MYC as a Transcription Factor

Transcription is the first step in gene expression, during which a DNA template is used to generate a primary RNA transcript that codes for a protein molecule that execute biological function in the cell. In other scenarios, the transcribed RNA molecule themselves can themselves exert biological functions.

Transcription factors are regulatory proteins that are tightly controlled and have a crucial role in gene expression and RNA biology. They recognize specific consensus sequences on DNA, which allows them to remodel chromosomes and drive the transcription of target genes. By regulating the synthesis of RNA molecules, transcription factors play a central role in various biological processes (Kress et al., 2015; Lambert et al., 2018; Latchman, 1993).

MYC proteins are transcription factors that have a specific domain called the basic region/helix-loop-helix/leucine zipper (bHLHZip) at their carboxyterminus, which is essential for their ability to bind DNA at specific DNA sequences 5-CACGTG-3, known as E-boxes located in the promoter or enhancer regions of target genes. To bind DNA, bHLHZip proteins need to dimerize, but MYC homodimerization doesn't occur under normal conditions. Therefore, MYC binds with another bHLHZip protein called MYC-associated protein X (MAX) to form a stable heterodimer that can bind DNA at E-boxes (Figure 2) (Amati et al., 1992; Murre et al., 1989; Singh et al., 2022; Tu et al., 2015).

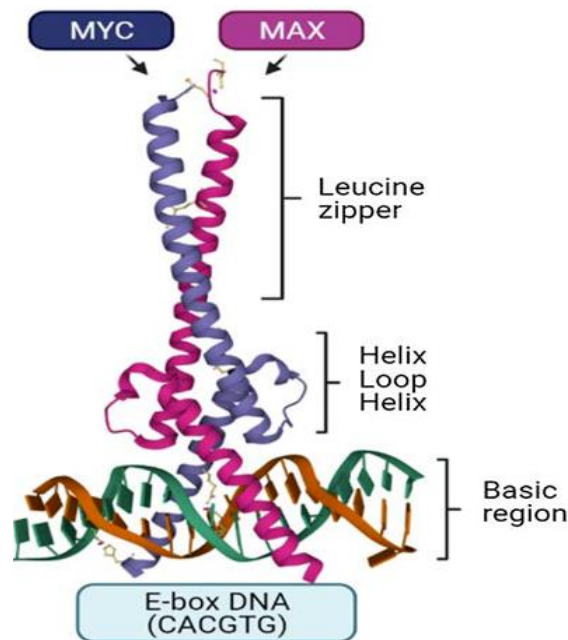


Figure 2: MYC:MAX Binding to E-boxes

This figure presents the crystal structure of the MYC:MAX heterodimer bound to E-box DNA sequences, illustrating the precise molecular interactions. MYC and MAX proteins are shown in their dimeric state, highlighting the essential bHLHZip domain for DNA binding. Reprinted from Ahmadi et al., (2021)

After binding to the chromatin, the MYC complex recruits a plethora of other transcription and chromatin regulatory factors, enabling the activation or repression of gene transcription (Frank et al., 2003). This wide range of protein-protein interaction networks allows MYC to regulate a vast range of biological processes such as cell cycle, apoptosis, metabolism, self-renewal, and differentiation (Figure 3). Thus, deregulation of MYC family of oncoproteins is considered a hallmark of cancer (Kalkat et al., 2018).

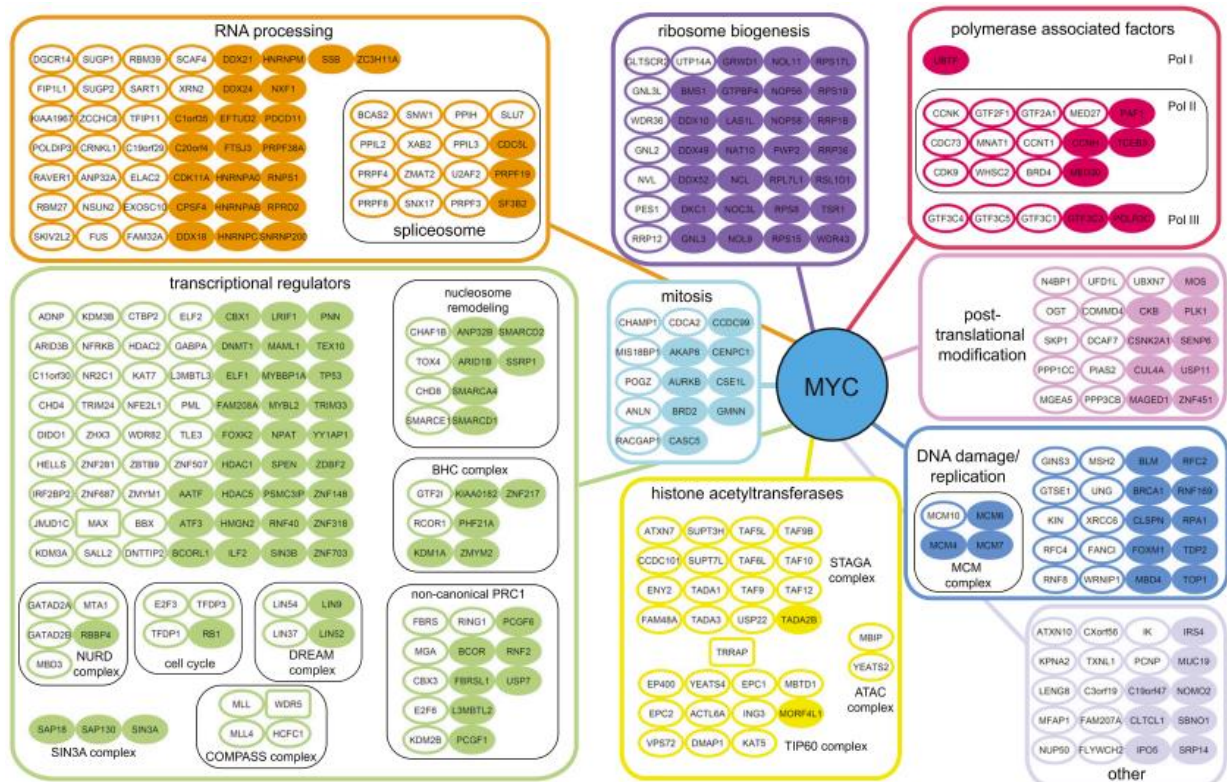


Figure 3: MYC interaction network

MYC regulates a wide range of cellular functions. It controls a large number of protein-coding or non-coding genes that participate in distinct cellular processes, including cell cycle, protein biogenesis, cell adhesion, metabolism, signal transduction, transcription, and translation, among others. Reprinted from Kalkat et al. (2018)

MYC is a versatile transcription factor that can activate and repress gene transcription by binding DNA and interacting with cofactors. In contrast to typical transcription factors, MYC not only stimulates gene expression by binding DNA sequences but also enhances active gene transcript production by controlling transcriptional pause release, recruiting elongation factors, and modifying chromatin. These actions impact essential cellular processes like proliferation, explaining MYC's extensive oncogenic role in cancer. MYC regulates 15% of human genes involved in cell cycle progression, growth, apoptosis, senescence, and metabolism. Thus, its deregulation can lead to tumorigenesis by disrupting the normal cellular balance (Conacci-Sorrell et al., 2014; Das et al., 2023; Fletcher & Prochownik, 2015; Lovén et al., 2012; Rahl et al., 2010; Rahl & Young, 2014).

In addition to its role in gene activation, MYC can also regulate gene repression. It can achieve this by several different mechanisms. One way is that MYC directly binds to the promoter of target genes, which can interfere with the binding of transcriptional activa-

tors or coactivators. MYC can also recruit co-repressor complexes that modify chromatin structure and inhibit transcription. For example, MYC can interact with the MIZ-1 protein to recruit repressive complexes that contain HDACs (histone deacetylases), leading to chromatin condensation and gene repression (Kleiner-Kohlbrecher et al., 2006). Also the Interaction between MYC and the tumor suppressor protein VHL utilizes this mechanism for gene repression (Hwang et al., 2012).

Moreover, MYC can regulate the expression of miRNAs that are involved in the repression of target genes, including those that play a role in cell cycle arrest and apoptosis (Gartel & Shchors, 2003).

1.3.2 MYC Structure and Interaction with Cofactors

The c-MYC protein consists of 439 amino acid residues and contains functional domains that facilitate interactions with various binding partners. Besides the bHLHZip domain at the C-terminus that binds DNA with MAX at E-boxes in a heterodimeric complex, as described above, MYC proteins have several conserved MYC homology boxes (Figure 4).

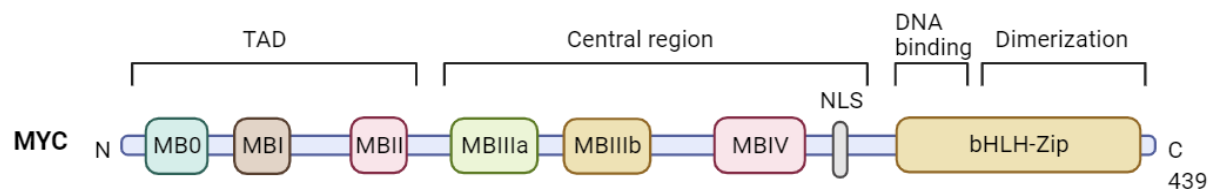


Figure 4: Schematic representation of MYC Structure

This schematic representation illustrates the structural features of the MYC protein, which spans 439 amino acids from its N-terminal to C-terminal regions. Key structural elements, including the MYC boxes and the basic helix-loop-helix leucine zipper (bHLHZip) domain, are highlighted. Created with Biorender.com.

The N-terminal region contains a transcription transactivation domain (TAD) with three MYC boxes (MB0, MBI, MBII) that play a crucial role in transcriptional regulation and protein degradation, critical for processes such as transformation, differentiation, and apoptosis. This region serves as a site for interactions with proteins involved in chromatin and histone modification, as well as proteins mediating ubiquitination and subsequent degradation (Barrett et al., 1992; Conacci-Sorrell et al., 2014; Dang & Lee W. M., 1988; Farrell & Sears, 2014; Kato et al., 1990; Y. Li et al., 2020; Tu et al., 2015).

Two phosphorylation sites, namely Threonine 58 (T58) and Serine 62 (S62), present within MBI, are crucial for regulating the stability and activity of MYC in response to cellular growth signals (Y. Chen et al., 2019; Farrell & Sears, 2014; Y. Li et al., 2020).

Both MB0 and MBII play important roles in MYC-mediated tumorigenesis. Specifically, MB0 is involved in interactions with transcription elongation factors, as it directly binds to the general transcription factor TFIIF, and it is a key factor in accelerating tumor growth. On the other hand, The MBII region within the MYC transactivation domain is a critical hub for interacting with various proteins, including histone acetyltransferase (HAT) complexes. These interactions promote histone acetylation and gene activation, making MBII important for most MYC activities. Additionally, MBII plays a role in MYC protein turnover by serving as a docking site for E3 ligases like SKP2 and Fbw7, which target MYC for degradation (Conacci-Sorrell et al., 2014; Hydbring et al., 2017; Kalkat et al., 2018).

A noteworthy MYC binding partner in this study is the S-phase kinase-associated protein 2 (SKP2), an F-box protein that forms a part of the SCF (Skp1-Cul1-F-box protein) family of ubiquitin ligase complexes. These complexes play an important role in regulating the stability of proteins by targeting them for degradation via the ubiquitin-proteasome pathway. SKP2 thereby modulates cell growth and division. SKP2-mediated ubiquitination of MYC has been shown to have a dual role, leading to its degradation as well as stimulating its transcriptional activity. This is critical for maintaining the balance between MYC stability and activity, which in turn affects cell growth and proliferation. SKP2 binds to specific regions within MYC, namely residues 129-147 in MBII in the amino terminus, and 379-418 in the bHLHZip region in the carboxy terminus. Furthermore, MYC itself upregulates the expression of SKP2 by binding to the E box in its promoter. The close interdependence between c-MYC and SKP2 in regulating transcription suggests a complex interplay between transcriptional activation and protein degradation. SKP2 is overexpressed in various human tumors and is linked to c-MYC overexpression and poor prognosis, making it a potential therapeutic target for cancer treatment (Bretones et al., 2011; Farrell & Sears, 2014; Hann, 2014; Hydbring et al., 2017; S. Y. Kim et al., 2003; von der Lehr et al., 2003).

The central region has a PEST domain, a nuclear localization sequence (NLS), and three other MYC boxes (MBIIIa, MBIIIb, and MBIV) that play a role in transforming activity, transcription, and apoptosis (Madden et al., 2021).

1.3.3 MYC and Cancers

The relationship between the *MYC* gene and cancer has been the subject of extensive research and has revealed intriguing insights into the molecular mechanisms underlying cancer development and progression. The aberrant activation or overexpression of the *MYC* oncogene, through mechanisms such as chromosomal translocation, gene amplification, increased transcription, and enhanced protein stability, has been demonstrated to be a major contributor to the development and progression of a wide range of human cancers, accounting for more than 50% of cases (Adhikary et al., 2005; Ahmadi et al., 2021; Tansey, 2014).

The discovery of MYC's role in cancer began with Burkitt's lymphoma, an aggressive B cell cancer. This cancer involves the *MYC* gene translocating from chromosome 8 to chromosome 14 (IGH locus), causing *MYC* overexpression due to disrupted regulation. This genetic change drives uncontrolled cell growth and proliferation by impacting crucial cellular processes, highlighting MYC's significant role in oncogenesis (Dalla-Favera et al., 1982; Taub et al., 1982).

The amplification of the *MYC* gene is another cause of MYC overexpression and was initially discovered in the human leukemia cell line HL60 in 1982 (S. Collins & Groudine, 1982). In fact, c-*MYC* stands out as the most frequently amplified oncogene in human cancer, contributing to its prevalent overexpression in various cancer types (Beroukhi et al., 2010; Duffy et al., 2021; Nakagawa et al., 2015; Nesbit et al., 1999).

MYC rarely undergoes point mutations, except in lymphomas. However, apart from amplification and translocation, MYC deregulation can occur through various mechanisms (Poole & van Riggelen, 2017). This includes deregulation of upstream signaling pathways, such as constitutive activation of growth factor signaling, loss of checkpoint components like p53, and loss of E3 ubiquitin ligases that target MYC. Studies indicate that over 50% of human cancers exhibit increased or deregulated MYC expression. Furthermore, MYC overexpression is frequently associated with aggressive and poorly differentiated tumors, leading to a poor prognosis and low survival rate in patients (Gabay et al., 2014; Kalkat et al., 2017).

1.3.4 MYC as a Cancer Target

Abnormal activation of transcription factors (TFs) is a common feature in cancer and can result in dysregulation of critical cellular signaling pathways, leading to uncontrolled cell growth and invasion. As a result, TFs have become an attractive target for developing new cancer therapies (Carroll et al., 2018; Kress et al., 2015; Lambert et al., 2018).

Deregulated expression of *MYC* is frequently observed in various tumor types and is associated with unfavorable prognostic outcomes and increased resistance to chemotherapy. MYC exerts control over a wide array of genes involved in critical cellular processes that contribute to cancer cell survival, proliferation, and invasiveness. As a result, MYC has emerged as a promising target for cancer therapy (H. Chen et al., 2018; Dang, 2012; Duffy et al., 2021; C. Wang et al., 2021).

MYC downregulation has been shown to result in growth arrest, increased apoptosis, differentiation, and tumor regression in various human cancer models (Felsher, 2010). However, despite extensive efforts over the past 20 years, there is currently no approved MYC inhibitor for cancer treatment. MYC has been considered an undruggable protein for several reasons. Firstly, it lacks a binding pocket typically targeted by drugs in the process of drug discovery. Secondly, inhibiting MYC, given its broad effects on essential cellular processes, may lead to

toxicity and significant side effects on normal cells and tissues, which can hinder the success of candidate compounds in clinical trials. Thirdly, MYC is a nuclear transcription factor with an intrinsically disordered structure until it forms a dimer with its partner protein, making it challenging to target (Carabet et al., 2019; Whitfield et al., 2017).

Despite these challenges, several direct and indirect strategies have been developed to target MYC (Figure 5). These strategies include disrupting MYC stabilization and dimerization with MAX, inhibiting MYC transcription, and interfering with processes induced by MYC (Whitfield et al., 2017). Some of these approaches are discussed below.

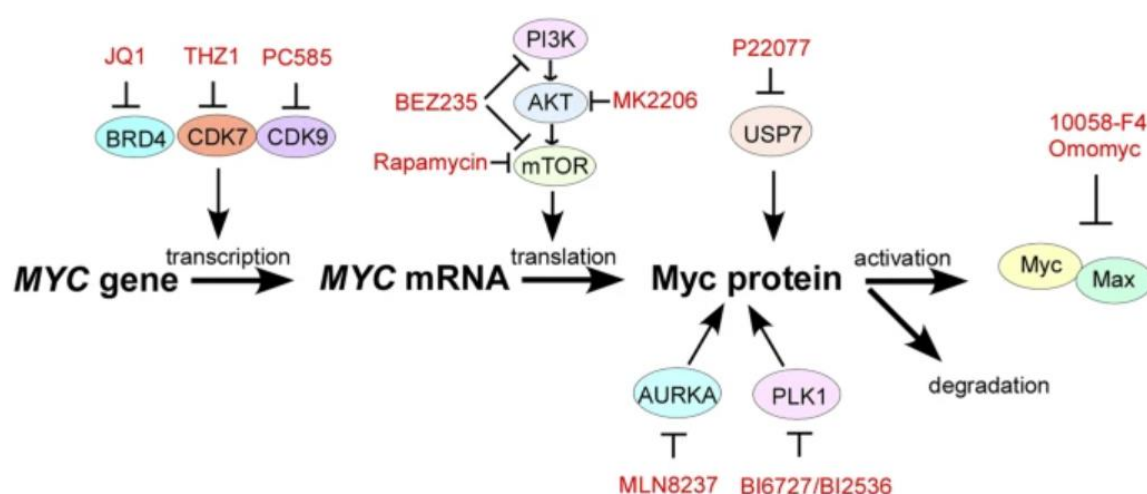


Figure 5: Targeting MYC - Multifaced Approaches

Overview of diverse strategies to target Myc at multiple levels of Myc regulation. Several inhibitors, including BRD4, CDK7, and CDK9 inhibitors, act to inhibit MYC expression at the transcriptional level. The PI3K/AKT/mTOR pathway inhibitors block MYC translation, disrupting protein synthesis. Inhibitors targeting USP7, AURKA, and PLK1 destabilize Myc at the post-translational level. Compounds like 10058-F4 and Omomyc function to interrupt the Myc–Max dimeric complex. Reprinted from H. Chen et al., (2018)

Indirect strategies to target MYC involve disrupting its transcription and protein stability. Bromodomain and extra-terminal domain (BET) inhibitors bind to bromodomains of BET-proteins, like BRD4, inhibiting MYC transcription (Brondfield et al., 2015; Ghurye et al., 2015; Puisant et al., 2013). Numerous BET inhibitors are in phase I-II clinical trials for various cancers (Doroshov et al., 2017).

Modulating MYC stability through PP2A activation shows promise across cancer types, alone or combined with other methods (Allen-Petersen et al., 2019; Kauko et al., 2018).

Targeting proteins in MYC's ubiquitination and deubiquitination for degradation is also being investigated as a strategy to regulate MYC protein stability (Schauer et al., 2020; Tavana et al., 2016; Y. Zhang et al., 2018).

Another approach are MYC:MAX inhibitors. Several small molecules inhibiting MYC-MAX dimerization are under investigation. These molecules aim to disrupt the interaction between MYC and MAX, which is crucial for their functionality. These inhibitors have shown promise in affecting cancer cell growth and immune responses. Improved versions of these molecules have exhibited enhanced effectiveness. This approach holds potential for altering MYC's role in cancer and modulating immune cell behavior (Castell et al., 2018; Chauhan et al., 2014; Han et al., 2019; Hart et al., 2014; Müller et al., 2014; H. Wang et al., 2007; Whitfield et al., 2017).

One promising MYC:MAX inhibitor is OmoMYC, a 90-amino acid peptide derived from MYC's bHLH-Zip domain with specific modifications (Soucek Vall D' & Nasi, 2002). It forms selective heterodimers with MAX or homodimers, acting as a dominant negative to obstruct MYC's DNA binding to E-boxes (Beaulieu et al., 2019; Demma et al., 2019). As the first MYC-targeted drug to complete phase I, OmoMYC is prepared to advance to phase II in the near future (clinicaltrials, 2022; Massó-Vallés et al., 2022; Peptomyc, 2022).

1.4 E3 Ubiquitin Ligases

Ubiquitin ligases exert control over cellular processes by modifying proteins through the addition of ubiquitin molecules to lysine residues or to the N-terminus. This post-translational modification directs signals to various cellular processes, including protein degradation via the proteasomal pathway, thus influencing most of cellular processes such as cell cycle progression, transcriptional regulation, DNA repair, and signal transduction. Ubiquitination plays a crucial role in these processes by modulating protein function and fate, thereby contributing to the overall regulation of cellular activities (Morreale & Walden, 2016).

The process of protein ubiquitination is a highly controlled enzymatic pathway that involves three different enzymes with distinct roles. These enzymes include the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the E3 ubiquitin ligase (E3). In humans, there are over 600 known E3 ubiquitin ligases (George et al., 2018; Meng et al., 2021).

E3 ubiquitin ligases, also known as E3 ligases, can be categorized into three distinct families based on their structure and function. These families include the RING finger family (RING E3s), the HECT (homologous to the E6-associated protein carboxyl terminus) domain family, and the RING in-between RING (RBR) E3 ubiquitin ligases. The RING E3s within the RING finger family catalyze the direct transfer of ubiquitin from the E2 enzyme to the substrate. In contrast, the HECT domain family and RBR E3 ligases indirectly transfer ubiquitin to the substrate (Buetow & Huang, 2016; Meng et al., 2021). (Figure 6)

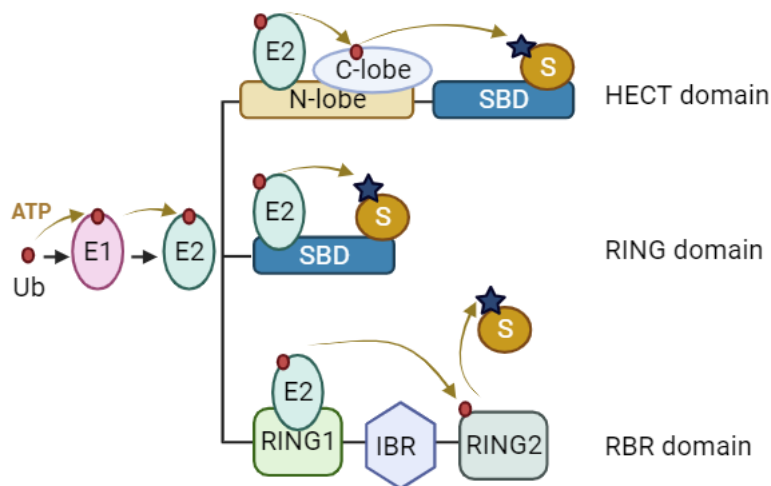


Figure 6: Mechanisms of Ubiquitination by E3 Ligase Families

This figure provides an overview of three major E3 ubiquitin ligase families: RING finger, HECT, and RBR. Each family employs distinct mechanisms to catalyze ubiquitination. RING finger ligases directly facilitate ubiquitin transfer from E2 enzymes to target substrates by acting as scaffolds, bringing them into proximity for direct ubiquitin transfer. HECT ligases initially accept ubiquitin from E2 enzymes onto a conserved cysteine in their N-lobe. They then indirectly catalyze ubiquitination by transferring ubiquitin to the substrate. RBR ligases blend RING finger and HECT features. They accept ubiquitin from E2 enzymes onto a cysteine in their RING1 domain, followed by HECT-like ubiquitin transfer to the substrate. Created with Biorender.com.

The VHL E3 ligase serves as an example within the RING finger family. It forms stable complexes with elongin B, elongin C, Cul2, and Rbx1/ROC1/Hrt1, which are crucial for its function (Meng et al., 2021; M. Wang et al., 2020). VHL acts as a substrate recognition unit and is known for targeting specific proteins for polyubiquitination. Its prominent role is the degradation of hypoxia-inducible factor (HIF) (Ivan & Kaelin Jr., 2001).

1.5 The von Hippel-Lindau (VHL) Tumor Suppressor Protein

1.5.1 von Hippel-Lindau Disease and clear cell Renal Cell Carcinoma (ccRCC)

von Hippel-Lindau disease (VHL disease) is a hereditary form of cancer that follows an autosomal dominant pattern of inheritance (Dwyer & Tu, 2017; Gossage et al., 2015). It is caused by the loss of both functional copies of the *VHL* gene, located on the short arm of chromosome 3. The *VHL* gene plays a role in regulating cellular processes, including angiogenesis and oxygen sensing. Dysfunctional VHL protein can lead to abnormal blood vessel growth and contribute to tumor formation (Chittiboia & Lonser, 2015; Hudler & Urbancic,

2022). This genetic alteration leads to the development of various types of tumors, including clear cell renal cell carcinoma (ccRCC) (Chittiboina & Lonser, 2015; Dwyer & Tu, 2017; Gnarra et al., 1994; Gossage et al., 2015; Nabih et al., 2022).

The initial signs of VHL disease were first described in 1894 by Collins (E. T. Collins, 1894). Subsequently, Eugen von Hippel and Arvid Lindau provided further characterization of the disease in 1904 and 1927, respectively, leading to the canonical classification of VHL disease (Lindau, 1926; von Hippel, 1904). The estimated frequency of the genetic mutation responsible for VHL disease is approximately 1-9 newborns per 100,000 births. While symptoms can manifest in childhood, most individuals begin experiencing symptoms between the ages of 20 and 30 years (Findeis-Hosey et al., 2016; rarediseases.org, 2021). The delayed onset can be explained by the requirement of homozygous loss of the *VHL* gene for disease onset. Patients with the hereditary predisposition to VHL disease experience symptom onset when the remaining functional copy of the gene undergoes a loss-of-function mutation event (Chittiboina & Lonser, 2015; Dwyer & Tu, 2017; Gnarra et al., 1994; Gossage et al., 2015; Nabih et al., 2022).

It is important to note that VHL disease can also occur sporadically in individuals without a family history of the condition. In sporadic cases, the random loss of both *VHL* gene copies in healthy individuals who are not carriers of the hereditary predisposition can lead to the development of non-hereditary clear cell renal cell carcinomas. These sporadic cases account for approximately 80% of all ccRCC cases (Chittiboina & Lonser, 2015; Dwyer & Tu, 2017; Gnarra et al., 1994; Gossage et al., 2015; Nabih et al., 2022).

Clear cell renal cell carcinoma (ccRCC) is the most common and aggressive form of kidney cancer, accounting for 75% of all cases. It accounts for the sixth most common cancers diagnosed in men and the ninth in women (Siegel et al., 2022). Approximately 90% of all cases of ccRCC involve the loss of the *VHL* tumor suppressor gene, making it the most common genetic mutation associated with this type of kidney cancer (Lindgren et al., 2017). The *VHL* gene produces the pVHL protein, which regulates the response to low oxygen levels. In ccRCC, VHL mutations lead to the accumulation and activation of hypoxia-inducible factors (HIFs). This results in the upregulation of genes involved in angiogenesis, cell growth, and metabolism, promoting tumor development and progression (Gnarra et al., 1994; Kaelin, 2022; Kaelin & Farber Professor, 2019; H. S. Kim et al., 2018; W. Y. Kim & Kaelin, 2004; Semenza, 2021).

However, while loss of VHL function is a critical factor in the development of clear cell renal cell carcinoma (ccRCC), it is not sufficient on its own to drive tumorigenesis. Additional genetic and epigenetic alterations are typically required for the progression of ccRCC (Jonasch et al., 2021; H. S. Kim et al., 2018).

For instance, in addition to the loss of chromosome 3p, focal amplification of the *MYC* gene at 8q24 has been identified in clear cell renal cell carcinomas (ccRCCs) (Beroukhim et al., 2009; Creighton et al., 2013). Amplification of *MYC* is a common genetic alteration observed in ccRCC (approximately 15%), suggesting its potential role in the development and progression

of the disease. MYC amplification may contribute to the dysregulation of cellular processes and promote tumor growth and aggressiveness in ccRCC (Bailey et al., 2017; Kalkat et al., 2017; Shroff et al., 2015).

