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A BC-box domain-related mechanism for VHL protein degradation

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LIST OF ABBREVIATIONS

APC: Anaphase Promoting Complex

aPKC: atypical Protein Kinase C

ATP: Adenosine tri-phosphate

BAG-1: Bcl-2-associated athanogene-1

BC-box: ElonginB/C box

BSA: Bovine Serum Albumine

BTB: Broad complex, Tram-track, Bric-a-brac

β-Trcp: beta-transducin repeat-containing protein

CAIX: Carbonic Anhydrase IX

CAND1: Cullin-Associated Nedd8-Dissociated 1

Card9: Caspase recruitment domain-containing protein 9

ccRCC: clear-cell renal cell carcinoma

CELO: Chicken Embryo Lethal Orphan

CFTR: Cystic-fibrosis trasmembrane-conductance regulator

CHIP: C-terminus of hsp70-interacting protein

CIS: Cytokine-inducible SH2-containing protein

CLL: Chronic Lymphocytic Leukemia

CRL: Cullin-RING Ligase

CSN: COP9 signalosome

CSN5: COP9 signalosome 5

Cul2: Cullin2

Cul5: Cullin5

Cys: Cysteine

DAPI: 4',6'-diamino-2-phenylindole

DDB1: DNA-damage-binding protein 1

ddH_2O: double-distilled H_2O

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DOX: Doxycycline

DRB: 5,6-dichlorobenzimidazole

DUB: De-ubiquitinating enzyme

E2-EPF: E2 Endemic pemphigus foliaceus

EBNA1: Epstein-Barr virus nuclear antigen 1

EBNA3C: Epstein-Barr virus nuclear antigen 3C

EBV: Epstein-Barr virus

EGFP: Enhanced GFP

ERK1/2: Extracellular signal-regulated kinase 1/2

F-box: cyclin F box

Fbw7: F-box and WD repeat domain-containing 7

FEM1B: Feminization 1 homolog B

FIH-1: Factor inhibiting HIF-1

FITC: Fluorescein isothiocyanate

G1: Gap 1 (a phase of cell cycle)

G76: Glycine 76

GA: Geldanamycin

Gam1: Gallus ante mortem 1

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

GRF: Guanine nucleotide-releasing factor

GSK3: Glycogen synthase kinase 3

GSK-3β: Glycogen synthase kinase 3β

GST: Glutathione S-Transferase

HBS: HEPES-buffered solution

HDAC1: Histone Deacetylase 1

HECT: Homologous to E6-associated protein C-terminus

HEK: Human Embryonic Kidney

HERC: HECT and RCC1-like domains containing protein

HHV8: Human Herpesvirus 8

HIF: Hypoxia-Inducible Factor

HIF-1: Hypoxia-Inducible Factor 1

HIF-1α: Hypoxia-Inducible Factor 1α

HIF-1β: Hypoxia-Inducible Factor 1β

Hip: Hsc70-interacting protein

His: Histidine

HIV-1: Human Immunodeficiency virus type 1

Hop: Hsc70-Hsp90 organizing protein

HPV: Human papilloma virus

HPV E6: HPV early protein 6

HPV16 E7: HPV type 16 early protein 7

HPV18: HPV type 18

HRE: Hypoxia Responsive Element

Hs: Hours

Hsp40: Heat shock protein 40 kDa

Hsp70: Heat shock protein 70 kDa

Hsp90: Heat shock protein 90 kDa

HSV-1: Herpes Simplex virus 1

ICP0: Infected Cell Polypeptide 0

IκB: Inhibitor of NF-κB

IP: Immunoprecipitation

IPTG: Isopropil β-D-1-tiogalattopiranoside

IVT: *In vitro* translated

JAMM: JAB1/MPN/Mov34 metalloenzyme

JAK: Janus kinase

K48; K63: Lysine 48; Lysine 63

kDa: kilo Dalton

KSHV: Kaposi's Sarcoma-associated Herpesvirus

LANA: Latency-associated Nuclear Antigen

LB: Luria-Bertani medium

LPS: lipopolysaccharide

LRR-1: Leucine-rich repeat containing protein 1

Luc: Luciferase

M: Mitosis

Mdm2: Murine double minute 2

MdmX: Murine double minute X

mRNA: messenger Ribonucleic acid

Nedd4: Neural precursor cell-expressed developmentally downregulated 4

Nedd8: Neural precursor cell-expressed developmentally downregulated 8

NEM: N-ethylmaleimide

NP-40: Nonyl Phenoxypolyethoxylethanol-40

NT: Not treated

Nur77: Nuclear receptor 77

p27: protein 27 kDa

p53: protein 53 kDa

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PFA: paraformaldehyde

PI3K: Phosphoinositide 3 kinase

PML: Promyelocytic leukemia protein

PMSF: Phenylmethanesulfonylfluoride

pRb: Retinoblastoma protein

Puro: Puromycin

Q-PCR: Quantitative-Polymerase Chain Reaction

RanGAP1: Ran GTPase-activating protein 1

Ras: rat sarcoma protein

Rbp1: RNA-binding protein 1

RBX1: RING box 1

Rcf: Relative centrifugal force

RING: Really Interesting New Gene

RLU/s: Relative Light Units per second

Roc1: Regulator of cullins 1

RT: Room temperature

RTA: R transactivator

RT-PCR: Reverse Transcription-Polymerase Chain reaction

RTK: Receptor tyrosine kinase

SAE1: Sumo Activating Enzyme 1

SAE2: Sumo Activating Enzyme 2

SCF: Skp, Cullin, F-box containing complex

SDS: Sodium Dodecyl Sulphate

SDS-PAGE: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

shRNA: short hairpin RNA

Skp1: S-phase kinase-associated protein 1

Skp2: S-phase kinase-associated protein 2

SOCS-box: Suppressor of cytokine signaling box

SOCS1: Suppressor of cytokine signaling 1

SOCS2: Suppressor of cytokine signaling 2

SOCS3: Suppressor of cytokine signaling 3

Src: Sarcoma protein

SSAT2: Spermidine/Spermine-N^1-Acetyltransferase 2

STAT: Signal Transducer and Activator of Transcription

STAT1: Signal Transducer and Activator of Transcription 1

STAT2: Signal Transducer and Activator of Transcription 2

Sti1: Stress-inducible protein 1

SUMO: Small Ubiquitin-like Modifier

SV5: Simian virus 5

TAE: Tris-acetate-EDTA buffer

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline Tween-20

TD-NEM: transcription-dependent nuclear export motif

Tet: Tetracycline

TPR: tetratricopeptide repeat

TRiC: TCP-1 ring complex

TSA: Trichostatin A

Ub: Ubiquitin

UBC9: Ubiquitin-conjugating enzyme 9

U-box: Ufd2 box

USP7: Ubiquitin specific peptidase 7

V: Volts

VBP-1: von Hippel-Lindau binding protein 1

VHL: von Hippel-Lindau

Vif: Viral infectivity factor

WB: Western blot

WT: Wild-type

FIGURES.

ABSTRACT

Viruses need to hijack cellular machineries both for their replication and propagation and to overcome cellular defenses. Since they often reconvert the functions of host proteins for their purposes, viruses are also powerful tool to better understand some molecular mechanisms underlying cellular processes.

Gam1 is an early protein of the avian adenovirus CELO and it possesses a BC-box domain to interact with the host adaptor heterodimer ElonginB/C and, hence, to act as a substrate receptor. It reconstitutes active Cullin2- and Cullin5-based E3 ligase complexes to ubiquitylate the host SAE1 protein, a subunit of SUMO E1 activating enzyme, inducing its proteasomal degradation.

Von Hippel-Lindau (VHL) protein is a cellular substrate receptor that associates to Cullin2 and ElonginB/C to specifically target the α subunits of HIF (Hypoxia-Inducible Factor) transcriptional factors for degradation. VHL is a tumor suppressor protein and its loss leads to the von Hippel-Lindau syndrome, characterized by the onset of renal cell carcinoma and other highly vascularized tumors.

We analyzed the possible effects on VHL due to the hijacking of host Cullin2 E3 ligase complexes by Gam1. Interestingly, we observed that Gam1 leads to VHL proteasomal degradation and to the consequent stabilization and activation of HIF-1.

Further experiments revealed that VHL protein degradation was not dependent on Gam1-related E3 ligase activity. Rather, the simple binding of Gam1 and other cellular and viral BC-box proteins to ElonginB/C was enough to induce VHL degradation, probably due to the reduced availability of free ElonginB/C complex that is essential for VHL stability.

Indeed, since unbound VHL undergoes misfolding, we are currently investigating the possible involvement of heat shock proteins and chaperone-dependent CHIP E3 ligase in affecting VHL stability upon BC-box proteins overexpression, as suggested by our preliminary data.

If verified, this will be an additional mechanism uncovered by the initial contribution of a viral protein.

INTRODUCTION

Viruses are obligate parasites, needing both to usurp cellular machineries for their replication and propagation and to overcome cellular defenses.

Since they often reconvert the functions of host proteins for their purposes, viruses are also powerful tools to better understand some molecular mechanisms underlying cellular processes.

Depending on the functions of these target proteins, viruses may alter some cellular pathways and, if they destroy tumor suppressors or if they induce the activation of proto-oncogene products, they can lead to cancer onset.

Therefore, understanding how and why viral proteins affect certain proteins belonging to the cellular apparatus is a very important research topic.

In particular, the Ubiquitin system seems to be a favorite target for viral hijacking, because it can regulate several pathways that can either contrast or sustain viral replication and infection.

The Ubiquitylation pathway.

Ubiquitin is a 76-aminoacid protein well conserved in eukaryotic organisms and totally absent in prokaryota. As the name suggests, Ubiquitin is ubiquitously expressed in all tissues in metazoan and was the first protein found to post-translationally modify other proteins (Ciechanover et al., 1980a; Hershko et al., 1980; Wilkinson et al., 1980). Throughout the years, ubiquitylation has emerged to control a plethora of different mechanisms, ranging from proteins degradation (Ciechanover et al., 1980a; Hershko et al., 1980), to endocytosis (Bonifacino and Traub, 2003; Dunn and Hicke, 2001),

signaling transduction (Kanayama et al., 2004; Sun and Chen, 2004; Sun and Allis, 2002), and DNA repair (Hoege et al., 2002).

Ubiquitylation is a reversible post-translational modification that consists in the creation of an isopeptide bond between the carboxyl group of the last glycine (G76) of activated-Ubiquitin and the ε-amino group of a lysine residue on the substrate (Ciechanover et al., 1980a; Hershko et al., 1980). It occurs through the subsequent and coordinated activity of three different kinds of enzymes, generally called Ubiquitin-activating enzyme E1, Ubiquitin-conjugating enzyme E2 and Ubiquitin ligase E3 (figure 1).

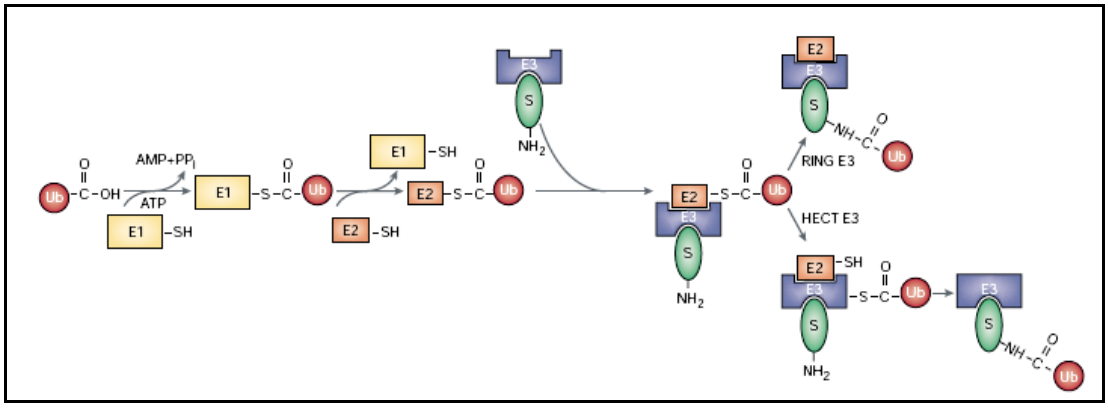

Figure 1. The ubiquitylation pathway.
(Figure taken from (Weissman, 2001)). The E1 enzyme activates Ubiquitin (Ub) to form an adenylated intermediate. Then Ubiquitin is transferred to E2 enzymes, which promote substrates ubiquitylation in association with several E3s (Weissman, 2001).

Ubiquitin is removed from the substrates by several de-ubiquitinating enzymes (Hershko et al., 1983). Multiple genes produce Ubiquitin as a series of immature precursors in a unique polypeptide, so it needs to be cleaved by a specific protease, belonging to the group of de-ubiquitinating enzymes (Wiborg et al., 1985). Key features of Ubiquitin protein are the carboxy terminal glycine residue, essential for the covalent binding to the substrates, and seven lysine residues, that can be differentially linked by other Ubiquitin moieties in order to target protein substrates for diverse fates. For example, K48-linked chains (containing at least four Ubiquitin moieties) drive protein substrates into proteasome-dependent degradation (Glickman and Ciechanover, 2002) and K63-linked chains are involved in vesicles trafficking (Haglund et al., 2003; Hofmann and Pickart, 1999, 2001) (figure 2).

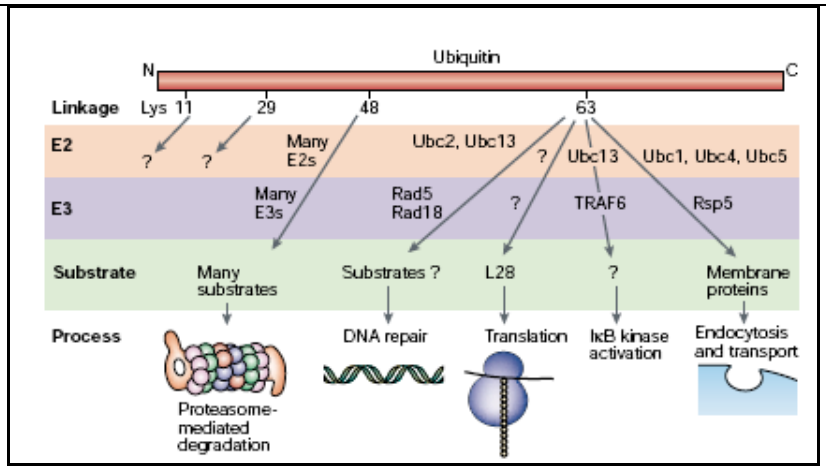

Figure 2. Different Ubiquitin linkages correspond to different fates.

(Figure taken from (Weissman, 2001))Schematic representation of different protein fates depending on the Ubiquitin lysine residue used to form the chain (Weissman, 2001).

Moreover, beside polyubiquitylation, there are two other kinds of modification by Ubiquitin: multiubiquitylation, which refers to the addition of Ubiquitin to several lysines in the same protein (Richly et al., 2005), and monoubiquitination, which refers to a single Ubiquitin protein modification (Haglund et al., 2003; Hicke, 2001; Mosesson et al., 2003).

The enzymes involved in the ubiquitylation cascade are diverse, ranging from the two E1s identified so far to the hundreds E3s present in humans. It is thus clear that it must be a tightly controlled pathway. Beside Ubiquitin, other Ubiquitin-like proteins, such as SUMO and Nedd8, are subjected to the same kind of activation process (reviewed in (Schwartz and Hochstrasser, 2003)).

A single gene, that generates two different E1 isoforms by alternative translation start sites, encodes the Ubiquitin E1. Since it is the only gene codifying E1 protein, its absence is lethal, as shown in the yeast *S. cerevisiae*. E1 protein requires magnesium ion and ATP as co-factors to generate an Ubiquitin adenylate intermediate. Then, this activated intermediate forms a high-energy thioester bond with the cysteine present in the active site of the E1 enzyme (Haas et al., 1982).

Ubiquitin is next transferred to the cysteine on the active site of E2 conjugating enzyme, through a transthiolation reaction.

Ubiquitin E2s, about 35 in humans, are characterized by the presence of a highly conserved ubiquitin-conjugation domain (reviewed in (Van Wijk, 2010)). They interact with both E1 and E3, connecting Ubiquitin activation to covalent proteins modification. Moreover, they select the Ubiquitin lysine residue to build the chain on (reviewed in (Van Wijk, 2010)); therefore they determine the fate of the target

substrates. In a few cases, the Ubiquitin moiety is linked to the substrate without the action of E3 enzymes, thus E2s are able to select some specific targets by themselves (Hoeller et al., 2007).

In most instances, however, the specific selection of the target substrates is determined by E3 ligases. In humans, the estimated number of these enzymes is over 600, highlighting the vast range of proteins that can be ubiquitylated. They cooperate with E2 enzymes to transfer Ubiquitin to the substrate, forming an isopeptide bond. E3 ligases activity is tightly regulated, and their interaction with E2 establishes the hierarchical organization of the ubiquitylation pathway (reviewed in (Ardley and Robinson, 2005)).

Ubiquitination can be reversed by the action of different de-ubiquitinating enzymes (DUBs), proteases that specifically cleave ubiquitin bonds. In the human genome about 100 DUBs have been identified, and they are divided into five different families, depending on their analogy with other cysteine proteases and on their different characterizing domains (reviewed in (Reyes-Turcu et al., 2009)). The roles of DUBs in the ubiquitylation pathway can be grouped in four main activities: 1) the cleavage of Ubiquitin precursors, both to resolve the Ubiquitin multiple copies into monomers and to remove the additional residue at the carboxy terminus in order to expose the last glycine (Wiborg et al., 1985); 2) the removal of Ubiquitin intermediates from incorrect thioester bond, occurring with enzymes not involved in the ubiquitylation pathway (Pickart and Rose, 1985); 3) the reverse of proper ubiquitylation, that can result into the inhibition of substrates degradation (Wilkinson, 1997), and 4) the recycle of Ubiquitin monomers from unbound polyubiquitin chains (Amerik et al., 1997; Piotrowski et al., 1997).

When not removed from the target substrates, the K48-linked polyubiquitin chain leads proteins to degradation by the proteasome (Glickman and Ciechanover, 2002). Proteasomes are multisubunits protein complexes found both in prokaryota and in eukaryota, distributed both in the cytoplasm and in the nucleus (Wojcik and DeMartino, 2003). The 26S proteasome consists in the 20S core particle and in the 19S regulatory subunits. Four heptameric rings, which are further subdivided in two inner rings, containing the active site of the proteases involved in proteins degradation, and two outer rings, forming the pores for the passage of the substrates to degrade, create the 20S core (Groll et al., 1997; Liu et al., 2003; Piwko and Jentsch, 2006). The regulatory ATPase subunits are located at both sides of the core particle and they are required for the recognition of ubiquitinated proteins and for their subsequent unfolding to allow the entrance into the proteolytic machinery (Elsasser and Finley, 2005; Lam et al., 2002).

The Ubiquitin/proteasome system has an essential role in maintaining the correct levels of cellular proteins, determining their turnover, and when an event alters its activity, it can have a detrimental impact on the cell or even on the entire organism.

Given the high versatility of ubiquitylation, some pathogens and viruses exploit the Ubiquitin system for their advantage. For example, viruses can encode E3 ligases (Boutell et al., 2002; Yu et al., 2005), as well as substrate receptors (Blanchette et al., 2004; Boggio et al., 2007; Cai et al., 2006; Cheng et al., 2007a; Elliott et al., 2007; Knight et al., 2005; Yu et al., 2003; Yu et al., 2004) and control (or even encode) some de-ubiquitinating enzymes (Holowaty et al., 2003). The herpes simplex virus (HSV-1) ICP0 protein is an example of viral E3 ligase that targets several host proteins for degradation, such as PML protein, Sp100, p53, and cyclin D3 (Boutell et al., 2002), whereas Epstein-Barr virus (EBV) EBNA1 interacts with the de-ubiquitinating enzyme USP7, contrasting its own ubiquitylation and prevents de-ubiquitylation of p53 protein through binding competition (Holowaty et al., 2003). Finally, the high-risk HPV E6 protein represents a pivotal example of a viral adaptor protein. It assembles with the host E3 ligase E6-AP complex to induce the degradation of p53 protein (Scheffner et al., 1993; Scheffner et al., 1990).

Ubiquitin E3 Ligases.

The Ubiquitin E3 ligases are a vast and heterogeneous group of proteins that act in the third step of ubiquitylation. They recruit the proteins to be ubiquitylated and they can either directly modify the target substrates or assist the E2 conjugating-enzyme to do that. Based on the diverse modes of action and on their different characteristic domains, E3 ligases are divided into three main families. These refer to the HECT (Homologous to E6-associated protein C-terminus), the RING (Really Interesting New Gene)-finger and the U-box domains. The U-box is a modified RING motif that cannot bind Zn^{2+}. All these domains are docking sites for E2 conjugating-enzymes.

The variable parts of these E3 ligases include the substrate-binding site that recognizes a defined pattern of modification on the substrate itself. There are, for example, E3 ligases that interact only with the phosphorylated forms of some proteins, others that bind just the acetylated or hydroxylated ones, and so on. Given the defined role of E3 ligases, they are finely regulated for timing and localization in

cells. Moreover, E3 ligase subunits are often subjected to self-ubiquitylation as a further mechanism to limit their activity in time and space (Galan and Peter, 1999; Zhou and Howley, 1998).

HECT domain E3 ligases family.

The HECT domain is usually present at the carboxy terminus of these E3 ligases and it was first identified in E6-AP, which associates with the papillomavirus protein E6 (Huibregtse et al., 1995; Scheffner et al., 1993). The main feature of this domain is the presence of a cysteine residue that binds the Ubiquitin intermediate before transfering it to the substrate (Huang et al., 1999; Scheffner et al., 1995).

The amino terminus determines the possible association with lipids or other proteins (Dunn et al., 2004; Plant et al., 2000), including the target substrates, and, based on its structure, HECT E3 ligases are further divided into three groups, the Nedd4 family, the HERC family and the other HECTs.

Several mammalian Nedd4 family members are involved in the regulation of proliferative signaling pathways (Cao et al., 2008; Lin et al., 2000), in vesicle sorting (Belgareh-Touze et al., 2008) and endocytosis (Dupre et al., 2004). Moreover, they display an important role in the immune response; in particular, loss of Nedd4 member ITCH induces some immune defects mostly related to an impaired self-tolerance (Fang et al., 2002; Perry et al., 1998; Venuprasad et al., 2006).

HERC family E3 ligases are often localized in proximity to vesicles and to the Golgi apparatus (Garcia-Gonzalo and Rosa, 2005a; Hochrainer et al., 2008), and they are distinguished in large HERCs, which act also as guanine nucleotide-releasing factor (GRF) (Garcia-Gonzalo et al., 2005b), and small HERCs. Because of their association with vesicles and endocytic proteins, some HERCs may be involved in vescicular sorting (Garcia-Gonzalo and Rosa, 2005a).

The remaining HECT ligases do not form further families and, among them, there is E6-AP, which interacts with the HPV E6 protein to target the tumor suppressor p53 for degradation (Scheffner et al., 1993; Scheffner et al., 1990).

Each HECT ligase consists of a single polypeptide that sustains its enzymatic activity. However, in some situations, HECT ligases can be regulated or supported by several other proteins that act as adaptors, for example in the case of HPV E6.

RING domain E3 ligases family.

The RING E3s represent the largest group of Ubiquitin E3 ligases.

The RING domain is a cysteine/histidine-rich domain that is able to bind two atoms of zinc (Borden and Freemont, 1996; Freemont et al., 1991). The RING sequence has six to seven conserved cysteines and one or two conserved histidines separated by variable stretches of other amino acid residues (Cys-x_2-Cys-$x_{(9-39)}$-Cys-$x_{(1-3)}$-His-$x_{(2-3)}$-Cys/His-x_2-Cys-$x_{(4-48)}$-Cys-x_2-Cys) (Freemont et al., 1991) (figure 3).

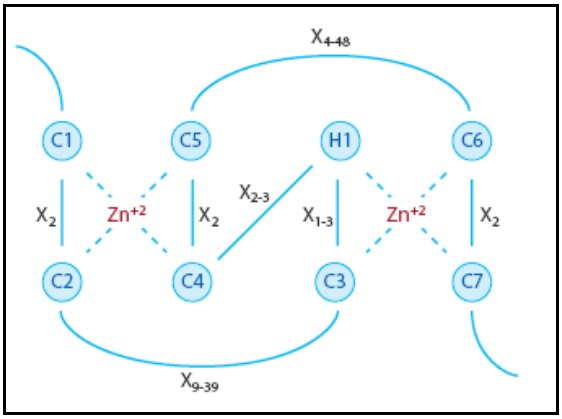

Figure 3. The RING finger domain.

(Figure taken from (Deshaies and Joazeiro, 2009)). Schematic organization of the conserved RING finger domain contained in RING E3 ligases and in other proteins. C refers to cysteine residue, whereas H to histidine residue. X refers to any amino acid residue and the associated numbers correspond to the number of amino acids in the spacer regions between the conserved residues (Deshaies and Joazeiro, 2009).

The presence of cysteine or histidine at the fifth conserved position is important to determine the correct recognition of the ubiquitin-conjugating E2 enzymes. Indeed, the substitution of the cysteine with the histidine often impairs the E2-E3 binding (Ardley et al., 2001).

The mechanism of substrate ubiquitination is completely different from the HECT one. Indeed, RING E3 ligases rather act like a scaffold to keep in close proximity the E2 thioesterified with Ubiquitin (E2~Ub) and the protein substrate. Evidence indicates that E2~Ub undergoes a conformational change when it interacts with the RING domain, facilitating the Ubiquitin transfer to the target substrate (Seol et al., 1999).

RING E3 ligases can be either single chain or multisubunits complexes. Parkin is a single chain RING E3 ligase (Shimura et al., 2000), c-Cbl works as a homodimer (Kozlov et al., 2007), whereas Mdm2 is

often found in association with MdmX to form a heterodimer (Linares et al., 2003). However, the large majority of RING E3 ligases are multisubunit complexes, like Cullin-RING Ligases (CRLs) and the Anaphase-Promoting Complex (APC). In these latter examples the RING domain is part of a single protein subunit, whereas the other subunits have structural functions and contain the acceptor site for the substrates to ubiquitinate.

Parkin may play a more general role in the removal of abnormally folded or damaged protein (Imai et al., 2000; Olzmann and Chin, 2008). Mutated Parkin loses its activity and it appears to be the main cause underlying the pathogenesis of Parkinson disease, especially for the juvenile form (Beasley et al., 2007; Safadi and Shaw, 2007). c-Cbl is required for proper endocytosis of the activated form of receptor tyrosine kinases (RTKs) and to promote their subsequent degradation (Penengo et al., 2003; Waterman et al., 1999). Mdm2 is involved in the regulation of p53 (Fuchs et al., 1998; Haupt et al., 1997), and association with MdmX seems to increase its activity (Linares et al., 2003). APC complex tightly controls late phases of mitosis assuring accurate chromosome segregation (Engelbert et al., 2008) and M/G1 transition through the sequential degradation of cyclins, securin, and other proteins involved in the progression of mitosis (reviewed in (Baker et al., 2007)). CRLs, instead, participate in multiple pathways, ranging from cell cycle regulation to the control of cell homeostasis.

Cullin-RING Ligases.

Cullin-RING Ligases represent the largest group of E3 ligases. They all contain a small RING protein that constitutes the docking site for the E2 enzymes, and a Cullin subunit that forms the E3 ligase backbone and connects the RING protein to the adaptors. The adaptor proteins usually interact with the substrate-receptor subunits that specifically recognize the proteins to target for ubiquitination.

The RING protein is the same for all Cullins, called RING BOX 1 (RBX1) or Regulator of cullins 1 (Roc1), and it binds the Cullin carboxy terminus and the E2 enzyme (Kamura et al., 1999a).

In humans, seven different Cullins (Cullin1, 2, 3, 4A, 4B, 5 and 7) have been identified. As already mentioned, they bind RBX1 at the carboxy terminus, whereas at the amino terminus they are linked to the adaptor protein. Cullin1 and Cullin7 assemble with the adaptor protein Skp1 (Dias et al., 2002; Lyapina et al., 1998), whereas Cullin2 and Cullin5 use ElonginB/C heterodimer as adaptor (Pause et al., 1997; Yu et al., 2003). Cullin3 binds to BTB (Broad complex, Tram-track, Bric-a-brac) domain

protein (Xu et al., 2003) and Cullin4A interacts with DDB1 (DNA-damage-binding protein 1) protein (He et al., 2006) (figure 4).

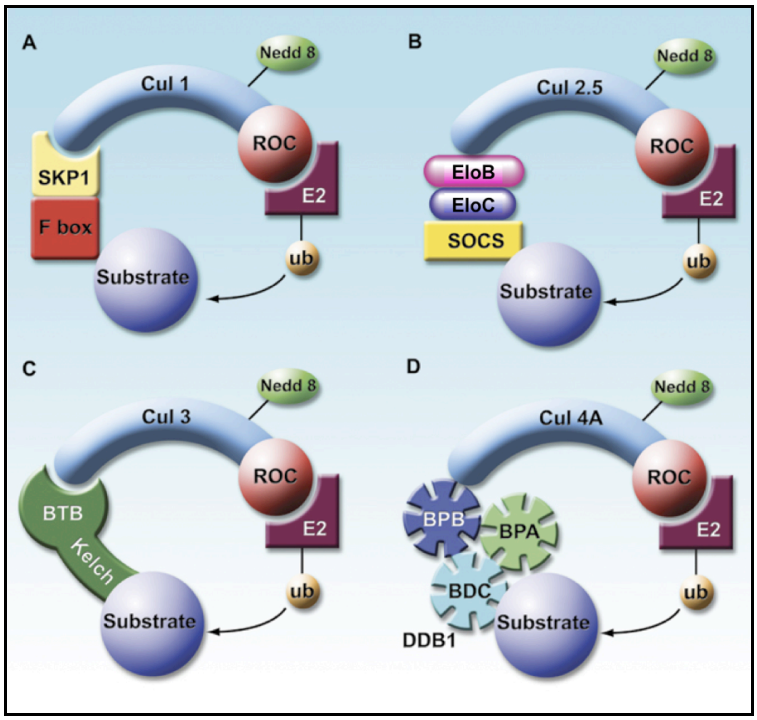

Figure 4. Cullin RING ligases.

(Figure taken from (Barry and Fruh, 2006)). Schematic representation of the general Cullin-RING ligases structures. (A) SCF complex consists in Cullin1, RBX1, Skp1 and F-box protein subunits; (B) Cullin2 and Cullin5 interact with the same adaptor ElonginB/C, RBX1 and a SOCS-box protein subunits; (C) Cullin3 recruits RBX1 and a BTB-domain proteins that act both as adaptor and substrate receptor; (D) Cullin4A binds RBX1 and the substrate adaptor DDB1 (Barry and Fruh, 2006).

The adaptor recognizes the substrate-receptor through a specific conserved sequence, the F-box domain in the case of the Cullin1-associated Skp1 (S-phase kinase-associated protein 1) (Bai et al., 1996), whereas the BC-box domain for ElonginB/C (Kamura et al., 1998). Virtually, all proteins that contain these motifs can interact with the relative adaptors to form different combinations of E3 ligase complexes. Given the high versatility of these complexes, it is evident why they represent the most numerous group of E3 ligases.

Cullin-RING Ligases activity is regulated by Nedd8 post-translational modification (Hori et al., 1999) and by the association of some other protein complexes, such as the CSN (COP9 signalosome) (Cope and Deshaies, 2003; Wolf et al., 2003; Zhou et al., 2003) and the CAND1 (Cullin-Associated Nedd8-Dissociated 1) protein (Min et al., 2003).

Nedd8 is an Ubiquitin-like protein that covalently attaches to other proteins following a similar enzymatic cascade as Ubiquitin (reviewed in (Schwartz and Hochstrasser, 2003)). However, Nedd8 is not usually involved in protein degradation, rather it regulates the activity of its substrates, for example in the case of Cullins (Ohh et al., 2002). Nedd8 modifies all Cullins at a conserved lysine residue in their carboxy terminus. Cullin harboring mutation in the lysine residue display reduced activity, underlying the importance of this modification for the correct function of these E3 ligase complexes (Pan et al., 2004). On the other hand, Nedd8 preferentially modifies Cullin-RING ligases that still bind the target protein, indicating that, the presence of substrates stimulates Cullins activation (Chew and Hagen, 2007). Nevertheless, only Cullins that are complexed with RBX1 can be neddylated, and mutations in the RING domain of RBX1 abolished Cullin neddylation, suggesting that RBX1 may act as Nedd8 E3 ligase (Furukawa et al., 2000; Kamura et al., 1999b; Sufan and Ohh, 2006).

CAND1 associates with Cullin1, 2, 3, 4A and 4B (Min et al., 2003), but the precise regulatory mechanism was mainly described for Cullin1. CAND1 preferentially binds the deneddylated form of Cullin1 at the amino terminus, thereby preventing the assembling to the adaptor proteins (Oshikawa et al., 2003; Zheng et al., 2002a). This explains how CAND1 negatively affects Cullin1 E3 ligase activity.

The COP9 signalosome complex, instead, facilitates the removal of Nedd8 from Cullins (Lyapina et al., 2001). CSN5 is the catalytic subunit of CSN complex and it contains a conserved metalloprotease motif called JAMM (Cope et al., 2002). This domain is directly involved in deneddylation activity. Differently from CAND1, CSN complex binds to completely assembled CRLs and keeps them in a protected and inactivated state (Wee et al., 2005). Indeed, neddylation and deneddylation cycles are essential for the proper function of CRLs, since CSN inactivation reduces CRLs activity (Cope and Deshaies, 2003). This inactivation might be due to the increased autoubiquitination of the substrate-receptors in continuously active CRLs.

This activation and deactivation cycles may favor substrate receptors switch in order to rapidly create new combinations of CRLs in response to some stimuli or cellular conditions.

In some cases, Nedd8 can mediate CRLs dimerization, as demonstrated for Cullin3-based complexes, further contributing to the activity of these enzymes (Wimuttisuk and Singer, 2007).

As previously mentioned, substrate receptors that contain the same binding domain, such as the F-box and the BC-box, correspond to a specific adaptor that, in turn, corresponds only to certain Cullins. Instead, BTB domain, which associates with Cullin3, includes in the same protein both the adaptor domain (BTB) and the substrate-receptor one (Xu et al., 2003). Therefore, different kinds of BTB

proteins exist and they possess different protein-protein interaction domains at the carboxy terminus (reviewed in (Perez-Torrado et al., 2006)).

Hence, the vast range and the different types of substrates that can be ubiquitylated by CRLs are determined by the substrate-receptors subunits.

The F-box, so called because first identified in cyclin F protein (Bai et al., 1996), is a conserved 50 amino acids long domain, located at amino terminus of some substrate receptors (Zheng et al., 2002b). The F-box domain interacts with the Skp1 adaptor (Bai et al., 1996). These proteins present other protein-interaction domains at their carboxy terminus, usually for the binding of phosphorylated substrates (Skowyra et al., 1997). The most known F-box proteins are Skp2, which targets the S phase regulator p27 for degradation (Carrano et al., 1999; Tsvetkov et al., 1999), β-Trcp, which induces IκB and β-catenin ubiquitination (Winston et al., 1999), and Fbw7, which controls cell cycle progression driving cyclin E for degradation (Koepp et al., 2001).

The BC-box is a 10 amino acid long domain that specifically associates to ElonginB/C heterodimer and is usually located at the carboxy terminus of proteins (Kamura et al., 1998). The BC-box is generally included in a larger domain, such as the SOCS-box or the VHL-box, which establishes Cullin selectivity. Indeed, SOCS-box often contains a central LPϕP (where ϕ identifies any hydrophobic residue) motif that determines the recruitment of Cullin5, whereas the VHL-box associates preferentially with Cullin2 (Kamura et al., 2004; Mahrour et al., 2008).

SOCS (Suppressor of Cytokine Signaling) were the first proteins in which SOCS-box domain was identified and associated to ElonginB/C binding (Hilton et al., 1998; Kamura et al., 1998; Zhang et al., 1999). The most studied members are SOCS1 and SOCS3, involved in the control of cytokines signaling (Endo et al., 1997; Nicholson and Hilton, 1998). Their expression is driven by STAT (Signal Transducer and Activator of Transcription) transcription factors (Naka et al., 1997) that become active upon JAKs (Janus Kinases) activation by cytokines (Adams et al., 1998). Therefore, SOCS proteins act in a negative feedback loop, which re-establishes cellular starting conditions.

VHL-box, as the name suggests, was identified in VHL (von Hippel-Lindau) protein, the most known substrate receptor that associates with Cullin2 (Pause et al., 1997). VHL recognizes hydroxylated proline residues on α subunits of HIFs (Hypoxia Inducible Factors) and targets them for degradation in normoxic conditions (Cockman et al., 2000; Groulx and Lee, 2002; Tanimoto et al., 2000). HIF-α destabilization is reverted by the establishment of hypoxia (Minet et al., 1999; Wang and Semenza,

1993) or upon certain other conditions, which can include immune response (Blouin et al., 2004; Cramer et al., 2003; Haddad and Harb, 2005), intracellular acidification (Mekhail et al., 2004), oxidative stress (Huang et al., 2009) and cancer (He et al., 2004; Khacho et al., 2008; Maxwell, 2005; Tang et al., 2007).

As for HECT E3 ligases, also RING domain E3 ligases can be encoded or exploited by viruses, and this is particularly true for CRLs, because of their modular composition (reviewed in (Barry and Fruh, 2006)). It is evident that reconversion of CRLs functions can have a detrimental impact on cells not only for the establishment of a favorable viral environment, but also for the impairment of essential host pathways. For instance, the paramyxovirus SV5 V protein binds to DDB1 and hence with Cullin4A E3 ligase to eliminate both STAT1 and STAT2, thereby overcoming the interferon α/β antiviral response (Parisien et al., 2002; Precious et al., 2005); the EBV (Epstein-Barr virus) EBNA3C protein recruits the SCF complex to target pRb for degradation (Knight et al., 2005); whereas the HPV16 E7 and Adenovirus type 5 E4orf6 (in association with E1B-55k) contain BC-box domains allowing them to interact respectively with Cullin2- and Cullin5-based E3 ligases to eliminate the tumor suppressors pRb and p53 (Huh et al., 2007; Querido et al., 2001).

U-box domain E3 ligase family.

The U-box is a domain consisting in 75 amino acids, first identified in the yeast protein Ufd2, which is involved in the elongation of Ubiquitin chains (Koegl et al., 1999). For this reason U-box E3 ligases were initially classified as Ubiquitin E4. However, it was demonstrated that they are able to act as proper E3 ligases without the aid of another E3. The U-box domain structure is related to RING, but it does not bind zinc atoms, rather it uses salt bridges to stabilize its folding (Aravind and Koonin, 2000). Nevertheless, the ubiquitination mechanism is very similar to the one utilized by RING E3 ligases, since they function as protein scaffolds to allow interaction between E2~Ub and the selected substrates. In humans, the best characterized U-box E3 ligase is CHIP (Carboxy terminus of hsp70-binding protein) (Ballinger et al., 1999). This E3 ligase mediates the degradation of substrates carried by hsp70 and hsp90, for this reason CHIP activity is considered chaperone-dependent and is involved in the quality control pathway that assists protein folding or promotes the degradation of misfolded and damaged proteins (Ballinger et al., 1999; Connell et al., 2001; Rosser et al., 2007).

The U-box of CHIP is located at its carboxy terminus, whereas a tetratricopeptide repeat (TPR) motif is present at the amino terminus (Ballinger et al., 1999). The latter domain is involved in protein-protein interaction, and more precisely it is required for the recruitment of heat shock protein (Lamb et al., 1995).

CHIP forms a multimeric E3 ligase complex in association with hsp70, hsp90, and BAG-1. Some accessory proteins, like Hip (Hsc70-interacting protein) (Hohfeld et al., 1995) and Hop (Hsc70-Hsp90 organizing protein) (Smith et al., 1993), can be recruited in the complex to increase the affinity for the substrates or to facilitate the cooperation between hsp70 and hsp90 (Ballinger et al., 1999).

CHIP ubiquitylates misfolded CFTR (cystic-fibrosis trasmembrane-conductance regulator) (Meacham et al., 2001), and heat-denaturated Luciferase through the combined action of hsp70 and hsp40, whereas native Luciferase is not affected by the presence of CHIP (Murata et al., 2001). Moreover, CHIP induces the clearance of expanded polyglutamine proteins, conferring some protection against neurological diseases (Jana et al., 2005).

CHIP also regulates the recover of inducible hsp70 protein level after heat shock, thus re-establishing the initial conditions (Qian et al., 2006). Indeed, heat shock triggers hsp70 expression to protect cells from toxic aggregates that can occur after protein misfolding (Lovell et al., 2007). Upon depletion of misfolded proteins, hsp70 protein must return to physiological level and CHIP has an active role in hsp70 degradation (Qian et al., 2006).

Differently from HECT and RING domain E3 ligases, no viral protein was found to directly interact with or substitute U-box E3 ligases, even if several viruses induce the expression of heat shock proteins (Glotzer et al., 2000; Young et al., 2008b), encode their own chaperones (Brodsky and Pipas, 1998; Gober et al., 2005; Kuciak et al., 2008), or interact with host chaperones for their functions (Liu et al., 1998; Park and Jung, 2001; Ujino et al., 2009). Therefore it could be possible that viral proteins can modulate CHIP activity indirectly, through the regulation of molecular chaperone proteins.

The BC-box and the SOCS-box domains.

Cullin2 and Cullin5 adaptor proteins ElonginB and ElonginC form a complex well conserved through evolution. ElonginB possesses an amino terminal Ubiquitin-like domain that directly interacts with its

partner ElonginC (Brower et al., 1999). ElonginB seems to have a chaperone-like function in maintaining the folding, and thus the stability, of ElonginC (Aso et al., 1995). ElonginC, in turn, seems to prevent the degradation of its binding partners, as demonstrated in *S. cerevisiae* (Hyman et al., 2002). ElonginC is the Skp1 homolog (Bai et al., 1996; Garrett et al., 1994).

ElonginB/C heterodimer is found in several complexes, such as the elongation factor SIII (Aso et al., 1995; Conaway et al., 1993; Garrett et al., 1994) and in some E3 ligases that contains Cullin2 or Cullin5 as scaffold. It specifically binds a domain that is called BC-box, with a degenerate consensus sequence (xLxxxCxxx[A,I,L,V]) (Aso et al., 1996; Duan et al., 1995b; Kamura et al., 1998; Kibel et al., 1995). In Gam1 viral protein an alanine residue substitutes the conserved cysteine (Boggio et al., 2007). The crystal structure of the ternary complex VHL-ElonginB/C revealed that the interaction of VHL with ElonginC occurs between the conserved leucine of BC-box and the hydrophobic pocket of ElonginC (Ohh et al., 1999; Stebbins et al., 1999; Takagi et al., 1997). It is expected that the same interaction exists between all BC-box containing proteins and ElonginC. The BC-box is usually contained in larger domains, such as SOCS-box and VHL-box (Kamura et al., 2004; Zhang et al., 1999), even if there are proteins that display just the conserved BC-box like MUF-1 (Kamura et al., 2001) and ElonginA (Aso et al., 1996).

The VHL-box was identified in the von Hippel-Lindau (VHL) protein and is present in other proteins that associate with Cullin2 complexes, such as LRR-1 and FEM1B (Kamura et al., 2004).

Besides SOCS proteins, also members of Ras, WD-40 repeat, ankyrin repeat, and SPRY domain families contain a SOCS-box domain that allows them to interact with Cullin5 E3 ligases (Kamura et al., 2004). Since such a great number of proteins interact with ElonginB/C heterodimer, it is logical to suppose the existence of some mechanisms regulating ElonginB/C recruitment. Differently to F-box proteins that are subjected to autoubiquitination when extensively complexed with the same E3 ligase (Galan and Peter, 1999; Zhou and Howley, 1998), BC-box proteins show a greater stability when bound to ElonginB/C (Hyman et al., 2002; Kamura et al., 2002; Schoenfeld et al., 2000). However, at least for SOCS proteins, there is evidence supporting the existence of cross-modulatory mechanisms between these substrate receptors. Indeed, SOCS2 expression occurs later than CIS, SOCS1 and SOCS3 and is maintained longer in time (Starr et al., 1997). Therefore, it can compete with them for the association to the E3 ligase complex. Indeed, SOCS2 was shown to bind Cullin5 with a higher affinity than CIS, SOCS1 and SOCS3 and to target them for proteasomal degradation (Babon et al., 2009; Piessevaux et al., 2008; Piessevaux et al., 2006). Finally, SOCS3 presents a further regulatory

mechanism; indeed the Jak-dependent phosphorylation of tyrosine residues present on the BC-box domain impairs the interaction with ElonginB/C and enhances SOCS3 degradation (Haan et al., 2003).

The von Hippel-Lindau (VHL) protein.

The von Hippel-Lindau protein (VHL) protein is the most studied substrate receptor that assembles with Cullin2-based E3 ligases (Pause et al., 1997).

VHL gene was cloned in 1993 and, when mutated, it causes the VHL sydrome, an autosomal inherited multiple cancer syndrome, characterized by highly vascularized tumors (Chen et al., 1995; Latif et al., 1993). The frequency of this disease is 1 per 36000-45000 live births and about 70% of VHL patients develop clear-cell renal cell carcinoma (ccRCC) (Maddock et al., 1996). VHL protein is a tumor suppressor, since its re-expression in VHL-defective cells inhibits their ability to form tumors in nude mice (Gnarra et al., 1996; Iliopoulos et al., 1995).

VHL gene consists of three exons encoding for a unique mRNA that produces two main functional protein isoforms arising from different internal translation sites. The shorter isoform has an apparent molecular mass of 19 kDa, whereas the longest of 30 kDa (Iliopoulos et al., 1998; Schoenfeld et al., 1998).

Analysis of VHL protein sequence and structure has identified at least three different regions: an acidic domain at the amino terminal end of the 30 kDa isoform (not present in the shorter isoform), with no fully defined functions yet, the β domain located in the central region and in the carboxy terminal end consisting in seven β-strands and one α-helix, the C-terminal α domain with three α-helices that bind ElonginB/C complex (Stebbins et al., 1999) (figure 5).

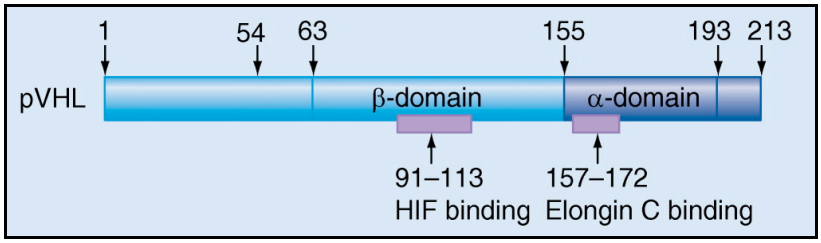

Figure 5. VHL protein domains.

(Figure taken from (Rathmell and Chen, 2008)). VHL has three different domains: the acidic domain (residues 1-63), the β domain, which is the acceptor sites for several substrates and interactors, such as α subunits of HIF, and the α domain that binds ElonginC (Rathmell and Chen, 2008).

VHL α domain contains the BC-box and the residues involved in Cullin2 interaction (Ohh et al., 1999). Several inactivating mutations can arise in this region impairing ElonginB/C binding, thus leading to VHL destabilization and loss of function (Schoenfeld et al., 2000). Interaction with ElonginB/C allows VHL to act as substrate receptor in the context of Cullin2-RING E3 ligases (Lisztwan et al., 1999; Lonergan et al., 1998; Pause et al., 1996), and stabilization of VHL/ElonginC association enhances the ubiquitylation of the target substrates, as demonstrated by the recruitment of SSAT2 (Spermidine/Spermine-N^1-Acetyltransferase 2) (Baek et al., 2007). Furthermore, VHL assembly into the E3 ligase complex promotes Cullin2 neddylation and thus its activity (Sufan and Ohh, 2006; Wada et al., 1999). VHL α domain is also the acceptor site for Nur77, an orphan nuclear receptor induced by several growth factors (Williams and Lau, 1993; Yoon and Lau, 1994). Nur77 competes with ElonginC for the binding of VHL, leading to the stabilization of VHL substrates and to the impairment of other VHL related functions. Therefore, Nur77 negatively regulates VHL activity by simply binding the α domain, further underlying the necessary role of ElonginB/C in VHL complexes (Kim et al., 2008).