Interestingly, there is a complex interplay between VHL and MYC in ccRCC. pVHL hinders the expression of c-MYC by enlisting HDACs to the c-MYC promoter, leading to transcriptional repression. This interaction is critical for the auto-suppression of c-MYC and maintaining proper levels of its expression. In cases of clear cell renal cell carcinoma (ccRCC), VHL gene mutations frequently occur, causing the activation of the MYC pathway and the development of highly metastatic and vascularized cancer. This interplay between VHL and MYC further fuels tumor growth, contributing to the aggressiveness of ccRCC (Bailey et al., 2017; Hwang et al., 2012).

Recent findings and initial data from the Larsson laboratory suggest that the interaction between the MYC oncoprotein and the VHL tumor suppressor protein/E3 ligase plays a crucial role in regulating MYC-target genes. Understanding this MYC-VHL interaction could reveal vulnerabilities in MYC-driven cancers that can be targeted therapeutically.

1.5.2 Structure of VHL

The *VHL* gene, identified as a tumor suppressor gene in 1993 (Latif et al., 1993), is located on the shorter arm of chromosome 3 at position 3p25 (Dwyer & Tu, 2017; Kondo & Kaelin, 2001). It encodes three isoforms of the VHL protein: pVHL213, pVHL160, and pVHL172. These isoforms differ in their amino acid length (Figure 7). While pVHL213 and pVHL160 have tumor suppressive functions, pVHL172, on the other hand, has been found to be enriched in certain clear cell renal cell carcinomas (ccRCCs) and has a tumor-enhancing function (Blankenship et al., 1999; Hascoet et al., 2017; Hudler & Urbancic, 2022; Iliopoulos et al., 1998; Schoenfeld et al., 1998).

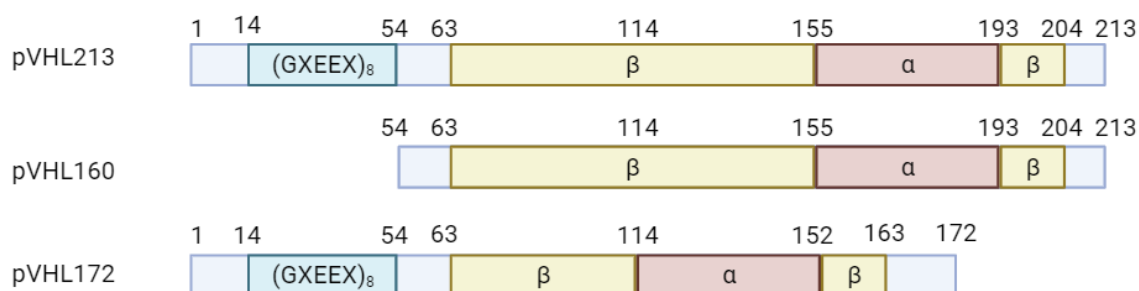


Figure 7: VHL Protein Isoforms

This figure showcases the three primary isoforms of the von Hippel-Lindau (VHL) protein: VHL213, VHL160, and VHL172. It depicts the VHL protein structure, highlighting key components including the (GXEEX)₈ repeat motif (in blue), the α-domain (in red), and the β-domain (in yellow). Created with Biorender.com.

All three isoforms of the VHL protein are part of a larger E3-ubiquitin ligase complex called the VCB-CR complex, or VHL complex. This multiprotein complex includes the substrate recognition subunit pVHL (VHL), the adaptor proteins elongin B and elongin C, as well as the catalytic RING protein Rbx1 with the adapter protein Cullin-2 (Figure 8). The VHL complex belongs to the group of cullin-RING E3 ubiquitin ligase complexes, which have the ability to target specific proteins for degradation by poly-ubiquitination, ultimately leading to their degradation by the 26S proteasome. This process is facilitated by the E2 ubiquitin-conjugating enzyme ((Berndsen & Wolberger, 2014; Cai & Yang, 2016; Sarikas et al., 2011; Zimmerman et al., 2010).

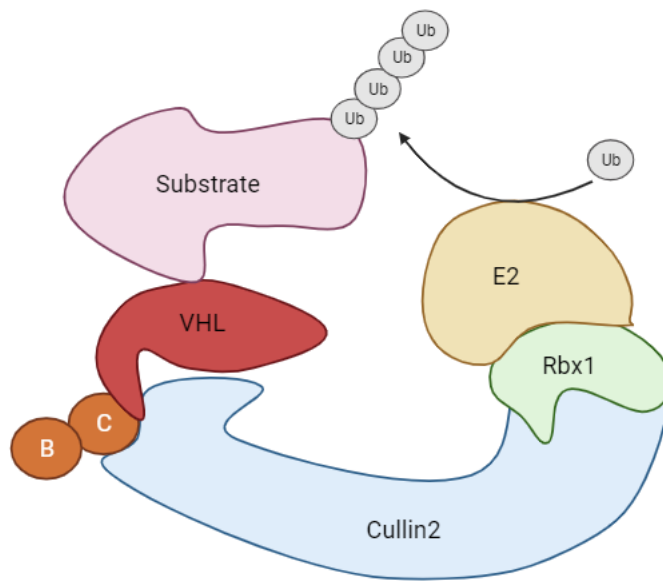


Figure 8: VHL Complex

This figure illustrates the molecular components of the VCB-CR (VHL complex). Cullin2 (Blue) serves as a scaffold and forms the structural framework for the complex. Elongin B and C (Orange) are integral components of the complex. VHL (Red) is the substrate recognition subunit and crucial for identifying and binding target proteins. Substrate (Pink) represents the protein undergoing ubiquitination. Rbx1 (Green) plays a role in recruiting the E2 ligase to facilitate ubiquitin transfer. E2 Ligase (Yellow) participates in the ubiquitination process. Ubiquitin molecules (Grey) are attached to the substrate as part of the ubiquitination process. Created with Biorender.com.

The VHL protein is comprised of distinct domains, namely the α -domain and the β -domain. The α -domain, situated at the N-terminal region, adopts a helical conformation characterized by multiple α -helices. It serves as a pivotal component by harboring critical binding sites essential for protein-protein interactions, such as with Elongin B/C. It also mediates its E3 ubiquitin ligase activity. Conversely, the β -domain is located at the C-terminal region and exhibits a β -sheet arrangement. This specific β -sheet architecture facilitates the engagement of the VHL protein with various partner proteins, like HIF-1 α and p53, thereby playing a crucial role in the overall functionality of the VHL complex and indispensable for its tumor suppressor function (Hwang et al., 2012; Lewis & Roberts, 2004; Tyers & Rottapel, 1999).

Gaining insight into the structure of the VHL protein is essential for comprehending its functions, its relevance to conditions such as Von Hippel-Lindau syndrome, and for guiding potential therapeutic avenues

1.5.3 VHL Function and Its Role in Cancer

The VHL protein serves as a tumor suppressor and plays a vital role in regulating various cellular processes, including oxygen sensing, cell division, and the degradation of specific proteins. Mutations in the VHL gene can lead to the loss or dysfunction of pVHL, disrupting its normal functions and contributing to the initiation and progression of several types of cancer ((Dwyer & Tu, 2017; Hudler & Urbancic, 2022; Kaelin & Farber Professor, 2019; E. Kim & Zschiedrich, 2018; H. S. Kim et al., 2018; W. Y. Kim & Kaelin, 2004).

One of the primary functions of pVHL is its involvement in the cellular response to low oxygen levels, a process known as hypoxia. Under normal conditions (Normoxia), pVHL targets HIF-proteins (HIF-1 α and HIF-2 α) for degradation. HIF-1 α and HIF-2 α contain an oxygen-dependent degradation domain (ODDD), which undergoes structural changes triggered by oxygen via PHD enzymes. This alteration facilitates their binding to the VHL-elongin B/C complex, leading to ubiquitination and subsequent degradation through the proteasomal pathway (Clifford et al., 2001; Haase, 2009; Kaelin, 2008; Kuznetsova et al., 2003; Y. Li et al., 2020; Mendoza et al., 2023). HIF-proteins play a critical role in adapting cells to low oxygen environments by activating genes involved in angiogenesis (formation of new blood vessels) and glucose metabolism. The regulatory pathway involving pVHL-mediated degradation of HIF-1 α and HIF-2 α is crucial for suppressing genes activated by hypoxia. However, the absence of oxygen during hypoxia prevents PHDs from hydroxylating HIF- α 's, leading to their stabilization. Consequently, pVHL loses its ability to target them for degradation. This disturbance results in the accumulation of HIF- α 's, leading to abnormal activation of HIF-dependent genes and the promotion of tumor growth (Arjumand & Sultana, 2012; W. Y. Kim & Kaelin, 2004; Y. Li et al., 2020; Maxwell et al., 1999; Mendoza et al., 2023; Minervini et al., 2020; Semenza, 2021). In conditions like clear cell renal cell carcinoma (ccRCC), VHL dysfunction causes targeted HIF-2 α accumulation. This disruption drives ccRCC's development, highlighting the vital role of the VHL-HIF axis in disease progression (Hoefflin et al., 2020; Jonasch et al., 2021).

In addition to its pivotal role in oxygen regulation, the VHL protein engages in a diverse spectrum of cellular processes ((Jonasch et al., 2021; M. Li & Kim, 2011; J. Zhang & Zhang, 2018). TBKIt interacts with various molecules and aids in DNA repair (An & Rettig, 2005; Espana-Agusti et al., 2017; Guo et al., 2016; Hu et al., 2020; Metcalf et al., 2014; Peri et al., 2013; Scanlon et al., 2018; H. Yang et al., 2007). Its absence can impact pathways related to cell growth and control (An & Rettig, 2005; J. Zhang et al., 2018). VHL also manages cellular structures, like intercellular junctions and extracellular matrix assembly, contributing to stability and proper cellular division (Calzada et al., 2006; Hell, Duda, et al., 2014; Hell, Thoma, et al., 2014; Hergovich et al., 2003; Ohh et al., 1998; Thoma et al., 2009). Moreover, it serves to counteract aggressive cancer behavior (Guo et al., 2016; Janku et al., 2018; Jonasch et al., 2021; J. Zhang et al., 2018; J. Zhang & Zhang, 2018). Furthermore, VHL's influence might extend to transcriptional

regulation beyond oxygen-dependent processes, potentially affecting gene elongation control (Kuznetsova et al., 2003; Mikhaylova et al., 2008). Its interactions with specific cellular proteins imply a role in cell survival and proliferation as well as oncogenic events involving fibroblast transformation (Berra et al., 1997; Diaz-Meco et al., 1996; Fedorov et al., 2002; Haase, 2009; Minervini et al., 2020). Thus, VHL's diverse roles go beyond established functions, suggesting involvement in diverse cellular processes and potential contributions to oncogenesis (Jonasch et al., 2021; Kuznetsova et al., 2003; Minervini et al., 2020).

The impact of VHL mutations is particularly evident in ccRCC. The *VHL* gene is mutated in the majority of ccRCC cases, leading to the accumulation of HIFs and subsequent activation of downstream pathways that promote tumor development. Understanding the role of VHL in ccRCC has not only shed light on the molecular mechanisms underlying this specific cancer type but has also provided valuable insights into general principles of tumor biology (Arjumand & Sultana, 2012; E. Kim & Zschiedrich, 2018; W. Y. Kim & Kaelin, 2004).

Significantly for this thesis, VHL has been found to bind and interact with MYC, through its β -substrate-binding domain, leading to non-proteasomal ubiquitination of MYC (Hwang et al., 2012). This interaction results in altered levels of MYC expression, which typically triggers a negative feedback loop that represses MYC transcription. Interestingly, VHL is proposed to participate in this auto-repressive mechanism by recruiting histone deacetylases (HDAC's) to the c-MYC promotor. This interaction is critical for maintaining proper levels of c-MYC expression. In ccRCC, *VHL* gene mutations frequently occur, causing the activation of the MYC pathway and the development of highly metastatic and vascularized cancer. Targeting pVHL in ccRCC could potentially serve as a therapeutic strategy (Hwang et al., 2012).

Ultimately, understanding the VHL-MYC interaction not only provides insights into gene regulation and the cell cycle, but also into VHL disease and ccRCC. Studying this interaction could reveal dysregulated processes in VHL-related diseases and ccRCC development. Therefore, the study of the VHL-MYC link is crucial for the understanding and treatment of these complex diseases.

1.6 Hypothesis and Aim of the Study

As previously mentioned, amplification of the *MYC* gene at 8q24, along with the loss of *VHL* on chromosome 3p, is commonly observed in clear cell renal cell carcinomas (ccRCCs) (Beroukhi et al., 2009; Creighton et al., 2013). These genetic alterations potentially disrupt cellular processes, driving tumor growth and aggressiveness in ccRCC. (Bailey et al., 2017; Kalkat et al., 2017; Shroff et al., 2015).

Interestingly, a complex interplay between VHL and MYC has been reported in ccRCC. VHL's role in suppressing c-MYC expression through HDAC recruitment to the c-MYC promoter is crucial for auto-suppression and maintaining appropriate expression levels. However, VHL mutations in ccRCC activate the MYC pathway, contributing to highly metastatic and vascularized cancer growth (Bailey et al., 2017; Hwang et al., 2012).

Previous findings from the Larsson laboratory underscore the pivotal role of the MYC-VHL interaction in regulating MYC-target genes. While MYC and VHL form a complex, particularly in the nucleus, with VHL involved in the non-proteolytic ubiquitylation of MYC, the precise outcomes of this interaction remain unclear in normal and malignant cells. The aim of this study was to investigate possible cross-talk between VHL and MYC and its impact on MYC protein function.

At the outset of this study, the primary objective was to confirm the MYC-VHL interaction in cells. Although the MYC-VHL interaction has been previously reported (Hwang et al., 2012), establishing this interaction in our laboratory holds importance for the subsequent phases of this project.

One of the key objectives of this project is to explore the interplay between VHL and MYC and assess the role of SKP2 (S-phase kinase-associated protein 2) in modulating this interaction. SKP2 is another E3-Ubiquitin Ligase and leads to the degradation of MYC, but at the same time SKP2 stimulates MYC's transcriptional activity (S. Y. Kim et al., 2003; von der Lehr et al., 2003). Previous studies have indicated that both SKP2 and VHL bind to specific regions of MYC, including the MYC box 2 and specific amino acids at the C-terminus. Our research hypothesis proposes a competitive binding scenario between SKP2 and VHL for MYC, implying that an increased abundance of SKP2 in cells would result in a reduced interaction between MYC and VHL. To address this aim, I employed co-immunoprecipitation assays to examine the physical association between VHL, MYC, and SKP2. These experiments provide valuable insights into the binding dynamics of these proteins and shed light on the potential competition for MYC binding.

My secondary goal is to explore how VHL mutations affect its interaction with MYC and the subsequent functional outcomes. Using site-directed mutagenesis, I altered specific amino acids in VHL, potentially important for binding to MYC's bHLHZ region. I then assessed the extent of interaction by performing co-immunoprecipitation assays, measuring if the mutated

VHL-MYC interaction was significantly reduced or lost. Identifying key amino acids in VHL-MYC binding was crucial for our goal. By creating VHL mutations that disrupt MYC interaction while preserving HIF binding, we can confirm if gene expression changes are solely due to the VHL-MYC interaction, independent of hypoxia-driven HIF changes.

By exploring the complex interplay between MYC and VHL, this research aims to shed light on the underlying mechanisms and functions of the MYC-VHL interplay and how that impact tumor development.

MATERIAL AND METHODS

In order to ensure accuracy and reproducibility, all experiments were conducted following the prescribed protocols. Any deviations from the protocol were noted and taken into account during data analysis. Chemicals and materials were handled according to the instructions provided by the manufacturers to ensure safety and proper use.

2.1 Cell Line and Cell Culture

In this study, the Cos7 cell line was used, which is derived from African green monkey kidney tissue and has a fibroblast-like morphology. Cos7 is a widely used cell line to study protein overexpression and interactions, thus, Cos7 cells are suitable for the experimental objectives. Cos7 cells were stably transfected with an origin-defective SV40 mutant encoding wild-type T antigen. Their fibroblast-like properties and strong adherence to surfaces facilitate in vitro culture and experiments (Nozaki, 2018). In particular, the exceptional transfection efficiency of the Cos7 cell line, especially with plasmid DNA, facilitated gene overexpression and enabled efficient co-immunoprecipitation (co-IP) studies to analyze protein interactions.

The Cos7 cell line was cultured in Dulbecco's DMEM medium containing 4,5g/L Glucose, L-Glutamine and Pyruvate, supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Cat.no.: 41966-052). The cells were maintained in an incubator at 37°C with 5% CO₂ and >90% relative humidity, with fresh media provided every second to third day. Cells were passaged at around 70% confluency by washing with PBS and subsequent trypsinization, with the cells being split and reseeded in fresh medium at the appropriate ratio. Cell viability of >95% was indicative of healthy cells to start experiments.

2.2 Transfection

The experimental timeline involved seeding the Cos7 cells at a density of 0.5×10^6 cells per dish in 6 cm² dishes and 1.5×10^6 cells per dish in 10 cm² dishes. The cells were then allowed to grow for 24 hours in a standard cell culture incubator at 37°C, reaching 70-90% confluency. In the initial experiments, transfection was carried out with the Invitrogen™ Lipofectamine™ 2000/3000 transfection reagent, following the provided protocols (Invitrogen, 2013, 2016b). However, for all subsequent and relevant experiments, the FuGene® HD Transfection Reagent (cat.no.: E2311/E2312) was utilized according to the specified protocol (Promega, 2023). Following transfection, the cells were cultured for 24 hours to allow for gene expression and protein production. After this incubation period, the cells were harvested for further analysis.

2.3 Western Blot

Cells were harvested by gently scraping them from the culture dish using a cell scraper in cold 1XPBS. Cells were collected by centrifugation at 1500rpm for 5 mins. The cell pellet was immediately placed on ice for subsequent cell lysis. For cell lysis, the cell pellets were gently resuspended in 75µL of 1x RIPA buffer supplemented with protease and phosphatase inhibitors by pipetting up and down gently. After cell lysis, the lysate was subjected to sonication three times for 30 seconds at 80kHz. Following sonication, the lysate was centrifuged at 12,000 rpm for 15 minutes at 4°C, and the resulting supernatant was carefully transferred into a new Eppendorf tube. Total protein concentration was determined using the Pierce™ BCA Protein Assay Kit by Thermo Scientific™ (Cat.no.: 23225). The absorbance of the lysate was measured at 568 nm using a luminometer. Approximately 10-20 µg of protein was loaded onto Invitrogen™ NuPAGE™ 4-12% Bis-Tris polyacrylamide gels (Cat.no.: NP0323) or onto Invitrogen™ Bolt™ 4-12% Bis-Tris Plus polyacrylamide gel (Cat.no.: NW04127). To prepare the protein samples, NuPAGE™ LDS Sample Buffer (4x) (Cat.no.: NP0007) and NuPAGE™ Sample Reducing Agent (10x) (Cat.no.: NP0009) were added. Thermo Scientific™ PageRuler™ Prestained Protein Ladder (Cat.no.: 26616) ranging from 10 to 180 kDa was used for molecular weight determination. The electrophoresis was conducted in 1X MOPS running buffer (Cat.no.: NP000102). The gels were run at 100-120V using an appropriate gel apparatus. Subsequently, the gels were transferred onto nitrocellulose membranes (Bio-Rad Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Packs, Cat.no.: 1704158 or Bio-Rad Trans-Blot Turbo Midi 0.2 µm Nitrocellulose Transfer Packs, Cat.no.: 1704159) using the Trans-Blot Turbo™ Transfer System by Bio-Rad for

transfer. After the transfer, the membranes were stained with Ponceau S solution for 5 minutes to visualize the transferred proteins. The membranes were then washed with PBS and blocked in 5% skim milk for 1 hour at room temperature (RT) to prevent non-specific antibody binding. Following blocking, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were washed three times for 10 minutes each with PBS-Tween20 (0.1%) and incubated with secondary antibodies for 1 hour at RT. After three additional washes of 5 minutes each with PBS-Tween20 (0.1%), the membranes were developed for 5 minutes using Bio-Rad Clarity™ Western ECL Substrate (Cat.no.: 170-5061) and imaged using the Bio-Rad Gel Doc XR+ imaging system. Complete list of antibodies used In this study Is provided In table 3.

Table 1: List of Antibodies used in this Study

Target	Type	Manufacturer	Cat. No.	Application
Actin	Mouse	Sigma-Aldrich	A5441	Western Blot
FLAG	Mouse	Sigma-Aldrich	F1804	Western Blot, Co-IP
HA	Rabbit	abcam	AB9110	Western Blot
HA	Rat	Roche	11867423001	Co-IP
HA	Rabbit	Santa Cruz	sc-805	Western Blot
c-MYC (9E11)	Mouse	Abcam	ab56	Western Blot
c-MYC (Y69)	Rabbit	Abcam	ab32072	Western Blot
Dnk pAb to Goat IgG	Donkey	Abcam	ab6885	Western Blot
Goat pAb to Rb IgG	Goat	Abcam	ab97080	Western Blot
pVHL	Mouse	BD Pharmingen	556347	Western Blot
Rb pAb to Ms IgG	Rabbit	Abcam	ab97046	Western Blot
SKP2	Goat	Santa Cruz	Sc-1567	Western Blot

2.4 Co-Immunoprecipitation (co-IP)

Co-immunoprecipitation (co-IP), was carried out using the Cos7 cell line in accordance with established protocols. $1,5 \times 10^6$ cells were seeded in 10 cm² cell culture dishes until they reached 80-90% confluency before transfection. For the initial experiments, Invitrogen™ Lipofectamine™ 2000/3000 transfection protocol (Invitrogen, 2013, 2016b) was used, whereas for all subsequent and relevant experiments, Promega FuGENE® HD Transfection Reagent protocol (Promega, 2023) was utilized.

After transfection, cells were harvested by gently scraping them from the culture dish using a cell scraper in cold 1x PBS, then centrifuged at 1500g for 5 minutes. The cell pellet was subsequently collected by aspirating the PBS and stored on ice until further processing. The cells were lysed using a "low-stringency lysis" buffer, also referred to as WIMAN buffer. The buffer composition included 1% NP-40, 1M Tris pH 8.5M NaCl, and 0.5M EDTA,. Additionally, Pierce™ Phosphatase and protease inhibitors (Cat. No.: A32961) were added to the buffer. A volume of 320 µl of the lysis buffer was used for each 10 cm cell culture dish. The lysis process was performed on ice for a duration of 30 minutes. Following lysis, the cell pellet was resuspended by gently pipetting up and down several times. Subsequently, the lysate was subjected to centrifugation at 16000g for 20 minutes at 4°C, and the resulting supernatant was promptly transferred into a new tube. For short-term storage, the lysate was kept at -20°C, while for longer storage periods, it was stored at -80°C if the procedure was not continued the same day.

A total of 250-500 µg of protein was utilized for co-IP. co-IP was conducted with magnetic protein G beads using Dynabeads™ Protein G (Cat.no.:10004D) in accordance with the Invitrogen Dynabeads protocol (Invitrogen, 2016a). To perform coIP, a preparation was made using 50µL of Dynabeads™ protein G and the desired antibody (typically FLAG or HA antibodies, as well as SKP2 and VHL initially for testing). The antibody was linked to the magnetic beads at room temperature for 15-30 minutes. Then, the antibody-coupled magnetic beads were incubated with the protein sample. The co-IP protocol was conducted following the Invitrogen Dynabeads™ protein G protocol, with some modifications. Some co-IPs were carried out overnight at 4°C to promote binding of the antigen to the antibody, while others, particularly the more recent ones, were carried out at room temperature for 15 minutes to 2 hours depending on the protein concentration in the lysate. After conducting several washing steps and

performing a final elution step to extract the protein of interest from the Dynabeads™, the eluted sample was transferred to a new tube. Loading dye (NuPAGE™ LDS Sample Buffer (4x) (Cat.no.: NP0007) and NuPAGE™ Sample Reducing Agent (10x) (Cat.no.: NP0009)) was added to the sample, and it was heated at 96°C for 10 minutes using a heat block. Following heating, the sample was loaded onto a gel, alongside an Input control. The samples were then subjected to further processing in accordance with the Western blot procedure.

2.5 Quantification of Protein Expression and Interactions

To quantify protein expression in the input samples, a normalization process was applied. The target protein band was normalized to the corresponding actin band to address potential loading variations. Subsequently, values were normalized to a reference sample, either the sample containing VHL + MYC and no SKP2 (for competition experiments), or the sample containing wild-type VHL in mutation experiments. The purpose of this normalization was to assess changes in protein expression across the samples, accounting for potential loading differences and ensuring reliable quantification.

To quantify protein abundance in the IP samples, a slightly different approach was used due to the unavailability of actin as a direct loading control. Instead, band intensities of the protein of interest were measured in IP samples and normalized to the reference sample. This approach allowed the assessment of changes in protein abundance within the IP samples relative to the reference sample, facilitating the direct comparison assessment of changes in immunoprecipitated protein levels and protein expression.

In most experiments, the goal was to achieve, compare, and assess changes in the interaction affinity between two proteins (MYC, VHL, SKP2). To quantify this interaction, the protein abundance of the co-immunoprecipitated protein was divided by the abundance of the immunoprecipitated protein, yielding a ratio representing the relative abundance of co-immunoprecipitated protein to immunoprecipitated protein. The same ratio was calculated for input samples to assess protein expression relative to the protein to be immunoprecipitated. These values were then normalized to the reference sample to detect differences that could indicate changes in interaction affinity.

Additionally, to account for potential loading or transfection differences between IP and input samples, the normalized ratios of IP samples were divided by the normalized ratios of input samples. Subsequently, these values were normalized to the reference sample to directly assess changes in potential interaction affinities across the samples.

It's important to note that observed changes could also be influenced by other factors, such as variations in the co-IP steps or differences in co-IP efficiency.

2.6 Transformation

Plasmids for transfection experiments were amplified in NEB® 10-beta Competent *E. coli* cells and extracted with NucleoBond® Xtra Midi prep (Cat.no.: 740410.50) or Zyppy™ Plasmid Miniprep (Cat.no.: D4036)/ ZymoPURE™ Plasmid Miniprep (Cat.no.: D4209) kits according to the manufacturer's instructions. The competent bacteria were retrieved from the -80°C freezer and thawed on ice for 20 minutes. For each transformation, 1µg of DNA was gently mixed with 10µL of competent cells by flicking the tube. The mixture was incubated on ice for 30 minutes. Subsequently, each transformation tube was heat shocked at 42°C using an Eppendorf ThermoMixer®C for 30 seconds, followed by immediate return to ice for 2 minutes. To allow bacterial growth, 1mL of the provided outgrowth media was added to each tube and incubated for 45 minutes at 37°C in a shaker incubator. After the incubation, the transformed cells were plated onto two 10cm LB agar plates containing Ampicillin as selection marker. The plates were then incubated overnight at 37°C. On the following day, a single colony was selected and transferred into an Erlenmeyer flask, where it was cultured in 100 mL LB media overnight in a shaker incubator at 37°C. The bacterial cells were harvested by centrifugation, and plasmid extraction and purification were performed according to the protocols of the NucleoBond® Xtra Midi prep or Zyppy™ Plasmid Miniprep/ ZymoPURE™ Plasmid Miniprep kits. The concentration and purity of the extracted plasmid DNA were assessed using the NanoDrop™ 1000 spectrophotometer. The purified plasmid DNA was stored at 4°C in purified water. A complete list of plasmids is provided in table 2 and 3.

While all regular plasmids were amplified and tested, five were selected for the experiments, while others were not utilized due to functional issues. The five plasmids I worked with in this study were pcDNA3 and the ones containing *HA-MYC*, *FLAG-VHL*, *SKP2* and *HA-MYC-ΔN3*.

Table 2: Amplified Vectors - Regular Plasmids

Plasmid Name	Description	Purpose/Function
pcDNA3	Empty Vector	Negative Control
pcDNA3- <i>HA-MYC</i>	HA tag fused with <i>c-MYC</i> gene	Analyze protein expression levels, protein interactions, Competition experiments
pcDNA3- <i>FLAG-VHL</i>	FLAG tag fused with <i>VHL</i> gene	Analyze expression and function of VHL protein, protein interactions, Competition experiments, Template for Mutagenesis
pcDNA3- <i>SKP2</i>	<i>SKP2</i> gene inserted	Study Protein interactions, Competition experiments
pcDNA3- <i>HA-MYC-ΔN3</i>	HA tag fused with <i>MYC</i> deletion mutant $\Delta N3$	Study Protein interactions and their Specific binding sites
pcDNA3- <i>HA-MYC-ΔN4</i>	HA tag fused with <i>MYC</i> deletion mutant $\Delta N4$	Study Protein interactions and their Specific binding sites
pcDNA3- <i>FLAG-MYC</i>	FLAG tag fused with <i>MYC</i> gene	Analyze protein expression levels, protein interactions, Competition experiments
pcDNA3- <i>HA-VHL</i>	HA tag fused with <i>VHL</i> gene	Analyze expression and function of VHL protein, protein interactions, Competition experiments, Template for Mutagenesis
pcDNA3- <i>HA-MYC-V394D</i>	HA tag fused with <i>MYC</i> mutant <i>V394D</i>	Study Protein interactions and their Specific binding sites
pcDNA3- <i>GFP</i>	<i>GFP</i> gene inserted	Negative Control and Transfection control

Table 3: Generated Mutant Plasmids

Plasmid Name	Mutation(s)	Parent Plasmid	Purpose/Function
pcDNA3-FLAG-VHL-M1	M1	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-M2	M2	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-M3	M3	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-M4	M4	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-M5	M5	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-M6	M6	pcDNA3-FLAG-VHL	Investigate effect on protein interaction

pcDNA3-FLAG-VHL-D1	D1 (M1 + M5)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D2	D2 (M2 + M5)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D3	D3 (M3 + M4)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D4	D4 (M5 + M6)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D5	D5 (M4 + M5)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D6	D6 (M3 + M6)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D7	D7 (M3 + M5)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction
pcDNA3-FLAG-VHL-D8	D8 (M4 + M6)	pcDNA3-FLAG-VHL	Investigate effect on protein interaction

2.7 Glycerol Stock

To prepare the glycerol stock, 500 μ l of 50% glycerol solution was mixed with 500 μ l of the bacterial culture (*E. coli*) that had been grown in LB broth. The resulting mixture was then carefully transferred into a glycerol tube, ensuring proper sealing, and promptly placed in a -80°C freezer for long-term storage.

2.8 Site-Directed Mutagenesis

Site directed mutagenesis to generate specific mutation in the VHL protein was carried out using the Q5® Site-Directed Mutagenesis Kit (Cat. No.: E0554S), following the manufacturer's provided protocol (New England BioLabs, 2020).

For the primer design, the specific amino acid of interest was determined by analyzing the amino acid sequence available in the PubMed database. The corresponding nucleotide sequence was then examined to identify the specific codons responsible for encoding the amino acid. After identifying the target codon positions, the codons were modified by introducing one or more base substitutions according to the desired changes. The NEB Base Changer tool was utilized to generate the appropriate mutagenesis primers based on the specified substitutions. To ensure the accuracy of the primers, a careful verification process was followed. This involved confirming the correctness of the primer sequences and checking that

they aligned correctly with the target sequences. Once the primers were validated, they were ordered and delivered for use in the mutagenesis experiments.

The target gene, which was the plasmid containing *Flag-VHL*, underwent amplification using a high-fidelity DNA polymerase in a polymerase chain reaction (PCR) assay. Mutagenic primers were employed in the PCR to introduce specific mutations. The PCR conditions, including annealing temperature and cycle parameters, were carefully optimized for each targeted substitution to ensure efficient mutagenesis and accurate amplification. The Q5® Site-Directed Mutagenesis Kit (Cat. No.: E0554S) was used for this purpose following the manufacturer's instructions. Following amplification, the PCR product underwent enzymatic treatments including kinase, ligase, and DpnI digestion for 5 minutes. The resulting mutated DNA was then transformed into NEB 5-alpha competent *E. coli* cells, and colonies were selected for further analysis. Plasmid DNA was extracted from the bacterial colonies using Zyppy™ Plasmid Miniprep/ ZymoPURE™ Plasmid Miniprep kits and subjected to sequencing to confirm the presence of the desired mutations. For expression and functional analysis, the mutated gene constructs were introduced into the appropriate expression system, specifically Cos7 cells, using the FuGene® HD Transfection Reagent (Cat. No.: E2311/E2312) according to the provided protocol (Reference).

Due to confidentiality agreements and the proprietary nature of the mutations introduced, specific details including the primer sequences and mutagenesis strategies cannot be disclosed in this work. The mutations were designed and carried out following established protocols provided by New England Biolabs. While detailed primer sequences and mutagenesis information are restricted from this publication, the procedures followed were in accordance with standard practices for introducing targeted mutations.

2.9 Sanger Sequencing

Sanger DNA analysis of the constructs was outsourced to KIGene, an internal sequencing service provider at KI (Karolinska Instituted), using their provided protocol (KIGene, 2023). Sequencing was performed using the ABI 3730 PRISM®DNA Analyser. The ABI 3730 instruments were used in either 96-well or 384-well format with a 48x36cm or 50cm capillary array. For the sequencing process, the constructs were provided to KIGene in PCR strip-well tubes. The plasmids submitted for sequencing contained 150-200 ng of DNA. For the sequencing process, the universal T7 promoter primer was used, conveniently available at the

sequencing facility. Subsequent analysis of the obtained sequences enabled the identification of the desired genetic alterations.

RESULTS

The primary objective of this study was to investigate the interaction between MYC and VHL, aiming to unravel vulnerabilities in MYC-driven tumors. The significance of this investigation derives from the widespread dysregulation of the MYC oncogene, observed in over 50% of human cancers.

3.1 Confirmation of Plasmid Expression

The initial step in this study involved the validation of specific plasmids critical to this study through overexpression experiments followed by Western blot analysis. This was necessary to confirm both the identity of the plasmids, ensuring that the correct genes of interest were present and correct proteins were expressed, and the integrity of the plasmids, verifying that they remained structurally intact without alterations affecting protein expression.

To initiate this process, the plasmids of interest, provided by my laboratory group, underwent a transformation into *E. coli* cells, subsequent expansion, and extraction of those plasmids. These steps aimed to yield a substantial quantity of plasmid DNA suitable for transfection experiments. After measuring the DNA concentration and testing the purity, I proceeded with the transfection procedures.

The experimental approach involved seeding Cos7 cells initially, allowing their growth to 70-80% confluence, and subsequently transfecting them with the respective plasmid DNAs. In each case, a crucial negative control was included, represented by the empty vector pcDNA3. This vector serves as the backbone for the aforementioned plasmids but lacks an insert. The negative control was of importance in the subsequent Western blot analysis to ensure that the

observed bands or signals on the blot do indeed represent the expression of the protein encoded by the plasmid and not due to non-specific bands or other anomalies. Following 24 hours of protein overexpression, the harvested cells underwent lysis. To fragment DNA, disrupt any interactions and cellular membranes effectively, sonication was used. After removal of cell debris, protein lysates were obtained and their concentrations measured. To ensure uniform protein loading, a standardized amount of protein (20 µg) was then mixed with loading dye. The proteins were subsequently subjected to western blot analysis. In the western blot procedure, protein samples were first separated on a gel matrix and then transferred onto a membrane. The membranes were subsequently incubated with primary antibodies, specific for the target proteins under investigation. Following primary antibody probing, the membranes were probed with secondary antibodies, which are exclusively directed against the species in which the primary antibodies were produced. This strict specificity ensures that secondary antibodies do not bind to other proteins on the membrane, enhancing the accuracy of the Western blot analysis. Finally, protein bands were visualized using the ChemiDoc imaging system. Western Blot images of proteins encoded by the plasmids, relevant for this study, are presented in Figure 9.

In the beginning, I tested several plasmids with different TAGs and used different antibodies in Western blot analysis to determine which plasmids and which antibodies were best suited for my experiments. In the end, I used four plasmids for this study. These plasmids were HA-c-MYC, FLAG-VHL, SKP2 and the MYC deletion mutant HA-c-MYC-ΔN3.

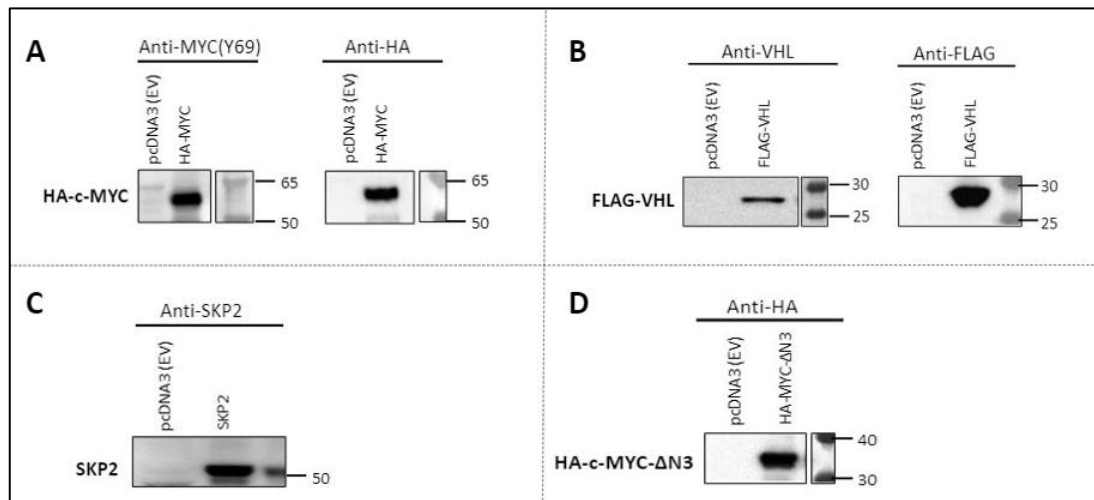


Figure 9: Confirmation of Plasmid Overexpression by Western Blot Analysis. Cos7 cells were transfected with a plasmid containing the protein of interest. The empty vector pcDNA3 was used as negative control. After 24h of overexpression western blot analysis was carried out. PageRuler Prestained was used as ladder. A: Confirmation of HA-c-MYC. Membranes were stained with an anti-MYC antibody (left) to confirm the protein expression and an anti-HA antibody to confirm the correct TAG (right). B: Confirmation of FLAG-VHL. Membranes were stained with an anti-VHL antibody (left) to confirm correct protein expression and an anti-FLAG antibody to confirm the correct TAG (right). C: Confirmation of SKP2. Membranes were stained with an anti-SKP2 antibody to confirm correct protein expression. D: Confirmation of the MYC deletion mutant HA-c-MYC-ΔN3. Membranes were stained with an anti-HA antibody to confirm correct protein expression and the correct TAG. I used specific antibodies for each protein. To enhance clarity and presentation, some images have been edited to remove certain lanes, marked by small gaps, as they are not relevant to this illustration.

Figure 9 shows that the expression of all plasmids, including HA-MYC, FLAG-VHL, SKP2 and HA-MYC-ΔN3 was confirmed in Cos7 cells. This marked the cells for suitable systems for my experiments. Figure 9A shows the HA-MYC band, which is observed at the expected size of 62kDa, detected both by c-MYC- and HA-antibodies. Similar observations can be made in Figure 9B for the FLAG-VHL protein. This protein migrates at around 27kDa. Additionally, both SKP2 (~53 kDa) and the deletion mutant HA-MYC-ΔN3 (which runs at approximately 35 kDa), could be confirmed, both running at their respective expected sizes (Figure 9C and D).

3.2 Confirmation of MYC Interactions with VHL and SKP2

Previous work at the LGL lab and others suggested possible protein-protein interactions between MYC and the two E3-Ub ligases, VHL and SKP2. To this end, the first crucial step in advancing towards the primary objectives of this study was to confirm whether an interaction indeed exists between these MYC proteins.

To explore this, I first focused on confirming the interactions of MYC with both VHL and SKP2. To obtain this dual confirmation, a co-transfection approach using COS-7 cells was adopted, which are known for their ease of transfection and their possession of the SV40 large T antigen. This antigen, coupled with the presence of the SV40 origin of replication, facilitates plasmid amplification in these cells over several days, which formed the basis for my studies. The core of this investigation relied on the precise co-immunoprecipitation (co-IP) assays, which are well-known for their precision in studying protein interactions. These assays provide the key to unraveling the complex interactions between MYC and these important partners. Firstly, an HA-antibody was employed to selectively pull down the MYC protein, to assess whether VHL and SKP2 could be co-precipitated with MYC.

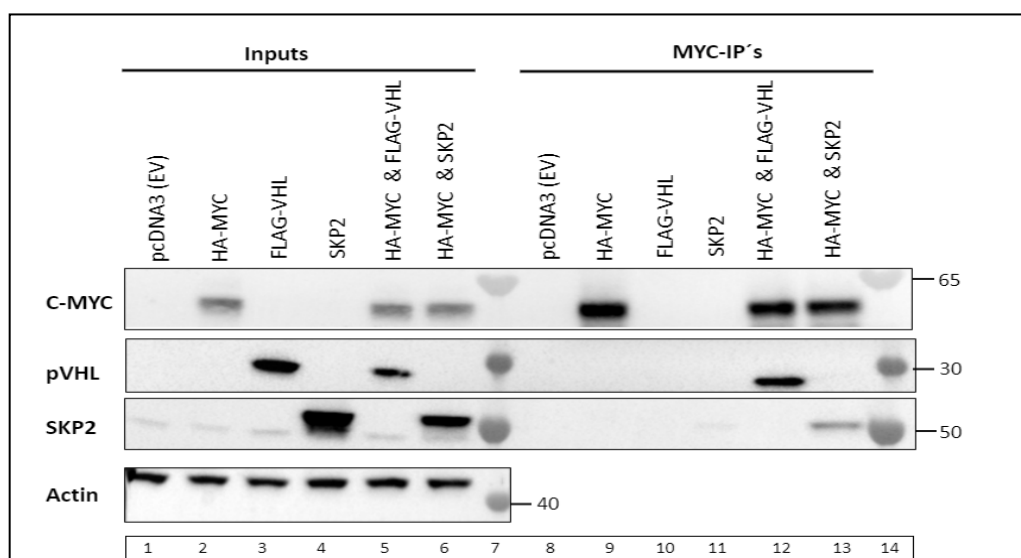


Figure 10: Confirmation of MYC Interactions with VHL and SKP2 through HA-Co-IP: Western Blot Analysis
Co-immunoprecipitation (Co-IP) experiments were conducted using COS-7 cells transfected with plasmids for MYC, VHL, SKP2, as well as co-transfections of MYC+VHL and MYC+SKP2. MYC was pulled down using an anti-HA antibody, with 500 µg of protein used for each Co-IP. Western blot analysis was performed, and the membranes were probed with antibodies against SKP2, VHL, MYC, and Actin. Input samples are shown on the left, while corresponding Co-IP samples are displayed on the right.

For reliability, I performed 3 replicates of this experiment.

As shown in Figure 10, COS7 cells expressed the MYC, VHL and SKP2 proteins in single and combinatorial transfections. Co-immunoprecipitation using HA-antibody to pull-down HA-MYC protein construct was proven to be efficient as shown in Figure 10 HA-MYC lane. Notably, anti-VHL western blot on HA-IP samples revealed that VHL was co-immunoprecipitated with MYC. Additionally, anti-SKP2 antibodies, when applied to HA-IP samples, demonstrated the co-immunoprecipitation of SKP2 with MYC, as illustrated in the SKP2 lane of figure 10. The presence of bands in Western blot analysis serves as direct evidence of interactions between MYC and these proteins, confirming their ability to form protein complexes. Therefore, I have successfully established the interactions between MYC and both VHL and SKP2.

To provide robust confirmation of the observed interactions, two additional replicates of the same experiment were conducted, each involving procedural adjustments to address troubleshooting requirements. Both replicates independently confirmed the interactions between MYC and VHL and MYC and SKP2, supporting the results of the first experiment.

As part of a comprehensive assessment of the MYC-VHL interaction, I performed additional co-immunoprecipitation experiments utilizing an anti-Flag antibody to selectively pull down VHL. The primary aim was to provide a complementary perspective on the MYC-VHL interaction.

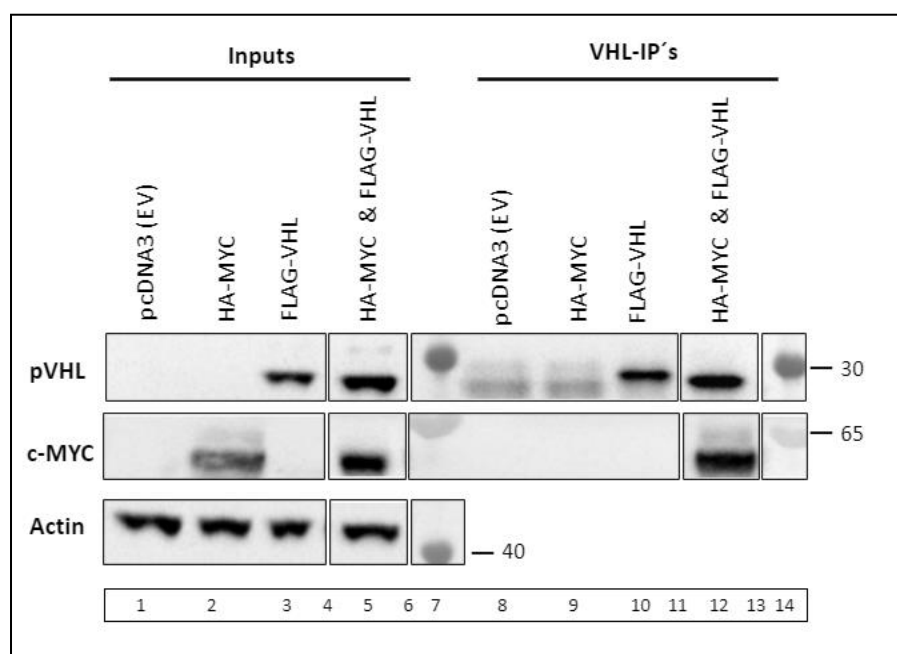


Figure 11: FLAG co-Immunoprecipitation Confirms MYC-VHL Interaction - Western Blot Analysis

co-immunoprecipitation (co-IP) experiments were conducted using COS-7 cells transfected with plasmids for MYC and VHL as well as co-transfections of MYC+VHL. VHL was pulled down using an anti-FLAG antibody, with 250 μ g of protein used for each co-IP. Western blot analysis was performed, and the membranes were probed with antibodies against VHL, MYC, and Actin. Input samples are shown on the left, while corresponding co-IP samples are displayed on the right. For the sake of clarity and better presentation, certain sections of the images have been edited to remove lanes 4, 6, 11, and 13, which are indicated by small gaps. These excluded lanes contain data that is not relevant to the specific illustration. Importantly, it should be noted that the sections shown together always originate from the same gel and membrane, ensuring consistency and accuracy in the representation.

As depicted in Figure 11, VHL was effectively precipitated in both single and co-transfection samples. Notably, the MYC band was exclusively observed in the co-transfection sample, indicating the successful co-pulldown of MYC with VHL in the co-transfection. This suggests that the interaction of MYC and VHL and their ability to bind each other could be confirmed in both Co-IPs.

3.3 Characterizing the Dynamic Interplay of MYC, VHL, and SKP2: Insights into Binding Competition

To gain a deeper understanding of the complex interplay among SkKP2, VHL, and MYC, I employed co-immunoprecipitation (co-IP) assays with specific antibodies targeting each protein. This technique allowed me to capture both MYC and VHL proteins from cellular lysates, enabling the identification of protein complexes formed by MYC and VHL, as well as their interactions with SKP2. Following this, I conducted Western blot analyses on the immunoprecipitated samples to determine the presence and relative abundance of SKP2, VHL, and MYC. Any competition between SKP2 and VHL for MYC binding would be reflected in changes in the affinity of these interactions, as seen by alterations in the intensity of the bands observed in the Western blot images.

However, the experimental process encountered challenges over five months, including unexpected variations in plasmid tags and antibody functionality. These obstacles required precise troubleshooting to resolve technical inconsistencies.

Having established the interactions of MYC with VHL and SKP2, I further explored whether VHL and SKP2 compete for MYC binding. In these competition experiments, I performed five transfections in Cos7 cells. To establish a baseline and validate the interactions, I conducted control transfections, including a negative control with an empty vector (pcDNA3) and a positive control with co-transfection of *MYC* (4ug) and *VHL* (4ug). Additionally, I conducted triplicate transfections, maintaining constant MYC and VHL levels while varying the concentrations of *SKP2* plasmid DNA (initially 2 ug, 4 ug, and 8 ug, later adjusted to 1 ug, 3 ug, and 9 ug) to assess SKP2's impact on interactions.

For the co-IP experiments, I used two approaches. Firstly, I used the anti-HA antibody to immunoprecipitate MYC, followed by Western blot analysis to investigate interactions with VHL and SKP2. Secondly, I employed the anti-Flag antibody to pull down VHL, providing an alternative perspective on competition.

Here, I present one representative experiment that effectively illustrates the overall outcomes of all conducted experiments (Figure 12).

In some experiments, when titrating *SKP2* at 2 ug, 4 ug, and 8 ug, challenges arose as no clear gradient of SKP2 emerged. To address this, I adjusted the *SKP2* quantities to 1 ug, 3 ug, and 9 ug, effectively tripling the amount of *SKP2* DNA transfected. This adjustment aimed to provide a clearer gradient, facilitating a more precise evaluation of SKP2's impact on the

expression of MYC and VHL, as well as their interactions. Following transfection, co-IP procedures were conducted using an anti-FLAG antibody to facilitate the pull-down of VHL. Figure 12 presents the Western blot analyses, illustrating the results of this experiment.

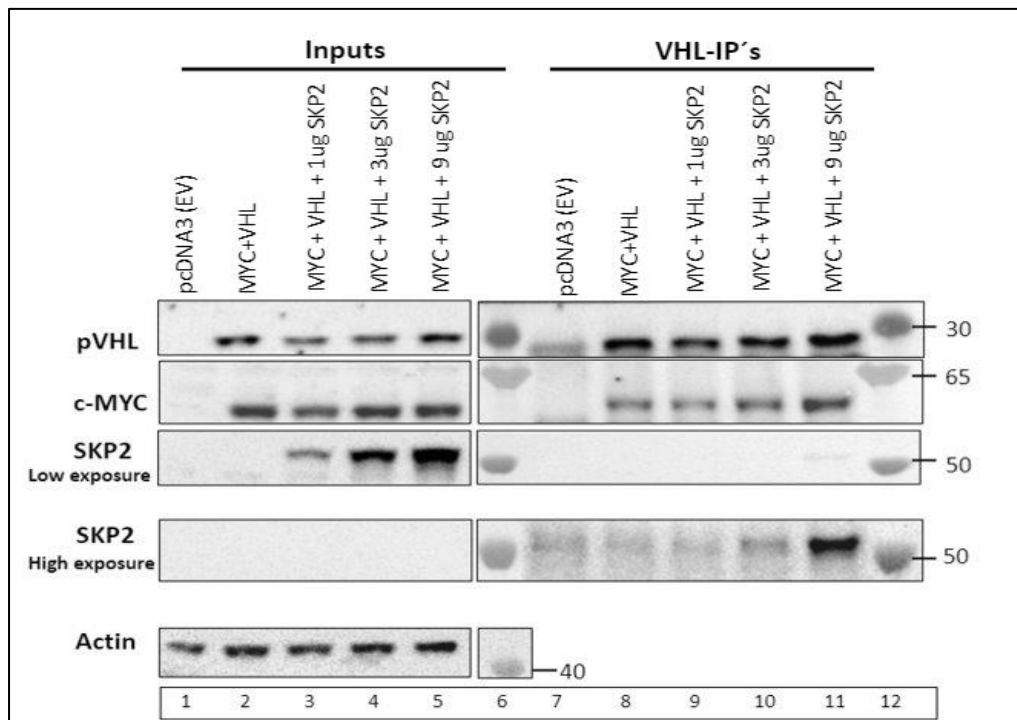


Figure 12: Investigation of Competition Behavior Between SKP2 and VHL for MYC Binding through VHL-Co-IP: Western Blot Analysis

co-IP experiments were conducted using COS-7 cells transfected with plasmids for pcDNA3 (negative control), as well as co-transfection of *MYC*+*VHL* (positive control) and triple transfections with titrated *SKP2*. VHL was pulled down using an anti-FLAG antibody, with 300 µg of protein used for each co-IP. Western blot analysis was performed, and the membranes were probed with antibodies against SKP2, VHL, HA(MYC), and Actin. Input samples are shown on the left, while corresponding co-IP samples are displayed on the right. co-IP samples of SKP2 are presented with varying exposure times for enhanced clarity. Input lanes at high exposure were covered. The small gap in the images indicates that they were edited for clarity. Three lanes were removed, which contained input samples with single transfections of SKP2 (1 µg, 3 µg, and 9 µg). This was done to evaluate the behavior of SKP2 protein expression without MYC and VHL. Removing these lanes increased clarity and better highlighted the relevant results. The exclusion of these lanes does not affect the interpretation of the data presented. For reliability I performed this experiment 4 times.

Western blot analysis of VHL successfully confirmed VHL protein expression in all VHL-transfected samples. Notably, the input lanes (lanes 1-5) showed relatively consistent VHL expression levels, although lanes 3 and 4 exhibited slight variations, which could be attributed to differences in transfection efficiency or potential biological diversity. As expected, the negative control (lane 1) displayed no VHL expression. In the co-IP samples (lanes 8-11), VHL expression

patterns closely mirrored those observed in the input samples. Regarding MYC protein expression, its presence could be observed in all corresponding samples (lanes 2-5), with minor fluctuations, specifically a slight reduce in sample with 1 μ g *SKP2*. Intriguingly, I observed an increase in the co-immunoprecipitated MYC with VHL as the *SKP2* titration level increased (lanes 8-11). However, this trend did not hold in lane 9, containing 1 μ g *SKP2*. This discrepancy may be attributed to lower total MYC expression in this sample, possibly due to transfection-related issues. *SKP2* protein expression exhibited a clear trend, increasing in tandem with *SKP2* titration (lanes 3-5), which aligned with the higher levels of *SKP2* transfection. Unexpectedly, *SKP2* bands emerged in the co-IP samples (lanes 9-11), suggesting an unforeseen interaction with VHL. In terms of actin expression, analysis of input samples (lanes 1-5) revealed consistent protein loading across all samples.

To gain a deeper insight into the relationships between changes in protein expression and the levels of co-immunoprecipitated proteins, which can indicate differences in protein interactions, I conducted a quantification analysis. Initially, I measured the intensity of bands in the input samples for MYC, VHL, and *SKP2* proteins. To ensure accuracy, I normalized these intensities to their corresponding actin bands to account for any variations in protein loading. Following this, I further normalized these ratios to the reference sample, MYC + VHL without *SKP2*. This allowed me to examine how the expression levels change in samples with varying *SKP2* titration compared to the reference sample. Thus, we could gain insights into how increasing *SKP2* affects the expression of VHL and MYC. The graphical representation is shown in Figure 13.

I also conducted a comparable analysis on the IP samples, where I calculated the relative levels of immunoprecipitated proteins (Figure 14). This was achieved by normalizing the band intensities of the proteins of interest to the reference sample. This approach allowed for comparison between the relative expression levels and the relative levels of immunoprecipitated proteins across these samples.

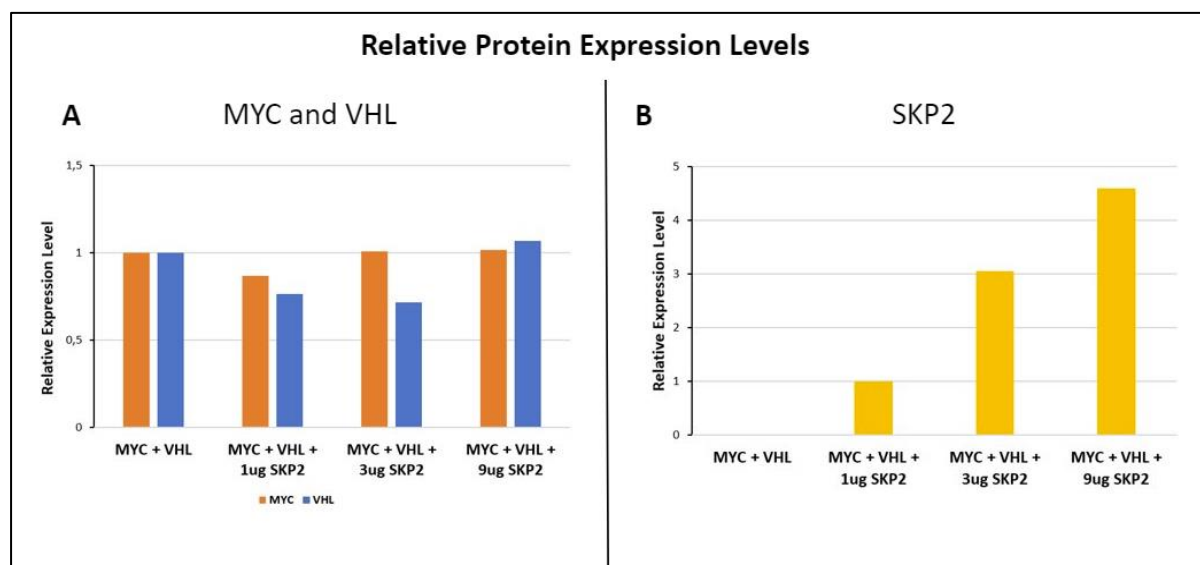


Figure 13: Relative Protein Expression Levels MYC-VHL-SKP2
 Graph A: Protein expression levels of MYC (orange) and VHL (blue) in input samples. Graph B: Protein expression levels of SKP2 in input samples, presented separately due to its high fold change with increasing titration of SKP2. Band intensities were normalized to corresponding actin bands and then further normalized to the reference sample (MYC+VHL without SKP2).