VHL β domain is the acceptor site for multiple and different proteins ranging from substrates to ubiquitylate, for example the α subunits of HIFs (Hypoxia-Inducible Factors) transcription factors (Maxwell et al., 1999; Ohh et al., 2000), Rbp1 (the large subunit of RNA polymerase II) (Mikhaylova et al., 2008) and aPKC (atypical Protein Kinase C) (Okuda et al., 2001), to simple interactors, such as fibronectin (Bluyssen et al., 2004), collagen IV (Kurban et al., 2008), Sp1 (Cohen et al., 1999; Mukhopadhyay et al., 1997), Card9 (Yang et al., 2007), FIH-1 (Mahon et al., 2001), p53 (Roe et al., 2006), p400 and pRb (Young et al., 2008a), and microtubules (Hergovich et al., 2003). Moreover, this domain contains the binding sites for the chaperonine TRiC and hsp70, which assist VHL folding (Hansen et al., 2002; Melville et al., 2003), and the nuclear export sequence that interacts with Ran (Bonicalzi et al., 2001; Groulx et al., 2000).

Given the numerous proteins that associate with the same VHL domain, it appears evident that VHL can be subjected to post-translational modifications and other regulatory mechanisms controlling its proper distribution among these different complexes.

For example, VHL neddylation on lysine 159 acts as a molecular switch to temporary detach VHL from ElonginB/C, and thus from the E3 ligase complex, and to promote association with fibronectin (Russell and Ohh, 2008; Stickle et al., 2004), as well as phosphorylation of the acidic domain (Lolkema et al., 2005). Fibronectin and collagen IV deposition was identified as one of the main VHL tumor suppressor functions (Kurban et al., 2008; Ohh et al., 1998). Indeed, VHL-deficient cells fail to form a proper extracellular matrix, thus being more prone to develop metastatic invasion.

Phosphorylation of serine 68 by GSK3 (glycogen synthase kinase 3), instead, impairs VHL-induced microtubules stabilization (Hergovich et al., 2006), maybe resulting in some reorganization of microtubule apparatus. More adverse effects on spindle orientation and chromosomes stability come from the total loss of VHL protein functions, suggesting another possible mechanism for cancer onset in VHL patients (Thoma et al., 2009). Moreover, VHL, together with GSK-3β, seems to play a role in the signaling pathway for the maintenance of the primary cilium in kidney cells, which is an essential structure in controlling kidney cells proliferation (Thoma et al., 2007).

Furthermore, it was recently demonstrated that elevated Src kinase activity induces phosphorylation of VHL tyrosine 185 and this modification leads to VHL degradation also under normoxic conditions, thereby increasing the stability of HIF-1α. In this way, activated Src can drive a hypoxia-like response thanks to the expression of HIF-1 target genes and can contribute to cancer establishment. Since Src phosphorylation affects a residue of VHL α domain, it was suggested that this modification displaces VHL protein from ElonginC, thus facilitating its destabilization (Chou, 2010; Schoenfeld et al., 2000). Indeed, even under native conditions, the unbound VHL assumes a molten globule structure that is prone to misfolding and aggregation, because of the exposure of hydrophobic residues on its surface (Sutovsky and Gazit, 2004). Therefore, the combined action of the chaperonine TRiC and the hsp70 protein in assisting nascent VHL protein appears to be essential. VHL release occurs only in the presence of available ElonginB/C heterodimer, otherwise VHL is retained in the folding complex, as observed for some VHL mutants that were unable to interact with ElonginC (Feldman et al., 1999; Melville et al., 2003). However, since these mutants show a reduced half-life compared to wild-type VHL (Schoenfeld et al., 1998), TRiC and hsp70 might play a role in mutated VHL degradation.

Indeed, a chaperone-dependent VHL degradation mechanism was described in yeast. A molecular chaperone complex containing both hsp70 and hsp90, together with Sti1 (which human homolog is Hop), recruits the unbound VHL (McClellan et al., 2005). Accordingly, hsp70 displays a double action on VHL protein: in association with TRiC it assists VHL folding (Melville et al., 2003), and together with

hsp90 it determines VHL degradation (McClellan et al., 2005), pointing out an essential role of this protein towards VHL stability.

Other cancer-related mechanisms can limit or inhibit VHL expression at different regulatory levels, such as the repression of *VHL* gene transcription due to the hypermethylation of VHL promoter (Herman et al., 1994), or the negative control of *VHL* mRNA translation by microRNA miR-92-1 expression in B-Chronic Lymphocytic Leukemia (CLL) cells (Ghosh et al., 2009) and the enhanced degradation of VHL proteins induced by the Ubiquitin-conjugating enzyme E2-EPF, which is particularly elevated in some cancer types or under certain stress conditions (Jung et al., 2006; Lim et al., 2008; Tedesco et al., 2007). VHL can be also inactivated by nuclear sequestration under acidosis (Mekhail et al., 2004) and by SUMO-induced VHL proteins aggregation during hypoxia (Cai et al., 2010). Nevertheless, the latter examples correspond to reversible physiological mechanisms that operate to assure a rapid activation of HIFs.

Indeed, one of the most studied effects of VHL inactivation relates to the stabilization and the activation of HIF transcription factors, since α subunits of HIFs are favorite VHL-based E3 ligase substrates under normoxic conditions (Cockman et al., 2000; Maxwell et al., 1999). HIFs have two different helix-loop-helix subunits: the α subunit is normally targeted by VHL, whereas the β subunit is constitutively present in cells (Wang et al., 1995). Under hypoxia, HIF migrates into the nucleus where it recognizes and binds a specific bipartite sequence on DNA, called HRE (Hypoxia Responsive Element) (Semenza and Wang, 1992). Thus, it can drive the transcription of several genes that are involved in glucose metabolism and uptake (Zelzer et al., 1998) and the establishment of neoangiogenesis (Forsythe et al., 1996). This might explain why VHL deficient tumors are highly vascularized. Under normoxic conditions, however, HIF-α is post-translationally modified by a class of enzymes, called prolyl hydroxylases, on two highly conserved proline residues, at position 402 and 564 (Ivan et al., 2001; Jaakkola et al., 2001). These are specifically recognized by VHL that binds HIF-α and promotes its ubiquitylation (Cockman et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Maxwell et al., 1999) (figure 6).

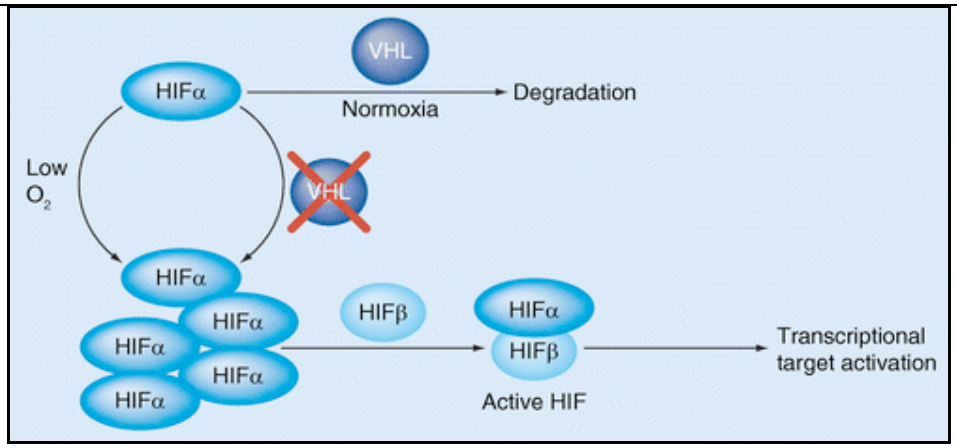

Figure 6. HIFα subunits regulation.

(Figure taken from (Rathmell and Chen, 2008)). HIFα subunits are efficiently degraded in normoxic conditions by the action of VHL-related E3 ligase. However, under hypoxia or upon VHL loss, HIFα becomes more stable, migrates into the nucleus and associates with HIF-1β to form an active transcription factor. HIF regulates the expression of several genes involved in glucose methabolism and neoangiogenesis (Rathmell and Chen, 2008).

VHL interacts also with a hydroxylated proline residue on Rbp1, the large subunit of RNA polymerase II, targeting it for degradation upon DNA damage in the context of the transcription-coupled nucleotide excision repair (Aune et al., 2008; Kuznetsova et al., 2003; Mikhaylova et al., 2008).

HIF-α ubiquitylation and degradation occurs mainly in the cytoplasm, whereas VHL-dependent Rbp1 ubiquitylation occurs in the nucleus. This may explain why VHL proper activity is so strictly related to its ability to shuttle between the nucleus and the cytoplasm (Lewis and Roberts, 2003). VHL trafficking and activity are transcription-dependent, as demonstrated by actinomycin D and DRB (5,6-dichlorobenzimidazole) treatments, which inhibit RNA polymerase II transcriptional activity, inducing nuclear retention of VHL protein (Groulx and Lee, 2002; Khacho et al., 2008; Lee et al., 1999). Moreover, when comparing the VHL sequence to other proteins that display the same behavior, it was possible to identify a novel motif called TD-NEM (transcription-dependent nuclear export motif) (DxGxxDxxL) (Khacho et al., 2008).

The intrinsic relation between VHL and transcription, however, was initially connected to the possible inhibition of transcriptional elongation, due to the sequestration of ElonginA partners (ElonginB and C), as shown by *in vitro* experiments (Duan et al., 1995a). Nonetheless, this mechanism has never been further retrieved *in vivo*, suggesting that it was probably an artifact due to *in vitro* experimental conditions, even if VHL does inhibit the transcription of a subset of genes that are dependent on the activity of Sp1 transcription factor (Cohen et al., 1999; Mukhopadhyay et al., 1997).

Different viruses interact with VHL protein in either a positive or a negative relation. As part of Cullin-RING E3 ligase complexes, VHL was found to mediate the degradation of HIV-1 integrase, and, together with the prefoldin protein VBP-1 (von Hippel-Lindau binding protein 1), to promote the transcription of viral genes (Mousnier et al., 2007). In this case VHL protein seems to be essential for the propagation of HIV-1 infection. Conversely, VHL protein is actively degraded by KSHV (Kaposi's Sarcoma-associated Herpesvirus, called also Human Herpesvirus 8 (HHV8)) LANA (Latency-associated Nuclear Antigen). Indeed, LANA hijacks Cullin5-based E3 ligases by its SOCS-box motif that associates with ElonginB/C adaptor, and, in this way, it can target for degradation some host substrates, such as VHL and p53 tumor suppressors (Cai et al., 2006). This may suggest that VHL protein has an antiviral function.

The avian adenoviral protein Gam1.

Gam1 (Gallus Ante Mortem 1) is an early protein of the avian adenovirus CELO (Chicken Embryo Lethal Orphan) with no significant sequence homology with other cellular proteins. However, a low degree of homology was found with other Fowl Adenoviral ORFs (Ojkic and Nagy, 2000).

When CELO genome was completely mapped (Chiocca et al., 1996) Gam1 was identified during a screen to determine novel viral anti-apoptotic proteins that can mimic cellular Bcl-2 or adenoviral E1B 19K protein activity (Chiocca et al., 1997). In the same study, some Gam1 carboxy terminal mutations were inserted in order to modify the presumptive protein-protein interaction domain and to observe the resulting phenotype. A double leucine residues mutant (L258, 265) displayed altered Gam1-related functions, although still retaining a wide nuclear distribution (Chiocca et al., 1997). The Gam1 mutant impaired functions implied the existence of some interacting proteins that are necessary for Gam1 activity. Indeed, our group demonstrated that Gam1 carboxy terminal domain contains a BC-box motif that enables Gam1 to act as viral substrate receptor in association with both Cullin2 and Cullin5 E3 ligases (Boggio et al., 2007). The recruitment of both ElonginB/C-related CRLs relies on the absence of a canonical Cullin-box that could discriminate between the two scaffold proteins (Boggio et al., 2007).

Gam1 usurps the host Ubiquitin E3 ligase machinery to redirect the ubiquitylation and the subsequent degradation of SAE1 (SUMO Activating Enzyme 1) subunit. SAE1 loss determines the destabilization of its interacting partner SAE2 that, together with SAE1, forms the SUMO E1 heterodimer complex. Moreover, Gam1 induces SUMO E2 (UBC9) degradation by a still unknown mechanism (Boggio et al., 2004; Boggio et al., 2007).

Therefore, Gam1 leads to a strong inhibition of the SUMOylation pathway, as observed by the missed SUMOylation of PML proteins, resulting in the disappearance of PML nuclear bodies (Colombo et al., 2002), and in a wider cytoplasmic delocalization of SUMO-1 protein itself (Colombo et al., 2002).

Furthermore, Gam1 is able to directly bind HDAC1 (Histone Deacetylase 1), thereby inhibiting histone deacetylation in a TSA (trichostatin A)-like manner (Chiocca et al., 2002). Histone acetylation is a general regulator of chromatin status and usually results in enhanced transcriptional rate (reviewed in (Verdone et al., 2006)). HDAC1 and SUMOylation inhibition may explain how Gam1 is a potent activator of gene expression (Chiocca et al., 2002; Colombo et al., 2003).

TSA treatment was able to restore Gam1-defective CELO viral replication (Chiocca et al., 2002). A similar behavior was observed by the overexpression of hsp40 protein, and TSA upregulates both hsp70 and hsp40 (Chiocca et al., 2002; Glotzer et al., 2000). These effects parallel Gam1 functions. Indeed, Gam1 protein can induce a heat shock response by upregulating hsp70 and hsp40 proteins expression and promoting their nuclear translocation (Glotzer et al., 2000). Moreover, increased expression of hsp70 usually correlates with better cell survival under stress condition (Beere et al., 2000; Gabai et al., 1997; Mosser et al., 2000) and this may be linked to Gam1 survival effects (Chiocca et al., 1997).

Although Gam1 was initially described as an anti-apoptotic protein, a quite recent work shows that Gam1 seems to sensitize cancer cells to DNA-damaging and non-DNA-damaging agents promoting the activation of the apoptotic effector caspase-3 (Wu et al., 2007). The authors tried to explain such an opposite behavior suggesting that Gam1 expression in cancer cell lines can induce apoptosis (Wu et al., 2007), whereas in primary cells, where the initial experiments were carried out, it promotes survival (Chiocca et al., 1997).

Nevertheless, Gam1 is a useful tool to better understand the mechanisms underlying viral strategies and to unveil some cellular regulatory functions that may emerge only under certain conditions, as those triggered by viral proteins.

MATERIALS AND METHODS

Cell Culture.

Cell lines and culture conditions.

HeLa, Phoenix, U2OS, C33A and RCC4 VHL-/- cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cambrex) supplemented with 10% fetal bovine serum (Cambrex), 2 mM L-glutamine (Cambrex) and antibiotics (250 μg/ml penicillin and 25 μg/ml streptomycin). RCC4 VHL+/+ cell line was grown in the same medium as RCC4 VHL-/-, further supplemented with 1 mg/ml neomycin for the selection. CaSki cell line was grown in RPMI-1640 medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Cambrex), 2 mM L-glutamine (Cambrex) and antibiotics (250 μg/ml penicillin and 25 μg/ml streptomycin). HEK293T and HeLa Flp-In cell lines were generated by Flp-recombinase-mediated integration according to manufacturer's instructions (Invitrogen Flp-In System, San Diego, CA), and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cambrex) supplemented with 10% Tet-free fetal bovine serum (Cambrex), 2 mM L-glutamine (Cambrex), antibiotics (250 μg/ml penicillin and 25 μg/ml streptomycin), and 150 μg/ml hygromycin B for the selection.

All cell lines were cultured in a humidified incubator at 37°C containing 5% CO_2.

RCC4 VHL-/- and RCC4 VHL+/+ cell lines (Clifford et al., 2001) were kind gifts by Dr. M.S. Wiesner.

Drug treatments.

Proteasome inhibitor MG132 (Z-Leu-Leu-Leu-Al) (SIGMA) was used at the final concentration of 10 μM for the time indicated at each experiment. Deubiquitinating enzymes inhibitor NEM (N-ethylmaleimide) (SIGMA) was added to the lysis buffer at the final concentration of 5 mM in the experiments where Ubiquitin was detected. Hsp90 inhibitor geldanamycin (GA) (AG Scientific) was used at the final concentration of 1 μM for 20 hours where indicated. Puromycin (SIGMA) was used at the final concentration of 1 μg/ml for at least four days to select transfected HeLa cells where indicated. HEK293T and HeLa Flp-In cell lines were induced with 1 μg/ml doxycycline (SIGMA) for the time indicated at each experiment.

Calcium phosphate transfection.

The day before transfection cells are plated to be exponentially growing the day after. Just prior the transfection two different solutions were prepared.

First mixed solution:

- 5-20 μg of DNA (depending on different conditions and plasmids combinations)
- 61 μl of $CaCl_2$
- ddH_2O up to 500 μl

Second solution:

- 500 μl of 2X HBS (HEPES-buffered solution: 250 mM Hepes pH 7, 250 mM NaCl and 150 mM Na_2HPO_4)

The first mixed solution was added dropwise into the second one while bubbling and then leaves for 10 minutes at RT in order to form fine precipitates. They were then distributed on cells that were incubated for about 14 hours at 37°C. After that, cells were washed twice with PBS 1X and incubated in fresh medium for the remaining time before harvesting.

Lipofectamine™ 2000 transfection for luciferase assay.

HeLa cells were transfected through lipofectamine™ 2000 method according to the manufacture's (Invitrogen) instructions. 0.5 μg of pH3SVL-Luciferase reporter plasmid with 1 μg of either pSG9m-Gam1 wt or an empty vector as control was used to perform the transfection. After 4 hours, according to the manufacture's instruction, fresh medium was added to the cells.

Transfection with shRNA (short hairpin RNA) plasmids for RNA interference.

HeLa cells were transfected through the calcium phosphate method with 10 μg of the indicated plasmids. In the case of plasmids without any own particular eukaryotic resistance (from pSUPER vectors), 2 μg of pBabePURO vector were co-transfected. About 36 hours after transfection, cells were selected with puromycin, as previously indicated. When cells were subjected to a second different transfection, the selective medium was replaced with fresh one and cells were maintained without selection for the rest of the experiment.

The twenty-nucleotide long sequences for RNA interference are listed below, as control we used an unrelated shRNA sequence against *luciferase* (LUC) mRNA not expressed by human cells. SAE1, SAE2, UBC9 and LUC were cloned in pSUPER vectors digested with BglII and HindIII restriction enzymes. pMX-puroII-Cul2 and pMX-puroII-Cul5 vectors were kind gifts by Dr. K.I. Nakayama (Kamura et al., 2004).

SAE1: GTTCTTTACAGGAGATGTT

SAE2: AGTGGAACAGCTGGGTATC

UBC9: GAATACAGGAACTTCTAAA

CUL2: CATTTGCATAAGAGAGTTTT

CUL5: GCAGATATGGTAGCAGCTGC

LUC: CGTACGCGGAATACTTCGA

Heat shock treatment.

Flp-In Gam1-inducible HeLa cells were kept in a humidified incubator at 43°C for 90 minutes to induce heat shock and then they were immediately harvested and lysed.

Molecular Biology.

Bacterial transformation.

50 μl of calcium competent bacterial cells was thawed on ice and 1 μg of a plasmid, resuspended in ddH_2O, was added. The mixture was incubated for 30 minutes on ice, then kept for 1 minute at 37°C followed by other 2 minutes on ice. Then cells were grown at 37°C for 45 minutes in LB (Luria-Bertani medium: 10 g/l Casein hydrolysate peptone; 5 g/l yeast extract pH 7.5) using a thermomixer apparatus for shaking. Cells were plated on LB plates (LB plus 15g/l Bacto agar) in the presence of the selection antibiotic (100 μg/ml Ampicillin or 20 μg/ml Kanamicin). For plasmid preparation we used the bacterial strain *E. coli* DH5aF', instead for protein production and purification we used *E. coli* BL-21.

Analysis of DNA.

The concentration of purified DNA was determined by measuring the absorbance at 260 nm. A_{260}=1 corresponds to 50 μg/ml of double-stranded DNA, and high quality DNA preparations should have an A_{260}/ A_{280} ratio of 1.8-2.0. DNA samples were diluted in Acridine Orange dye for DNA (50% glycerol; 50% Acridine Orange stock (125 mg Orange; 40 ml ddH_2O)) and were analyzed by agarose gel electrophoresis. Agarose gels (1-2%) were prepared and run in TAE 1X buffer (0.04 M tris-acetate pH 8.5; 0.001 % EDTA). The DNA was visualized with Ethidium Bromide (0.5 μg/ml) and examined under UV light.

DNA preparation.

Small scale plasmid preparation (mini prep.): single colonies were inoculated in 3 ml LB plus antibiotics and grown for 8 hours at 37 °C on shaking. 1.5 ml of cell culture was harvested (5 minutes at 1700 rcf) and processed using the QIAGEN Plasmid Mini Kit according to manufacture's instructions.

High scale DNA preparation (maxi prep.): a little aliquot of bacterial glycerol stock was inoculated in 250 ml LB plus antibiotics and grown overnight (O/N) at 37 °C on shaking. Cells were harvested (15 minutes at 5500 rcf) and processed using the QIAGEN Plasmid Maxi Kit according to manufacture's instructions.

Enzymatic modification of DNA.

Plasmid DNA was subjected to restriction analysis both for colonies analysis and to prepare DNA fragments for subcloning. DNA was digested with restriction enzymes according to the manufacture's (New England Bio Labs) instructions.

Ligation was performed by mixing DNA vector and insert at a 1:5 ratio. Ligation buffer and enzyme (T4 DNA ligase) were supplied by Roche and used according to the manufacture's instructions.

An aliquot of the ligation mix was used for bacterial transformation (see above).

PCR for creation of deleted mutants.

Reaction conditions:

- 50 ng of DNA template
- 400 nM of each primer (forward and reverse)
- 80 μM of each dNTP (dATP, dTTP, dCTP, dGTP)
- 2 Units of Phusion™ (Finnzymes) DNA polymerase
- HF Phusion™ (Finnzymess) buffer (7.5 mM $MgCl_2$)
- ddH_2O up to 50 μl

PCR cycles:

- Step 1: 95°C 1 min
- Step 2: 95°C 30 sec
- Step 3: 58°C 1 min
- Step 4: 72°C 6 min 30 sec
- Step 5: 72°C 7 min

Steps 2 to 4 were generally repeated 30 times.

Primers:

- SOCS1 (172-212) forward: CGCTCGAGTTGTCCGGCCGCTGCA;
- SOCS1 (172-212) reverse: CCGGGATCCGGTCAGATCTGGAAGGGG;
- SOCS3 (186-225) forward: CGCTCGAGTTGTGGCCACCCTC;
- SOCS3 (186-225) reverse: CCGGGATCCGGTTAAAGTGGAGC.

Primers were purchased by SIGMA Genosys (purified by HPLC).

PCR products were obtained using pEF-FLAG-I-mSOCS1 and pEF-FLAG-I-mSOCS3 (kindly provided by Dr. D.J. Hilton (Nicholson et al., 1999)) as templates and they were then loaded on agarose gel (2%), the bands were excised and PCR products were purified with QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacture's instructions. Then they digested with XhoI and BamHI restriction enzymes to free expose their ends in order to be further ligated in pEGFP-C1 vector (a kind gift by Prof. P.G. Pelicci).

Quantitative RT-PCR.

RNA was extracted from cells using the QIAGEN RNEasy Protect Mini Kit. cDNA was generated by reverse transcription-PCR (RT-PCR) using the PE Applied Biosystems TaqMan® Reverse Transcription Reagents. Relative levels of specific mRNAs were determined using the SYBR Green I detection chemistry system (Applied Biosystems Foster City, CA). All quantitative RT-PCR reactions were performed using a 7500 Fast Real-Time PCR System (AB Applied Biosystem). For each PCR run with SYBR Green I detection, a melting curve analysis was performed to guarantee the specificity in each reaction tube (absence of primer dimers and other nonspecific products). Quantification was

performed using the comparative C_T method as described in the manufacturer procedures manual. GAPDH was used as a control gene for normalization.

The primers used were:

- GAPDH forward: GAAGGTGAAGGTCGGAGTC;
- GAPDH reverse: GAAGATGGTGATGGGATTTC;
- CAIX forward: GGGTGTCATCTGGACTGTGTT;
- CAIX reverse: CTTCTGTGCTGCCTTCTCATC;
- VHL forward: CAGCTACCGAGGTCACCTTT;
- VHL reverse: GCTGTCCGTCAACATTGAGA.