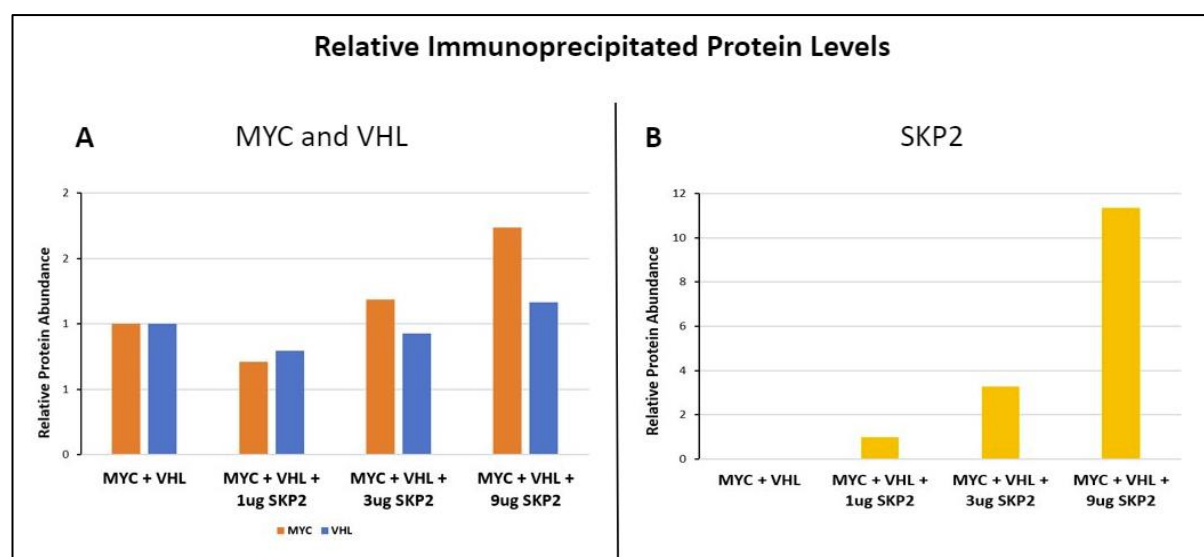


Figure 14: Relative Immunoprecipitated Protein Levels in IP samples MYC-VHL-SKP2
 Graph A: Displays immunoprecipitated MYC (orange) and VHL (blue) protein levels in VHL-IP samples. Graph B: Depicts immunoprecipitated SKP2 protein levels in VHL-IP samples, shown separately due to its significant fold change with increasing SKP2 titration. Protein band intensities in IP samples were normalized to the reference sample (MYC+VHL without SKP2).

Figure 13 reveals, as the protein expression levels of SKP2 increased (Figure 13B) across the samples, the levels of expressed VHL and MYC generally maintained a stable trend (Figure 13A). Quantitative analysis corroborated our observations from Western blotting, revealing that VHL expression levels in samples transfected with 1 and 3 μg of *SKP2* were slightly lower when compared to *SKP2*-free conditions. In contrast, in the presence of 8 μg of *SKP2*, VHL expression exhibited a slight increase relative to the *SKP2*-free condition. MYC expression remained relatively consistent across most samples, except for the 1 μg *SKP2*-transfected sample, where it demonstrated a modest decrease. These minor expression variations could potentially be attributed to factors such as transfection efficiency or genuine expression alterations. Overall, the expressions of MYC and VHL in the samples appeared relatively stable as SKP2 increases, with only minor variations. Identifying the exact cause of these variations is complex, as they could potentially result from both transfection-related issues and the influence of SKP2.

Figure 14 revealed that the protein levels of VHL precipitated in the co-IP samples generally maintained consistency across all samples, with slight variations. These observations were in alignment with the expression levels of VHL in the inputs, which display slightly lower protein quantities in samples transfected with 1 and 3 μg of *SKP2* and slightly higher quantities in the presence of 9 μg *SKP2* compared to the reference condition (without *SKP2*) (see Figure 14A). Interestingly, the amount of co-immunoprecipitated MYC showed an increase with increasing SKP2 concentrations (see Figure 14A). This deviated from MYC expression levels in the input, where MYC levels are not increasing. This suggests an augmented binding between MYC and VHL with increasing *SKP2* titration, indicating a specific stimulatory effect of SKP2 on this interaction. Notably, as was seen in the western blot image, an unexpected increase in co-precipitated SKP2 with VHL was observed as *SKP2* titration increases (Figure 14B), corresponding to the higher SKP2 expression in the input samples.

To clarify this further, I calculated the MYC/VHL and SKP2/VHL ratios in both the input and IP samples. This involved measuring the band intensities and dividing the co-IP protein values (MYC or SKP2) by the IP protein value (VHL). These ratios were then normalized to the reference sample ratio (without SKP2). This approach allowed for a comparison of the ratios in the IP samples to those in the input samples, providing possible insights into changes in protein interactions. It sheds light on the impact of increasing SKP2 on the interaction between MYC and VHL. The results are presented in Figure 15.

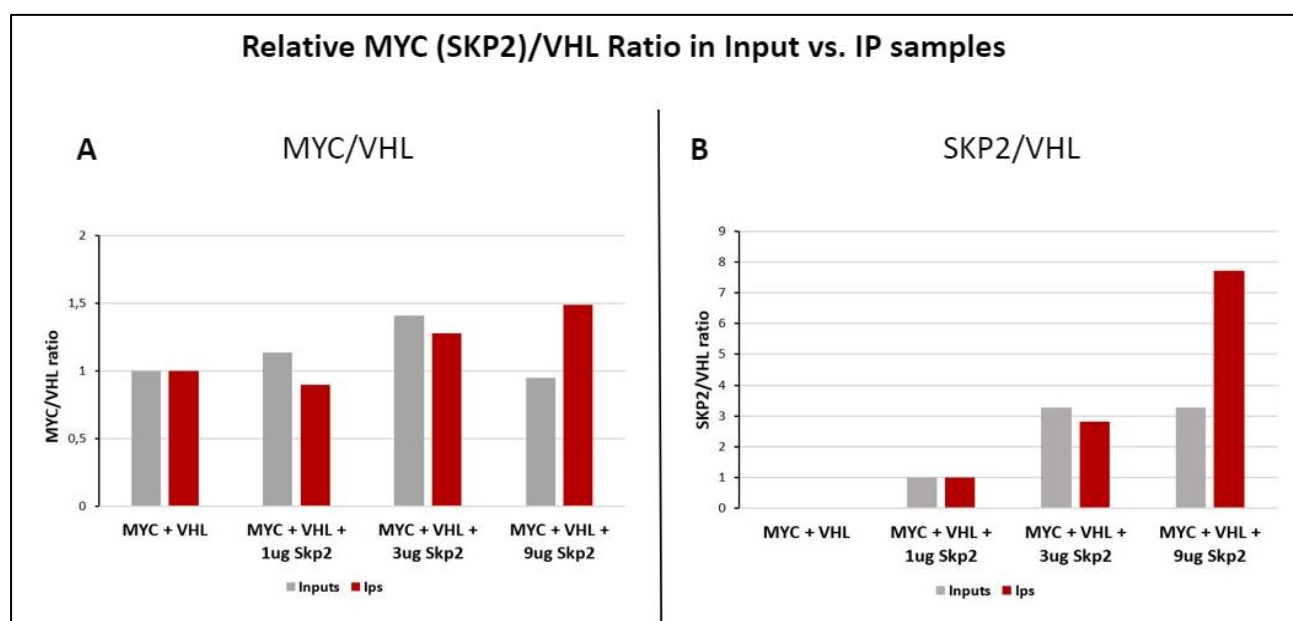


Figure 15: Relative MYC/VHL and SKP2/VHL ratios in Input vs. VHL-IP samples
 Graph A: Comparison of MYC/VHL Ratio in Input (grey) and IP (red) samples. Band intensities of MYC in Input and IP samples were normalized to the corresponding VHL band in each lane to determine the relative MYC levels relative to VHL.

Figure 15A depicts the MYC-to-VHL ratios in both input and IP samples. In the input samples, these ratios exhibited a slight increase from no *SKP2* to 1 μ g and 3 μ g *SKP2*, followed by a decrease at 9 μ g *SKP2*. Conversely, the MYC/VHL ratios increased in all IP samples except the 2 μ g *SKP2* sample, where a marginal decrease compared to the reference sample is observed. Notably, even in the 9 μ g *SKP2* sample with lower MYC/VHL ratio in input, an increase was seen in the IP sample. This suggests a potential enhancement in the interaction between MYC and VHL as SKP2 levels rise, supporting the stimulatory effect of SKP2 on MYC-VHL interaction.

In Figure 15B, the SKP2/VHL ratios are presented. In the input samples, these ratios significantly increased from 1 μ g to 3 μ g SKP2 and then stabilize at 9 μ g SKP2. However, the unexpected presence of SKP2 protein in the IP samples relative to VHL indicates a significant increase in SKP2-VHL interaction, particularly with higher SKP2 concentrations.

To provide further clarity, I computed the fold differences between the previously determined ratios (MYC/VHL and SKP2/VHL) in the co-IP and Input samples. These values may suggest an interaction affinity between the respective proteins, accounting for changes in protein expression across the samples. However, it's crucial to acknowledge that the observed alterations in these ratios in samples with titrated *SKP2*, compared to the reference sample without *SKP2*, could potentially result from various factors influencing the experiment. For

instance, variations in co-IP efficiency might contribute to these ratio changes. To derive these ratios, I divided the MYC/VHL ratio in the IP sample by the corresponding ratio in the input sample. The same calculation was applied to the SKP2/VHL ratios. This analysis aimed to offer insights into the potential impact of increasing *SKP2* on the interaction affinity between MYC and VHL. The resulting graph is depicted in Figure 16.

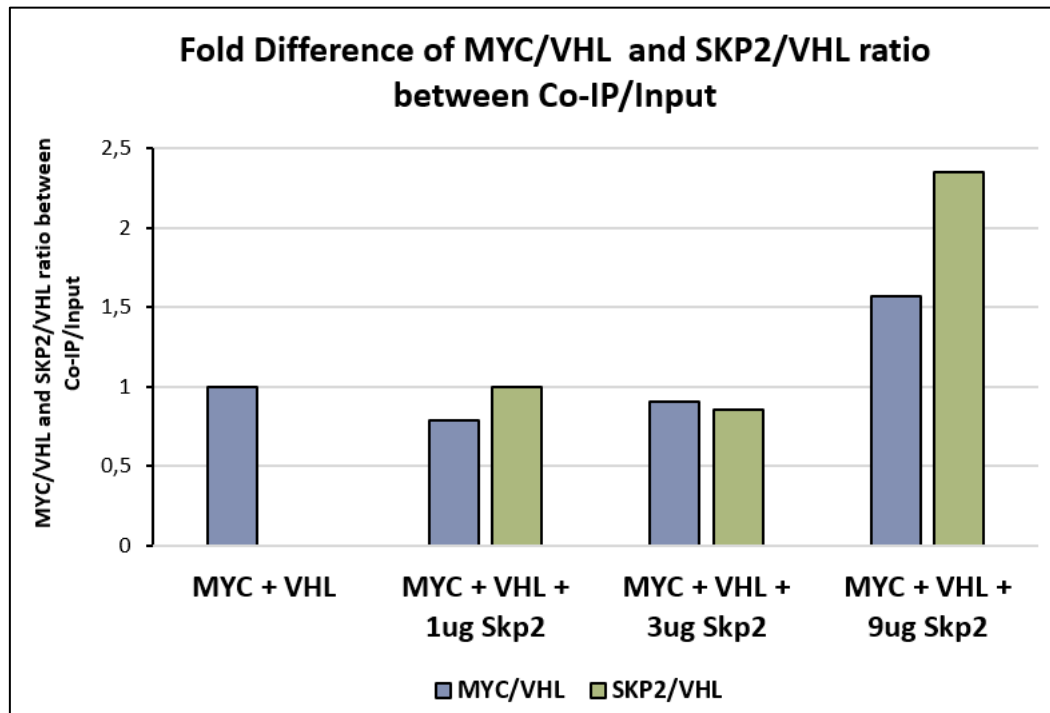


Figure 16: Fold Difference of MYC/VHL and SKP2/VHL ratio between co-IP/Input
 Grey bars indicate MYC/VHL interaction ratios, illustrating fold differences between IP and Input samples while accounting for potential expression level changes. These ratios were computed by comparing MYC/VHL ratios in the IP samples to their corresponding Input samples. Green bars represent SKP2/VHL interaction ratios, demonstrating fold differences between IP and Input samples while considering expression level changes. Similar to MYC, these ratios were calculated by comparing SKP2/VHL ratios in the IP samples to their corresponding Input samples.

Figure 16 illustrates that among the samples with titrated *SKP2*, the MYC/VHL interaction (grey bars) appears to increase. However, values for samples with 1 μ g and 3 μ g *SKP2* remain still lower than the value observed in the sample without *SKP2*. These values may indicate MYC/VHL interaction affinity, but might also be influenced by Co-IP efficiency. Notably, in the sample containing 9 μ g *SKP2*, the fold change in interaction was much higher than in the samples with lower amounts of *SKP2* or no *SKP2*. Additionally, the SKP2/VHL interaction was significantly higher in this sample compared to the samples with a low amount of *SKP2*. Interestingly, there was a slight decrease observed between the samples with 1 μ g *SKP2* and 3 μ g

SKP2. These results suggest a complex interplay between these proteins, indicating that as *SKP2* interacts more with VHL, MYC also shows an increased interaction with VHL. This supports the idea of a stimulatory effect between these proteins in binding to MYC.

For reliability, this experiment was replicated three times, all yielding consistent results. Across three replicate experiments, it was consistently observed that VHL expression levels exhibited variations under different *SKP2* titration conditions. Notably, VHL expression appeared lower in samples containing 1/2 and 3/4 μg *SKP2* compared to samples with no *SKP2* and those with higher *SKP2* concentrations (8/9 μg). This observation suggests a potential dose-dependent effect of *SKP2* on VHL expression, indicating potential regulatory dynamics between the two proteins. The immunoprecipitated VHL levels consistently mirrored the trends observed in the input samples across all experiments. Importantly, co-immunoprecipitated MYC consistently increased with higher titrated *SKP2* concentrations across all experiments, while MYC expression in the input samples remained relatively constant. Importantly, *SKP2* expression consistently mirrored the increased plasmid dosage across all experiments. Unexpectedly, *SKP2* bands also emerged in the co-IP samples, further supporting the unexpected finding of an *SKP2*-VHL interaction.

To provide an alternate viewpoint, I conducted the experiment using an anti-HA antibody for the co-IP, which effectively pulled down MYC. Subsequent Western blot analysis against VHL and *SKP2* highlighted changes in protein interactions. This approach allowed me to investigate the competitive dynamics between *SKP2* and VHL from another perspective.

To enhance the reliability of my findings, I performed this approach three times with the most representative result presented in the appendix. Its outcomes mostly align with those obtained from the FLAG-IP experiments, thereby supporting my findings. I observed consistent MYC expression levels, while co-immunoprecipitation of MYC increased with higher *SKP2* concentrations, suggesting a stimulatory effect. However, in this specific experiment, an interesting anomaly occurred with 8 μg *SKP2*, where co-immunoprecipitated *SKP2* and VHL did not increase despite higher MYC levels. Possible reasons for this phenomenon could include variations in transfection efficiency, saturation effects in protein interactions, or experimental variability.

3.4 Characterization of Single and Double VHL Mutants

The specific amino acid residues of VHL that are critical for MYC binding are still unknown.

This study is based on NMR analyses performed by Collaborators of our laboratory, namely the Zinzalla laboratory in Cambridge. NMR studies have been carried out to determine the molecular details of the binding of the VHL VCB (VHL, Elongin C and Elongin) complex to the c-MYC:MAX C-terminal (bHLHZip) heterodimeric apo complex. Full NMR assignment of the VHL VCB complex were previously determined by Dr Mark Bycroft and colleagues (Knauth et al., 2009), the crystal structure of the VHL VCB complex binding to a HIF-1 α peptide was also available (PDB: 1LM8, Figure A2 in the appendix). The Zinzalla lab has previously obtained the full assignment of the human c-MYC:MAX C-terminal apo complex as well as its crystal structure at very high resolution (Sammak et al., 2019; PDB = 6G6K, Figure A3 in the appendix). This has set the scene for them to carry out binding studies by NMR to map the interactions between these two master regulators of transcription. Amino acid residues that potentially drive the protein-protein interactions between the two complexes were identified by NMR. These were then further analysed by the Zinzalla lab, and mutants of VHL VCB and c-MYC:MAX that potentially would disrupt the formation of the VHL VCB/c-MYC:MAX complex were suggested. Combining the data obtained by NMR and the crystal structures previously obtained, structural analysis was carried out aided by computational tool Missense 3D (Ittisoponpisan et al., 2019; Khanna et al., 2021), to determine which mutations that would not be structurally damaging of the folding of each complex. Further, using the COSMIC database (Tate et al., 2019; Wellcome Sanger Institute, n.d.) each of the amino acids involved in the protein-protein interactions were analyzed to identify how frequently missense mutations of these residues are present in cancer. Several residues were thus identified to prepare mutants with a single or double mutations on VHL VCB that could fully inhibit or partially disrupt the binding (Table A1 in the appendix).

In this study, it was decided to investigate six specific amino acid residues. To accomplish this, single mutants of these six residues were initially generated, that we call M1-M6. Subsequently, I created double mutants derived from these single mutants, that we call D1-D8 (Table 4). The specific residues and corresponding mutations, as well as the primer sequences used for their generation, cannot be revealed at this point due to confidentiality reasons.

To achieve this goal, site-directed mutagenesis was performed using specific primers that introduced the selected mutations into the VHL gene. Through this technique, it was possible to introduce the desired mutations into the VHL gene. Subsequently, transformation into

competent *E. coli* bacteria was performed. After growing colonies of these bacteria, some colonies were selected to be expanded in LB media and the plasmid was extracted from these bacteria. The plasmids were sent to an internal Sanger sequencing facility to be sequenced. Upon receiving the decoded sequences, it was possible to confirm which colonies had the desired mutations in the VHL gene. If the mutations were introduced correctly, the plasmids were transformed again into *E. coli* cells to obtain only colonies carrying the targeted mutation. The bacteria were then expanded in a larger volume of LB broth and re-extracted to obtain larger amounts of the specific VHL mutant plasmids. The plasmids were then transfected into Cos7 cells, and overexpression experiments were performed using Western blot analysis. This was done to test whether specific mutations in the VHL gene had an effect on the expression level of VHL. Once this was tested, co-immunoprecipitations (co-IPs) were performed to investigate whether the mutations in VHL had effects on the interaction of VHL with MYC. Subsequent analysis of Western blot images determined whether or not the intended inhibition or prevention of interaction with MYC was achieved by the introduced mutations or not.

At the outset of this study, it was imperative to create specific mutagenesis primers tailored to introduce precise substitution mutations into the *FLAG-VHL* gene. These primers were custom-designed to target critical amino acid residues within VHL, allowing for the controlled introduction of desired mutations for subsequent investigations. The initial step involved identifying the target sequence, which in our case was the *VHL* gene. To achieve this, I referred to the PubMed gene database, which provided the amino acid sequence along with the corresponding CCDS sequence. Additionally, insights from NMR analysis conducted at the Zinzalla lab in Cambridge guided me in pinpointing the specific positions in VHL requiring modification and the corresponding amino acid substitutions. With both the amino acid and nucleotide sequences identified, I could determine the precise amino acid positions that needed alteration. By referring to the codon table, I identified the relevant codons. Knowing the target amino acid for substitution, I determined the specific base or bases requiring alteration. To streamline the primer design process, I used the NEBBaseChange tool for primer design. For substitutions, this inserted the desired mutated sequence directly into the forward (FWD) primer. After careful validation, I ordered these primers for use in site-directed mutagenesis experiments. For confidentiality reasons, I cannot disclose the specific primer sequences used. The summarized process is illustrated in Figure 17, using the *INSULIN* Gene as an example.

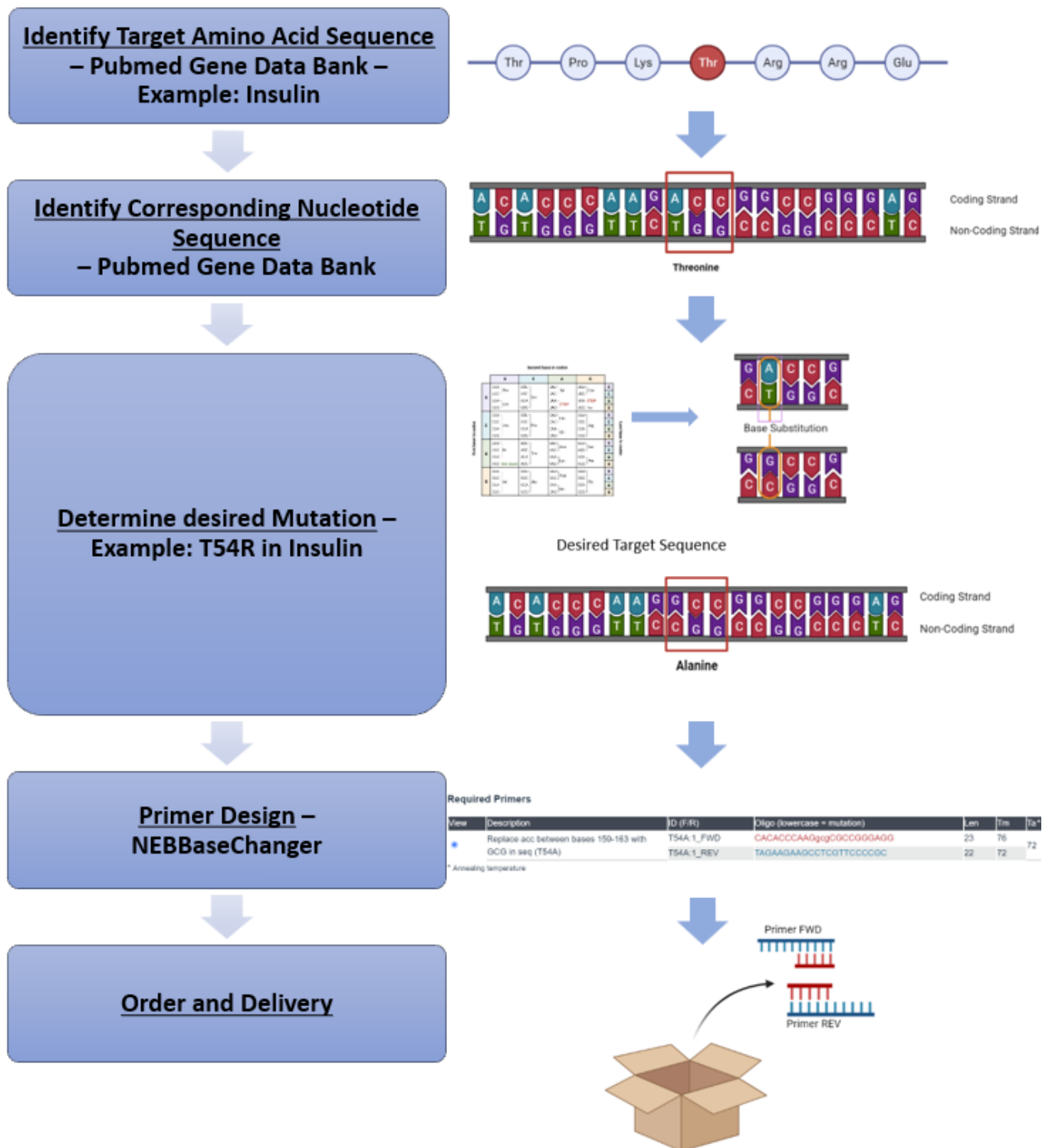


Figure 17: Primer Design Process

This figure outlines the process of primer design used in the study. The main text provides detailed explanations of each step.

Following the design and receipt of appropriate primers, I proceeded with site-directed mutagenesis to introduce specific mutations into the *VHL* gene. The process is illustrated in Figure 18. In this process, the plasmid containing *Flag-VHL*, which served as the target gene, underwent amplification through PCR. I used high-fidelity DNA polymerase and mutagenic primers tailored for each desired mutation. For site-directed mutagenesis, I employed the Q5® Site-Directed Mutagenesis Kit. After successful amplification, the PCR product underwent enzymatic treatments, including kinase, ligase, and DpnI digestion. The resulting mutated DNA was then transformed into competent *E. coli* cells, and colonies were selected for further analysis. Plasmid DNA was extracted from these bacterial colonies and subjected to sequencing to confirm the presence of the desired mutations. Notably, sequencing analysis confirmed the successful introduction of the desired mutations into the *VHL* gene.

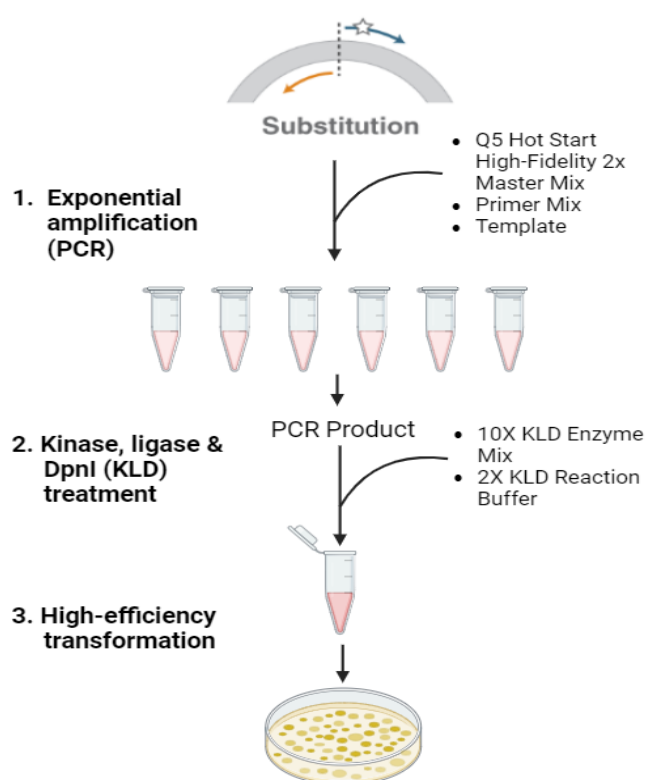


Figure 18: Site-Directed Mutagenesis

This figure visually represents the process of site-directed mutagenesis, which has been elaborated on in the main text. It provides an overview of the key steps involved in introducing specific mutations into the target gene.

I created alignment images to visually represent the amino acid changes within the VHL protein for both the wild type and mutant variants. These images demonstrate the specific amino acid sequences and the alterations at particular residues. Unfortunately, due to confidentiality constraints, these images cannot be displayed. However, Figure 19 showcases a similar image for the *INSULIN* gene, following the same design process as the VHL mutations.

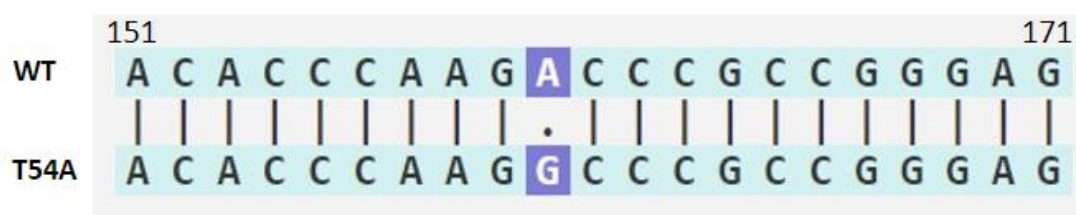


Figure 19: Alignment Sequence Example (Insulin wildtype and substitution)
This figure is an example illustrating the alignment sequence between the wildtype insulin gene and an inserted substitution. It is important to note that this insulin gene example was not actually generated but serves to demonstrate how VHL mutation alignment images appeared. Base substitutions for each amino acid alteration are depicted in purple.

It was confirmed that all mutations were successfully incorporated into the *VHL* gene, resulting in the generation of 6 single mutants and 8 double mutants of VHL. An overview of these mutations is provided in Table 4.

Table 4: VHL Single and Double Mutations

For confidentiality reasons, the specific amino acid changes and positions cannot be revealed.

<u>Single Mutations</u>	<u>Double Mutations</u>
M1	D1 (M1 + M5)
M2	D2 (M2 + M5)
M3	D3 (M3 + M4)
M4	D4 (M5 + M6)
M5	D5 (M4 + M5)
M6	D6 (M3 + M6)
	D7 (M3 + M5)
	D8 (M4 + M6)

After successfully generating the mutants, I proceeded with the interaction studies.

The purpose of the interaction studies was to assess the impact of VHL mutants on its interaction with MYC. Given that the mutations were specifically designed to disrupt interaction with MYC's bHLH-Zip region, a deletion mutant of MYC encompassing amino acids 221-439 was employed (Figure 20).

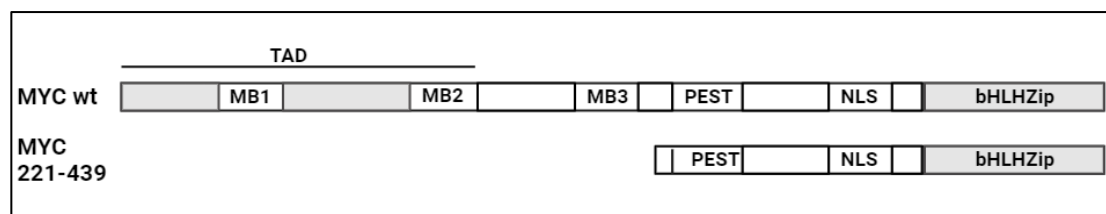


Figure 20: MYC-ΔN3

MYC Deletion mutant ΔN3 encompasses amino acids 221-439. (lower part)

To compare MYC wildtype is illustrated in the upper part of the figure.

This deletion mutant retained the bHLH-Zip region of MYC while excluding MYC box II. This distinction was crucial, as VHL interacts with both these regions of MYC. Including MYC Box II in the analysis would likely result in only small changes in interaction, making it challenging to observe differences between mutants and the wild type.

The initial step involved confirming the interaction between the HA-MYC-ΔN3 deletion mutant and FLAG-VHL. To achieve this, an Immunoprecipitation (IP) was performed using an anti-FLAG antibody to selectively capture FLAG-VHL and simultaneously co-immunoprecipitate HA-MYC-ΔN3. Results of the subsequent Western blot analysis are shown in Figure 21.

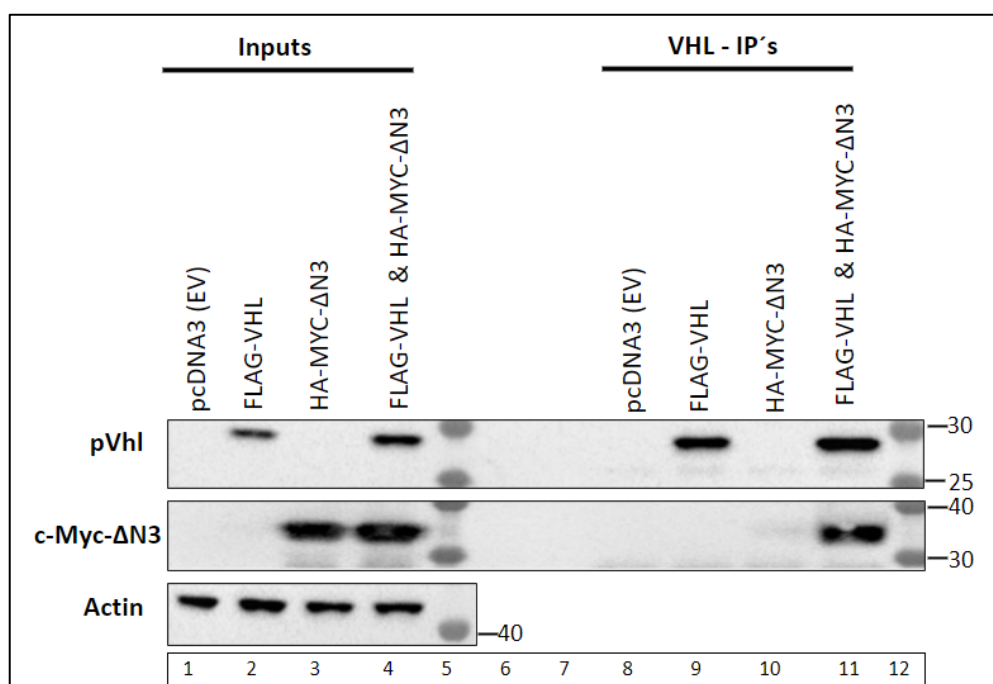


Figure 21: Confirmation of MYC- Δ N3 Deletion Mutant Interaction with VHL through Co-IP and Western Blot Analysis

Cos7 cells were subjected to four transfection conditions: three single transfections (including pcDNA3 as a negative control, Flag-VHL, and HA-MYC- Δ N3 as positive controls), as well as one double-transfection involving VHL and MYC- Δ N3. A total of 17 μ g of plasmid DNA, with 8.5 μ g for each plasmid, was used for transfection. Co-immunoprecipitation (Co-IP) was carried out using a FLAG antibody to capture VHL and its associated proteins. Subsequent Western blot analysis utilized an anti-HA antibody to detect MYC- Δ N3, a VHL antibody for VHL, and an Actin antibody for Actin. Input samples are presented on the left side (lanes 1-4), while co-IP samples are displayed on the right side (lanes 8-11)

Western blot analysis confirmed VHL expression in the respective samples (lanes 2 and 4) and successful VHL immunoprecipitation (lanes 9 and 11). Similarly, MYC- Δ N3 expression was validated (lanes 3 and 4), and the co-immunoprecipitation demonstrated the interaction of MYC- Δ N3 with VHL (lane 11). Actin served as a reliable loading control, with equal band intensities, ensuring the absence of loading issues. To ensure reliability, this experiment was replicated three times, consistently confirming the interaction between VHL and MYC- Δ N3.

With the interaction confirmed, I proceeded to assess the impact of mutants on their interaction with MYC- Δ N3 by conducting VHL co-IP experiments. This enabled a comparison of interaction patterns between VHL mutants and HA-MYC- Δ N3 with those involving wild-type VHL and HA-MYC- Δ N3.

The first experiment targeted the 6 single mutants M1-M6, encompassing a total of 8 transfections. Subsequently, a co-immunoprecipitation (co-IP) assay was carried out to capture VHL and its associated proteins, followed by Western blot analysis. The resulting image is presented in Figure 22.

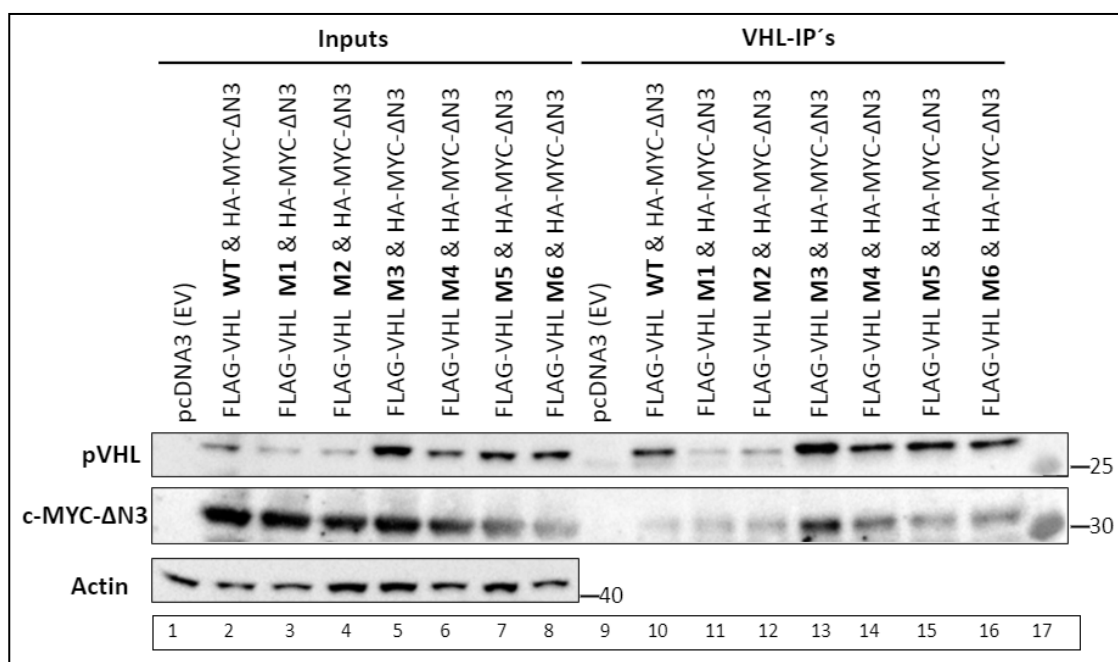


Figure 22: co-IP Analysis of VHL Single Mutants and VHL WT Interactions with MYC- ΔN3 Deletion Mutant
Cos7 cells were transfected with pcDNA3 (negative control), MYC-ΔN3 + VHL WT (positive control), and MYC-ΔN3 with VHL Single Mutants M1-M6. Each transfection utilized 8.5ug of plasmid DNA for a total of 17ug. co-IP was performed using an anti-FLAG antibody to capture VHL and its associated proteins. Western blot analysis employed anti-VHL, anti-HA (for MYC- ΔN3), and anti-Actin antibodies. Input samples are shown on the left (lanes 1-8), and co-IP samples on the right (lanes 9-16). Blotting issues affected MYC-ΔN3 bands in the input samples of MYC-ΔN3 co-transfected with mutants M5 and M6, as well as the IP samples with EV and the positive control with VHL WT. This experiment was only performed one time.

In lanes 2-8, Western blot analysis against VHL showed VHL expression in all respective samples. VHL expression in the Wildtype (WT) transfection was relatively low. Mutants M1 and M2 exhibited lower VHL expression, while mutants M3-M6 showed higher expression, with M3 having the highest. Lanes 9-16 display co-IP results, confirming VHL immunoprecipitation in all respective samples. The co-IP band pattern largely matched the one from the input samples. Mutants M1 and M2 had less pulled-down VHL compared to WT, while mutants M3-M6 showed similar or increased VHL pull-down. Mutant M3 had the highest amount of VHL pulled down. All this is consistent with the VHL expression levels in the samples.

Western blot analysis against MYC-ΔN3 confirmed the presence of MYC-ΔN3 in all samples, as evident in lanes 2-8. Notably, in co-transfections involving mutants M5 and M6, MYC-ΔN3 expression levels were notably lower, a phenomenon likely attributed to an observed blotting issue. This issue manifested as an air bubble on this specific region of the membrane. Lanes 10-16 show successful co-immunoprecipitation of MYC-ΔN3, indicating its interaction with VHL WT and all VHL mutants. Notably, the positive control displayed very low signal intensity, also attributed to the blotting issue during protein transfer. Pulled-down MYC-ΔN3 in M1 and M2 samples correlated with the immunoprecipitated VHL levels. Mutants M3 and M4 exhibited higher co-immunoprecipitated MYC-ΔN3, with M3 displaying the highest amount, consistent with VHL levels. However, it's important to note that in the M5 and M6 samples, reduced co-immunoprecipitated MYC-ΔN3 was observed. While this reduction might suggest a weakened VHL-MYC-ΔN3 interaction, additional factors should be considered. Specifically, there was also less protein expression observed in the input lanes for M5 and M6. This reduction in expression was initially attributed to a blotting issue. However, it's challenging to conclusively determine whether the reduced interaction is solely due to decreased binding affinity or if it's influenced by lower MYC-ΔN3 expression in the input samples. Therefore, it remains uncertain whether the interaction is genuinely reduced or if there is simply less MYC-ΔN3 available to bind with VHL.

In the Western blot analysis of actin, slight variations in band intensities were observed. Specifically, the MYC deletion mutant co-transfected with VHL mutants M3 and M2 exhibited the strongest signals, followed by slightly reduced signals in the sample containing M5. Further reductions were noted in the negative control, VHL wildtype (WT), M1, M4, and M6 samples, which displayed relatively consistent but slightly diminished band intensities compared to the previously mentioned samples.

Following investigation of the single mutants, I now investigated the behavior of the double mutants.

The initial focus was to assess the potential impact of double mutations on VHL protein stability and expression. To achieve this, an overexpression experiment was performed. The experiment was designed to reveal any changes in expression of VHL, as this would affect the co-IP experiments. The outcomes of this experiment provided valuable insights into the suitability of these mutants for subsequent co-immunoprecipitation (co-IP) assays, where their interaction with MYC was the primary point of interest.

For this purpose, transfection was performed, with 10 single transfections in Cos7 cells. The individual transfections included a negative control (*pcDNA3*), a positive control (VHL wt)

and the 8 double mutants VHL D1 – D8. After cell harvest, Western blot analysis was performed. The image of the overexpression experiment is shown in Figure 23.

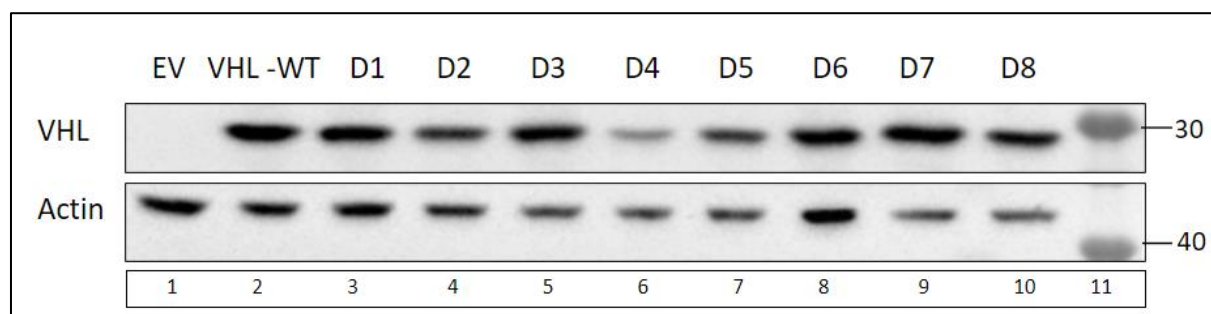


Figure 23: Protein Expression Analysis of Double Mutants
 Ten single transfections were conducted in Cos7 cells. pcDNA3 (EV) serves as the negative control, while VHL-WT represents the positive control. Lanes 3-10 correspond to the 8 Double Mutants under investigation. In the Western blot analysis, an anti-VHL antibody was utilized to visualize VHL expression (upper part of the figure). Additionally, an anti-actin antibody was employed to assess Actin expression and ensure there were no loading issues (lower part of the figure). The membrane is divided into upper and lower parts, with a small gap in between, to illustrate that it was cut into two parts for staining with different antibodies. Both parts originate from the same membrane. This experiment was performed one time.

Western blot analysis of VHL indicated that most of the mutants exhibited no significant change in VHL expression compared to the VHL wildtype. Notably, D4 (lane 6) showed a decrease in expression, while D2 and D5 displayed a slight decrease in expression relative to VHL-WT. In contrast, all other Double Mutants showed either no decrease or even a slight increase in expression. Significantly, the Western blot targeting actin indicated that compared to the negative control pcDNA3, there were subtle reductions in all other actin bands. Notably, the VHL wildtype and mutants D1 and D6 exhibited comparable band intensities, implying similar protein loads. In contrast, mutant D2 displayed a marginal reduction in the actin band. In the cases of mutants D3, D4, D5, D7, and D8, their intensities were further diminished, albeit these reductions remained relatively modest. These observations indicated that the reductions in VHL expression seen in some mutants could be attributed to a combination of potential transfection issues, instability, and variations in total protein load. These factors should be considered when interpreting co-IP results to assess changes in interaction with MYC.

Quantification of the resulting bands in the Western blot image was performed to accurately measure and objectively compare the differences. A graph representing the results is illustrated below in Figure 24.

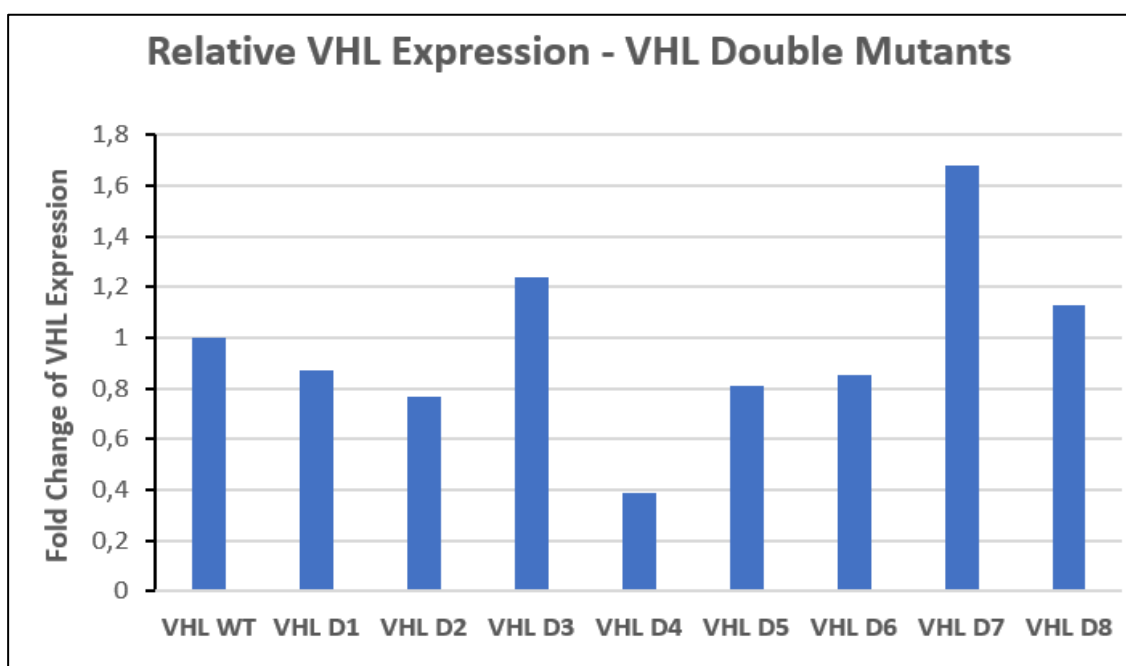


Figure 24: Relative VHL Expression in VHL Double Mutants
VHL bands were quantified and normalized to actin bands. The ratio of each mutant's expression relative to VHL-WT was calculated. The Y-axis represents the Fold Change of VHL Expression, while the X-axis represents the nine samples, including the wildtype as a positive control and the eight double mutants.

The quantification revealed a slight reduction in expression for mutants D1, D2, D5, and D6, compared to the wildtype. Mutant D4, as previously observed in the western blot image, displayed a significant drop by more than half in VHL expression. On the other hand, mutants D3, D7, and D8 showed an increase in VHL expression, indicating potential improvements in VHL stability and expression. It's important to consider the potential influence of transfection issues on the observed expression patterns. Thus, it remains uncertain whether the mutations alone are responsible for the observed changes in expression.

Given the results indicating no significant alterations in VHL expression, with the exception of a reduction in D4 and slight decreases in D1,2,5 and 6, I proceeded with the interaction studies involving VHL double mutants and MYC-ΔN3.

Due to challenges with mutagenesis and sequencing, the initial co-IP experiment was performed investigating only 4 mutants: D2, D3, D5, and D6. Six transfections were executed, followed by co-IPs and subsequent Western Blot analysis. Figure 25 displays the resulting image.

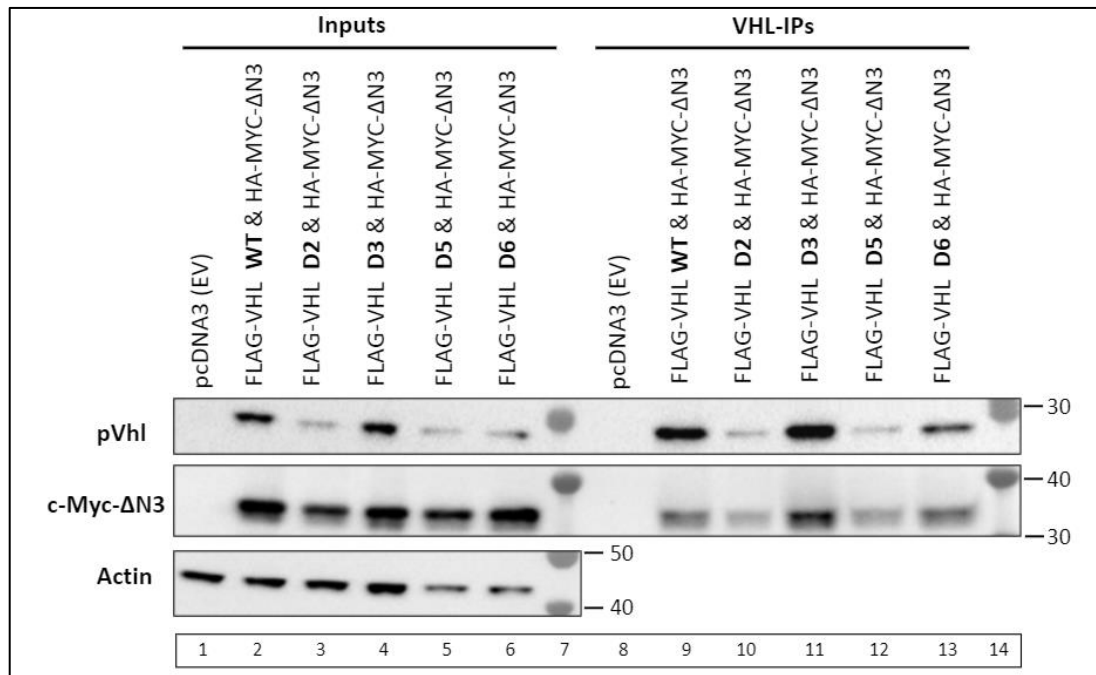


Figure 25: co-IP Analysis of VHL Double Mutants Interaction with MYC-ΔN3
Cos7 cells were subjected to six transfections, which included a single transfection with pcDNA3 as a negative control, a co-transfection of VHL WT + MYC-ΔN3 as a positive control, and four co-transfections involving MYC-ΔN3 along with the double mutations D2, D3, D5, and D6. A total of 17 μg of plasmid DNA was transfected. co-IP was performed using an anti-FLAG antibody to capture VHL and its associated proteins, with 250 μg of protein used for Co-IP and 15-minute incubation periods. Western blot analysis utilized anti-VHL, anti-HA, and anti-Actin antibodies. The left side of the image displays input samples to illustrate expression levels, while the right side shows VHL-IP samples for investigating potential interaction changes.

Western blot analysis targeting VHL revealed the presence of VHL expression in lanes 2-6. However, input samples of mutants D2, D5, and D6 showed reduced expression levels compared to the wildtype. These reductions could be attributed to several factors, including loading issues, particularly evident in the Actin bands of D5 and D6, or potential transfection issues. Additionally, double mutations could impact protein stability, as observed with D2 and D5 in a separate overexpression experiment where VHL alone exhibited a slight decrease. Notably, D6 appeared stable when transfected alone, but stability issues might arise in the presence of MYC-ΔN3 co-transfection. In lanes 8-13, the IP samples confirm successful VHL immunoprecipitation in all corresponding samples. Notably, signal intensities in samples containing mutants D2 and D5 are considerably low, consistent with their lower expression levels. The sample containing Mutant D3 shows a slightly stronger signal, mirroring its higher expression. Notably, the sample containing D6 exhibits a lower signal intensity compared to the wildtype, consistent with its low VHL expression level (lane 6). However, despite its lower expression, it demonstrates a higher amount of pulled-down VHL compared to samples with similar VHL expression levels, such as D2 and D5.

Western blot analysis of MYC- Δ N3 confirmed the expression of the protein in all samples (lanes 2-6). Samples containing mutants D2 and D5 displayed lower expression levels, aligning with the reduced VHL expression in these samples. This observation suggests potential lower transfection efficiency. Additionally, the lower protein load in D5 could also contribute to this pattern. Samples containing D3 and D6 have similar or even slightly higher expression levels. In lanes 9-13, MYC- Δ N3 was co-immunoprecipitated with VHL across all samples where it was transfected. Mutants D2 and D5 exhibited a reduced amount of co-immunoprecipitated MYC- Δ N3, consistent with the lower VHL expression levels in these samples. Mutant D6, despite exhibiting a low amount of immunoprecipitated VHL, displayed a MYC- Δ N3 signal intensity similar to the wildtype, indicating a strong interaction. The sample containing mutant D3 showed the highest amount of pulled-down MYC- Δ N3, indicating a robust interaction.

The Western blot analysis of actin showed slightly reduced expression levels in samples with mutants D5 and D6 (lanes 5 and 6), implying a lower total protein load in these samples. Conversely, all other transfections maintained a consistent level of Actin expression, indicating uniform protein loading.

When qualitatively assessing the differences in interaction, there seemed to be no significant overall reduction in interaction observed when evaluating the intensity of the MYC- Δ N3 bands in relation to the corresponding VHL bands and comparing this ratio in samples containing double mutants with the reference sample containing wild-type VHL.

For a better understanding of Changes in interaction between VHL Wildtype and VHL mutants, a quantification was performed. First, I quantified and calculated relative expression levels of MYC- Δ N3 and VHL, as well as the relative immunoprecipitated protein levels. The resulting graphs are presented in Figure 26 and 27.