Plasmids.

Eukaryotic expression plasmids:

pSG9m-Gam1 wt (myc-Gam1 wt) and pSG9m-Gam1 L258/265A (myc-Gam1 LL/AA) (Chiocca et al., 1997; Chiocca et al., 2002). pcDNA3-HA-VHL30 wt, pcDNA3-HA-VHL19 wt and pcDNA3-HA-VHL R167W were kindly provided by Dr. W. Krek (Lisztwan et al., 1999). pcDNA3.1-FLAG-VHL was generated by subcloning VHL from pcDNA3-HA-VHL wt into pcDNA3.1-FLAG frame C using BamHI/EcoRI restriction enzymes. pcDNA3-SV5-VHL was generated by subcloning VHL from pcDNA3-HA-VHL wt into pcDNA-Sv5-SAE1 vector, kindly provided by Prof. R.T. Hay (Desterro et al., 1999), using BamHI/EcoRI restriction enzymes. pcDNA3.1-FLAG-Ubiquitin was kindly provided by Dr. S. Polo. pcDNA3-HA-SUMO and pcDNA3-UBC9 were provided by Dr. F. Fiore. pSUPER-CTRL (shCTRL), pSUPER-SAE1 (shSAE1), pSUPER-SAE2 (shSAE2) and pSUPER-UBC9 (shUBC9) were generated by cloning the specific sequences into pSUPER vector. pMX-puroII-Cul2 and pMX-puroII-Cul5 were kind gifts by Dr. K.I. Nakayama (Kamura et al., 2004). pcDNA3-ElonginB and pcDNA3-ElonginC were generated by respectively subcloning ElonginB from pGEM3-ElonginB and ElonginC from pGEM3-ElonginC vectors (both kindly provided by Dr. M. Pagano) into pcDNA3 vector using HindIII/EcoRI (ElonginB) and HindIII/XbaI (ElonginC) restriction enzymes. pEF-FLAG-I-mSOCS1 and pEF-FLAG-I-mSOCS3 were kindly provided by Dr. D.J. Hilton (Nicholson et al., 1999). pEGFP-C1-mSOCS1(172-212) and pEGFP-C1-mSOCS3(186-225) were generated by cloning the PCR amplificates into pEGFP-C1 vector. pCMV-2B-FLAG-HPV16E7 was generated by subcloning HPV16E7 from pBabePURO-HPV16E7 (kindly provided by Dr. M. Tommasino (Giarre et al., 2001))

into pCMV-2B-FLAG-SENP2 vector (kindly provided by Dr. M. Dasso (Hang and Dasso, 2002)) using EcoRI/SalI restriction enzymes. pcDNA3.1-FLAG-E4orf6 was generated by subcloning E4orf6 from pcDNA3-HA-E4orf6 (kindly provided by Dr. R.T. Hay) into pcDNA3.1-FLAG-VHL vector (see above) using BamHI/EcoRI restriction enzymes. pH3SVL-Luciferase vector was kindly provided by Dr. D.M. Katschinski (Linden et al., 2003).

Bacterial expressing plasmids used for protein production and purification:

pGEX-2TK-Gam1 WT (GST-Gam1 WT) (Chiocca et al., 1997; Chiocca et al., 2002). pGEX-4T-VHL30 and pGEX-4T-VHL19 were generated by respectively subcloning VHL30 from pcDNA3-HA-VHL30 wt and VHL19 from pcDNA3-HA-VHL19 wt into pGEX-4T-SAE2 vector (generated by Dr. R. Boggio (Boggio et al., 2004)) using BamHI/EcoRI restriction enzymes.

Proteins and immunochemistry.

Protein extraction.

Lysis buffer Urea:

8 M Urea; 100 mM NaH_2PO_4; 10 mM Tris-HCl pH 8.0.

Lysis buffer E1A:

50 mM Hepes pH 7.5; 250 mM NaCl; 0.1% NP-40; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin.

Lysis buffer SDS:

Buffer I: 5% SDS; 150 mM Tris-HCl pH 6.8; 30% glycerol.

Buffer II: 25 mM Tris-HCl pH 8.3; 50 mM NaCl; 0.5% NP-40; 0.5% deoxycholate; 0.1% SDS; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin

The final solution is a 1:3 mixture of buffer I and buffer II respectively.

Reducing buffer 5X:

0.5 M Tris-HCl pH 6.8; 10% SDS; 20% glycerol; 5% β-mercaptoethanol; 150 ng/ml Bromophenol blue.

Cells were transfected with calcium-phosphate method. Then cells were harvested and lysed with Urea lysis buffer, when not different indicated. The lysates were sonicated twice for 15 seconds (30 seconds pause) and incubated on ice for 20 minutes. Cell fragments were removed by spinning for 20 minutes at 20000 rcf at 4 °C. The Bio-Rad protein assay was used to determine protein concentration, following manufacturer's instructions. A standard curve was made using bovine serum albumine (BSA) and used to evaluate the protein concentration in the samples. Samples were diluted in reducing buffer 5X before loading on gel.

Immunoprecipitation.

Immunoprecipitation.

Cells were transfected with calcium-phosphate method using indicated plasmids. 48 hours after transfection, cells were lysed in SDS lysis buffer. Prior to immunoprecipitations (IP), 2 mg of protein extracts were diluted in E1A lysis buffer (1:5 volume/volume ratio), then incubated with the indicated antibodies (2 μg/mg of crude extract) for 1.5 hours at 4 °C on rotation. Then protein-A/sepharose-beads (slurry 50%) were added to the samples for 2 hours at 4 °C on rotation. After 3 washes and the addition of reducing buffer, the samples were loaded on SDS-PAGE and immunoblotted (WB) with the indicated antibodies. 30 μg of total extracts (input) of indicated transfected cells was loaded as a control of the amount of transfected proteins.

Co-immunoprecipitation.

Cells were transfected with calcium-phosphate method using indicated plasmids. 48 hours after transfection, cells were lysed in E1A lysis buffer. Co-immunoprecipitations (co-IP) were done using 2 mg of protein extracts incubated with the indicated antibodies (2 μg/mg of crude extract) in E1A buffer for 1.5 hours at 4 °C on rotation and then protein-A/sepharose-beads (slurry 50%) were added to the samples for 2 hours at 4 °C on rotation. After 3 washes and the addition of reducing buffer, the samples were loaded on SDS-PAGE and immunoblotted (WB) with the indicated antibodies. 30 μg of

total extracts (input) of indicated transfected cells was loaded as a control of the amount of transfected proteins.

Sequential co-immunoprecipitation.

For the sequential co-IP, samples were lysed in E1A lysis buffer and 8 mg of extracts were immunoprecipitated with agarose-conjugated α-FLAG antibody (2 μg/mg of crude extract) and eluted with 2 μg/ml of FLAG peptide for 2 hours at RT on rotation. 2 mg of the samples was checked for protein precipitation, whereas the remaining eluted 6 mg were subjected to a further co-IP using α-myc antibody (2 μg/mg of crude extract) for 1.5 hours at 4 °C on rotation. Then protein-A/sepharose-beads (slurry 50%) were added to the samples for 2 hours at 4 °C on rotation. After 3 washes and the addition of reducing buffer, the samples were loaded on SDS-PAGE and immunoblotted (WB) with the indicated antibodies. 30 μg of total extracts (input) of indicated transfected cells was loaded as a control of the amount of transfected proteins. FLAG peptide was purchased by SIGMA.

SDS-PAGE.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was performed as described in (Sambrook and Gething, 1989) using a discontinuous buffer system (Laemmli, 1970).
SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a mass ratio of 1.4:1. Thus, proteins acquire a uniform negative charge, which is proportional to their size. SDS also disrupts protein tertiary structure, ensuring the linearity of proteins.
During electophoresis, an electric current is used to move the protein molecules across the polyacrilamide gel, which is a cross-linked matrix that acts as a molecular sieve. So, smaller molecules are able to migrate faster than bigger ones and they can be separated according to their molecular weight.
The gels were run in a BIORAD apparatus using a SDS running buffer (0.025 M Tris-base pH 8.8; 0.192 M glycine; 0.1% SDS).

Western blotting.

Western blotting was used to analyze protein expression, localization and modifications depending on the experiment. This method involves SDS-PAGE followed by immobilization of the proteins on a membrane by electroblotting. The proteins were detected using an antibody specific for the protein of interest. The proteins were transferred from the gel to a nitrocellulose membrane at 100 V for 1 hour. After electroblotting, the membranes were stained with Ponceau S to verify equal loading and transfer. Membranes were blocked in 5% low fat milk in TBS-T 1X for 1 hour then were incubated first with the primary antibody in TBS-T 5% milk for 1-12 hours depending on the antibody and after with a secondary antibody linked to the horse-radish peroxidase for 1 hour. The membranes were then washed and developed using enhanced chemiluminescence (ECL, Amersham).

Antibodies.

The following antibodies were used: anti-myc epitope (9E10, Calbiochem), anti-HA epitope (12CA5, Covance), anti-HA-probe (HA.11, Babco-Covance), anti-GST (home-made serum), anti-GFP (B-2, Santa Cruz), anti-GFP (home-made serum), anti-α-tubulin (Santa Cruz), anti-α-actin (SIGMA), anti-FLAG epitope (M2, Sigma), agarose-conjugated anti-FLAG anti-FLAG epitope (M2, Sigma), anti-DDDK (FLAG) (ab 1162, Abcam), anti-V5 tag (SV5-Pk1 clone, Serotec), anti-SAE1 (Abcam), anti-SAE2 (Abcam), anti-Vinculin (Santa Cruz), anti-RanGAP1 (N-19, Santa Cruz), anti-UBC9 (N-15, Santa Cruz), anti-Ubiquitin (P4G7, Abcam), anti-ElonginB (FL-118, Santa Cruz), anti-ElonginC (R-20, Santa Cruz), anti-Cullin2 (Zymed), anti-Cullin5 (H-300, Santa Cruz), anti-VHL (Cell Signaling), anti-HIF-1α (BD Bioscience), anti-Cdc25C (C-20, Santa Cruz) and anti-hsp70 (W-27, Santa Cruz).

Recombinant proteins production and extraction.

Lysis buffer for GST-protein purification:

Soluble GST-fusion proteins:

50 mM Tris-HCl pH 7.9; 500 mM NaCl; 0.5% NP-40; 10% glycerol; 10 mM DTT; 4 mM EDTA pH 8; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin.

Unsoluble GTS-fusion proteins:

20 mM Tris-HCl pH 7.9; 500 mM NaCl; 0.5% NP-40; 10% glycerol; 10 mM DTT; 4 mM EDTA pH 8; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin.

Hunt wash buffer:

20 mM Tris-HCl pH8; 100 mM NaCl; 1 mM EDTA; 0.5% NP-40; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin.

Recombinant GST-fusion proteins were induced in *E.coli* BL-21 strain (protease negative) with 0.3 mM IPTG for 3 hours at room temperature. After centrifugation cells were lysed with lysis buffer according to their solubility (see above) and the supernatant was directly used for binding to glutathione Sepharose 4B beads 50% slurry. After 2 hours of binding, washes were performed in Hunt wash buffer (see above) and in PBS 1X. After purification, the proteins were eluted with 30 mM reduced glutathione.

In vitro binding.

Binding buffer:

50 mM Hepes pH 7.5; 200 mM NaCl; 1 mM MgCl; 10% glycerol; 0.5% NP-40; 100 μg/ml PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotin.

Wash buffer:

50 mM Hepes pH 7.5; 200 mM NaCl; 1 mM MgCl; 1 mM $CaCl_2$; 10% glycerol; 1 mM EDTA pH 8; 1 mM EGTA pH; 1mM DTT; 0.5% NP-40.

Recombinant GST-Gam1, GST-VHL30 and GST-VHL19 proteins were produced in *E.coli* BL-21 strain. After purification using sepharose beads, 10 μg of these proteins were incubated in 500 μl of binding buffer (see above) with 50 μl of *in vitro* translated (IVT) HA-VHL30, HA-VHL19 or myc-Gam1 as indicated in the experiment for 1 hr at RT on rotation. HA-VHL30, HA-VHL19 and myc-Gam1 were *in vitro* translated (IVT) in a rabbit reticulocyte lysate system kit (Promega), according to manufacture's instructions. After 3 washes in wash buffer (see above), the samples were loaded in 15% poly-

acrilamide gel and immunoblotted with the indicated antibodies. 5 μl *in vitro* translated VHL and Gam1 were loaded as input.

Luciferase assay.

HeLa cells were transfected through lipofectamine™ 2000 method, as described above. After 24 hours, cells were harvested and lysed in Luciferase Cell Culture Lysis Reagent (Promega) according with the manufatcure's instructions. 30 μg of each lysate were mixed with 100 μl of Luciferase Assay Reagent (Promega) to proceed with Luciferase assay according with the manufatcure's instructions. Luciferase activity was measured on a luminometer (GDV).

Imaging.

Immunofluorescence microscopy.

HeLa and RCC4 VHL-/- and VHL+/+ cells were plated on coverslips and transfected through the calcium-phosphate method with the indicated plasmids. After 48 hours, cells were washed twice with PBS 1X and covered with 4% paraformaldehyde (PFA) fixing solution at RT for 15 minutes. After 3 washes with PBS 1X, cells were incubated with permeabilizing solution (0.1% Triton; 1% BSA in PBS 1X) at RT for 15 minutes. After two washes with PBS 1X, cells were incubated with 2% BSA blocking solution at RT for 10 minutes, and then coverslips were coated with the primary antibodies diluted in the blocking solution at RT for 1 hour. Cells were rinsed twice with PBS 1X and coated with the fluorescein isothiocyanate (FITC)-conjugated or Cy3-conjugated secondary antibodies at RT for 1 hour. DNA was stained with DAPI (4',6'-diamino-2-phenylindole) (SIGMA). Images were acquired with a wide field BX61 (Olympus) motorized fluorescence microscope and analyzed with open source ImageJ computer program developed by the American National Institute of Health (NIH).

RESULTS

The adenoviral Gam1 protein is able to bind the ElonginB/C heterodimer through its BC-box domain and acts in the context of Ubiquitin E3 ligases as substrate-receptor. In this way, Gam1 can reconvert the functions of Cullin2/5-based E3 ligases towards the non-physiological degradation of the SUMO Activating Enzyme 1 (SAE1) (Boggio et al., 2004; Boggio et al., 2007).

It was also shown, by gel filtration analysis, that Gam1 expression induces a clear change in the distribution of Cullin2/5-based E3 ligase subunits towards higher molecular weight complexes, in particular for the heterodimer ElonginB/C (Boggio et al., 2007).

This may indicate that the ElonginB/C complex could be displaced from the usual interactors (in most cases substrate receptors) and this event could lead to secondary effects.

There are several other viral proteins containing a BC-box domain that act as substrate receptors associated to Cullin2- or Cullin5-based E3 ligases (Blanchette et al., 2004; Cai et al., 2006; Huh et al., 2007; Mehle et al., 2004; Querido et al., 2001; Yu et al., 2003; Yu et al., 2004). However, the effort in characterizing these viral Ubiquitin ligases is mostly directed to the identification of their specific targets and it rarely considers other possible side effects that could arise from the hijack itself.

We decided to approach this aspect using Gam1 as a model for a BC-box viral protein and VHL as the cellular counterpart whose E3-related functions may be altered by the presence of Gam1.

Gam1 induces HIF-1α stabilization and activation.

HIF-1α is ubiquitously and constitutively produced in cells, but, in normoxic conditions, it is immediately ubiquitylated by VHL-containing E3 ligase complexes and then targeted to degradation

(Cockman et al., 2000; Kamura et al., 2000; Tanimoto et al., 2000), leaving its protein level barely detectable.

However, the half-life of HIF-1α can be markedly increased when not degraded by VHL.

This situation may occur either when HIF-1α is no longer associated to VHL (during hypoxia (Jaakkola et al., 2001), or upon treatment with cobalt chloride (Yuan et al., 2003), or in the presence of VHL mutations that impair the binding to HIF-1α itself (Clifford et al., 2001; Khacho et al., 2008; Miller et al., 2005)), or when VHL association to the E3 ligase complex is compromised (upon normoxic acidosis-induced nucleolar sequestration of VHL (Mekhail et al., 2004), or when the interaction with ElonginB/C is prevented either by the action of other binding partners (Kim et al., 2008) or by the presence of VHL mutations in the binding domain (Clifford et al., 2001; Rathmell et al., 2004; Stebbins et al., 1999)).

We thus assessed whether Gam1 could impair VHL-related Ubiquitin E3 ligase functions by analyzing the protein level of HIF-1α.

Either wild-type or BC-box mutated (L258,265A) Gam1 were transiently expressed in HeLa cells in normoxic conditions and, after 48 hours, whole-cell extracts were analyzed by western blot to detect Gam1 and HIF-1α.

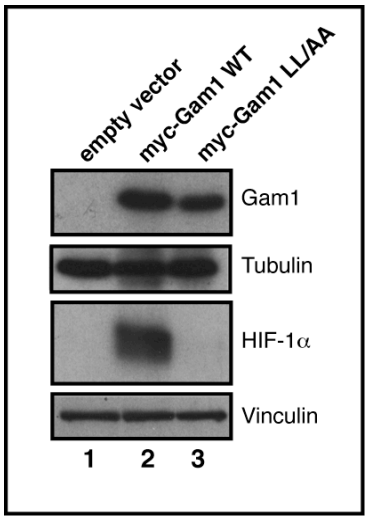

Figure 7. HIF-1α is stabilized in the presence of Gam1 wild-type.

Either myc-tagged Gam1 wild-type (WT) or mutant L258,265A (LL/AA) were expressed in HeLa cells. Forty-eight hours after transfection cells were lysed in denaturing Urea lysis buffer. 40 μg of lysates were loaded to detect myc-Gam1 and HIF-1α. Tubulin was used as loading control for myc-Gam1 (15% poly-acrylamide gel) and Vinculin as loading control for HIF-1α (8% poly-acrylamide gel).

As shown in figure 7, wild-type Gam1, but not the mutant (unable to bind to ElonginB/C), induced a clear stabilization of HIF-1α protein. This result may indicate that Gam1 could impair the VHL-HIF pathway, possibly by its ability to bind ElonginB/C- and Cullin2/5-based complexes.

To assess whether, in the presence of wild-type Gam1, HIF-1α forms an active transcription factor in association with HIF-1β, we analyzed the mRNA level of *Carbonic Anhydrase IX* (*CAIX*). This gene is a direct target of HIF-1 and a marker of established hypoxia (Loncaster et al., 2001). The level of *CAIX* mRNA was analyzed by quantitative RT-PCR technique using total retrotranscribed RNA extracts from HeLa cells previously transfected with wild-type Gam1 plasmid or empty vector as a control.

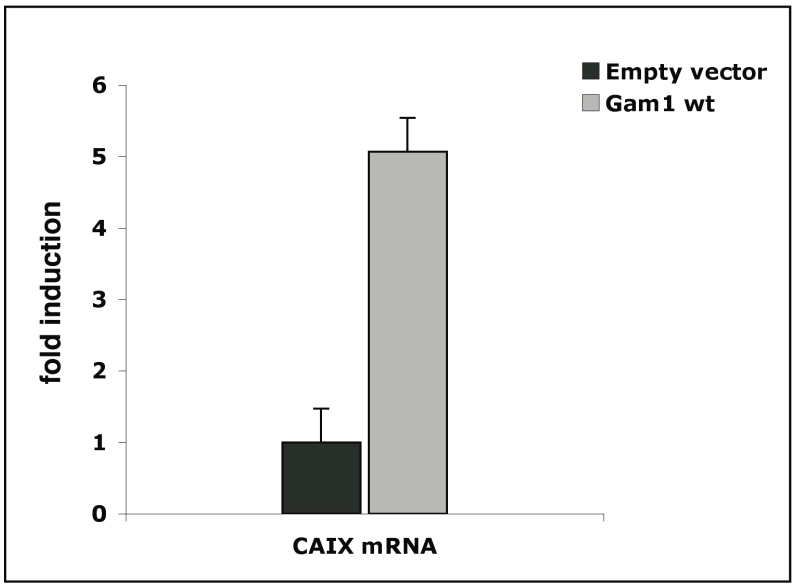

Figure 8. Gam1 leads to enhanced transcription of Carbonic Anhydrase IX gene.
Total RNA was retrotranscribed and analyzed by quantitative RT-PCR. Results are reported as fold induction of *CAIX* mRNA amount relative to the control (empty vector) and normalized relatively to *GAPDH* mRNA (to which the arbitrary value of 1 was assigned). They represent the average of three independent experiments with error bars corresponding to the standard deviations.

The results showed more than four-fold increase in CAIX transcription rate compared to the control, indicating that HIF-1 is active in cells expressing Gam1 protein (figure 8).

To further confirm the activity of HIF-1, we co-transfected HeLa cells with a reporter plasmid carrying the Luciferase gene under the control of a bipartite Hypoxia Responsive Element (HRE)-promoter and with the wild-type myc-Gam1 expressing plasmid or empty vector as a control. After 48 hours, cells were harvested and assayed for Luciferase activity.

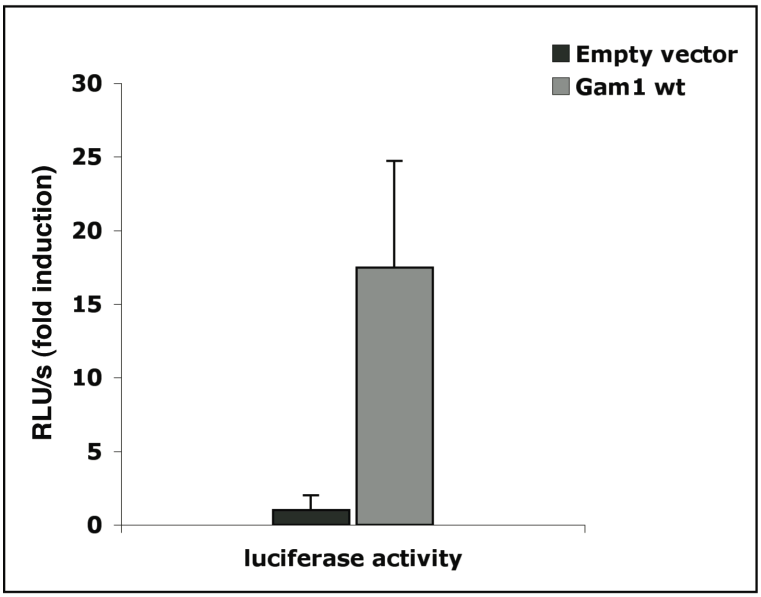

Figure 9. Gam1 leads to increased expression of luciferase protein driven by HRE-promoter.
Lysates of HeLa cells transfected with HRE-Luciferase reported plasmid together with either empty vector or myc-Gam1 plasmid were subjected to a luciferase assay and the results are reported as fold induction of Luciferase activity in relative light units per second (RLU/s) normalized to the control (empty vector). They correspond to the average of three independent experiments with error bars representing standard deviation.

The results illustrated in figure 9 showed an eighteen-fold induction of the activity of Luciferase in the presence of Gam1. The increase in the Luciferase activity correlates to a higher expression of the Luciferase protein itself, demonstrating that HIF-1 is active upon Gam1 expression.

Taken together, these data suggest that the wild-type, but not the BC-box mutant, Gam1 is able to induce a hypoxia-like response when expressed in human cells, thanks to the stabilization of HIF-1α and the transactivation of HIF-1.

VHL protein level decreases upon Gam1 expression.

As previously mentioned, the stability of HIF-1α, in normoxic conditions, is mostly related to VHL substrate-receptor functions. These functions could be impaired by the recruitment, on the behalf of Gam1, of the same E3 ligase complex for VHL, maybe disrupting their association. Therefore, we analyzed VHL protein level upon Gam1 expression.

Interestingly, we noticed that VHL decreased in the presence of wild-type, but not mutant, Gam1 (figure 10, compare lane 2 to lane 3).

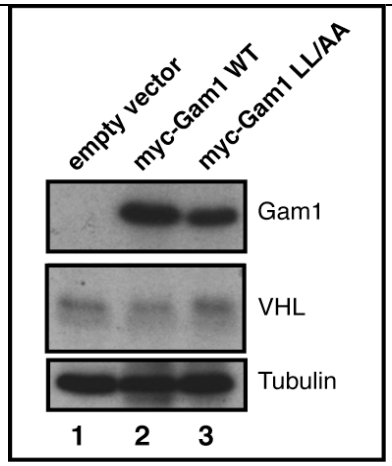

Figure 10. VHL protein level decreases upon wild-type Gam1 expression.