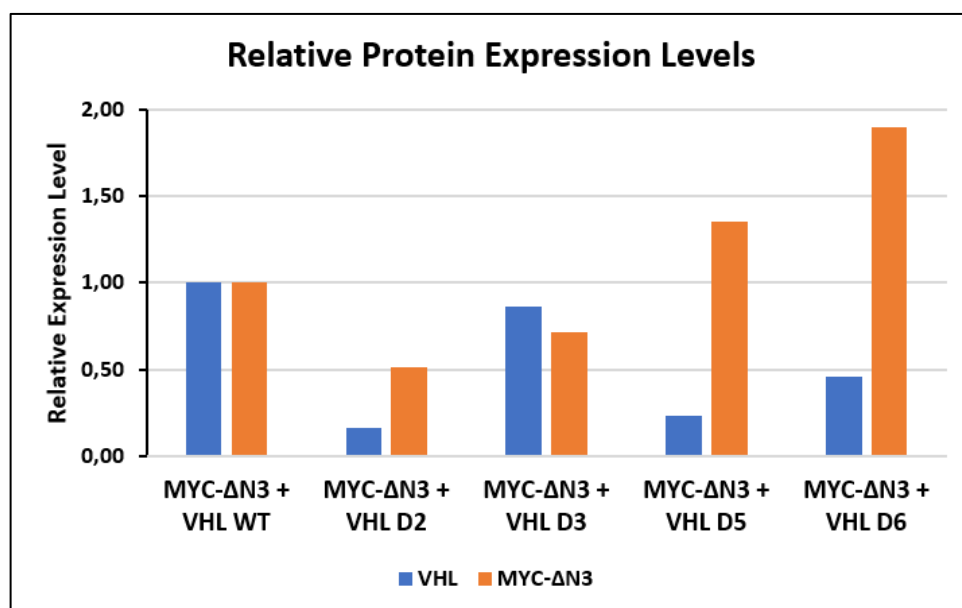


Figure 26: Relative Expression Levels of VHL and VHL Mutants D2, D3, D5, D6 and MYC-ΔN3

The Y-axis indicates the Relative Expression level, and the X-axis lists the five samples being investigated. MYC-ΔN3 + VHL WT was used as a reference sample. The intensities of the bands in the input samples were quantified using ImageJ. Both MYC-ΔN3 and VHL bands were normalized to their corresponding Actin bands to correct for potential loading issues. Subsequently, the resulting ratios were further normalized to the reference sample, allowing the determination of changes in expression levels for samples containing mutants relative to the reference sample

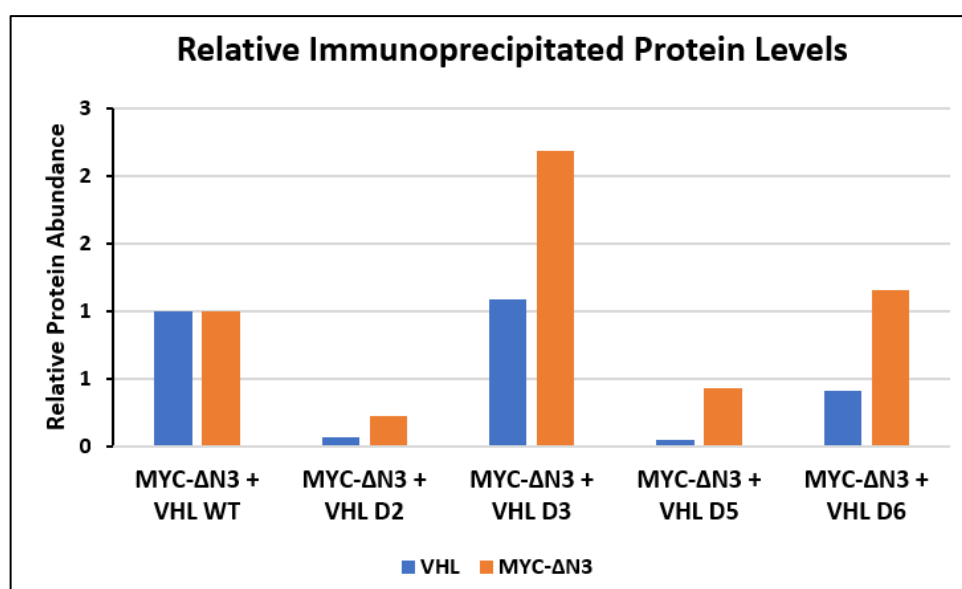


Figure 27: Relative Immunoprecipitated Protein Levels in VHL-IP samples of VHL and VHL Mutants D2, D3, D5, D6 and MYC-ΔN3

The Y-axis indicates the Relative Protein Abundance in VHL- IP samples, and the X-axis lists the five samples being investigated. MYC-ΔN3 + VHL WT was used as a reference sample. The intensities of the bands in the IP samples were quantified using ImageJ. Both MYC-ΔN3 and VHL bands were normalized to the reference sample, allowing the determination of changes in levels of immunoprecipitated protein for samples containing mutants relative to the reference sample

The graph in figure 26 reveals that VHL expression was significantly lower in samples with mutants D2, D5, and D6 compared to the reference sample. Conversely, mutant D3 showed a relatively less reduced VHL expression level, consistent with the qualitative assessment. In the case of MYC-ΔN3, samples containing mutants D2 and D3 display decreased expression, while those with mutants D5 and D6 exhibited increased expression. These quantitative findings provided a more nuanced understanding of protein quantities, especially

considering that the qualitative assessment led to different interpretations regarding MYC- Δ N3 expression.

The graph in Figure 27 illustrates significant differences in the amount of immunoprecipitated VHL across various samples, aligning with the qualitative assessment. Samples containing VHL D2 and D5 exhibited notably lower immunoprecipitated VHL levels, which can be attributed to their reduced VHL expression. Similarly, VHL D6 showed a slight decrease in immunoprecipitated VHL, mirroring its expression level. Interestingly, VHL D3 displayed slightly higher immunoprecipitated VHL than the wildtype, despite a slightly lower expression level, potentially indicating a more efficient immunoprecipitation process. When considering the levels of co-immunoprecipitated MYC- Δ N3, they generally followed the expression level patterns, except for the sample containing mutant VHL D3. In this case, there was a significant increase in MYC- Δ N3 co-immunoprecipitation compared to the reference.

To gain deeper insights into the interaction dynamics between MYC- Δ N3 and VHL, I have also calculated the relative MYC/VHL ratios for both input and IP samples, relative to the reference sample (Figure 28).

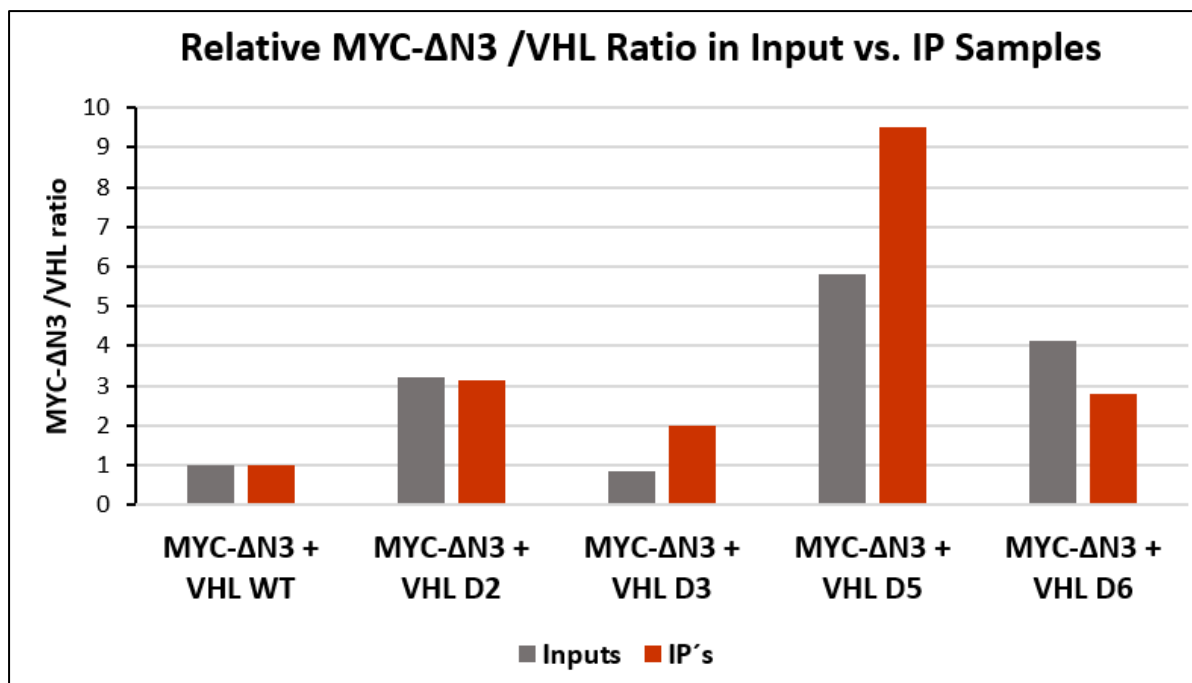


Figure 28: Relative MYC- ΔN3 /VHL Ratio in Input vs.VHL-IP Samples (D2,D3,D5,D6)

The Y-axis represents the Relative MYC- ΔN3/VHL ratio, which has been normalized to the reference sample, while the X-axis lists the five samples under investigation. The reference sample used is MYC-ΔN3 + VHL WT. To calculate this ratio, the intensities of the MYC-dN3 bands in the input samples were divided by the corresponding VHL band intensities, indicating the relative presence of MYC compared to VHL. The same calculation was performed for the protein bands in the IP (immunoprecipitation) lanes. Subsequently, both the input and IP ratios were normalized to the reference sample.

The graph illustrates changes in the MYC-ΔN3/VHL ratio compared to the reference condition in both input and IP samples. By comparing these ratios between IP and input samples, it became possible to determine if there was an increase or decrease in interaction. The input ratio provided valuable insights into the relative abundance of these proteins in the input samples, serving as a baseline reference for their expression levels. However, it is important to note that the input ratio does not directly account for their interaction; it primarily reflects protein abundance. To evaluate changes in the interaction between MYC and VHL, I focused on the IP ratio. This ratio is calculated from the co-immunoprecipitated proteins in the IP samples, where MYC and VHL are brought together. By comparing the IP ratio to the input ratio, I could discern whether there were alterations in their interaction under different conditions. This approach allowed me to isolate changes in interaction from variations in overall protein

expression levels, providing a comprehensive understanding of MYC-VHL interaction dynamics.

In the graph, it was evident that the IP ratios closely resembled the ratios in input samples for the Wildtype and D2 sample, indicating no change in interaction. However, D3 and D5 samples exhibited an increased IP ratio compared to the input ratio, suggesting a potential increase in interaction. Interestingly, the D6 mutant sample displayed a lower IP ratio compared to input, indicating a possible reduction in interaction affinity.

For increased clarity, the previously obtained IP ratios have been normalized by their corresponding Input ratios. This normalization allows for the evaluation of changes that may suggest alterations in interaction affinity while considering fluctuations in protein expression levels. The resulting graph is displayed in Figure 29. It's important to acknowledge that these observed changes could be influenced by various factors, such as the efficiency of the IP process, which could potentially explain the results.

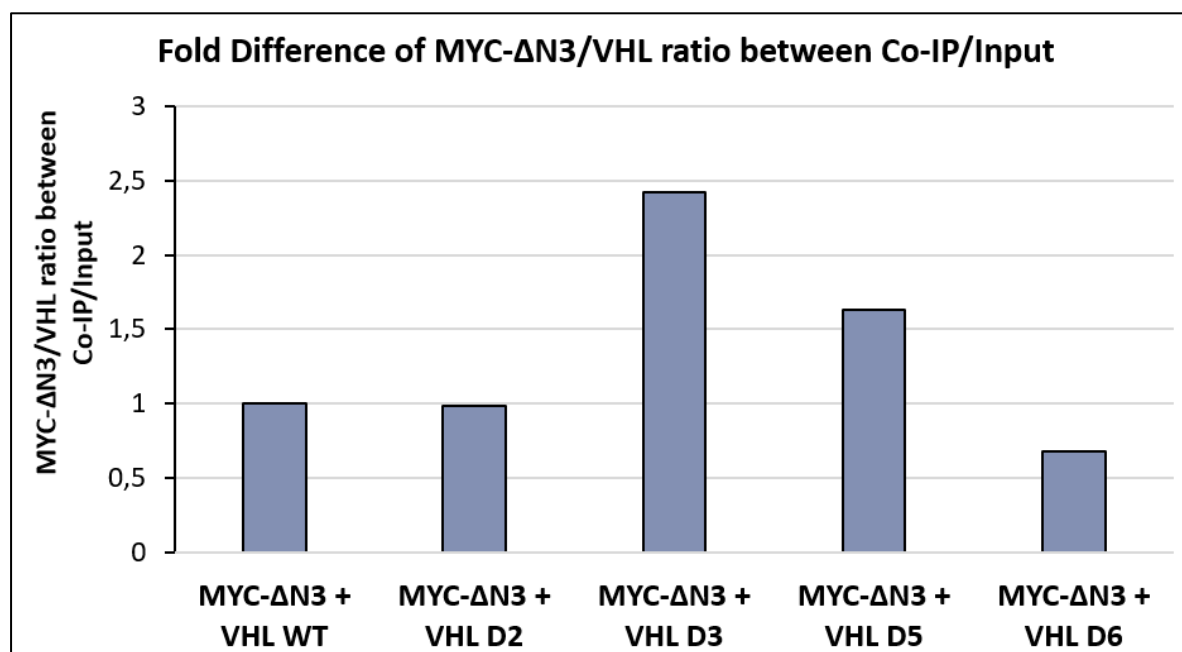


Figure 29: Fold Difference of MYC-ΔN3/VHL ratio between Co-IP/Input (D2, D3, D5, D6)
The Y-axis displays the MYC-ΔN3/VHL ratio of Co-IP/IP, accounting for variations in expression levels. The X-axis enumerates the samples examined. The reference sample is MYC-ΔN3 + VHL WT. This ratio was calculated by dividing the MYC-ΔN3/VHL ratio of IP samples by the corresponding ratios of the input samples.

The graph consistently showed higher ratio values (possibly reflecting interaction affinity) in all mutant samples compared to the wildtype, with the exception of the D6 sample, which exhibited a slight decrease. Overall, in line with the qualitative assessment, there was no

complete abolishment of interaction. Only a minimal reduction was observed in the D6 mutant sample, which might be due to a slight decrease in interaction or other factors during the co-IP process.

However, it's important to note that this quantification did not provide an absolute measurement of the interaction affinity itself. Additional experiments or assays may be necessary for a more precise quantification of interaction strength.

Following the assessment of the four initial double mutants, a decision was made to reevaluate D2 and D5 alongside the remaining double mutants that were now available for analysis. For the assessment of the effects of mutations D1, D2, D4, D5, D7, and D8, an additional co-IP experiment was conducted. Western blot analysis was subsequently performed, and the outcomes are visualized in Figure 30 for qualitative evaluation.

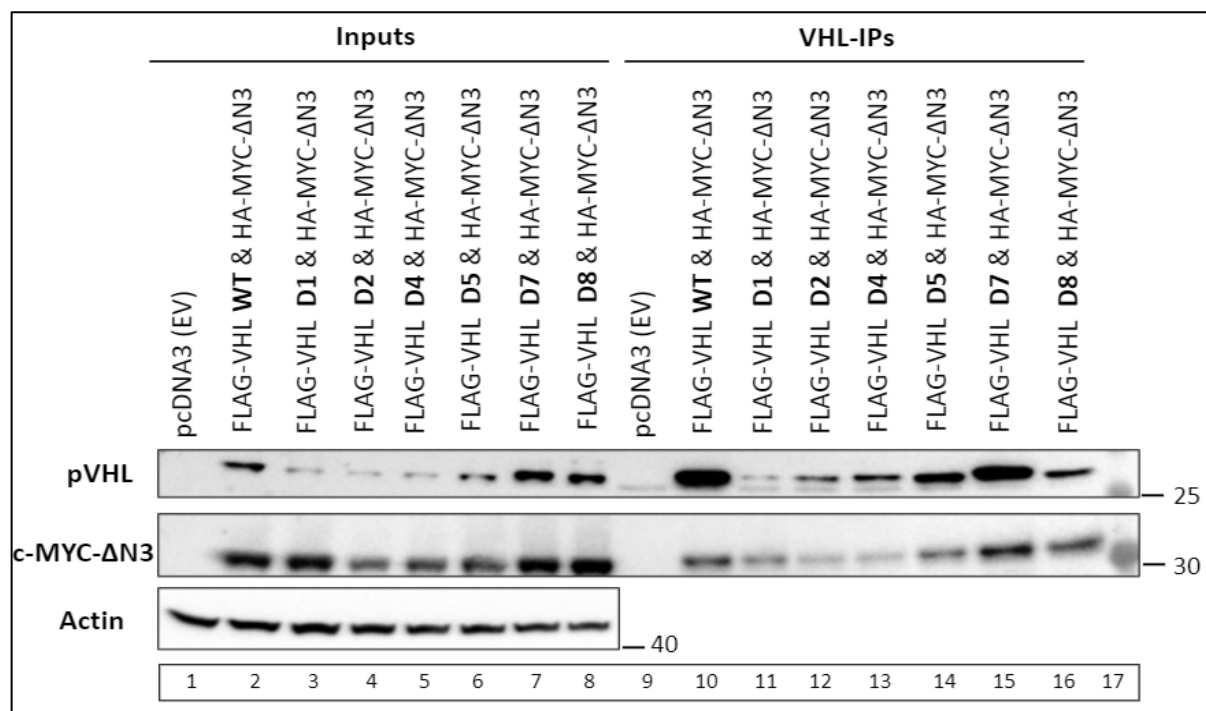


Figure 30: Co-IP analysis of Interaction between VHL Double Mutants with MYC-ΔN3
Eight transfections were conducted in Cos7 cells, including a single transfection with pcDNA3 serving as the negative control, co-transfection of MYC-ΔN3 as the positive control, and six additional co-transfections involving MYC-ΔN3 and VHL double mutants (D1, D2, D4, D5, D7, and D8). A total of 17 μg of plasmid DNA was used for transfection. The co-immunoprecipitation (Co-IP) was performed using an anti-FLAG antibody to capture VHL and its associated proteins, with 250 μg of proteins and an incubation time of 15 minutes. Western blot analysis was carried out using anti-VHL, anti-HA, and anti-Actin antibodies. The left side of the image (lanes 1-8) represents input samples for assessing expression levels, while the right side (lanes 9-16) displays the IP samples for investigating interaction changes.

Western blot analysis confirmed the expression of VHL in all samples (lanes 2-8). Mutants D1, D2, and D4 exhibited significantly lower VHL expression compared to the wildtype, while mutant D5 showed an expression level lower than the wildtype but higher than the aforementioned mutants. Mutants D7 and D8 displayed similar or slightly increased VHL expression levels. co-IP results in lanes 10-16 revealed successful VHL pull-down in all samples. However, all mutants exhibited reduced VHL pull-down compared to the wildtype. Among the mutants, D1 displayed the lowest pull-down, followed by D2 and D4, while D8 showed a slightly higher pull-down than D4. D5 exhibited intermediate pull-down, and D7 displayed the highest pull-down, although still lower than the VHL wildtype sample.

The Western blot analysis of MYC- Δ N3 confirmed its expression in all samples (lanes 2-8). Notably, samples containing mutants D2, D4, and D5 exhibited lower expression levels compared to the wildtype VHL sample. It's possible that these observations are influenced by factors such as transfection issues or protein stability concerns. The remaining samples showed similar expression levels. In lanes 10-16, the co-IP results demonstrated co-immunoprecipitation of MYC- Δ N3 in all respective samples. Interestingly, samples containing mutants D2 and D4 displayed a reduced amount of co-pulled-down MYC- Δ N3, indicating reduced interactions with VHL. Samples containing D1 and D5 exhibited lower pull-down than the wildtype, with D1's pull-down correlating with the amount of immunoprecipitated VHL, while D5 exhibited more VHL pull-down, suggesting a potential decrease in interaction. Mutant D8 exhibited a similar amount of co-immunoprecipitated MYC- Δ N3, despite having less VHL pulled down, implying an increased interaction. In contrast, the sample containing D7 showed a higher amount of pulled-down VHL than the wildtype VHL sample, indicating a robust interaction.

The Western blot analysis against actin revealed generally consistent expression levels across all samples, indicating no significant loading issues. However, it's worth noting that in lanes containing D4 to D8, a subtle difference in band size was observed. Overall, qualitative assessment suggests that none of the double mutants completely prevented the binding to MYC. Some reduction in interaction strength is apparent in D2, D4, and D5, although this requires further confirmation through quantitative analysis.

Therefore, I first evaluated the expression levels of all samples containing VHL mutants compared to the reference sample containing the VHL wild type. I also calculated the relative immunoprecipitated protein levels. The resulting graphs are represented in figure 31 and figure 32.

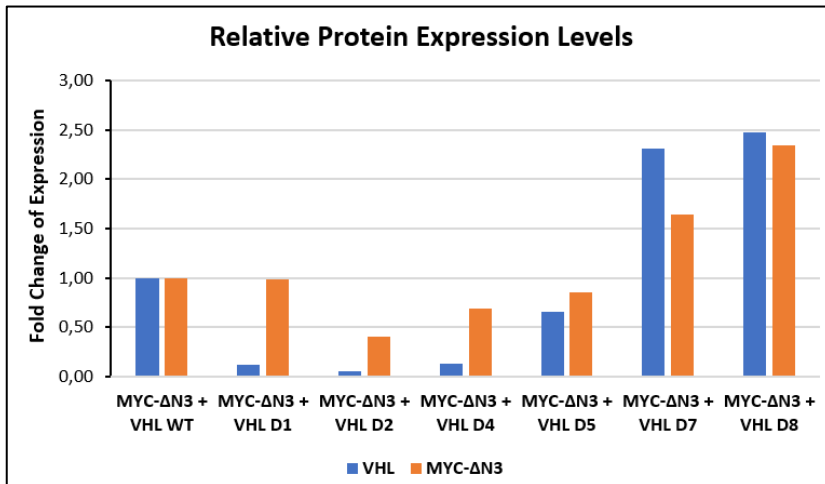


Figure 31: Relative Protein Expression Levels (VHL D1, D2, D4, D5, D7, D8)

The Y-axis indicates the Relative Expression level, and the X-axis lists the samples being investigated. MYC-ΔN3 + VHL WT was used as a reference sample. The intensities of the bands in the input samples were quantified using ImageJ. Both MYC-ΔN3 and VHL bands were normalized to their corresponding Actin bands to correct for potential loading issues. Subsequently, the resulting ratios were further normalized to the reference sample, allowing the determination of changes in expression levels for samples containing mutants relative to the reference sample

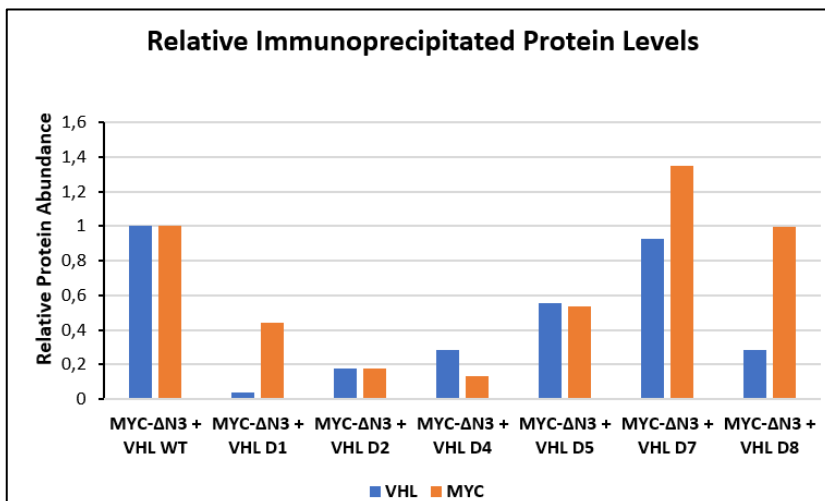


Figure 32: Relative Immunoprecipitated Protein Levels in IP samples (VHL D1, D2, D4, D5, D7, D8)

The Y-axis indicates the Relative Protein Abundance in the VHL- IP samples, and the X-axis lists the samples being investigated. MYC-ΔN3 + VHL WT was used as a reference sample. The intensities of the bands in the IP samples were quantified using ImageJ. Both MYC-ΔN3 and VHL bands were normalized to the reference sample, allowing the determination of changes in levels of immunoprecipitated protein for samples containing mutants relative to the reference sample

Figure 31 depicts the expression levels of VHL and MYC-ΔN3. VHL expression was significantly lower in samples with mutants D1, D2, D4, and D5 compared to the wildtype, consistent with our qualitative observations. Conversely, D7 and D8 samples exhibited notably higher VHL expression. Similar trends were seen in MYC-ΔN3 expression, with D2, D4, and D5 showing lower levels, and D7 and D8 displaying increased MYC-ΔN3 expression. These expression differences may be attributed to various factors, including potential transfection or stability issues.

In Figure 32, we delved into protein abundance in IP samples. Across all samples, there was lower immunoprecipitated VHL compared to the reference sample. D7 most closely resembled the WT, D5 exhibits roughly half the VHL immunoprecipitation, D4 and D8 displayed

even greater reductions, D2 even more so, and D1 has the least VHL immunoprecipitated. These findings aligned with our qualitative assessments. MYC-ΔN3 abundance mirrors this pattern, with D1, D2, D4, and D5 exhibiting reduced levels, D8 showing similar levels to the wildtype, and D7 demonstrating increased MYC-ΔN3 co-immunoprecipitation.

To provide a clearer understanding, I've calculated the relative MYC-ΔN3/VHL ratio in both input and IP samples, as illustrated in Figure 33.

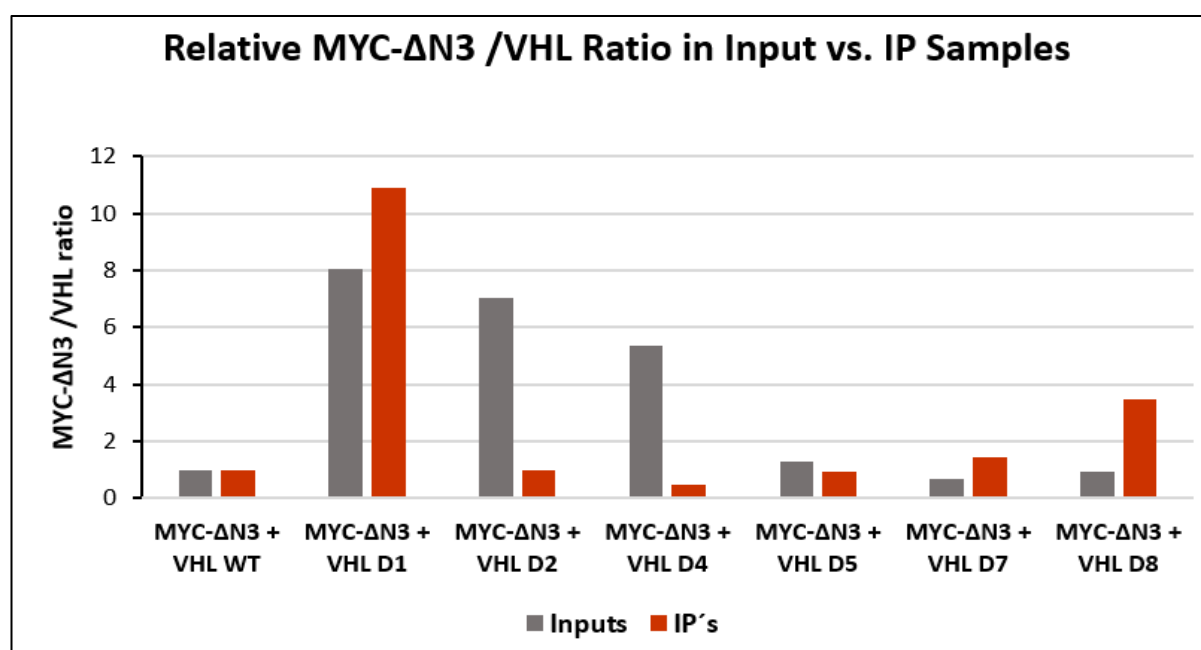


Figure 33: Relative MYC- ΔN3 /VHL Ratio in Input vs. IP Samples (D1, D2, D4, D5, D7, D8)
The Y-axis represents the Relative MYC- ΔN3/VHL ratio, which has been normalized to the reference sample, while the X-axis lists the samples under investigation. The reference sample used is MYC-ΔN3 + VHL WT. To calculate this ratio, the intensities of the MYC-dN3 bands in the input samples were divided by the corresponding VHL band intensities, indicating the relative presence of MYC compared to VHL. The same calculation was performed for the protein bands in the IP (immunoprecipitation) lanes. Subsequently, both the input and IP ratios were normalized to the reference sample.

The graph displays variations in the MYC-ΔN3/VHL ratio between IP and input samples. Notably, samples containing mutants D1, D7, and D8 exhibited a higher MYC-ΔN3/VHL ratio in IP samples compared to input samples, suggesting a potentially stronger interaction affinity in these cases. In contrast, mutants D2, D4, and D5 displayed a lower ratio in IP samples compared to input, indicating a potentially weaker interaction affinity. Notably, the ratios for D2 and D4 were significantly reduced compared to input, while D5 showed a more moderate decrease.

Certainly, it's crucial to emphasize that changes in IP ratios may not solely reflect alterations in interaction affinity. Various other factors and issues during the co-IP process could impact these values. To accurately determine the interaction affinity, additional experiments and assays are needed.

To provide a more comprehensive understanding of these interactions, I've calculated the ratio between MYC-ΔN3/VHL IP and Input, considering expression changes. This ratio is illustrated in Figure 34.