Either myc-tagged Gam1 wild-type (WT) or mutant L258,265A (LL/AA) were expressed in HeLa cells. Forty-eight hours after transfection cells were lysed in denaturing lysis buffer. 40 μg of lysates were loaded to detect myc-Gam1 and VHL respectively. Tubulin was used as loading control.

The pattern followed by VHL in figure 10 seems to inversely correlate to HIF-1α's one in figure 7. This may suggest that the increased stability of HIF-1α protein is just a consequence of the reduced level of VHL in Gam1 wild-type expressing cells.

It is to mention that in the results presented here we noticed some differences in the amount of VHL protein reduction upon Gam1 expression. They are likely related to the rate of transfection efficiency of Gam1-carrying plasmids. We measured that, on average, 60% Gam1-related transfection efficiency correlates with about 70% VHL protein reduction.

VHL is a tumor suppressor protein (Iliopoulos et al., 1995) and is involved in several other pathways beyond HIF-1α degradation (Bluyssen et al., 2004; He et al., 2004; Hergovich et al., 2003; Kurban et al., 2008; Lolkema et al., 2005; Ohh et al., 1998; Roe et al., 2006; Schermer et al., 2006; Thoma et al., 2007; Zhou et al., 2002), therefore Gam1-induced VHL reduction could have other consequences than just the impairment of its associated E3 ligase complex.

Gam1 does not alter *VHL* mRNA transcription.

Protein level can be controlled at different stages, among which through regulation of mRNA transcription. Gam1 was reported to be a global activator of cellular transcription, most likely because of inhibition of histone deacetylases activity and impairment of the SUMOylation pathway (Boggio et

al., 2004; Chiocca et al., 2002). Therefore, it may enhance the expression of some VHL negative regulators.

To determine whether Gam1 could affect *VHL* mRNA synthesis, we transfected HeLa cells either with myc-Gam1 plasmid or with an empty vector as a control. After 48 hours, cells were harvested and divided in two different pools: one was subjected to RNA extraction, the other was lysed and subjected to western blot analysis.

Total retrotranscribed mRNA was used to analyze *VHL* mRNA by quantitative RT-PCR. *VHL* mRNA level was normalized to *GAPDH* one, showing that no significant differences occurred in the presence of Gam1 compared to the control (empty vector) (figure 11A). Nevertheless, VHL protein decrease was still detectable upon Gam1 expression (figure 11B).

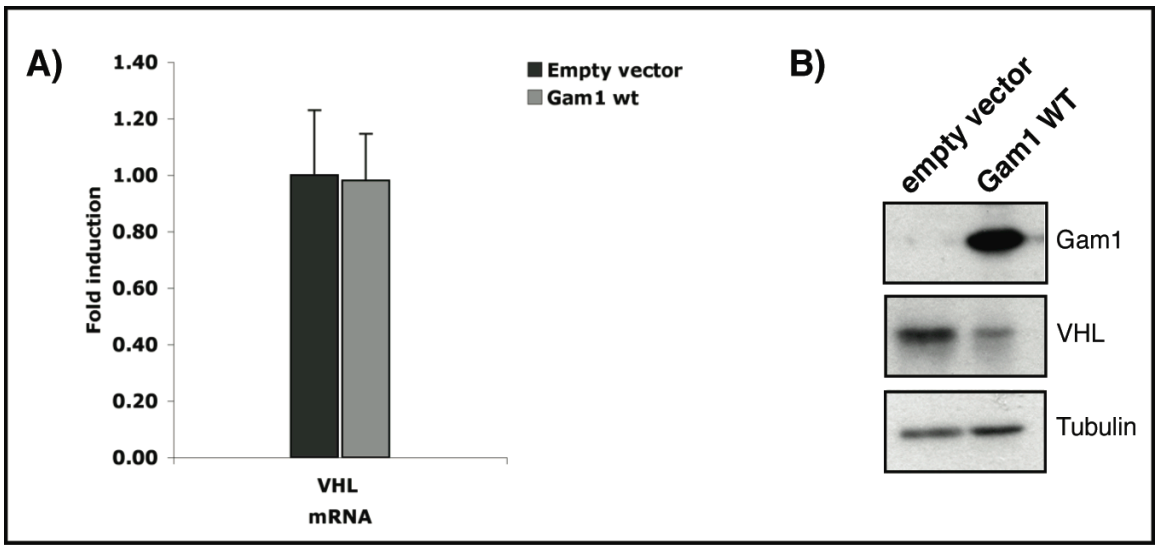

Figure 11. Gam1 does not affect *VHL* mRNA transcription.

A) Quantitative RT-PCR analysis of *VHL* mRNA derived from retrotranscribed total RNA extracted from HeLa cells transfected with myc-Gam1 plasmid or an empty vector. Results are presented as fold induction of *VHL* mRNA amount relative to the control (empty vector) and normalized to *GAPDH* mRNA. They represent the average of three independent experiments with error bars corresponding to standard deviations. B) 40 μg of lysates was loaded to detect VHL protein. The figure is representative of one of the three independent experiments carried out to verify the level of *VHL* mRNA in the presence of Gam1 protein. Tubulin was used as loading control.

These results suggest that VHL decrease upon Gam1 expression is due a post-transcriptional regulatory mechanism.

Gam1-induced VHL protein decrease depends on proteasome activity.

Proteasomal degradation is the major mechanism to rapidly degrade proteins and control their turnover.

Since we verified that Gam1 does not regulate VHL at the transcriptional level, we decided to assess whether proteasome activity could affect VHL protein in this situation.

First, we checked the kinetics of VHL disappearance upon wild-type Gam1 expression using a Flp-In HEK 293T Gam1-inducible cell line created by our group. This cell line is very suitable to perform time-course experiments upon Gam1 expression, overcoming the temporal restriction given by common transfection techniques. A Tet-on promoter drives myc-Gam1 transcription, thus Gam1 protein expression can be simply induced by addition of 1 μg/ml doxycycline in the culture medium and it starts to be easily detected 3 hour after induction (figure 12, lane 2).

We observed that VHL protein starts to diminish between three and six hours upon doxycycline addition, just after Gam1 expression, and it continues to decrease until 24 hours after Gam1 induction (figure 12, lanes 2-6). Then VHL protein level remains stable (figure 12, lanes 6-8). Whereas, the pattern of Gam1 protein expression shows a peak around six and twelve hours after induction followed by a slight decrease that determines the final Gam1 expression (figure 12, compare lanes 3-5 with lanes 6-8). This experiment suggests that the first hours upon Gam1 expression are important in defining a new equilibrium for both VHL protein and Gam1 protein.

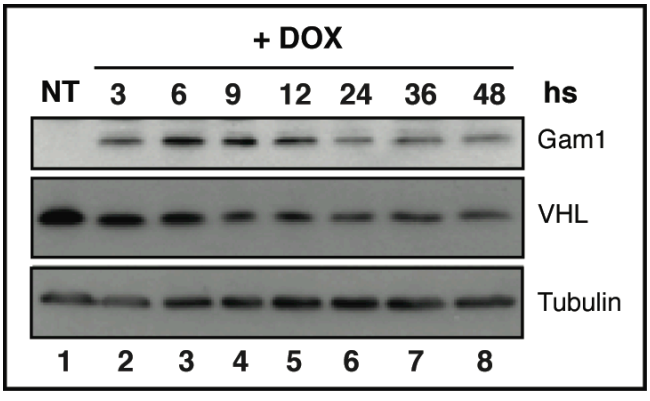

Figure 12. VHL protein starts to decrease immediately after Gam1 expression.

Gam1 expression was induced by the addition of 1 μg/ml doxycycline (DOX) to the medium of a Tet-on inducible HEK 293T cell line. Cells were collected at the indicated times (after induction) and 40 μg of whole-cell extracts were analyzed by immunoblot against myc-tag (Gam1) and VHL. Tubulin was used as a loading control.

NT refers to not treated cells.

Once the kinetics of VHL decrease had been established, we treated the same kind of cells with 10 μM of proteasome inhibitor MG132 (Z-Leu-Leu-Leu-Al) in order to block protein degradation. Gam1 expression had been induced one hour before MG132 treatment by the addition of 1 μg/ml doxycycline. Cells were harvested 13 hours after and whole-cell extracts were subjected to western blot analysis.

VHL protein level was completely rescued when proteasome was inhibited, even in the presence of Gam1 (figure 13, lanes 3 and 4).

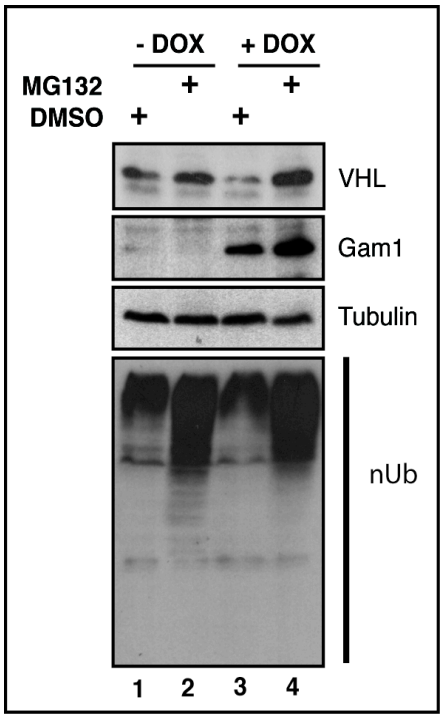

Figure 13. Inhibition of the proteasome activity rescues VHL protein level even in the presence of Gam1.

Myc-Gam1 expression was induced by the addition of 1 μg/ml doxycycline (DOX) to the medium of a Tet-on inducible HEK 293T cell line. After 1 hour cells were treated with either 10 μM MG132 or DMSO as control for other 13 hours. 40 μg of whole-cell extracts were analyzed by immunoblot for the indicated proteins. VHL and myc-Gam1 levels were analyzed by immunoblotting using the corresponding antibody. The level of conjugated Ubiquitin (nUb) was used as a positive control for proteasome inhibition. Tubulin was used as loading control.

This result suggests that proteasome may be actively involved in VHL protein degradation upon Gam1 expression.

VHL protein is highly ubiquitylated in the presence of wild-type Gam1.

Proteins targeted to proteasome for degradation are usually ubiquitylated. If VHL is degraded by this mechanism in the presence of Gam1, it will be likely subjected to this modification.

To test this hypothesis, HA-tagged VHL protein was expressed in Phoenix cell line either alone or together with FLAG-tagged Ubiquitin and myc-Gam1 (both wild-type and BC-box mutant L258,265A). Cells were treated with 10 μM MG132 for 2 hours before harvesting and lysed in a denaturing SDS-based lysis buffer in order to maintain only covalent bindings. Lysates were immunoprecipitated against the HA tag and resolved by SDS-PAGE. The immunoprecipitation results showed a strong ubiquitylation signal for VHL protein in the presence of wild-type Gam1 and FLAG-tagged Ubiquitin (figure 14, lane 5) and a faint ubiquitination smearing when VHL was co-expressed with FLAG-Ubiquitin and myc-Gam1 mutant (figure 14, lane 7).

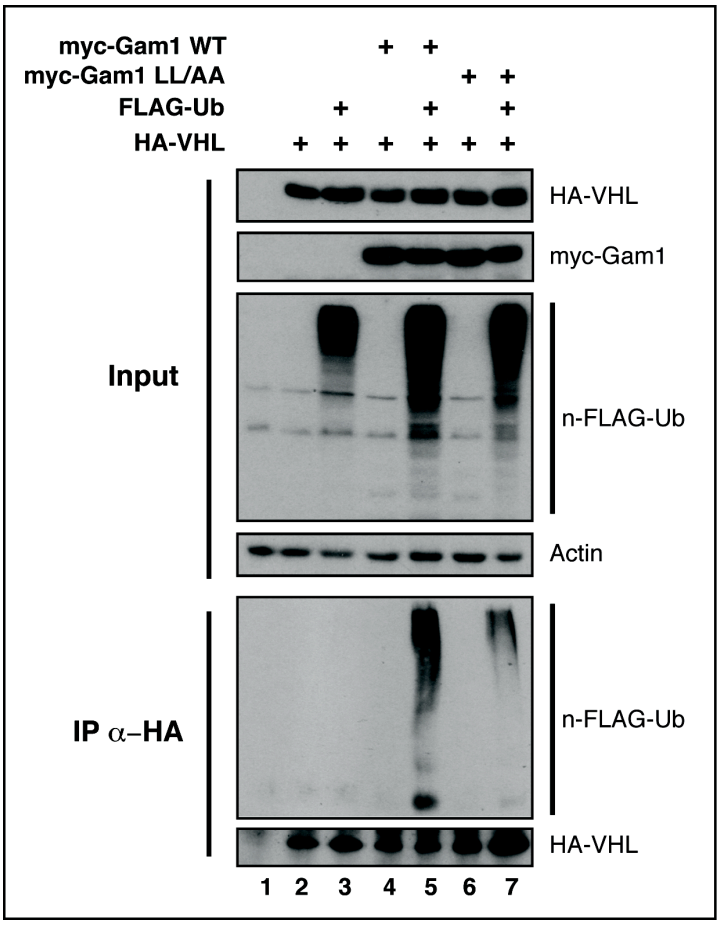

Figure 14. VHL is highly ubiquitylated in the presence of wild-type Gam1.

Overexpression of HA-VHL, myc-Gam1 either wild-type (WT) or mutant L258,265A (LL/AA), and FLAG-Ubiquitin (Ub) in Phoenix cell line. Cells were harvested 48 hours after the transfection and lysed in a denaturing SDS-lysis buffer. 1 mg of crude extract was immunoprecipitated against the HA tag and analyzed by

immunoblotting. α-FLAG antibody determined the level of ubiquitinated VHL (n-FLAG-Ub). 30 μg of lysates were used for the input analysis. Actin was used as loading control of the input.

Although endogenous VHL protein level seemed to be not affected by the presence of Gam1 L258,265A (figure 10, lane 3), it could be possible that the mutant Gam1 can slightly induce VHL protein ubiquitylation and it can be detectable only upon VHL overexpression.

The analysis of whole-cell lysates showed that the level of FLAG-Ubiquitin was almost identical in the presence of both forms of Gam1 and VHL (figure 14, lanes 5 and 7), whereas it was less expressed with HA-VHL alone (figure 14, lane 6). Nevertheless, we believed that VHL is not massive ubiquitinated in control conditions, whereas a strong VHL ubiquitylation occurs after expression of wild-type Gam1.

Gam1 and VHL directly interact both *in vitro* and *in vivo*.

The previous data (figures 13 and 14) indicate that upon Gam1 expression VHL is ubiquitinated by a still unidentified E3 ligase and then targeted to degradation *via* proteasome.

Since Gam1 acts as a substrate-receptor, we questioned whether VHL is degraded by the action of Gam1-associated Ubiquitin E3 ligases.

Therefore, we decided to verify whether Gam1 and VHL proteins directly interact, and we performed an *in vitro* protein binding analysis.

Either 50 μl of *in vitro* translated HA-VHL30 (or HA-VHL19) (figure 15A) or 50 μl of *in vitro* translated myc-Gam1 (figure 15B) were incubated with GST, GST-Gam1 and GST-VHL30 (or GST-VHL19).

GST, GST-Gam1 or GST-VHL (both 30 kDa and 19 kDa isoforms), were pulled-down by Glutathione-Sepharose beads and analyzed by immunoblotting (experiment made by Agnese Collino).

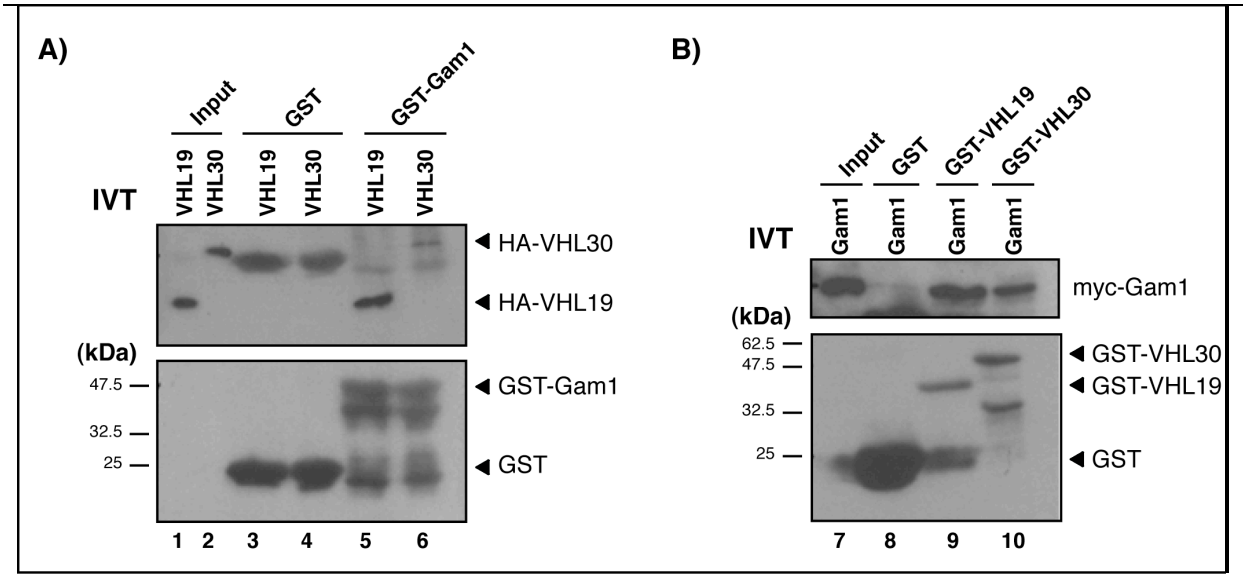

Figure 15. Gam1 and VHL proteins associated *in vitro*.

A) 50 μl of *in vitro* translated either HA-VHL30 or HA-VHL19 was subjected to a pull-down analysis in the presence of 10 μg of bacterially produced GST-Gam1 or GST as a control. 5 μl of *in vitro* translated reactions were used as controls of input. Proteins were detected by immunoblotting of the corresponding tags. The bands appearing in lanes 3-4 (upper panel) are artifacts due to the presence of a high amount of GST protein. B) 50 μl of *in vitro* translated myc-Gam1 was subjected to a pull-down analysis in the presence of bacterially produced GST-VHL (both 30 kDa and 19 kDa isoforms) and GST as a control. 5 μl of *in vitro* translated reaction was used as control of input.
Arrowheads indicate the corresponding proteins.

We demonstrated that Gam1 was able to directly bind both VHL protein isoforms (figure 15A, lanes 5 and 6) and *vice versa* both VHL isoforms pull-down myc-Gam1 protein (figure 15B, lanes 9 and 10), suggesting that the N-terminal acidic domain of VHL is not involved in this interaction.

This may be an indication that VHL could be a substrate for Gam1, however it was necessary to verify that the same interaction occurs also *in vivo* and whether they associate to the other subunits of E3 ligases complexes.

Thus, we performed co-immunoprecipitation experiments in order to observe this binding. We overexpressed either wild-type or R167W mutant HA-tagged VHL and myc-Gam1 in Phoenix cells.

VHL R167W mutant is impaired in binding ElonginB/C complex (Duan et al., 1995b), and therefore in assembling an active VHL-associated E3 ligase. It was expressed in order to discriminate the role of VHL from substrate receptor (able to directly bind E3 ligase subunits) to Gam1-recruited substrate (in this case, we expected to observe an interaction between mutant VHL and E3 ligase subunits only in the presence of Gam1).

Cells were harvested 48 hours later and lysed in a non-denaturing lysis buffer in order to preserve all the non-covalent interactions. 2 mg of each lysates were immunoprecipitated using 4 μg of antibody against HA-tag or myc-tag respectively and the precipitates were then analyzed by immunoblotting.

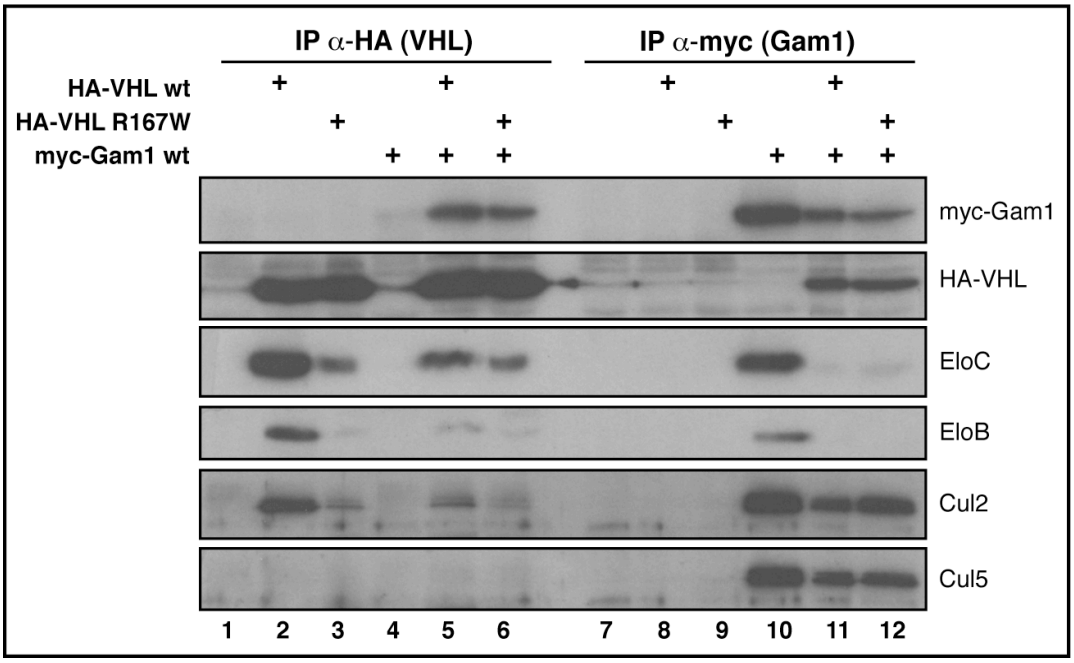

Figure 16. Gam1 and VHL proteins interact *in vivo*.

Phoenix cells were co-transfected either with wild-type or R167W mutant HA-VHL and myc-Gam1. 48 hours post transfection cells were collected and 2 mg of each lysate were subjected to co-immunoprecipitation against HA (VHL) or myc (Gam1) tags. The samples were resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

Even if VHL and Gam1 continued to interact also *in vivo*, their binding affinity towards ElonginB/C and Cullins drastically decreased when they were co-expressed (figure 16, compare lane 2 to lane 5 and lane 10 to lanes 11 and 12), possibly indicating that the binding to the E3 ligase subunits and VHL is mutually exclusive for Gam1 and *vice versa*.

Further analysis of whole-cell extracts underlines the striking difference in Gam1 expression in the presence of HA-VHL (figure 17, lanes 4-6).

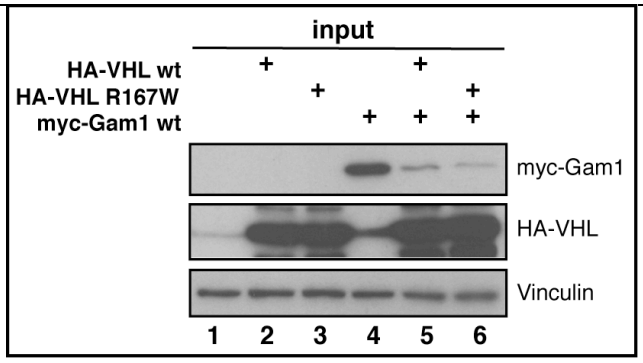

Figure 17. Gam1 protein markedly decreases in the presence of overexpressed VHL.

Phoenix cells were co-transfected as above. 30 μg of whole-cell lysates were analyzed by immunoblotting against the indicated tags. Vinculin was used as loading control.

A similar scenario was already reported when the viral protein LANA was co-expressed with VHL (Cai et al., 2006). Cai and colleagues hypothesized that VHL may act as a substrate-receptor for LANA, as well as LANA targets endogenous VHL for degradation, by recruiting Cullin-based E3 ligase complexes.

However, since VHL and Gam1 displayed lower binding affinity towards the E3 ligase complexes when they associated, this made us exclude this possibility. Rather, this may suggest that VHL and Gam1 mutual regulation could depend on their relative amounts and maybe on their reciprocal binding, without involving their E3 ligase functions.

In order to better investigate the possible effects of VHL and Gam1 interaction, we questioned whether they undergo to a subcellular localization change when they interact. Therefore, we performed an immunofluorescence analysis on HeLa cells co-transfected with HA-VHL and myc-Gam1. Moreover, to investigate whether their association could lead to protein degradation, we treated cells with the proteasome inhibitor MG132. Indeed, it could be possible that we cannot appreciate nuclear or cytoplasmic localization of a certain protein because of its degradation in that compartment.

It has already published that VHL protein is both nuclear and cytoplasmic (Duan et al., 1995b; Lee et al., 1999; Ye et al., 1998), whereas Gam1 displays a nuclear signal (Chiocca et al., 1997; Colombo et al., 2002), as confirmed by this analysis (figure 18, A2 and C3 respectively).

However, we noticed that Gam1 localization was restricted to some subnuclear aggregates when the proteasome was inhibited (figure 18, D3 and F3). Furthermore, the co-expression of Gam1 and VHL in cells treated with MG132 led the nuclear, but not the cytoplasmic, VHL fraction to rearrange in the

same structures (figure 18, F2). On the contrary, when VHL was solely expressed, it did not display this behavior neither in the presence of MG132 (figure 18, B2).

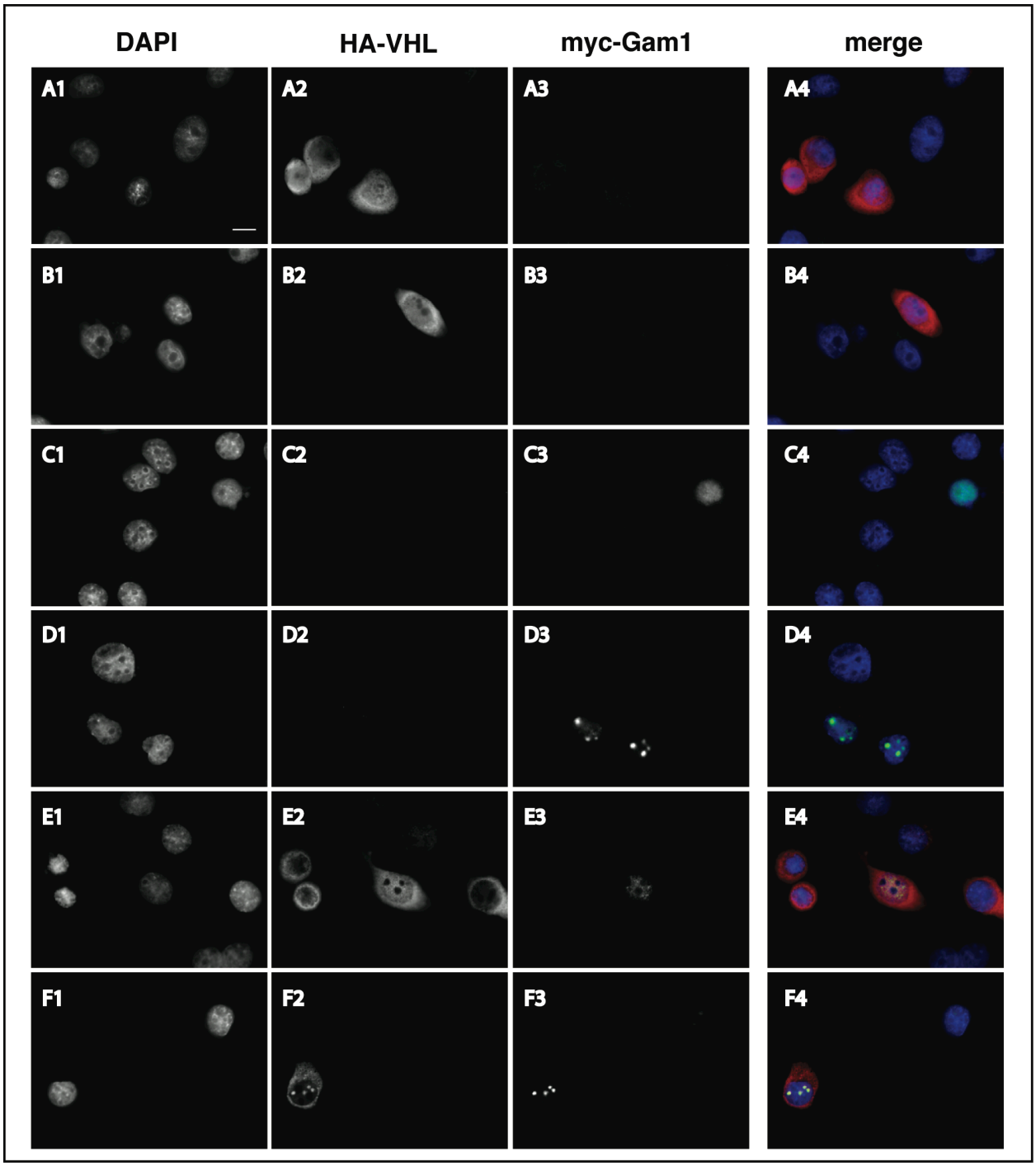

Figure 18. VHL and Gam1 co-localize in subnuclear structures upon proteasome inhibition.

Immunofluorescence analysis of about 10000 HeLa cells expressing HA-VHL and/or myc-Gam1 with 60% transfection efficiency. B, D and F panels showed the localization of the indicated proteins upon proteasome inhibition through the addition of 10 μM MG132 for 3 hours, whereas the other panels (A, C and E) represented the distribution of HA-VHL and myc-Gam1 in cells treated with DMSO as control. The transfected cells shown here are representative of all transfected cells analyzed in this experiment. Nuclei were stained with DAPI. Cells were analyzed using a wide-field microscopy. Scale bar: 20 μm.

VHL expression is dispensable for the localization and the activity of Gam1 protein.

To address whether the presence of VHL protein could be a prerequisite for the formation of these Gam1-containing structures, we compared the distribution of Gam1 protein in a renal carcinoma cell line deficient for VHL (RCC4 VHL-/-) and in its stably VHL-transfected counterpart (RCC4 VHL +/+) (Maxwell et al., 1999). Indeed, other cell lines were not suitable to well-discriminate this aspect because, even if HA-VHL was not co-transfected with Gam1, the endogenous VHL is rescued when cells are treated with MG132, as also shown in figure 13.

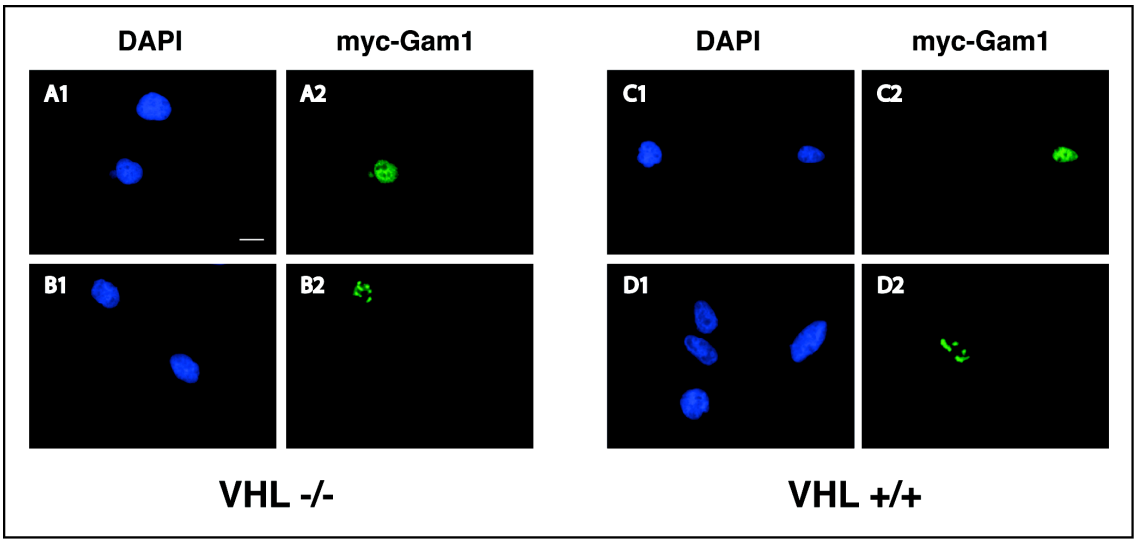

Figure 19. Gam1 rearranges in subnuclear aggregates upon proteasome inhibition even in the absence of VHL protein.

Renal carcinoma cell line (RCC4), deficient for VHL expression (VHL -/-), and its stably VHL-transfected counterpart (VHL +/+) were transiently transfected with myc-Gam1 and treated with 10 μM MG132 (B and D panels), or DMSO as control (A and C panels), for 3 hours before immunofluorescence analysis. Nuclei were stained with DAPI. Cells were analyzed using a wide-field microscopy. Scale bar: 20 μm.

The immunofluorescence analysis revealed that expression of VHL protein was not necessary for nuclear localization of Gam1 protein in normal conditions (figure 19, panel A2 and C2) or to rearrange Gam1 in these subnuclear structures when proteasome was inhibited (figure 19, panels B2 and D2).

Rather these data suggested that Gam1 drives overexpressed VHL in these new structures and not *vice versa*.

Moreover, western blot analysis of whole-cell extracts verified that Gam1-related E3 ligase activity, in particular towards SAE1, was not dependent on the presence of VHL protein (figure 20, lanes 2 and 4). Furthermore, this analysis highlighted the fact that VHL decrease upon Gam1 expression (figure 20, lane 4) did not depend neither on the regulation of VHL cellular promoter nor on the direct inhibition of *VHL* mRNA, since RCC4 cells express VHL starting from its cDNA sequence whose transcription is driven by the promoter present on the pcDNA3 vector (Maxwell et al., 1999).

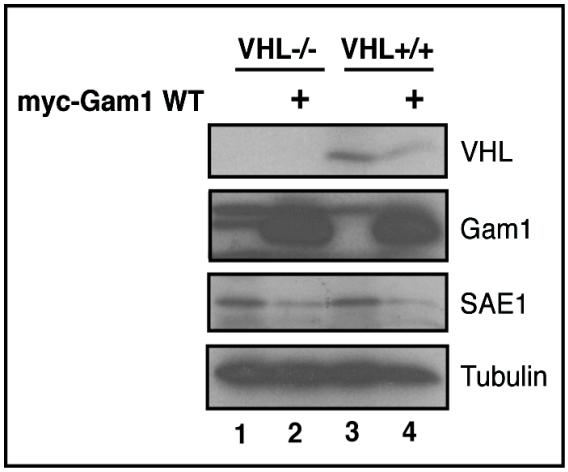

Figure 20. Gam1-related E3 ligase activity does not depend on VHL expression.

Both VHL-deficient (VHL-/-) and VHL-expressing (VHL +/+) RCC4 cells were transfected with wild-type (WT) myc-Gam1 plasmid and 40 μg of crude extracts were analyzed by immunoblotting. Tubulin was used as loading control.

These results clearly indicate that VHL decrease is related to some mechanisms that directly affect VHL at protein level and it is not just a consequence of a diminished VHL gene expression.

VHL and Gam1 form a complex that does not involve ElonginB/C and Cullin2/5.

To determine whether Gam1 and VHL form a complex that is distinct from the one they constitute with the E3 ligase subunits, we performed two consecutive immunoprecipitations to separate

overexpressed VHL-associated Gam1 pool from the group of other overexpressed VHL-interactive proteins.

Phoenix cells were transfected with FLAG-tagged VHL either alone or with myc-Gam1 and harvested after 48 hours. Cells were lysed in a non-denaturing lysis buffer and 8 mg of crude extracts were subjected to a first co-immunoprecipitation against FLAG-tagged VHL.

FLAG-VHL and the co-immunoprecipitated proteins were then eluted from the α-FLAG antibodies by competition using 2 μg/ml of FLAG peptide, and 1/4 of the eluted proteins were loaded for a first analysis (figure 21A, central panel). The remaining elution fraction was instead subjected to a second immunoprecipitation, this time against the myc tag (Gam1).

Concurrently, a further immunoprecipitation against myc-Gam1 was performed, as a control for Gam1 binding towards E3 ligase subunits, starting from 2 mg of crude extracts (figure 21B, lane 3).

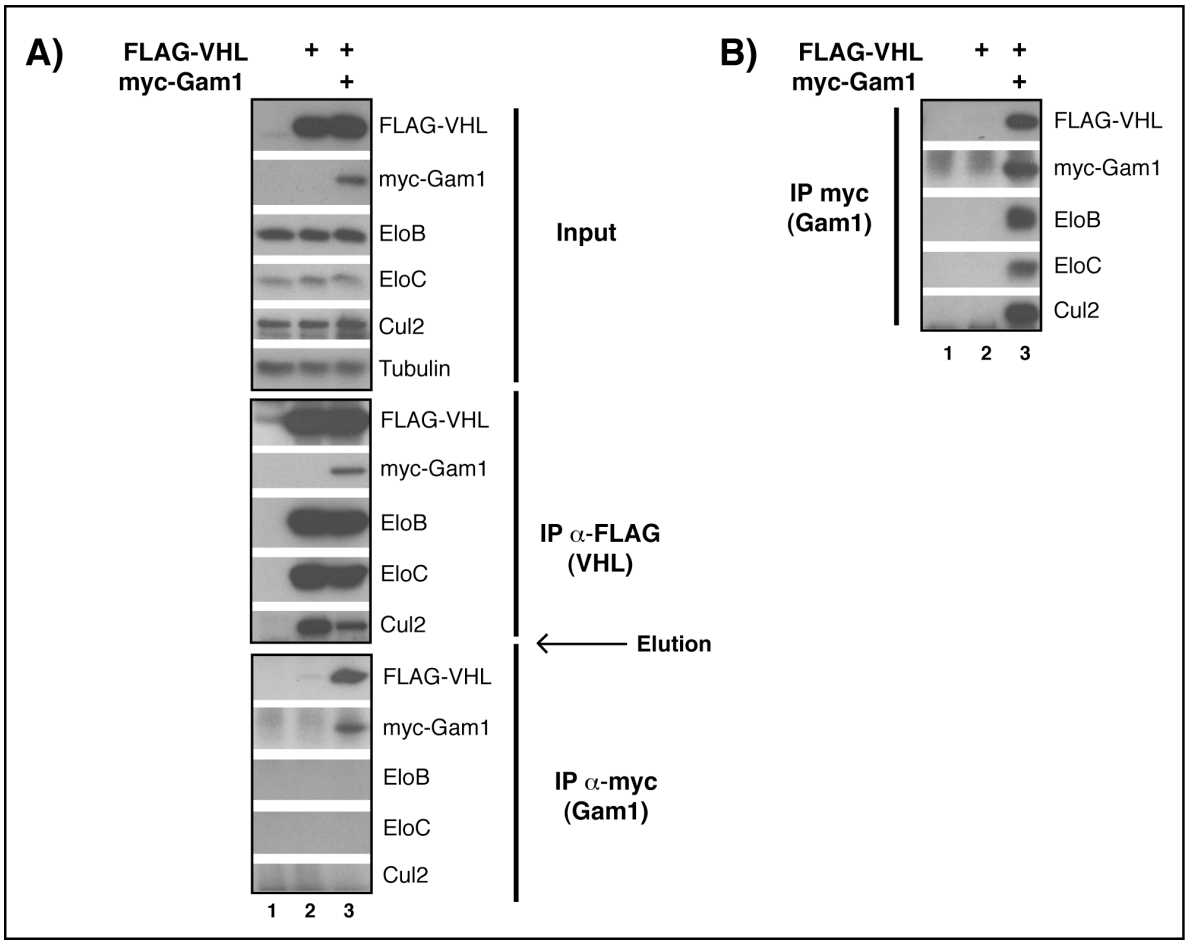

Figure 21. VHL and Gam1 interaction does not involve Elongins and Cullins.

A) Phoenix cells were transiently transfected with the indicated plasmids and harvested 48 hours later, followed by lysis in non-denaturing E1A lysis buffer and subjected to two sequential immunoprecipitation (IP) against the indicated tags. 8 mg of crude extracts were used for the first immunoprecipitation, then 1/4 of elution was loaded and the remaining eluted fraction was subjected to the second immunoprecipitation. 30 μg of whole-cell

extracts were loaded as the input and Tubulin was used as loading control. B) Immunoprecipitation against myc-tag starting from 2 mg of crude extract.

Although FLAG-VHL was co-eluted both with ElonginB/C and Cullin2 and with Gam1 (figure 21A, central panel, lanes 2 and 3), Gam1 interacted with VHL only after the second immunoprecipitation (figure 21A, lane 3, bottom panel) even if, starting from the initial lysates, myc-Gam1 was still able to associate with ElonginB/C and Cullin2 (figure 21B, lane 3).

These results highlight the fact that VHL and Gam1 interaction does not require the presence of other E3 ligase subunits (figure 15A and B), since it occurs in a completely separate pool.

The last data suggest that Gam1 does not drive directly VHL to degradation, rather it may activate an intracellular mechanism that leads to this effect.

VHL protein can be SUMOylated, but inhibition of SUMOylation does not alter its stability.

Gam1 is known to block cellular SUMOylation by inducing the degradation of both SUMO E1 (SAE1/SAE2) and SUMO E2 (UBC9) enzymes (Boggio et al., 2004; Boggio et al., 2007).

SUMOylation is a post-transcriptional modification that controls the activity of several transcription factors (Fogal et al., 2000; Goodson et al., 2001; Muller et al., 2000; Yang et al., 2003) and other proteins (Colombo et al., 2002; Kirsh et al., 2002; Shiio and Eisenman, 2003), influences protein stability (Cheng et al., 2007b; Lallemand-Breitenbach et al., 2008; Lin et al., 2003; Tatham et al., 2008) and localization (Mahajan et al., 1997; Matunis et al., 1996) and contributes to repair damaged DNA (Cheng et al., 2008; Galanty et al., 2009; Morris et al., 2009; Ouyang et al., 2009). The great variety of functions of SUMOylation locates this mechanism among the central cellular regulatory pathways.

To verify that VHL could be really modified by SUMO, Phoenix cells were transfected with plasmids carrying respectively V5-VHL, HA-SUMO1 or UBC9 and harvested 48 hours later. Immunoblotting analysis showed that indeed VHL could be SUMOylated at a unique site (figure 22, lane 4) (this VHL residue was identified and the role of VHL SUMOylation was described in a recent report published by Cai and colleagues) (Cai et al., 2010).

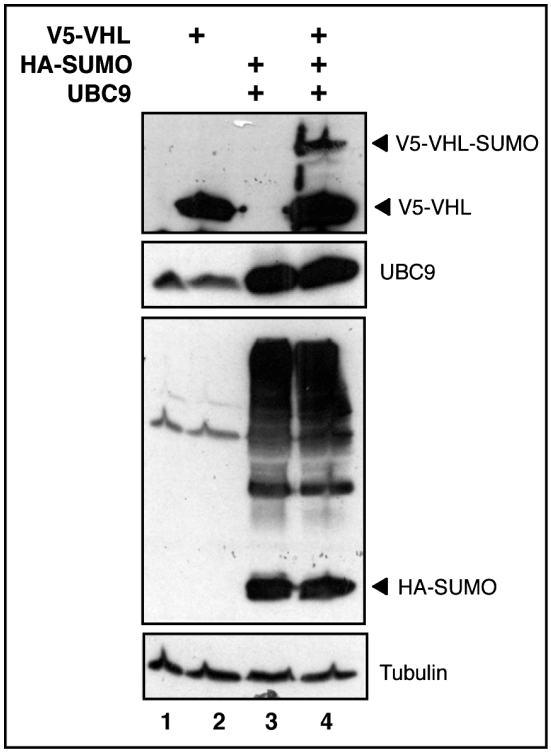

Figure 22. VHL can be SUMOylated at a unique residue.

HA-SUMO and UBC9 leads to SUMOylation of V5-tagged VHL when simultaneously expressed in Phoenix cells. Only a little fraction of VHL was SUMOylated and it displayed a unique site of modification. 30 μg of whole-cell extracts were loaded and Tubulin was used as loading control. Arrowheads indicate the corresponding proteins. Smearings above HA-SUMO bands correspond to SUMOylated proteins.

Having established that VHL can be subjected to SUMOylation, we assessed whether Gam-1induced SUMOylation inhibition could affect VHL protein stability. Thus, to phenocopy this effect, we depleted SAE1, SAE2 and UBC9 using short hairpin RNA (shRNA) interference approach. HeLa cells were transfected with plasmids carrying the shRNA sequences to efficiently knock-down our target proteins and, after six days under puromycin selection (1 μg/ml), cells were harvested and samples were examined by immunoblotting.

Western blot analysis showed that SAE1, SAE2 and UBC9 were successfully depleted and the presence of the unmodified form of RanGAP1 protein assessed the extent of SUMOylation inhibition (figure 23).

However, VHL protein stability was not minimally affected.

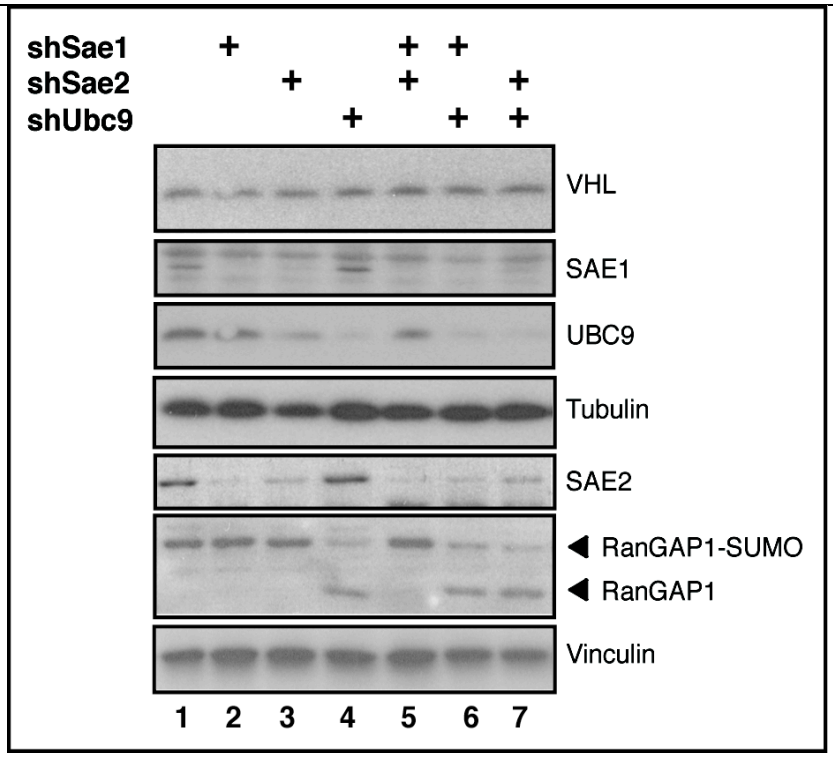

Figure 23. SUMOylation inhibition does not affect VHL protein stability.

Depletion of SAE1, SAE2 and UBC9 was controlled by immunoblotting using the relative antibodies. RanGAP1 was used as internal control of SUMOylation inhibition. 40 μg of whole-cell extracts were analyzed by immunoblot for the indicated proteins. Tubulin and Vinculin were used as loading control respectively for 15% and 8% poly-acrylamide gels.

This data clearly indicates that this is not the pathway involved in Gam1-induced VHL protein decrease and it prompted us to evaluate other mechanisms that may be responsible for VHL degradation.

Gam1-related Ubiquitin E3 ligase activity is not involved in VHL decrease.

Considering the results described above, we verified that VHL degradation in the presence of wild-type Gam1 does not directly involve Gam1-based E3 ligase action, but we cannot exclude that it may be somehow implicated. It was published that Gam1 E3 ligase activity can be efficiently counteracted by depletion of Cullin2 and Cullin5 (Boggio et al., 2007).

Therefore, we assessed whether VHL decrease can be related to this Gam1 function using a similar approach.

HeLa cells were initially transfected with plasmids containing shRNA sequences against Cullin2 or Cullin5 transcripts and kept under selection for eight days with 1 μg/ml puromycin. Thereafter they were transfected with wild-type myc-Gam1 and harvested 48 hours later.

However, neither single nor simultaneous depletion of Cullin2 and Cullin5 rescued VHL protein when also Gam1 was expressed (figure 24), thus ruling out the possibility that Gam1 E3 ligase activity can anyway be involved in VHL degradation.