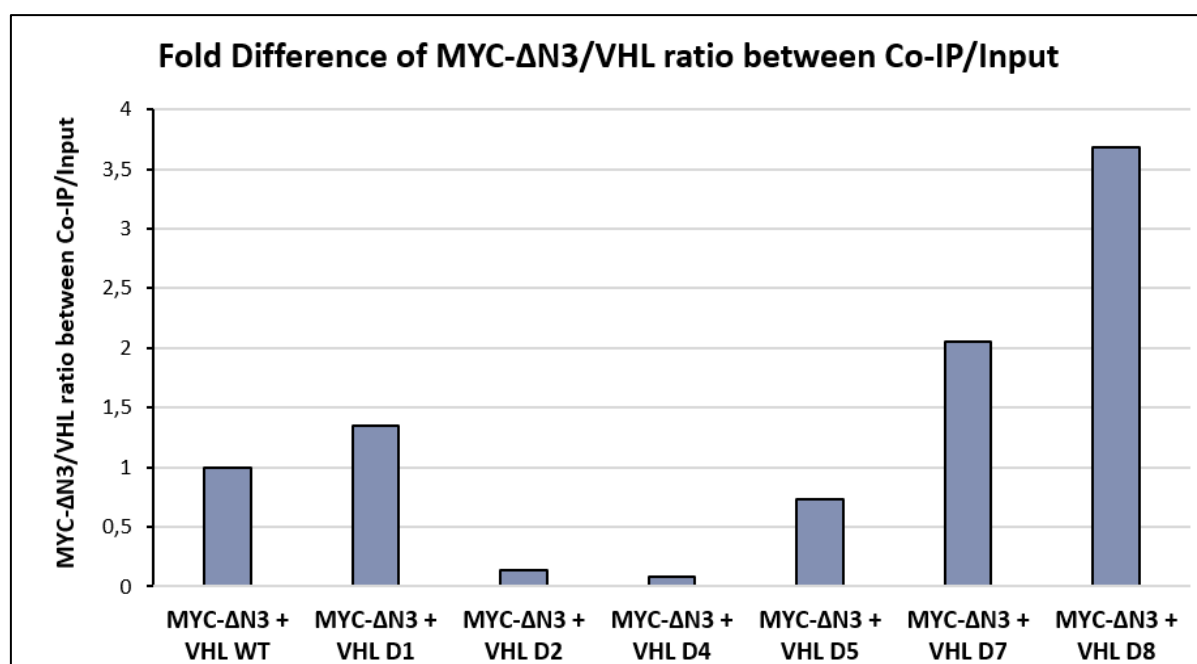


Figure 34: Fold Difference of MYC-ΔN3/VHL ratio between Co-IP/Input (D1, D2, D4, D5, D7, D8)
The Y-axis displays the MYC-ΔN3/VHL ratio of co-IP/IP, accounting for variations in expression levels. The X-axis enumerates the samples examined. The reference sample is MYC-ΔN3 + VHL WT. This ratio was calculated by dividing the MYC-ΔN3/VHL ratio of IP samples by the corresponding ratios of the input samples.

As anticipated from the previous graph, the relative interaction affinity in samples containing D2, D4, and D5 appeared lower compared to the sample containing VHL WT, potentially indicating reduced interaction affinity with MYC-ΔN3. Among these samples, D4 showed the most significant decrease, while D3 also exhibited a substantial reduction in interaction. D5 showed a milder decrease. However, it's crucial to note that these values may be influenced by co-IP efficiency and various other factors, and they might not solely reflect interaction affinity. Conversely, samples containing D1, D7, and D8 displayed higher values than the wildtype

sample, indicating potentially enhanced interaction affinity. These findings aligned with the data from previous graphs and qualitative assessments.

In summary, the western blot analysis revealed that none of the mutants exhibited a complete abolishment of interaction with MYC- Δ N3. However, a slight reduction was observed in mutant D5, which contradicts the initial experiment and could be influenced by other factors during co-IP. To ensure the reliability of these results, further experiments are necessary. More substantial reductions were observed in mutants D2 and D4, suggesting potential influences on the interaction with the MYC bHLHZip domain. Nevertheless, it is imperative to replicate these experiments in the future to establish the robustness of these findings.

DISCUSSION

MYC, a central transcription factor, governs crucial cellular processes in both normal and cancer cells. In normal cells, it maintains cellular homeostasis, regulating proliferation, differentiation, and survival. However, in cancer, MYC's role shifts dramatically, promoting uncontrolled cell division, inhibiting differentiation, and enhancing malignant cell survival, rendering it a potential therapeutic target (Ahmadi et al., 2021). Notably, MYC's diverse functions in normal and malignant cells are regulated by a network of interacting partners whose intricate interplay modulates its activity and highlights the complexity of its role in cellular processes (Dang, 2012).

One of these interaction partners is the E3 ubiquitin ligase subunit VHL. Previous studies, coupled with preliminary findings from the LGL laboratory, have already indicated a bi-directional crosstalk between these two pivotal proteins (Bailey et al., 2017; Hwang et al., 2012; Sharifi et al. in prep).

The VHL protein, functioning as an E3 ubiquitin ligase subunit, is primarily recognized for its involvement in ubiquitinating and degrading HIF- α transcription factors under normoxia, thereby contributing to the maintenance of cellular homeostasis (Minervini et al., 2020). In addition to its role in HIF degradation, VHL exerts a significant influence on various cellular functions and engages in interactions with a wide array of proteins and molecules (Jonasch et al., 2021; M. Li & Kim, 2011; J. Zhang & Zhang, 2018). Some interactions involve several kinases, such as NEK8, where VHL plays a role in downregulating NEK8 to preserve the primary ciliary structure in kidney cells. VHL also demonstrates interaction with the tumor suppressor protein p53, facilitating its transactivation by recruiting co-activators like p300 and Tip60. Furthermore, VHL is involved in the ubiquitination and degradation of RNA Pol II subunits RBP1 and RBP7. Another intriguing interaction involves VHL forming a complex with the androgen receptor (AR), where it acts to inhibit AR's transcriptional activity (Minervini et al., 2020). Hence, VHL is recognized for its diverse impact on a wide spectrum of cellular processes. This includes

contributions to DNA repair, control of cell growth, maintenance of cellular structures, and even extends to transcriptional regulation, which forms a central focus of investigation in this study.

The role of the interaction between VHL and MYC in cellular regulation is currently not entirely clear. Hwang et al. (2012) revealed that VHL binds to MYC through its β -substrate-binding domain, leading to non-proteasomal ubiquitination of MYC and altering its expression levels. This relationship introduces complexity through a negative feedback loop. Increased MYC expression typically triggers a repressive auto-response, down-regulating *MYC* transcription. Notably, VHL is suggested to play a role in this auto-repressive mechanism by recruiting histone deacetylases (HDACs) to the *MYC* promoter, maintaining proper MYC expression levels (Hwang et al., 2012). This molecular interplay is gaining importance in the context of clear cell renal cell carcinoma (ccRCC), as Bailey et al. (2017) showed. In clear cell renal cell carcinoma, *VHL* gene mutations frequently occur, which create the conditions for activation of the MYC signaling pathway. The result is the development of a highly metastatic and vascularized cancer.

Preliminary data from our lab (Sharifi et al. in prep.) suggests a regulatory role of VHL, characterized by non-proteolytic interactions that fine-tune the transcriptional activity of MYC to a subset of genes. It is important to note that prior publications did not investigate how VHL regulates MYC's pro-tumorigenic target genes. Interestingly, data from our laboratory indicates that VHL acts as a repressor for MYC-dependent gene transcription, although the specific mechanism remains unclear. We hypothesize that non-proteolytic ubiquitination of MYC may recruit co-repressors, thereby inhibiting MYC's activity. A more detailed study of the interaction of MYC and VHL could further unravel interesting mechanisms that contribute to the targeted modulation of MYC and could be of great importance in cancer treatment.

The primary goal was to ensure the successful overexpression of selected genes in Cos7 cells, a crucial preparatory step for subsequent investigations. I conducted a series of experiments involving the overexpression of all genes in Cos7 cells. Subsequently, I assessed their identity, integrity, verified the correct protein expression, and confirmed the presence of respective tags through western blot analysis. These analyses not only confirmed the successful overexpression but also established the stability of all plasmids utilized in my research.

My second task involved the confirmation of the interaction between the two proteins. Confirming this interaction was an important first step in this study, serving as a validation of my experimental approach and ensuring the reliability of subsequent investigations of MYC and VHL interactions. To accomplish this, an in vitro study was carried out, involving the overexpression of these proteins in Cos7 cells. Following this, co-IP assays were conducted to examine their interaction. I successfully demonstrated protein-protein interaction between MYC and VHL.

It is worth noting that this interaction was observed within highly overexpressed systems, which are not typically encountered in normal cells. Future investigations should consider assessing this interaction under normal expression levels in wild-type cells to provide a more physiologically relevant context. These findings align with previous studies that have reported successful interactions between these proteins (Hwang et al., 2012; Sharifi et al. in prep). This set the stage for me to proceed with the core objectives of this study.

The interaction between MYC and E3 ubiquitin ligases, as well as their regulatory roles on MYC's activity and stability, was not a novel discovery. Previous studies had already demonstrated that MYC interacts with a minimum of 18 E3 ubiquitin ligases, including the oncogenic E3 Ubiquitin ligase subunit SKP2, which is another protein of interest in this study (Sun et al., 2021). Initial investigations into the impact of E3 ubiquitin ligases on MYC's behavior suggested that these proteins played a role in ubiquitinating MYC and subsequently facilitating the recruitment of the 26S proteasome complex for MYC degradation (Farrell & Sears, 2014). Among these E3 ubiquitin ligases, SCF-SKP2 was the first to be discovered interacting with MYC. Interestingly, SKP2 is known to interact with MYC and promote its ubiquitination and subsequent degradation. However, SKP2 was unexpectedly shown to stimulate MYC's transcriptional activity during the transition from G1 to S phase (Hydbring et al., 2017; S. Y. Kim et al., 2003; von der Lehr et al., 2003). The proposed mechanism involves recruitment of SKP2 to the MYC promoter, initiating MYC ubiquitination. This recruits the 19S subunit of the proteasome (with or without the rest of the proteasome) and potentially ubiquitin-binding cofactors. The 19S subunit interacts with MYC via the growing ubiquitin chain. MYC also interacts with the positive elongation factor p-TEFb and subunits of RNA polymerase II. Subsequently the recruited 19S subunit together with co-factors stimulate transcription initiation and elongation. SKP2 may also ubiquitinate p-TEFb, further enhancing transcription elongation. MYC ubiquitination continues, leading to proteasomal degradation by the 26S proteasome. As this happens, SKP2, associated co-factors, p-TEFb and the proteasome dissociate from MYC and the promoter. Pol II exits the promoter after transcription termination, enabling the initiation of a new cycle with newly synthesized MYC. In that sense the degradation is believed to be necessary to restart another cycle and enabling continued cycles of transcription. The ubiquitination and degradation are required for MYC-driven transcription in this scenario, where the initial non-proteolytic ubiquitination promotes activities before the proteasome terminates the process (Hydbring et al., 2017). This unique characteristic in the regulation of MYC by an E3 ubiquitin ligase sets it apart from E3 ligases like FBXW7, which are negative regulators of MYC.

SCF-FBXW7 ligase was the second to be discovered interacting with MYC during the G1 and early S phase. It controlled MYC levels by attaching K48-linked polyubiquitin chains to it, ultimately leading to its proteasomal degradation. This mechanism, employed by FBXW7, effectively diminished MYC's transcriptional activity (Farrell & Sears, 2014; Welcker et al., 2022).

The E3 ubiquitin ligase TRUSS follows a similar pattern by interacting with MYC, resulting in MYC ubiquitination, subsequent degradation, and ultimately a reduction in MYC's transcriptional activity. It's important to note that this regulatory pattern isn't unique to TRUSS. Several other ligases, including CHIP, NEDD4, UBR5, TRIM32, ELL, and MAGI3, have demonstrated interactions with MYC in a comparable fashion (Farrell & Sears, 2014; Nicklas et al., 2019; Schukur et al., 2020; Sun et al., 2021; Thomas & Tansey, 2011; H. Wang et al., 2022; Q. Yang et al., 2021).

Additionally, FBX29 binds to MYC at the same binding sites as SKP2, specifically within the MYC Box II (MBII) located in MYC's transactivation domain (TAD) and the basic helix-loop-helix-leucine zipper (bHLHZ) domain. Like SKP2, FBX29 induces MYC ubiquitination and degradation. However, unlike SKP2, FBX29's action results in a reduction in MYC's transcriptional activity, aligning with the effects observed with many other ligases that target MYC for ubiquitination (Farrell & Sears, 2014; Koch et al., 2007).

SKP2, sharing the same binding sites on MYC with FBX29, suggests a potential competition between these ligases (Farrell & Sears, 2014; Koch et al., 2007). Moreover, there is a correlation between the overexpression of SKP2 and the reduction of TRUSS levels in human cancers (Jamal et al., 2015). These findings, along with other related studies, collectively imply that an intricate interplay exists among ubiquitin ligases, tightly regulating MYC during various stages of the cell cycle.

On the contrary, some E3 ubiquitin ligases have been discovered to ubiquitinate MYC in a manner that does not lead to proteasomal degradation but instead stabilizes MYC or enhances its activity (Farrell & Sears, 2014; Sun et al., 2021).

For instance, SCF- β -TRCP is known to counteract SCF-FBXW7 by ubiquitinating MYC on lysines K33/K63/K48 in a mixed fashion, thereby opposing FBXW7 and stabilizing MYC protein during subsequent cell cycle phases (Popov et al., 2010). Another F-box protein, FBXL16, acts in opposition to FBXW7 and increases MYC stability by inhibiting FBXW7-mediated ubiquitination of MYC. Interestingly, FBXL16 does not compete with FBXW7 for binding to MYC but may form a complex with both FBXW7 and MYC, directly suppressing FBXW7 ligase activity toward MYC (Morel et al., 2020). HECTH9 and HUWE1 ligases belonging to the HECT-domain family interact with MYC and enhance MYC's transcriptional activity by recruiting co-factors such as p300, which possesses histone acetyltransferase (HAT) activity and activates MYC transcription (Adhikary & Eilers, 2005; Farrell & Sears, 2014; Sun et al., 2021). Similarly, the ligase FBX028 can ubiquitinate MYC and recruit the p300 co-activator, consequently increasing MYC's activity (Cepeda et al., 2013).

Similar to these ligases, VHL belongs to the E3 ligases responsible for the non-proteolytic ubiquitination of MYC. However, preliminary data from our laboratory indicate a distinct mechanism: unlike other E3 ligases, VHL appears to repress MYC's transcriptional activity rather than enhancing it.

In previous studies, it has been demonstrated that both SKP2 and VHL bind to the same regions of MYC (S. Y. Kim et al., 2003; von der Lehr et al., 2003; Sharifi et al. in prep), yet they seem to exert opposite effects on MYC regulation. This suggests that the interplay between these E3 ubiquitin ligases plays a crucial role in tightly controlling MYC, with each ligase having distinct impacts on MYC activity.

We hypothesize that MYC, SKP2 and VHL form a network involved in a complex interplay between ubiquitination, proteasomal degradation, and transcriptional regulation. SKP2's ubiquitination and degradation of MYC paradoxically stimulate its transcriptional activity, while VHL's non-proteolytic ubiquitination seems to repress MYC activity. Disruptions in this network could have implications for cancer pathogenesis and for cancer treatment.

We envision the following scenario: During the transition from G1 to S phase, SKP2 facilitates MYC ubiquitination, marking it for proteasomal degradation and simultaneously stimulates MYC's transcriptional activity. Conversely, VHL engages in non-proteolytic ubiquitination of MYC, leading to the repression of MYC's transcriptional activity. This is presumably part of a tumor suppressor mechanism designed to down-regulate MYC's transcription of genes that are crucial for tumor development. In cancer, the loss of VHL disrupts this regulation, resulting in uncontrolled transcription of specific MYC target genes. The precise mechanism behind this process is not yet fully understood.

One potential mechanism is that VHL associates with MYC on the promoter and recruits histone deacetylases (HDACs), leading to the repression of MYC-induced transcription, including its own transcription. This negative feedback loop mechanism has been observed on the MYC promoter upstream of the *MYC* gene (Hwang et al., 2012). Additionally, VHL may ubiquitinate MYC in a K48/K63 manner and recruit co-repressors like MIZ1 to further suppress transcription (Sharifi et al., in prep.).

In cancer, MYC is frequently overexpressed, and elevated levels of SKP2 contribute to enhanced MYC-driven transcription (Bretones et al., 2011). Conversely, the loss or downregulation of VHL in renal and other cancers may lead to disruption of this negative feedback loop, impeding VHL's ability to repress MYC-target genes. This disruption could potentially lead to uncontrolled transcription of specific MYC target genes.

Previous research has demonstrated that when two E3 ligases share identical binding sites for a target protein, they can compete for the interactions with that target protein (Boyer et al., 2020; Yoo et al., 2013). Additionally, Kitagawa et al. (2008) revealed that SKP2 competes with p53 for binding to p300, resulting in the inhibition of p53's transcriptional activity. This competitive interaction between SKP2 and p53 for p300 binding suppresses p53-mediated transcription and promotes cell survival in cancer cells. These findings underscore the notion

that SKP2, when engaging in competition with a tumor suppressor protein for binding to a specific target protein, can exert oncogenic effects.

Considering that SKP2 and VHL interact with MYC through the same domains, namely the MBII and bHLHZip domains (Hwang et al., 2012; S. Y. Kim et al., 2003; von der Lehr et al., 2003; Sharifi et al. in prep.), but seem to exert opposing effects on MYC regulation, we hypothesized that these two E3-Ub ligases might engage in competitive binding to MYC.

To validate this hypothesis, I conducted a series of competition experiments, steadily increasing the expression levels of SKP2 while keeping the levels of VHL and MYC constant. Using co-immunoprecipitation experiments, I successfully captured VHL or MYC along with their respective associated proteins. This method enabled me to closely monitor any changes in interactions as I titrated increasing amounts of *SKP2*. As a result, I could evaluate the impact of elevated SKP2 levels on the MYC-VHL interaction, assessing whether a reduction in their interaction occurred and thereby indicating the possibility of competitive interactions between these proteins.

The western blot analysis yielded intriguing findings. During immunoprecipitation to capture VHL, I observed that the quantity of co-immunoprecipitated MYC actually increased, rather than decreased, as the levels of transfected SKP2 were elevated. Similarly, when I reversed the experimental approach and conducted immunoprecipitation to capture MYC, a similar trend emerged. The levels of immunoprecipitated MYC actually rose with the increasing amount of SKP2. Moreover, I noticed an interesting trend in the co-immunoprecipitated VHL levels. In the sample with 4ug of transfected *SKP2*, there was a substantial increase in co-immunoprecipitated VHL. However, in the sample containing 8ug of *SKP2*, I observed a decrease in co-immunoprecipitated VHL. Furthermore, despite the increased expression of SKP2 in the input samples, the levels of co-immunoprecipitated SKP2 did not rise accordingly in the 8ug sample. These observations suggest that specific factors in the sample with 8ug of *SKP2* might have disrupted the interactions between MYC, VHL, and SKP2. Several factors could contribute to this pattern, such as the saturation of binding sites on MYC or potential conformational changes induced by elevated SKP2 levels. However, this issue was unique to this particular sample and was not replicated in subsequent experiments. Therefore, aside from this isolated occurrence, the results strongly indicate that increasing levels of SKP2 do not significantly diminish the MYC-VHL interaction; instead, they seem to stimulate it. These findings suggest that despite VHL and SKP2 binding to the same regions of MYC, there was no observed competition between these proteins for MYC binding. Notably, the VHL IPs revealed an increasing amount

of co-immunoprecipitated MYC. This suggests that SKP2 may have a stimulatory effect on the interaction between MYC and VHL.

Moreover, another unexpected observation emerged from the VHL IPs, where SKP2 was co-immunoprecipitated along with VHL, indicating an interaction between SKP2 and VHL. This interaction could potentially be direct, involving a physical binding between SKP2 and VHL, or it could be indirect, with both proteins binding to different motifs on MYC simultaneously. In the latter scenario, all three proteins would form a complex, with MYC serving as a scaffold between SKP2 and VHL.

These observations suggest a complex interplay between the two E3 ubiquitin ligases in the regulation of MYC. Interestingly, other studies have also indicated an interplay between two E3 ubiquitin ligases in the regulation of MYC (Jamal et al., 2015; Morel et al., 2020; Popov et al., 2010).

Our experimental results challenge our previously hypothesized competition between SKP2 and VHL for MYC binding. Instead, our findings suggest a more intricate regulatory mechanism governing MYC activity involving cooperative interactions between SKP2 and VHL. Contrary to our initial expectations of competition, our data revealed an unexpected augmentation in the interaction between MYC and VHL as SKP2 levels increased. This suggests that SKP2 might play a stimulating role in facilitating the MYC-VHL interaction. While the exact mechanism behind this enhancement remains to be explored, it's conceivable that SKP2, in addition to its role in MYC ubiquitination, acts as a co-factor that promotes VHL's binding to MYC. This facilitation could involve inducing conformational changes in MYC or fostering cooperative interactions among SKP2, MYC, and VHL.

Furthermore, the presence of SKP2 in the VHL immunoprecipitates suggests a potential interaction between SKP2 and VHL. This interaction might arise from their concurrent binding to distinct motifs on MYC. These intriguing findings raise questions about the precise nature of this interaction—whether SKP2 and VHL physically interact or if MYC acts as a scaffold, bringing them into proximity.

In light of these discoveries, I propose a model in which SKP2 and VHL interact within a complex regulatory network to fine-tune MYC activity. In this model, SKP2 and VHL could work together in a highly coordinated manner. During the transition from G1 to S phase, SKP2 enhances MYC activity through ubiquitination, as previously described. However, in subsequent phases, SKP2's role in activating MYC-induced transcription diminishes. One intriguing possibility is that VHL acts as a repressor of SKP2-enhanced MYC-driven transcription. SKP2

may remain associated with MYC, but the recruitment of VHL could result in VHL acting as a co-repressor by binding to different motifs on MYC, potentially forming a triplex complex.

In a study by J. Wang et al. (2014), it was demonstrated that VHL possesses an intriguing ability to enhance the de-ubiquitination of its target, the androgen receptor (AR), ultimately resulting in a reduction of AR's transcriptional activity. Remarkably, despite its compact size, VHL displayed the capability to de-ubiquitinate various types of poly-ubiquitination, including K6-, K48-, and even K29-linked poly-ubiquitination. The mechanism behind VHL's de-ubiquitination function may involve indirect pathways, potentially through the recruitment of other proteins equipped with de-ubiquitination activity, so called DUBs. Alternatively, VHL might leverage its E3 ligase activity to induce ubiquitination and degradation of other specific E3 ligases responsible for MYC ubiquitination. Another possibility is that SKP2 and VHL target different lysines in MYC for ubiquitylation with different consequences for MYC activity or stability. MYC contains 22 lysines that all could be targets for E3 ligases, which offer a high number of possibilities for MYC regulation.

Additionally, another study by Roe et al. (2011) uncovered a physical interaction between VHL and SKP2, particularly through the β -domain of VHL, which is in line with my findings. Intriguingly, VHL was found to destabilize SKP2, notably inducing DNA synthesis during the S phase of the cell cycle. Unfortunately, I did not have time to investigate this possibility in the system used here. This could be studied by titrating the concentration of VHL against a fixed concentration of SKP2. The destabilization of SKP2 observed by Roe et al. (2011) was not dependent on the E3 ubiquitin ligase activity of VHL, leaving the exact mechanism of action still unclear. These findings shed light on VHL's tumor suppressor function in hindering S-phase progression, effectively inhibiting cell proliferation in response to DNA damage.

Additionally, the mechanism outlined by Hwang et al. (2012), where MYC directly interacts with its promoter and recruits HDACs to inhibit transcription, could also be considered. This could suggest that following the G1-S transition, VHL binds to MYC and SKP2 in a triplex formation, repressing SKP2's ubiquitination activity and recruiting co-factors that inhibit MYC-induced transcription on the promoter or counteract SKP2-induced ubiquitination of MYC.

In a separate study (Morel et al., 2020), another F-box protein known as FBXL16 displayed effects similar to those we propose for VHL and SKP2. FBXL16 acted antagonizing to FBXW7, which is responsible for MYC ubiquitination and degradation. Notably, it was observed that FBXL16 and FBXW7 were not engaged in competition for MYC binding. Instead, FBXL16 hindered FBXW7-mediated ubiquitination of MYC. FBXW7 functions as a tumor suppressor, while FBXL16 exhibits oncogenic properties, reinforcing the notion of antagonistic actions

between these ligases. Given the absence of competition, it was suggested that these proteins might form a triplex to enable FBXL16's antagonistic effects. This proposed model aligns with our hypothesis, suggesting that VHL and SKP2 may counteract each other bidirectionally, possibly through triplex formation. In the context of my results, SKP2 seems to stimulate the interaction between VHL and MYC. This could be due to increasing SKP2 availability, therefore increasing binding to MYC, so that VHL's binding to both proteins intensifies, facilitating its antagonistic effects.

These discoveries favors the concept of a triplex formation involving VHL, SKP2, and MYC. In this model, VHL might inhibit SKP2 by interfering with its ubiquitination activity and using co-factors to inhibit MYC-induced transcription at the promoter. Alternatively, VHL might counteract SKP2-induced ubiquitination of MYC by recruiting E3 ligases that target SKP2 for degradation or by harnessing other co-factors with de-ubiquitination activity to reverse MYC ubiquitination.

This dynamic interplay between SKP2 and VHL could ensure precise control over MYC levels, with implications for oncogenic and tumor-suppressive functions. In the context of ccRCC, where VHL is often lost, SKP2's functions would become less repressed, leading to uncontrolled MYC-induced transcription. This could create a positive feedback loop, as SKP2 is a direct target of MYC, amplifying the uncontrolled expression of both SKP2 and MYC.

Hence, this model proposes that SKP2 and VHL, both E3 ligases involved in MYC regulation, establish an interactive relationship in modulating MYC activity. SKP2 facilitates MYC transcription when required, while VHL acts to inhibit SKP2's role, ensuring that MYC's transcriptional activation is precisely controlled.

The precise mechanisms governing how VHL and SKP2 together regulate MYC's transcriptional activity present intriguing questions that require further investigation. To elucidate whether these three proteins form a triplex, future studies should consider utilizing cell lines that closely mimic physiological conditions rather than relying solely on overexpression systems. Rather than transient transfections, stable cell lines based on inducible expression systems with more physiological expression levels could be used. Additionally, employing techniques like *in situ* Proximity Ligation Assay (isPLA) could enhance sensitivity in detecting endogenous protein interactions and potential triplex formations, even for subtle interactions. Analyzing endogenous proteins in their native context may provide more physiologically relevant insights. To assess the impact of the interplay between SKP2 and VHL on MYC-driven transcription, experiments measuring RNA expression by quantitative polymerase chain reaction (qPCR) or RNA sequencing (RNA-seq) could prove invaluable. These approaches would

help dissect the specific contributions of each protein and their combined influence on MYC-mediated transcription, shedding light on the intricacies of their regulatory roles.

My model raises intriguing questions regarding the specific molecular mechanisms that govern SKP2-MYC-VHL interactions. Further studies are needed to dissect the domains and motifs involved and to decipher how these proteins collaborate to modulate MYC's diverse functions. This could shed light on possible targets in cancer. In cancer, alterations in MYC, SKP2, and VHL levels are commonly observed. MYC is frequently overexpressed, SKP2 levels are elevated, and VHL is often downregulated or lost in renal and other cancers. This perturbation of the MYC/SKP2/VHL network contributes to uncontrolled transcription of MYC target genes, fueling tumorigenesis. Future studies are needed to delve into the possibility of targeting this network in cancer therapies.

In conclusion, our findings challenge our initial assumption regarding the SKP2-VHL competition and point toward a more intricate regulatory mechanism in this network, which could shed light on potential new avenues for therapeutic interventions in cancer.

In the next part of the project, we aimed to understand in molecular detail how the VHL complex interacts with MYC. Since both VHL and MYC have multiple targets in the cell, specific disruption of the MYC-VHL complex without touching other interactions is crucial to understand the biological role of this complex. We therefore wanted to investigate whether specific mutations in the VHL protein could disrupt the binding to the MYC. To achieve this, our collaborators, the Zinzalla laboratory (Karolinska Institutet, and University of Cambridge, UK) conducted NMR (Nuclear Magnetic Resonance) studies of MYC and VHL in the context of the MYC:MAX and the VHL E3 ligase complexes.