Moreover, by this experiment, it appears that the reduced expression of Cullin2 is not a limiting factor for VHL stability (figure 24, lane 3), therefore the assembly of an active E3 ligase complex.

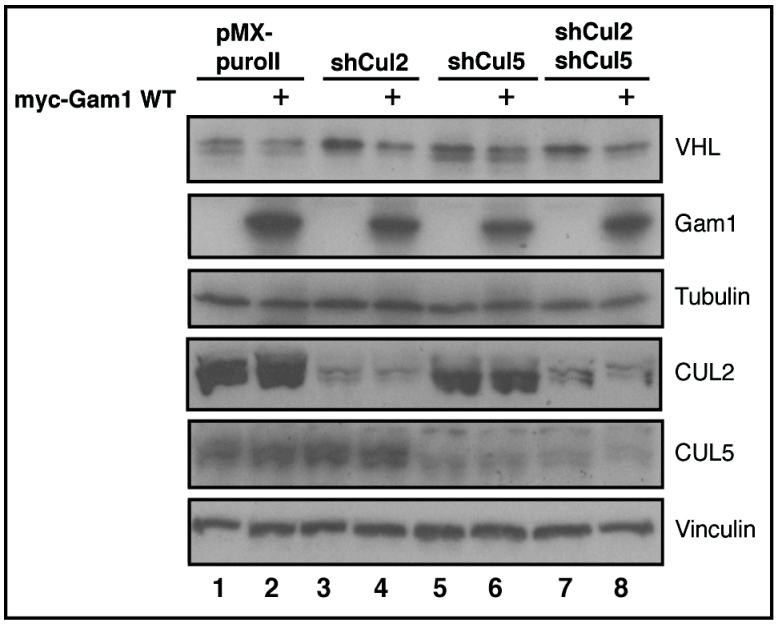

Figure 24. VHL protein decrease does not depend on Gam1-related E3 ligase activity.

HeLa cells were transfected with plasmids carrying the short-hairpin RNA (shRNA) sequence against Cullin2 (shCul2) or Cullin5 (shCul5) and put under selection with 1 μg/ml puromycin for eight days. Then they were further transfected with a plasmid carrying wild-type myc-Gam1 sequence and analyzed by immunoblotting 48 hours later. 40 μg of whole-cell extracts were analyzed by immunoblot for the indicated proteins. Tubulin was used as loading control for myc-Gam1 and VHL (15% poly-acrylamide gel) and Vinculin as loading control for Cullin2 (CUL2) and Cullin5 (CUL5) (8% poly-acrylamide gel).

Overexpression of ElonginB/C heterodimer partially rescues VHL protein level in the presence of Gam1.

Since neither E3 ligase activity nor Cullin2 or Cullin5 presence were directly related to VHL decrease upon Gam1 expression, we wondered which other wild-type specific Gam1 characteristics might be involved.

The main difference between Gam1 wild-type and Gam1 L258,265A mutant, that does not cause VHL reduction (figure 10, lane 3), resides in their protein sequence, thus in their ability to bind or not ElonginB/C heterodimer (Boggio et al., 2007).

ElonginB/C is very important for VHL stability as reported by Schoenfeld and colleagues. Indeed, they demonstrated that binding to ElonginB/C complex is necessary towards VHL protein proper folding, and this interaction prevents VHL degradation (Schoenfeld et al., 2000).

Moreover, Gam1 is present in large amounts when overexpressed in cells and it could recruit a large amount of ElonginB/C heterodimer, reducing its availability. In this way, the fraction of unbound VHL would get misfolded and could be subjected to ubiquitination and degradation. Our previous experiments are in agreement with this hypothesis, as shown in figures 16 and 18, suggesting that a sort of binding competition towards ElonginB/C exists between VHL and Gam1 and, maybe, their relative amounts can determine which of them associates to the E3 ligase subunits.

To test whether the availability of ElonginB/C could be a limiting factor, we decided to overexpress these proteins and Gam1 in HeLa cells and analyze the lysates by immunoblotting.

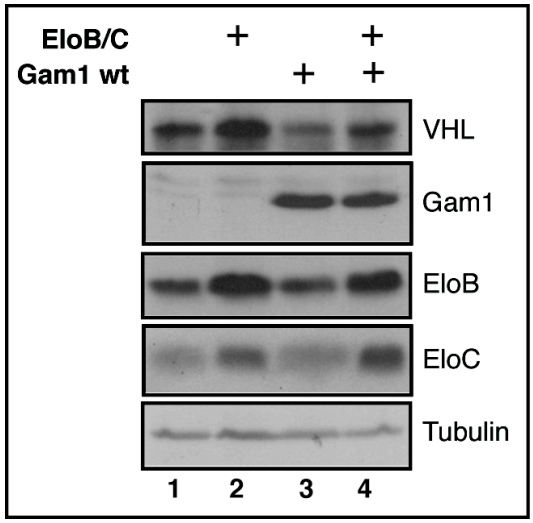

Figure 25. Overexpression of ElonginB/C heterodimer leads to increased stability of VHL protein.

ElonginB and ElonginC (EloB/C) were both overexpressed in HeLa cell line in combination with wild-type (wt) myc-Gam1. 40 μg of whole-cell extracts were analyzed by immunoblot for the indicated proteins. Tubulin was used as loading control.

These results showed that overexpression of ElonginB/C complex led to increased VHL protein level, but this increase was observed also with respect to the control (figure 25, lanes 1 and 2). Therefore, the partial rescue detected upon Gam1 and ElonginB/C co-expression could be due to a higher starting amount of VHL protein itself (figure 25, lanes 3 and 4).

Nevertheless, it must not be excluded that Gam1 could recruit more ElonginB/C complex for itself, thereby resulting in a partial, instead of total, rescue of VHL protein.

Other BC-box containing proteins induce VHL protein decrease.

If the reduced availability of ElonginB/C heterodimer is the key feature for the decrease of endogenous VHL protein level, it could be possible that the expression of other proteins that associate with this complex can lead to the same effect.

There are several proteins described to associate with ElonginB/C, among which the Suppressor Of Cytokine Signaling (SOCS) proteins (Kamura et al., 1998).

They are usually expressed in response to the signaling started by the presence of pro-inflammatory cytokines, and, as the name suggests, they regulate this signaling through a negative feedback mechanism (reviewed in (Croker et al., 2008)). Even if they were the first proteins whose SOCS-box domain (comprising the BC-box domain) was described and they were known for years to bind ElonginB/C, only recently their role as substrate-receptors has emerged (Babon et al., 2009).

Since SOCS proteins are expressed in response to an altered physiological condition, and since Gam1 seems to likely activate a cellular response that leads to VHL degradation, we believed that they could be good candidates to test whether other proteins beyond Gam1 could induce VHL decrease as well.

We transiently overexpressed either FLAG-tagged SOCS1 or SOCS3 in Phoenix cells and observed whether the level of endogenous VHL protein could be somehow affected.

We observed that when SOCS1 or SOCS3 were present, VHL protein markedly diminished (figure 26, lanes 2 and 3).

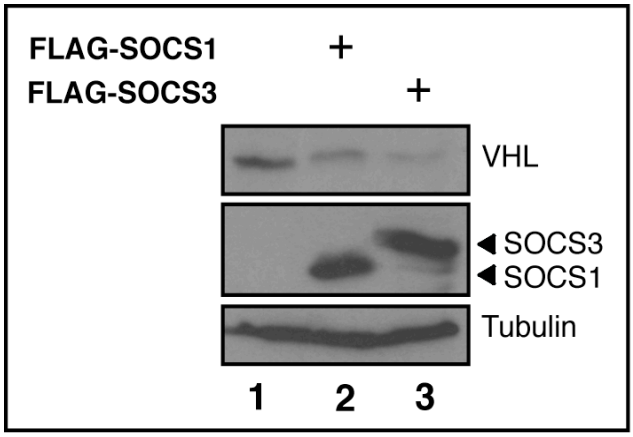

Figure 26. Overexpression of SOCS1 and SOCS3 leads to VHL protein decrease.

Phoenix cells were transfected with plasmid carrying FLAG-SOCS1 or FLAG-SOCS3 sequence and after 48 hours cells were harvested and lysates were subjected to western blot analysis. SOCS1 and SOCS3 were detected using a α-FLAG antibody and arrowheads indicate the corresponding proteins. 40 μg of whole-cell extracts were analyzed by immunoblot for the indicated proteins. Tubulin was used as loading control.

This may suggest that ectopically expressed cellular proteins containing a BC-box domain can induce VHL protein destabilization. However, SOCS1 and SOCS3 possess other domains besides the SOCS-box (Endo et al., 1997; Hilton et al., 1998; Starr et al., 1997) and they could affect VHL protein level by a different mechanism than Gam1.

Expression of the SOCS-box domain is sufficient to lead to VHL protein decrease.

To definitively demonstrate that the amount of available ElonginB/C complex for VHL binding is the limiting factor for VHL stability, and to overcome the possible effects given by the presence of other domains in full-length SOCS1 and SOCS3, we fused their SOCS-box domains to the carboxy terminus of EGFP protein and we expressed them in Phoenix cells.

We chose to use the SOCS-box domains of these two proteins instead of the Gam1 one because they were already reported to independently assume a correct folding (Babon et al., 2009), and we wanted to be sure to express a functional binding domain.

Specifically, Phoenix cells were transiently transfected with EGFP, EGFP-SOCS1(172-212) or EGFP-SOCS3(186-225) and after 48 hours they were harvested and lyzed in a non-denaturing buffer to

perform an immunoprecipitation against the EGFP protein, in order to further test whether these SOCS-box domains retained the ability to interact with ElonginC. We then analyzed both these samples and the crude extracts by immunoblotting. EGFP protein *per se* did not have any effect on VHL protein or HIF-1α stability (figure 27, lane 1), whereas EGFP proteins fused with the SOCS-box domains induced VHL decrease and HIF-1α stabilization (figure 27, lanes 2 and 3).

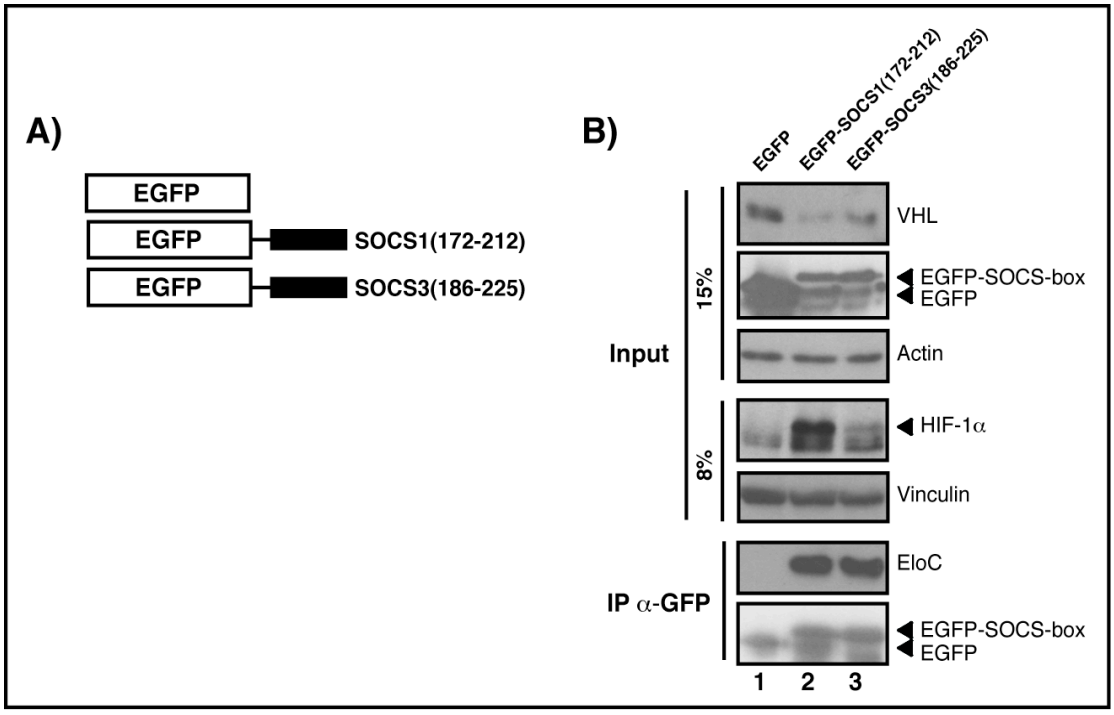

Figure 27. Overexpression of EGFP-SOCS1(172-212) or EGFP-SOCS3(186-225) led to VHL protein decrease.

A) Schematic representation of EGFP, EGFP-SOCS1(172-212) and EGFP-SOCS3(186-225). B) Phoenix cells were transiently transfected with EGFP pure or fused with the SOCS-box domains of SOCS1 and SOCS3. 2 mg of crude cell extracts were subjected to immunoprecipitation (IP) using 4 μg of α-GFP antibody and they were subsequently analyzed by western blot. 40 μg of crude cell extracts were loaded in 15% or 8% poly-acrylamide gels and immunoblotted against the indicated proteins. Actin was used as loading control for VHL and EGFP-containing proteins, whereas Vinculin was used as loading control for HIF-1α.

Moreover, immunoprecipitation against EGFP protein confirmed the interaction of both SOCS-box domains with ElonginC, whereas EGFP protein alone did not possess this ability.

The results clearly indicated that the expression of a properly folded SOCS-box domain is sufficient to induce endogenous VHL protein decrease, and that HIF-1α stabilization correlates with VHL reduction (figure 27, lanes 2 and 3).

Several viral BC-box containing proteins are able to affect VHL protein stability.

To further test to what extent VHL protein stability can be affected by the presence of BC-box containing proteins, we decided to express some viral proteins known to possess this domain and to interact with ElonginB/C complex.

Our choice comprised the human adenovirus type 5 protein E4orf6 (Blanchette et al., 2004; Cheng et al., 2007a) and the human papillomavirus 16 (HPV16) protein E7 (Huh et al., 2007). They were both described to associate with ElonginB/C, and either with Cullin5 or Cullin2 respectively, to form an active E3 ligase complex to degrade their target proteins (Huh et al., 2007; Querido et al., 2001). We transiently transfected Phoenix cells with a plasmid carrying the FLAG-tagged sequence of HPV16 E7 and we harvested the cells after 24 hours. We observed that it was the time point that correlates with the highest expression of E7 protein (data not shown) and, since we hypothesize that the stability of VHL is influenced by the relative amount of the antagonist protein in the cell, we considered it as the right moment to perform our investigation. Indeed, immunoblotting data revealed that VHL protein markedly decrease upon E7 expression (figure 28, lane 2) (samples provided by Daniele Frangioni).

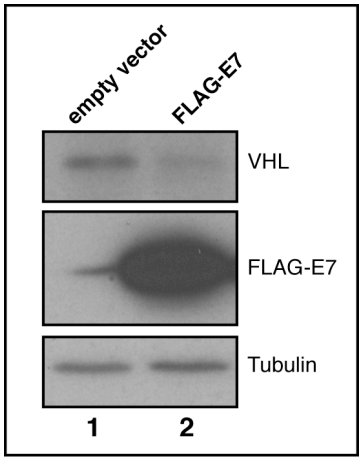

Figure 28. The expression of HPV16 E7 protein induces a marked decrease of VHL protein.

Phoenix cells were transfected with a plasmid codifying a FLAG-tagged HPV16 E7 (FLAG-E7) protein and they were harvested after 24 hours. 40 μg of whole-cell extracts were analyzed by immunoblotting using the relative antibodies. Tubulin was used as loading control.

It was widely demonstrated that both HPV16 E6 and E7 oncoproteins are tightly related to enhanced tumor angiogenesis *in vivo* (Bequet-Romero and Lopez-Ocejo, 2000) and to the upregulation of angiogenic factors *in vitro* (Lopez-Ocejo et al., 2000; Stoppler et al., 2001; Tang et al., 2007; Toussaint-Smith et al., 2004). In agreement with these previous observations, we showed that HPV16 E7 is able to induce VHL protein decrease and this may be considered another source of HPV16-associated angiogenesis.

Similarly, the expression of E4orf6 in U2OS cell line led to VHL protein decrease, although to a lesser extent (figure 29, lane 2). Even if the reduction of VHL was barely evident, we were still able to detect HIF-1α protein stabilization (figure 29, lane 2). It could mean that just a little variation in VHL protein amount may be enough to induce a hypoxia-like response.

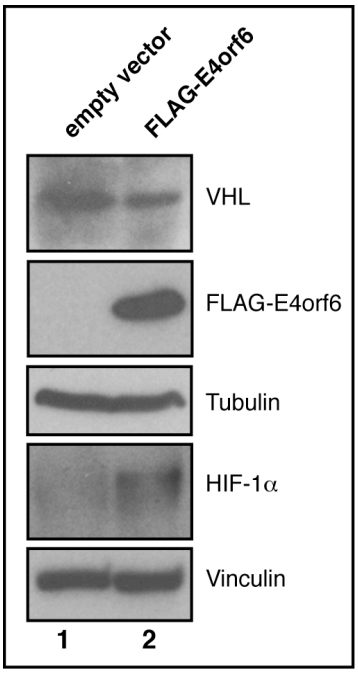

Figure 29. The expression of Adenovirus type 5 E4orf6 protein leads to a weak VHL decrease, but enough to stabilize HIF-1α protein.

U2OS cells were transiently transfected with a plasmid carrying the FLAG-E4orf6 sequence and they were harvested 48 hours later. 40 μg of whole-cell extracts were analyzed by immunoblotting using the relative antibodies. Tubulin was used as loading control for VHL and FLAG-E4orf6 (15% poly-acrylamide gel) and Vinculin was used as loading control for HIF-1α (8% poly-acrylamide gel).

Taken together, these data seem to support our model and they could indicate that other cellular and viral proteins containing a domain to bind ElonginB/C may lead to VHL protein decrease and, as a consequence, to HIF-1α stabilization.

Different cervical carcinoma-derived cell lines display a diverse VHL expression that may correlate with their cellular background.

However, since we supposed that both relative amounts (depending on the transcriptional rate and the protein turnover) and binding affinities towards ElonginB/C heterodimer could be important features for this model, we wondered whether VHL stability could be affected by the same proteins in a context of a naturally occurring infection.

In order to clarify this aspect, we analyzed (by western blot) VHL protein level in three different cervical carcinoma-derived cell lines, namely C33A (HPV negative), HeLa (HPV18 positive) and CaSki (HPV16 positive). C33A cell line was used as a control, whereas HeLa and CaSki were used to compare the possible effect on VHL protein of HPV18 (whose E7 does not seem to interact with Cullin2 and ElonginC (Huh et al., 2007)) and HPV16 (whose E7 contains a BC-box domain and it induces VHL decrease when overexpressed by transient transfection (figure 28, lane 2)). Previous results had already showed that HeLa cells are able to express VHL protein, as confirmed by this data (figure 30, lane 3). C33A cells displayed almost the same level of VHL expression as HeLa (figure 30, lane1), whereas CaSki cell line was VHL-deficient (figure 30, lane 2).

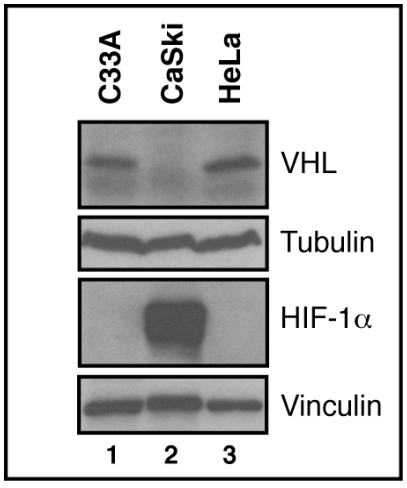

Figure 30. Analysis of three different cervical carcinoma-derived cell lines highlighted some differences in the expression of VHL and HIF-1α proteins.

Western blot analysis on 40 μg of whole-cell extracts from C33A (HPV negative), CaSki (HPV16 positive) and HeLa (HPV18 positive) cell lines derived from cervical carcinomas to detect VHL and HIF-1α protein levels. Tubulin was used as loading control for VHL (15% poly-acrylamide gel) and Vinculin was used as loading control for HIF-1α (8% poly-acrylamide gel).

In agreement with the previous observations (figure 28), we confirmed that CaSki cell line expressed HIF-1α protein at high level (figure 30, lane 2).

To rule out the possibility that the missed expression of VHL protein in CaSki cell line may due to transcriptional repression of VHL gene, we performed a quantitative RT-PCR analysis of *VHL* mRNA to compare its relative amounts in these cell lines (experiment made by Archana Varadaraj).

As shown by the graph, *VHL* mRNA level was lower in CaSki cell line than in HeLa or C33A (figure 31).

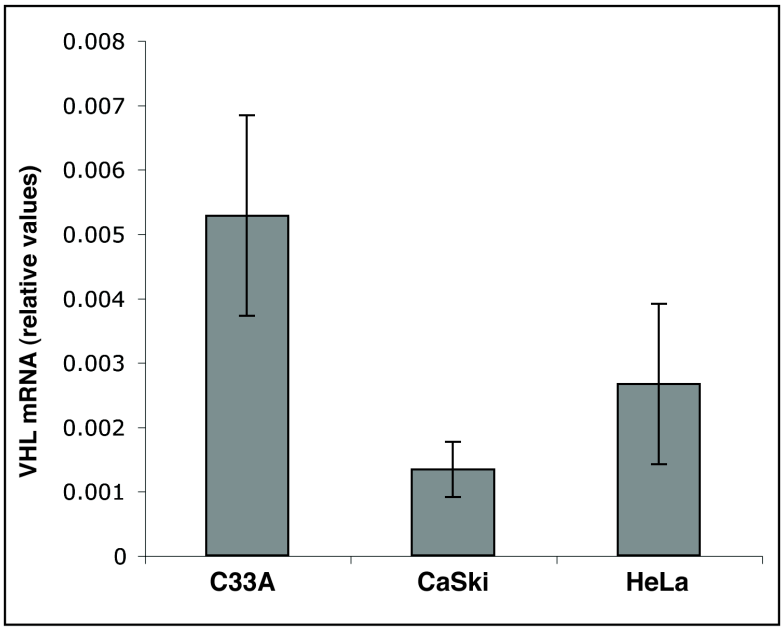

Figure 31. *VHL* mRNA relative amounts in three cervical carcinoma-derived cell lines.

C33A, CaSki and HeLa total mRNA was retrotranscribed and analyzed by quantitative RT-PCR. The graph describes the relative amounts of *VHL* mRNA normalized to *GAPDH* mRNA (to whom was assigned the arbitrary value of 1). The error bars correspond to the standard deviations.

However, the difference in *VHL* mRNA production in these cell lines was enough to justify the totally absent expression of VHL protein in CaSki cells. Since HeLa cells do not display alteration in VHL protein level, we can exclude that VHL disappearance in CaSki cells is due to dysregulation of p53 or pRb pathways. Rather, it suggests the involvement of ERK1/2 and PI3K in the induction of angiogenic factors upon HPV16 oncoproteins overexpression (Tang et al., 2007), leading to the possibility that these signaling pathways may affect also VHL expression. However, it could be possible that other mechanisms take part in VHL regulation in this context and we will try to elucidate better these aspects in the future.

HSP90-related mechanisms seem not involved in Gam1-induced VHL degradation.

Having determined that the expression of BC-box domain proteins, able to reduce the availability of free ElonginB/C, can lead to VHL decrease, we need to address the underlying mechanism. In other words, we wonder which proteins are directly involved in VHL degradation.

When VHL cannot bind to ElonginB/C complex, it has a misfolded conformation that can lead to VHL degradation by the action of hsp70 and hsp90 complex (McClellan et al., 2005).

Moreover, it has been already shown that Gam1 enhances the expression of hsp70 and hsp40 proteins. The overexpression of hsp40, but not hsp70, in cells infected with Gam1-negative CELO adenovirus is able to partially restore viral replication (Glotzer et al., 2000). This results support the idea that chaperone proteins may be important for some Gam1-related activities.

Therefore, we assessed whether hsp70 and hsp90 chaperones were directly involved in targeting VHL protein to degradation. Since hsp70 is also necessary to assist VHL correct folding (Melville et al., 2003), we chose to inhibit the activity of hsp90 using the selective hsp90 binding agent geldanamycin (GA). Geldanamycin disrupts the association between hsp90 and its client proteins, leading them to degradation (Minet et al., 1999; Nomura et al., 2005; Schulte et al., 1995). VHL is not an hsp90 client protein. Rather, the binding to hsp90 drives the misfolded VHL to degradation (McClellan et al., 2005). Therefore, we used geldanamycin to observe whether VHL protein was rescued upon Gam1 expression.