NMR is a powerful technique that allows to examine the molecular interactions between proteins, and it is particularly powerful in the case of complexes that are highly dynamic such as MYC:MAX and the VHL complex. To determine the binding epitope, nitrogen-15 (^{15}N) labelled proteins were prepared. In particular, they labeled the VHL VCB (VHL, Elongin C and Elongin) complex and observed its binding to the unlabeled c-MYC:MAX C-terminal (bHLHZip) heterodimeric apo complex to determine where c-MYC was binding on the VHL complex. Zinzalla then prepared ^{15}N -labelled c-MYC:MAX complex and observed its binding to the unlabeled VHL complex. This approach allowed them to map the regions of interaction on both proteins. It was possible to use this strategy as: 1. they had previously determine the full NMR assignment of the VHL VCB complex (Knauth et al., 2009) and the crystal structure of the VHL VCB complex binding to a HIF-1 α peptide is also available (PDB: 1LM8); and 2. they also have

previously obtained the full assignment of the human c-MYC:MAX C-terminal apo complex as well as its crystal structure at very high resolution (Sammak et al., 2019; PDB = 6G6K). Chemical shift changes and the disappearance of a few peaks were observed in the NMR spectra, indicating selective the amino acid residues involved in the interaction between VHL and c-MYC:MAX (Williamson, 2013). With this valuable information in hand, the Zinzalla lab identified candidate amino acid residues that should play a pivotal role in mediating the protein-protein interactions. These key residues were then considered for mutation. However, it was essential to select mutations that would disrupt the binding without causing structural damage to the overall folding of the proteins. In order to do this, the Zinzalla laboratory used a computational tool called Missense 3D (Ittisoponpisan et al., 2019; Khanna et al., 2021), which aided in predicting the structural impact of potential mutations. This step was crucial to ensure that the mutated proteins would still maintain their basic structural integrity. Additionally, the researchers delved into the COSMIC database (Tate et al., 2019), which contains information on genetic mutations in cancer. They analyzed the frequency of missense mutations in the identified amino acid residues, helping to prioritize which mutations were most likely to be biologically relevant and potentially impactful in disrupting the VHL-MYC interaction. Ultimately, a number of promising candidate amino acid residues were pinpointed for mutation in the VHL VCB complex, while three key residues were identified in the c-MYC:MAX complex. These mutated proteins were designed to either fully inhibit or partially disrupt the binding between VHL and MYC. In summary, the NMR studies provided critical insights into the molecular details of the VHL-MYC interaction. This information, combined with computational analysis and cancer mutation data, guided the selection of specific amino acid residues for mutation.

The goal of introducing these mutations was to disrupt the binding between VHL and MYC. This approach provides valuable insights into the essential binding domains and specific amino acid residues that play a critical role in this interaction. Understanding these molecular details is crucial because it can shed light on how MYC-driven gene expression is regulated at the molecular level, and VHL and MYC mutants unable to bind each other will shed light on the biological role of the interaction. This knowledge not only deepens our understanding of cellular processes but also has potential implications in the development of targeted therapies, especially in the context of ccRCC, where dysregulated gene expression is a hallmark. Thus, the study not only contributes to our fundamental understanding of molecular biology but also holds promise for future therapeutic strategies. Additionally, our research distinguishes between gene expression changes resulting from VHL-MYC interactions and hypoxia-induced HIF changes. This distinction is important for clarifying the different roles of VHL-MYC interactions

and HIF-related processes in gene regulation. If certain mutations can be identified that specifically disrupt the VHL-MYC interaction while preserving other functions of VHL, it might open up possibilities for developing targeted therapies aimed at modulating MYC-related signaling pathways.

VHL gene mutations are relatively rare, primarily due to their unique genetic inheritance patterns. The approach to introduce missense mutations into the *VHL* gene is an important approach to understand the functional consequences of these genetic changes. Inherited VHL mutations follow an autosomal dominant pattern, meaning that one mutated allele is sufficient to increase the risk of VHL syndrome. However, for VHL-related tumors to develop, both alleles of the *VHL* gene within specific cells must be mutated, a condition known as biallelic inactivation. The likelihood of both alleles becoming mutated, whether one is inherited and the other sporadic or both sporadic, is extremely low within the same individual. This rarity is a significant factor contributing to the infrequency of VHL-related tumors (Dwyer & Tu, 2017). Another crucial factor that contributes to the rarity of VHL mutations could be the essential role of VHL in maintaining oxygen homeostasis in cells. Mutations that disrupt this function can have severe physiological consequences, especially in tissues that rely heavily on oxygen supply, such as the kidney. The body is less likely to tolerate mutations that compromise oxygen regulation, making them less common (Haase, 2009). The rarity of VHL-related tumors can also be attributed to tissue-specific factors. Kidney tissue, in particular, is more susceptible to sporadic VHL mutations. This susceptibility is linked to the kidneys' distinct oxygen-sensing requirements, which make them more prone to these somatic mutations. The kidneys play a crucial role in regulating oxygen supply and responding to changes in oxygen levels. Consequently, sporadic VHL mutations can occur in various tissues, but their tumorigenic potential is more pronounced in oxygen-sensitive organs like the kidneys (Haase, 2009). In the context of clear cell renal cell carcinoma (ccRCC), more than 80% of cases are affected by VHL aberrations, including allelic deletion, promoter methylation, or mutations. Many of these mutations are missense mutations, which can affect pVHL stability and its binding ability. These mutations may have varying impacts on pVHL's interactions with different targets and pathways involved in tumor carcinogenesis (C. Razafinjatovo et al., 2016).

This study focused on identifying missense mutations that disrupt the binding of VHL to MYC, a critical step in understanding the regulation of MYC by VHL and VHL's tumor suppressor function within ccRCC.

The genetic aberrations in ccRCC involve the loss of VHL and overexpression of MYC. Identifying specific residues that interact with MYC and investigating the regulatory behavior

of these interactions are essential for gaining insights into the mechanisms underlying ccRCC. Furthermore, finding these residues could open avenues for studying specific molecules that can target these binding sites in ccRCC and mimic VHL to reconstitute its function, as seen in the case of PROTAC molecules that can restore VHL's E3 ligase activity (Diehl & Ciulli, 2022).

The process of mutating specific sites on the VHL protein involved the careful design and validation of specific primers. Subsequently, site-directed mutagenesis was performed, utilizing *FLAG-VHL* as the template. This approach, which introduces amino acid substitutions into a protein, has proven successful in numerous previous studies, including investigations of missense mutations within the *VHL* gene (Lanikova et al., 2013; Ma et al., 2022; C. F. Razafinjatovo et al., 2016; C. Yang et al., 2013). Site-directed mutagenesis, a widely employed scientific technique, was initially described by Michael Smith in 1978 (Kresge et al., 2006). It enables researchers to precisely introduce point mutations into a known oligonucleotide (wild type) to explore the effects of these mutations on specific aspects of the molecule. Over the years, this approach has gained increasing popularity in scientific research.

In this study, this method played a crucial role in generating mutant versions of the VHL protein. Site-directed mutagenesis emerged as the method of choice for introducing mutations due to its precision and ability to target specific DNA sequences (Carrigan et al., 2011). This technique perfectly aligned with our research objectives. Through the controlled introduction of mutations into the coding DNA sequences of VHL, I could directly evaluate their influence on binding, enabling the localization of essential amino acid residues and binding domains.

The process of site-directed mutagenesis involved several important steps. First, I designed specific primers that contained the desired mutations, flanked by regions complementary to the target DNA. These primers were used in a polymerase chain reaction (PCR) to amplify the entire plasmid containing the *VHL* gene while incorporating the mutations. Subsequently, the original, non-mutated plasmid DNA was removed using a restriction enzyme, leaving only the newly synthesized, mutated DNA. Finally, the mutated plasmid was transformed into bacterial cells, and cells expressing the mutant genes were selected for further analysis.

To confirm the success of the mutagenesis process and ensure the desired mutations were introduced, I employed Sanger sequencing. This gold-standard sequencing technique allowed for the sequencing of the entire gene region of interest and enabled me to verify the presence of mutations at specific positions. By comparing the sequencing results to the known DNA sequence of the wild-type gene, I could definitively confirm the introduction of the mutations.

While the site-directed mutagenesis method proved effective in introducing specific mutations into the DNA sequences of the *VHL* gene, a minor limitation was encountered during the colony selection process. The bacterial colonies grown on the selection plates displayed heterogeneity, meaning not all colonies contained the mutated *VHL* gene. Consequently, to ensure the selection of colonies with the desired mutation, it was necessary to test and sequence multiple colonies. This additional step increased the time and effort required for mutant plasmid identification and amplification. However, it's important to note that this limitation did not compromise the overall success of the study, as the desired mutant plasmids were ultimately obtained, and the mutations were confirmed through sequencing. In contrast, no significant issues were encountered with primer design, gene size, or mutation detection using Sanger sequencing.

In my research, I chose site-directed mutagenesis due to its precision in adding specific mutations to *VHL* DNA sequences. However, it's worth acknowledging that alternative methods, such as CRISPR-Cas9 genome editing or the more recent LFEAP mutagenesis, could also be utilized for introducing mutations. CRISPR-Cas9 has the advantage of altering genes within their natural genomic environment (Gostimskaya, 2022), while LFEAP mutagenesis excels at introducing multiple mutations and handling large plasmids (Zeng et al., 2018). Nevertheless, our study primarily aimed to examine immediate effects on VHL-MYC interactions. Site-directed mutagenesis allowed us to make controlled changes to single or a few nucleotides in plasmids without fragmenting them, aligning perfectly with the precision needed for our research focus.

I was able to generate 6 VHL single missense mutants (M1-M6) as well as 8 VHL double missense mutants (D1-D8).

To investigate VHL binding to MYC's bHLHZip region, I utilized MYC-ΔN3, a deletion mutant covering MYC amino acids 221-439. This mutant lacks the N-terminal transactivation domain (TAD) containing the MBII region, which contributes to the MYC/VHL interaction (Salghetti et al., 1999). It was previously confirmed that VHL interact with the MYC-ΔN3 mutant (Sharifi et al. in prep.). Thus, we selected MYC-ΔN3 to explore VHL's specific amino acid residues crucial for the interaction between VHL and the bHLHZip domain of MYC and its impact on binding strength. Our aim was to identify amino acid mutations in VHL that could either completely abolish or partially disrupt this interaction.

The first step involved confirming the interaction between VHL and MYC-ΔN3. Through co-IP assays using *FLAG-VHL*, *HA-MYC-ΔN3*, and empty vector-transfected Cos7 cells, protein-protein interaction was observed between VHL and MYC-ΔN3. This finding aligns with Sharifi

et al.'s work and our laboratory's preliminary data, confirming the interaction. It's worth noting that these interactions occurred in highly overexpressed systems, not typically seen in normal cells. Future studies evaluating this interaction at normal cellular levels may provide a more representative context. This confirmation paved the way for further investigation involving VHL mutants and their interactions with MYC- Δ N3.

Utilizing Cos7 cells for expression and transfections, including *FLAG-VHL wt*, *FLAG-VHL M1-M6*, *D1-D8*, *HA-MYC- Δ N3*, and *pcDNA3* as a negative control, I conducted VHL co-IP experiments along with western blot analysis. The results demonstrated that all VHL mutants maintained an interaction with MYC- Δ N3. Notably, none of the VHL mutants completely disrupted the interaction with the bHLHZip region of MYC.

Initially, I assessed the VHL single mutants, but the western blot analysis indicated no significant reduction in interaction strength with MYC bHLHZip. Minor decreases in co-immunoprecipitated MYC- Δ N3 were observed for mutants M5 and M6, possibly indicating a slight interaction decrease. However, given the relatively diminished expression levels of MYC- Δ N3 in the lysate of these mutants, it's conceivable that VHL mutations might influence MYC- Δ N3 expression rather than the affinity of their interaction. A blotting issue, specifically the presence of an air bubble, introduced uncertainty when assessing the expression levels of samples containing M5 and M6. Consequently, it is challenging to determine definitively whether there is a decreased interaction between MYC and VHL in these samples. Without a clear view of the true expression levels of MYC and VHL, it remains unclear whether the ratio of MYC to VHL in the co-IP samples accurately reflects the ratio observed in the input samples. This ambiguity makes it challenging to draw firm conclusions regarding a reduction in interaction in these mutants. Quantification would have provided more precise insights, but the blotting issue during protein transfer hindered reliable comparisons. Unfortunately, time constraints prevented a repeat of the experiment. Future research should explore the interaction changes of VHL mutants M5 and M6 in more depth to assess their significance.

I then proceeded to investigate the VHL double mutants. Initially, I performed single transfections of all mutants and *wt VHL* into Cos7 cells, followed by western blot analysis. This step aimed to assess whether the mutations in VHL affected the protein's stability and expression, ensuring the reliability of subsequent co-IP results and avoiding potential false decreased interaction outcomes.

All VHL mutants were successfully expressed in Cos7 cells. However, mutant D4, which contained M5 and M6 mutations, exhibited significantly lower expression compared to other VHL mutants and *wt VHL*. This observation suggested that the D4 double mutation indeed

influenced VHL stability and expression, necessitating consideration when analyzing subsequent interaction patterns. Furthermore, mutants D2 (M2 + M5) and D5 (M4 + M5) displayed a minor decrease in expression levels.

I then proceeded with VHL co-IP experiments to capture VHL and assess the interaction affinity of VHL double mutants and the wildtype with HA-MYC bHLHZip.

In the first experiment, I tested mutants D2, D3, D5, and D6. None of these mutants completely abolished the interaction. Consistent with previous results from an overexpression experiment, mutants D2 and D5 exhibited lower VHL expression, indicating potential instability of these VHL mutants. However, quantification of these VHL mutants compared to wt VHL indicated that, for the most part, there was no substantial decrease in their interaction with the bHLHZip domain of MYC. However, one exception was observed in the sample containing D6, where quantification revealed a slight reduction in interaction. Another set of VHL-co-IP experiments involved mutants D1, D2, D4, D5, D6, D7, and D8, along with wt VHL. The results confirmed that none of the mutants completely abolished their binding to the MYC bHLHZip domain. Notably, mutants D1, D2, D4, and D5 exhibited reduced VHL expression levels, indicating potential effects on VHL stability. Interestingly, this trend was consistent with the first overexpression experiment. However, D2 and D5 consistently showed decreased VHL expression, while D1 displayed high expression when transfected alone. This suggests that mutations may have a more pronounced effect on VHL stability or degradation when co-transfected with MYC- Δ N3. One possible explanation is that MYC's bHLHZip region competes with VHL for essential binding partners or sites within the cell, potentially limiting VHL stability and expression. Additionally, the presence of MYC could induce cellular stress responses, leading to changes in protein expression levels as cells respond to stressors. Furthermore, MYC might influence post-translational modifications of VHL, potentially impacting its stability or activity.

Qualitative analysis indicated minor reductions in interaction for mutants D2, D4, and D5. Quantitative analysis further supported these observations, showing a significant reduction in interaction affinity for mutant D2 and D4, with a slighter reduction seen in mutant D5. These findings, however, contrast with our previous experiment, where D2 exhibited a more similar interaction affinity to wt VHL, and mutant D5's affinity was even higher. It's crucial to reiterate that the reduced values in this experiment may not solely reflect interaction affinity but can be influenced by various other factors. Mutant D4 displayed stability issues in previous experiments when transfected alone and in this experiment when co-transfected with MYC- Δ N3, as indicated by its low expression level. Thus, it would be beneficial to rule out stability issues as a contributing factor. Notably, in mutants D2, D4, and D5, despite the presence of

low VHL expression and high MYC- Δ N3 expression, a larger amount of VHL was immunoprecipitated, while co-immunoprecipitated MYC- Δ N3 levels were minimal. This indicates a mutation-induced partial disruption of the interaction, in addition to the effect on VHL stability.

Quantifying the IP samples encountered a limitation in directly accounting for protein loading and potential transfection issues. It is important to acknowledge that the IP process involves multiple steps, each introducing potential sources of error. Hence, we chose to normalize the amount of immunoprecipitated protein to the vehicle after completing all IP steps. This approach directly reflects the isolated protein of interest, aligning with the complexities of IP experiments, where each step, including washing and elution, can contribute to variability.

Notably, all the double mutants with reduced VHL expression across various experiments contained the M5 mutation. This observation strongly suggests that the M5 mutation adversely affects VHL protein stability, resulting in lower expression levels. This should be looked into. Low expression of specific VHL mutations can result from various factors, including misfolding, instability, degradation susceptibility, and disrupted interactions with stabilizing proteins or cellular stress responses.

However, contrary to our expectations, none of the created mutants completely eliminated the binding between VHL and MYC bHLHZ. However, one mutant, D4, did show a significant reduction in binding. This reduction may be due to partial disruption, but it's essential to note that the expression of D4 was notably low. This suggests that the mutation may affect VHL protein stability and expression, which requires further investigation. Mutants D2 and D5 also displayed reduced binding in one of the two experiments, indicating the possibility of partial disruption. Additional research is needed to clarify these interactions. In summary, while our mutants didn't fully abolish the interaction, there are hints of partial disruption, and we must explore the impact of these mutations on VHL protein stability and expression for a conclusive assessment.

NMR (Nuclear Magnetic Resonance) studies are a powerful and precise technique for examining molecular structure, dynamics, and interactions at the atomic level (Q. Li & Kang, 2020). The mutants' failure to disrupt interactions was likely due to various limitations, not the unreliability of NMR studies. I used Cos7 cells due to their efficient transfection and ease of maintenance in studying VHL mutations' impact on MYC bHLHZip interactions. However, Cos7 cells' high baseline expression levels may have caused non-specific or "sticky" binding of VHL mutants to MYC- Δ N3. For more reliable results, future experiments could explore stable cell lines or non-transformed human cells with lower baseline expression levels, closer to physiological conditions and less affected by overexpression.

I chose co-IP as the detection method, a common approach for studying protein-protein interactions. However, it has limitations like potential disruption of cellular structures altered protein interactions during protein extraction and limited resolution for single-molecule interactions. To address these issues, an alternative method, isPLA (in situ Proximity Ligation Assay), could be considered in the future. isPLA is performed on fixed cells, preserving cellular structures and native interactions. It's highly sensitive, capable of detecting weak interactions relevant to assessing changes in VHL mutant interactions with the MYC bHLHZ region. Importantly, isPLA provides quantitative data for better resolution in assessing mutation effects.

In conclusion, this study into how VHL mutations affect the interaction with the MYC bHLHZ region within Cos7 cells has offered valuable initial insights. Nevertheless, I acknowledge the need for further investigations using alternative cell lines and detection methods to validate and refine my findings. By addressing these considerations, we can enhance the reliability and depth of our understanding of these critical protein-protein interactions.

For future research, we also plan to produce more single and double mutants as suggested by the Zinzalla lab. If necessary, we will also consider generating triple mutants to abolish interactions.

Once the interaction is successfully abolished, the next research phase will focus on understanding how this disruption affects MYC transcriptional activity, MYC stability, gene expression patterns, and downstream signaling pathways. It's important to note that our mutations specifically target the binding to MYC while leaving interactions with HIF intact. This eliminates the need for HIF knockout cell lines, guaranteeing that all observed changes can be attributed solely to the MYC-VHL interaction.

A MYC-driven promoter/luciferase reporter assay can be employed to monitor alterations in MYC's transcriptional activity by measuring changes in luciferase activity. Western blot analysis can be conducted to evaluate MYC protein levels and assess its stability when transfected with the VHL mutant. For gene expression profiling, qPCR and RNA-Seq serve as valuable tools to investigate changes in gene expression patterns influenced by MYC, comparing VHL wildtype with MYC and VHL mutant conditions. Chromatin Immunoprecipitation (ChIP) analysis is used to examine changes in MYC's binding affinity to its promoter sites and how VHL-MYC disruption impacts this interaction. Cell viability and proliferation assays can measure the impact of altered MYC activity on cell growth and viability. To assess the influence on apoptosis, an apoptosis assay can be performed, potentially using a Caspase activity assay. Additionally, cell cycle analysis helps determine if MYC's role in cell cycle regulation is altered,

with flow cytometry being a valuable technique for this purpose. These experiments collectively provide essential insights into the role of the MYC-VHL interaction in tumorigenesis.

Furthermore, we can consider the generation of animal models to delve into the in vivo effects of the disrupted interaction and closely monitor its impact on tumorigenesis through a range of assays. Additionally, an in-depth analysis of the protein-protein network could be conducted. This analysis would allow us to identify other proteins affected by this disrupted interaction, shedding light on additional pathways and mechanisms implicated in cancer development.

Furthermore, we have the opportunity to explore drug screening as a means to investigate potential therapeutic strategies for addressing tumors with the disrupted MYC-VHL interaction. This may involve screening for small molecules or drugs designed to selectively target cells with this specific genetic alteration. High-Throughput Screening can be employed to rigorously assess the efficacy of various compounds or small molecules in targeting these cells.

There are many options for our future research, all of which hold great promise. As we move forward, it is clear that further investigation into the MYC-VHL interaction is essential to deepen our understanding and uncover potential therapeutic strategies for cancer treatment.

CONCLUSION

In conclusion, this research has provided valuable insights into the intricate molecular interactions governing the relationships between the MYC oncoprotein, the VHL tumor suppressor protein, and the E3 ubiquitin ligase subunit SKP2. Through a comprehensive array of techniques, including co-immunoprecipitation experiments, and site-directed mutagenesis, our primary objectives encompassed elucidating both how VHL competes or collaborates with SKP2 in regulating MYC and uncovering the intricate molecular details of the VHL-MYC complex.

My research has illuminated that contrary to our initial expectations of a competitive interaction between SKP2 and VHL for binding to c-MYC, our results indicate a more intricate relationship. Elevated levels of SKP2 appear to enhance, rather than diminish, the interaction between VHL and MYC, suggesting a potential role for SKP2 in facilitating the MYC-VHL interaction. Furthermore, the presence of SKP2 in VHL immunoprecipitates implies a direct or indirect interaction between these E3 ubiquitin ligases, possibly engaging in co-regulating MYC through a bidirectional crosstalk, potentially forming a triplex complex. We propose a model in which SKP2 and VHL interact within a regulatory network to finely modulate c-MYC's activity.

I also successfully generated a series of VHL mutants, including single and double missense mutants. While I did not achieve complete disruption of the VHL-MYC interaction, these mutants have shed light on the challenging task of selectively targeting this specific protein-protein interaction. Notably, mutants D2, D4, and D5 exhibited some indications of partial disruption, warranting further investigation into their potential as tools for modulating the VHL-MYC interaction.

Understanding this intricate network holds significant implications for cancer research and potential therapeutic interventions. Dysregulation of MYC, SKP2, and VHL is frequently

observed in many cancers, making this network a promising target for novel cancer therapies. As we look to the future, unraveling the exact molecular mechanisms governing these interactions and their impact on c-MYC-driven transcription remains a compelling challenge that demands further investigation. Future studies should aim to dissect the specific domains and motifs involved in these interactions and decipher how these proteins collaborate to finely modulate MYC's diverse functions.

My work represents a significant step towards understanding the role of VHL in regulating MYC-driven gene expression. It sets the stage for investigations into the intricate regulation of MYC by SKP2 and VHL, offering new directions for further exploration in these critical areas of molecular biology and cancer research. I have also paved the way for future research focused on evaluating the consequences of disrupted VHL-MYC interactions on MYC's transcriptional activity, stability, gene expression patterns, and downstream signaling pathways. This multifaceted approach expands our understanding of essential cellular processes and holds great promise for the development of targeted therapies, particularly in the context of ccRCC, where dysregulated gene expression plays a pivotal role.

In summary, this research not only challenges our initial hypotheses but also underscores the complexity of the molecular interactions within this network. It opens new avenues for future exploration and potential interventions in cancer treatment, highlighting the significance of elucidating these intricate cellular mechanisms.

As we move forward, it is important to further clarify the biological role of the MYC-VHL interaction in normal and cancer cells. The potential for future in-depth studies, including in vivo investigations, network analyses, and drug screening, offers exciting avenues for advancing our understanding and potentially translating these findings into clinical applications. Ultimately, my work contributes not only to the fundamental understanding of molecular biology but also holds promise for the development of innovative therapeutic interventions in the ongoing battle against cancer.

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A

AN APPENDIX

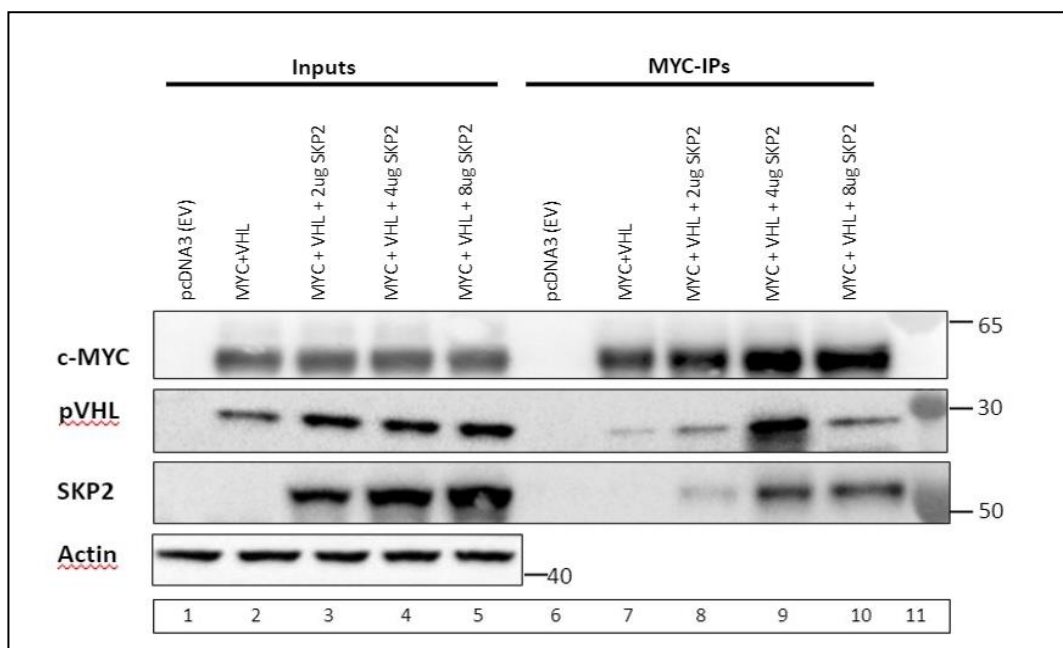


Figure A1: Investigation of SKP2 and VHL Competition for MYC Binding via MYC co-IP - Western Blot

Analysis

COS-7 cells were transfected with plasmids for pcDNA3 (negative control), co-transfected with MYC+VHL (positive control), and subjected to triple transfections with varying titrations of SKP2 (2 µg, 4 µg, and 8 µg). MYC was immunoprecipitated using an anti-HA antibody, with 500 µg of protein used for each co-IP. Western blot analysis was conducted, and the membranes were probed with antibodies against SKP2, FLAG(VHL), MYC, and Actin. Input samples are presented on the left, while corresponding co-IP samples are shown on the right. Key observations include consistent MYC expression in all MYC-containing samples (lanes 2-5) and an increase in immunoprecipitated MYC (lanes 7-10) across the samples. VHL expression was detected in all corresponding samples (lanes 7-10), with a slight reduction in the reference sample (no SKP2), possibly attributed to transfection issues or biological variability. Apart from this minor difference, VHL expression remains consistent. Co-immunoprecipitation of VHL with MYC reveals a decrease in samples without SKP2, reflecting its expression level in the input sample. Interestingly, there is an increase in co-immunoprecipitated VHL from 2 µg to 4 µg SKP2, which may suggest enhanced interaction but could also result from factors such as IP efficiency. Notably, despite the increase in MYC immunoprecipitation, the amount of co-immunoprecipitated VHL decreases in the 8 µg SKP2 sample. SKP2 expression levels in input samples align with the expected titration levels. However, in the co-IP samples, no increase was observed from the 4 µg to 8 µg SKP2 sample.



Figure A2: Crystal structure of a HIF-1a-pVHL-ElonginB-ElonginC Complex
This figure displays the molecular structure corresponding to Protein Data Bank (PDB) type 1LM8.



Figure A3: Crystal structure of the human c-MYC:MAX C-terminal apo complex
This figure displays the molecular structure corresponding to PDB type 6G6K.

Table A1: Residues on VHL VCB with the potential to fully inhibit or partially disrupt binding when mutated

For reasons of confidentiality, the exact residues cannot be specified. .

Entry	Residue	Structural stability by Missense 3D	Number of missense mutations by COSMIC (≤ 10)
1	R1_VHL	Damaging	///
2	R2_VHL	Damaging	///
3	R3_VHL	Damaging	///
4	R4_VHL	Damaging	///
5	R5_VHL	Damaging	///
6	R6_VHL	Neutral	< 10
7	R7_VHL	Neutral	< 10
8	R8_VHL	Damaging	///
9	R9_VHL	Neutral	> 10
10	R10_VHL	Neutral	< 10
11	R11_VHL	Neutral	< 10
12	R12_VHL	Neutral	< 10
13	R13_VHL	Neutral	< 10
14	R14_VHL	Damaging	///
15	R15_VHL	Damaging	///
16	R16_VHL	Damaging	///
17	R17_VHL	Neutral	-
18	R18_VHL	Damaging	///
19	R19_VHL	Neutral	< 10
20	R20_VHL	Damaging	///
21	R21_VHL	Neutral	< 10



2023

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Studies of the Regulation of the MYC Oncoprotein by the VHL Tumor Suppressor Protein