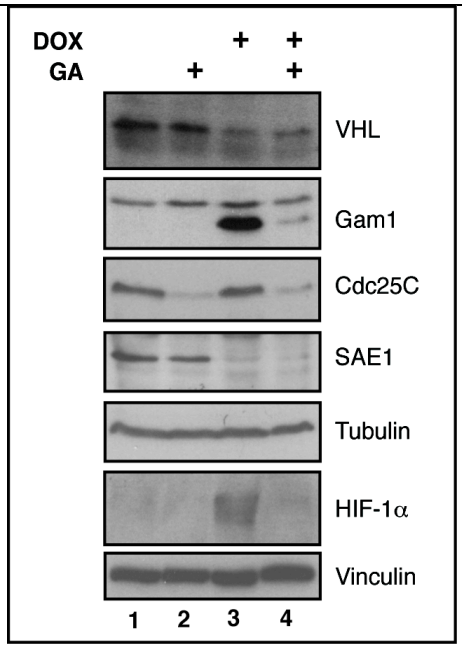

Figure 32. Hsp90 chaperone protein is not involved in VHL degradation induced by Gam1.

Western blot analysis of 40 μg of whole-cell extracts from Flp-In HeLa cells expressing myc-Gam1 by a Tet-on inducible promoter were treated with 1 μM geldanamycin (GA) for 20 hours where indicated. Gam1 expression was induced by the addition of 1 μg/ml doxycycline (DOX) for 20 hours. Cdc25C was used as control for hsp90 inhibition. SAE1 was used as control for Gam1-E3 ligase related activity. Tubulin was used as loading control for 15% poly-acrylamide gel proteins. Vinculin was used as loading control for HIF-1α.

Our experimental results revealed some interesting aspects. First of all, it clearly appeared that inhibition of hsp90 activity did not counteract VHL degradation upon Gam1 expression (figure 32, lane 4), even if Gam1 was expressed at low levels. However, Gam1 was still able to induce SAE1 degradation (figure 32, lanes 3 and 4), meaning that the observed amount of Gam1 was enough to recruit E3 ligase subunits and carry on its activities. Cdc25C protein decrease demonstrated that hsp90 inhibition given by the addition of geldanamycin was efficient (Senju et al., 2006) (figure 32, lanes 2 and 4), also confirmed by the disappearance of HIF-1α protein band, even if VHL protein level was still low (figure 32, lane 4). Indeed, HIF-1α is a known hsp90 client protein and this was an expected result (Mabjeesh et al., 2002).

In conclusions, the pathway underlying VHL degradation does not implicate the participation of hsp90 protein. However, we cannot exclude that VHL degradation may be driven by another chaperone-mediated mechanism.

Heat shock treatment strongly affects VHL stability.

Cells subjected to heat shock treatment express a set of inducible proteins called heat shock proteins (HSPs). These proteins were classified depending on their molecular weights, ranging from 27 to 110 kDa (Landry et al., 1982).

Besides hyperthermia, heat shock proteins are also induced by other protein-damaging stresses, such as oxidative stress (Baird et al., 2006; Wallen et al., 1997), UV irradiation (Shi et al., 2008; Zhou et al., 1998), and under pathological conditions, cancer (reviewed in (Ciocca and Calderwood, 2005)) and viral infections (Glotzer et al., 2000; Li et al., 2010; Young et al., 2008b). Their protective potential resides in their functions as molecular chaperones, required to assist nascent protein folding (Beckmann et al., 1990) or to refold misfolded proteins (Freeman and Morimoto, 1996; Schumacher et al., 1996). Highly damaged proteins, impossible to refold, are targeted for degradation by other heat shock proteins-associated complexes (Parag et al., 1987), such as CHIP E3 ligase and the auxiliary proteins hsp90 or hsp70/hsp40 (Connell et al., 2001; Murata et al., 2001).

Our results excluded the involvement of hsp90 in Gam1-dependent VHL protein degradation (figure 26). However, Gam1 triggers the expression of hsp70 and hsp40, but not of hsp90 (Glotzer et al., 2000), suggesting that other heat shock proteins could perhaps mediate VHL destabilization in the presence of Gam1.

To test this possibility, we undertook a more general approach, on the basis of heat-induced heat shock proteins (Glotzer et al., 2000; Landry et al., 1982) and on the observation that inducible hsp70 proteins play a pivotal role under these conditions. Flp-In Gam1-inducible HeLa cells were subjected to heat shock treatment at 43°C for 90 minutes or to doxycycline addition to express Gam1, then harvested and analyzed by western blot (experiment made by Archana Varadaraj).

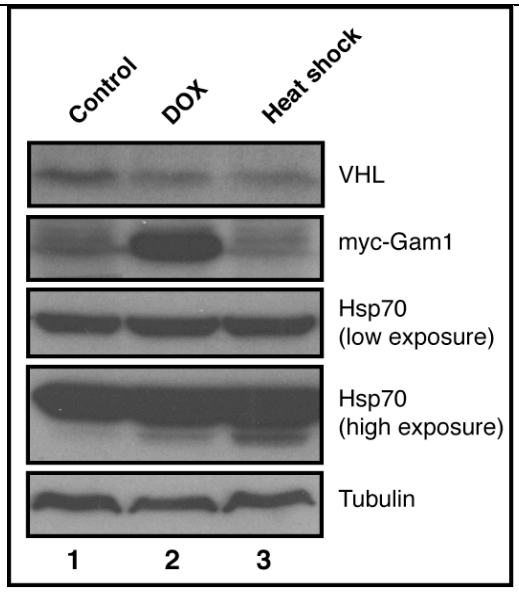

Figure 33. Heat shock treatment displays Gam1-induced similar effects on VHL protein stability.

Western blot analysis of 40 μg of whole-cell extracts from Flp-In HeLa cells expressing myc-Gam1 by a Tet-on inducible promoter were subjected to heat treatment (heat shock) at 43°C for 90 minutes where indicated. Gam1 expression was induced by the addition of 1 μg/ml doxycycline (DOX) for 20 hours. Hsp70 was used as a control for heat shock treatment. Tubulin was used as loading control.

VHL protein decreases to similar levels both after Gam1 expression and heat shock treatment (figure 33, lanes 2 and 3). As expected, Gam1 was expressed only after the addition of doxycycline and is not induced by heat shock (figure 33, lanes 2 and 3). Hsp70 protein levels slightly increased upon Gam1 expression and heat shock treatment (figure 33, hsp70 low exposure). However, upon higher exposure, we noticed an additional lower band, not present in the control (figure 33, compare lane 1 with lanes 2 and 3), which may correspond to the resolved hsp69/72 doublet that appears in response to heat shock treatment (Burns et al., 1989).

Although this data is not a conclusive evidence for a Gam1/heat shock proteins mechanism for VHL degradation, these preliminary results are very encouraging. Indeed, they highlight that VHL is sensitive to stress conditions that can alter its structure, hence its protein level dramatically decrease.

Further experiments will evaluate the possible roles of hsp70/hsp40 and chaperone-dependent Ubiquitin E3 ligase CHIP in affecting VHL stability upon BC-box containing proteins overexpression.

DISCUSSION

During the last few years several viruses were described to exploit the cellular ubiquitylation pathway by hijacking different host proteins to sustain their efficient infection and replication. Viral proteins often target Ubiquitin E3 ligases, the central enzymes in the ubiquitylation pathway, for the recognition of specific substrates.

Some of these viruses encode their own E3 ligases, such as KSHV RTA (Yu et al., 2005) or HSV-1 ICP0 proteins (Boutell et al., 2002), while others use viral adaptor proteins to recruit endogenous cellular E3 ligases, such as HPV16/18 E6, which forms a complex with the cellular E3 ligase E6AP (Scheffner et al., 1993; Scheffner et al., 1990), or Adenoviral E1B-55k and E4orf6 proteins or HIV-1 Vif, all acting as substrate-receptors associated to Cullin5-based E3 ligases (Blanchette et al., 2004; Querido et al., 2001; Yu et al., 2003).

Our group has recently demonstrated that the CELO early protein Gam1 belongs to this last group. Indeed, Gam1 possesses a degenerate SOCS-box domain that allows the interaction with the heterodimer ElonginB/C. Moreover, Gam1 recruits both Cullin2- and Cullin5-based E3 ligases, equally involved in the degradation of the SUMO E1 enzyme (SAE1/SAE2 heterodimer) (Boggio et al., 2007). Conversely, the SOCS-box mutant L258,265A is not able to bind to ElonginB/C and does not display any of the known effects of the Gam1 wild-type protein (Boggio et al., 2004; Boggio et al., 2007; Chiocca et al., 1997; Chiocca et al., 2002; Colombo et al., 2002), supporting the idea that the majority of Gam1-related effects depends on its ability to recruit these host E3 ligase complexes. Furthermore, the hijack of host complexes by viruses may develop also in the appearance of several side effects, which could somehow explain the multiplicity of pathways affected by the expression of a single viral protein. Gam1 is in fact another example of a multi functional viral protein that can simultaneously subvert distinct cellular pathways.

The goal of this thesis is to define a possible general mechanism occurring during Cullin-based E3 ligase aberrant recruitment.

Gam1 protein leads to stabilization of HIF-1α and activation of a hypoxia-like response.

Based on the previous finding from our group on the recruitment of Cullin2- and Cullin5-based E3 ligases by Gam1, we focused on the possible impairment of the normal activities related to these two E3 ligase complexes. Among the several substrate-receptors associating to ElonginB/C and to Cullin2 or Cullin5 subunits, we selected the well-studied von-Hippel Lindau (VHL) tumor suppressor protein.

We expected to observe an increase in VHL substrates if the association of Gam1 protein to ElonginB/C and Cullin2 subunits abrogates VHL-related E3 ligase activity.

The best known target of VHL-associated complex is the Hypoxia-Inducible Factor (HIF)-1α. This protein is part of a heterodimeric transcription factor (HIF-1) that drives the expression of a plethora of genes involved in glucose metabolism (Zelzer et al., 1998), angiogenesis (Forsythe et al., 1996), and erythropoiesis (Wang and Semenza, 1993). HIF-1α is constitutively produced, but, in normoxia, it is continuously subjected to ubiquitylation and degradation through the action of VHL-based E3 ligase complexes (Cockman et al., 2000; Maxwell et al., 1999). Further mechanisms leading to HIF-1α to degradation do exist (Koh et al., 2008; Liu et al., 2007; Luo et al., 2009; Ravi et al., 2000), however, under physiological conditions, VHL plays the major role.

HIF-1α is stabilized in hypoxia, upon VHL protein loss or VHL inactivation. Therefore, we investigated HIF-1α protein levels in cells transiently transfected with Gam1 and we found that HIF-1α is highly expressed (figure 1).

Moreover, the increased transcriptional rate of *CAIX* mRNA production and the positive outcome of the luciferase assay (figures 2 and 3) confirmed that HIF-1 was active, underscoring that Gam1 is also able to start a hypoxia-like response in cells. Indeed, Gam1-induced SUMOylation inhibition may actively promote HIF-1 transactivation (Bernardi et al., 2006; Cheng et al., 2007b; Huang et al., 2009) even in normoxic conditions.

However, since the binding to VHL itself and other binding partner, such as FIH-1 (Mahon et al., 2001), tightly regulate HIF-1α, we wondered how its activity as transcription factor could be strongly enhanced by Gam1.

Wild-type Gam1 induces VHL protein degradation.

When we investigated VHL protein levels in HeLa cells, we noticed that it markedly decreased in the presence of wild-type, but not L258,265A mutant Gam1, suggesting that the Gam1-induced HIF-1α stabilization may be a direct consequence of this effect. Moreover, HIF-1 transactivation might be enabled by both SUMOylation inhibition and VHL reduction.

However, this unexpected outcome prompted us to focus the causes of VHL reduction, in order to understand the underlying mechanism and the effects of this decrease. In other words, we aim to comprehend whether VHL decrease could be useful either for the host defense or for a successful viral infection.

We demonstrate that Gam1, in spite of its ability to regulated transcription (Chiocca et al., 2002; Colombo et al., 2003), does not alter the transcriptional control of *VHL* mRNA, indicating that VHL reduction occurs at post-transcripitional level. This is in agreement with our results, and with the fact that wild-type VHL usually exhibits a long half-life (Schoenfeld et al., 2000), whereas, after Gam1 expression, its protein levels start to diminish within 3 hours (figure 6).

Since Gam1 does not regulate VHL at the transcriptional level and VHL decrease seems to take place quite rapidly once Gam1 is present, we determined whether VHL decrease is due to proteasome-dependent degradation.

Indeed, we found that proteasome inhibition prevents VHL reduction even in the presence of Gam1 (figure 7), suggesting that this is the mechanism involved. Proteasome-dependent degradation often requires ubiquitylation of the target substrates; therefore we attempted to determine the level of VHL ubiquitination in cells co-transfected with VHL, Gam1 and Ubiquitin. We were able to confirm that VHL is much more ubiquitinated in the presence of wild-type Gam1 than in the presence of the mutant Gam1, and compared to the control (figure 8).

Considering the previous findings, we believe that Gam1 could affect VHL stability in a direct way, because of its Ubiquitin E3 ligase activity, as similarly reported for the viral protein LANA (Cai et al.,

2006). However, further experiments clearly showed that, although VHL and Gam1 are able to interact, they do not assemble in the same E3 ligase complex, indicating that, when interacting, VHL and Gam1 form a novel and independent complex, and binding to the E3 ligase complex subunits or to each other is mutually exclusive. Moreover, Gam1-induced SUMOylation inhibition does not affect VHL protein and Cullin2 and Cullin5 depletion, though inhibits Gam1-based E3 ligase activity, does not restore VHL protein level in the presence of Gam1. All together, these results indicate that substrate-receptor activity is not required for Gam1-induced VHL degradation.

VHL protein is sensitive to the presence of other BC-box containing proteins.

The L258,265A Gam1 mutant does not lead to VHL decrease, implying that a wild-type Gam1 intrinsic feature is essential to determine VHL protein reduction.

We thus asked whether the ability to bind the ElonginB/C heterodimer was the key of VHL instability upon wild-type Gam1 expression. The finding that VHL proteins harboring mutations in the ElonginB/C binding domain are unstable and rapidly degraded by the proteasome (Schoenfeld et al., 2000) support this idea. Moreover, Schoenfeld and colleagues demonstrated that higher availability of ElonginB/C increases the half-life, and hence the stability, of the VHL protein.

We further confirmed this by overexpressing ElonginB and ElonginC and observing that VHL protein level markedly increased. Unfortunately, we were not able to completely rescue VHL protein in the presence of Gam1, probably because, if there is extra availability of ElonginB/C complex, Gam1 will recruit it more.

Nevertheless, if our hypothesis is true, other BC-box containing proteins can have a similar effect on VHL protein stability and the Gam1-induced phenotype is based on cellular mechanisms. Therefore, one possibility is that VHL destabilization occurs in certain situations that can alter cell homeostasis. Indeed, apart from BC-box containing proteins normally expressed, there are others which expression is a consequence of stress stimuli. Consequently, the cytokine-induced SOCS1 and SOCS3 proteins were considered good candidates to test our hypothesis.

In agreement, overexpression of SOCS1 and SOCS3 led to an evident VHL decrease, further suggesting that aberrant recruitment of ElonginB/C reduces the half-life of VHL protein (figure 20).

The strong decreased of VHL upon the expression of EGFP-fused with the SOCS-box domains of SOCS1 or SOCS3 entirely validates this idea, indicating again that competition for ElonginB/C binding negatively affects VHL protein, and leads to stabilization of HIF-1α (figure 21).

Moreover, we demonstrated that the presence of other viral proteins, such as the HPV16 E7 and the Adenovirus type 5 E4orf6, known to possess a BC-box domain, behave as Gam1, SOCS1 and SOCS3, supporting the existence of a common and general cellular mechanism that drives VHL degradation in similar situations.

VHL protein levels in cervical-carcinoma derived cell lines may reflect their different viral (or non viral) background.

The comparison of VHL protein level in three different cervical carcinoma-derived cell lines, namely HPV-negative C33A, HPV18-positive HeLa and HPV16-positive CaSki, may support the previous idea. Indeed, the high-copy HPV16-positive CaSki cells do not display any detectable amount of VHL protein. As previously mentioned, HPV16 E7 was found to contain a BC-box domain and to associate to ElonginB/C and Cullin2 ubiquitin E3 ligase, unlike HPV18 E7. Indeed, HPV18-positive HeLa cells express VHL and not HIF-1α (figure 24). This discrepancy in VHL expression does not seem to be related to a different rate of *VHL* mRNA transcription (figure 25) nor dependent on either pRb or p53 pathways.

However, besides HPV16 E7, also HPV16 E6 was shown to induce HIF-1α stabilization when overexpressed in C33A or HeLa cells (Tang et al., 2007), indicating that some additional mechanisms could induce VHL decrease in CaSki cell line. Indeed, Tang and colleagues demonstrated that inhibition of the signaling cascades initiated by ERK1/2 and PI3K prevents HPV16 E6 and E7-induced HIF-1α stabilization and, thus, VEGF expression (Tang et al., 2007).

Nevertheless, we believe that Gam1-induced VHL degradation, and the subsequent HIF-1α stabilization, may be driven by a mechanism that is mostly related to the inability of VHL to bind ElonginB/C heterodimer when Gam1 is present.

Degradation of unbound VHL may be driven by chaperone-associated mechanisms, but they do not required hsp90 activity.

Sutovsky and Gazit were able to determine the structure of the unbound VHL by spectrometric analysis and to discover that VHL displays a molten globule configuration under native conditions (Sutovsky and Gazit, 2004). This implies that if VHL is not bound to ElonginB/C, it will present marginal stability also in physiological conditions of ionic strength, pH and temperature. Moreover, Feldman and colleagues showed that the unassembled form of VHL associates longer with the hsp70 and TriC chaperones folding complex (Feldman et al., 1999), which may be involved in the pathway of VHL degradation.

Furthermore, Gam1 upregulates the expression of hsp70 and hsp40 proteins (Glotzer et al., 2000), and thus, taken together, it might be that VHL destabilization in the presence of Gam1 could be driven by the molecular chaperone system. This hypothesis is also based on the finding that ectopical expression of VHL in *S. cerevisiae* leads to VHL degradation by a mechanism requiring both hsp90 and hsp70 (McClellan et al., 2005). Therefore, we assessed whether the same machinery, in human cell lines expressing wild-type Gam1, could degrade VHL. Since hsp70 is also necessary for VHL folding (Melville et al., 2003), we impaired hsp90 activity using a specific binding inhibitor called geldanamycin.

However, our results indicate that hsp90 does not affect VHL stability, since hsp90 inhibition does not rescue VHL protein level upon Gam1 expression. Nevertheless, as *S. cerevisiae* does not express any form of VHL protein, it could be possible that the yeast hsp90/hsp70 complex recognizes exogenous unfolded proteins to target for degradation, whereas, in human cells, as VHL is endogenously present, the mechanism may be different. In support of this hypothesis, McClellan and colleagues showed that, in yeast, Sti1, homolog of the mammalian Hop (Smith et al., 1993), plays a key role in VHL degradation (McClellan et al., 2005). However, Wegele and colleagues demonstrated that the mammalian hsp90 machinery differs from the yeast system in the activity of Hop (Wegele et al., 2006). This data may thus explain the differences we observed comparing the published data (McClellan et al., 2005) with our results on VHL stability in the presence of Gam1.

Moreover, VHL destabilization upon heat shock treatment (figure 27) further supports our idea of the involvement of chaperones machinery in VHL degradation upon Gam1 expression. This prompts us to further analyze this pathway in order to define precisely the mechanism of VHL degradation in the context of Gam1 and BC-box proteins expression.

Conclusions and future perspectives.

The data here presented strongly support that competition for the binding to ElonginB/C complex can result in the destabilization of VHL protein followed by stabilization of HIF-1α and activation of HIF-1 transcription factor, leading then to a hypoxia-like response.

Moreover, it underscores the existence of a general cross-regulatory mechanism towards proteins containing the BC-box domain, as already shown for SOCS protein family members (Babon et al., 2009; Piessevaux et al., 2006).

Although the mechanism is not completely clear, we believe that molecular chaperones have a key role in this process. Indeed, considering the likelihood that VHL, upon Gam1 expression, is not bound to ElonginB/C, it will remain associated to TRiC and Hsc70 chaperone complex (Feldman et al., 1999; Melville et al., 2003). Moreover, our data supports that upregulation of hsp70 and hsp40 upon Gam1 expression may favor VHL degradation (figure 27). Hsp40 comprehends a family of co-chaperone proteins that are intimately connected with hsp70 activity. They stimulate the hydrolysis of ATP molecules bound to the active site of hsp70, leading to an increased affinity of hsp70 towards its substrates. Hsp70 client proteins could thus be retained longer in hsp70-based complex, leading to VHL ubiquitylation by the chaperone-dependent Ubiquitin E3 ligase CHIP, as recently shown for hsp70-bound substrates (Stankiewicz et al., 2010). CHIP is a U-box Ubiquitin E3 ligase not present in yeast, underlying once again the difference between yeast and mammalian quality control system.

Molecular chaperones and the Ubiquitin E3 ligase CHIP, indeed, cooperate to promote the degradation of misfolded or damaged proteins, as fully described for mutant cystic fibrosis transmembrane conductance regulator (CFTR) (Meacham et al., 2001). Clearance of irreversible misfolded proteins prevents the formation of toxic protein aggregates and, since native VHL shows a

molten globule structure prone to aggregation, unbound VHL is likely targeted for degradation *via* the Ubiquitin/proteasome pathway through this mechanism.

Besides HIF-1α stabilization, the marked VHL reduction driven by high expression of BC-box proteins could have additional effects, mainly related to other pathways in which VHL is involved, such as microtubules destabilization (Thoma et al., 2009), reduced synthesis of fibronectin (Bluyssen et al., 2004), altered deposition of both fibronectin and collagen IV on the extracellular matrix (Kurban et al., 2008; Ohh et al., 1998), and impaired DNA damage response through reduced levels and low transcriptional activity of p53 protein (Roe et al., 2006).

VHL loss can also induce senescence, but only in those cells that maintain active pRb tumor suppressor and p400 chromatin remodeling proteins (Young et al., 2008a).

VHL degradation upon BC-box proteins expression may be an additional mechanism to rapidly activate HIF-1 in response to certain stimuli, such as inflammation (that induces the expression of SOCS proteins) and viral infections. Indeed, increasing evidences on the role of this transcription factor in the innate immune system have been lately emerging.

For example, Peyssonnaux and colleagues demonstrated that HIF-1 controls the transcription of cathelicidins, broad-spectrum antimicrobial peptides produced by keratinocytes. In fact, mice lacking *hif-1*α gene in skin cells are more susceptible to bacterial infections (Peyssonnaux et al., 2008). Other studies highlight the role of HIF-1 for the correct inflammatory response by myeloid cells (Cramer et al., 2003) and for increasing the lifespan of neutrophils when an infection occurs (Walmsley et al., 2005). Moreover, it was demonstrated that the gram-negative lipopolysaccharide (LPS) is able to activate HIF-1 in macrophages (Blouin et al., 2004; Frede et al., 2006) and, in turn, HIF-1 is essential for the development of LPS-induced sepsis (Peyssonnaux et al., 2007), thus resulting also detrimental rather than protective for the organism involved.

Viruses were also shown to induce HIF-1 activation, but the final outcome for the host can be different. On one hand, it was demonstrated that interferon-induced HIF-1α stabilization, as VHL loss, could cooperate in organizing host defenses (Hwang et al., 2006; Naldini et al., 1993), on the other hand, persistent infections given by some oncogenic viruses, such as hepatitis B and C viruses, human papillomavirus 16, Epstein-Barr virus and human herpesvirus 8 (known also as Kaposi Sarcoma-Associated Herpesvirus), display higher levels of HIF-1 that can sustain tumor growth by the stimulation of angiogenesis (Cai et al., 2006; Moon et al., 2004; Tang et al., 2007; Wakisaka et al.,

2004) . Finally, HIF-1α is stabilized in response to some cytokines and HIF-1 participates in the signaling cascade initiated by these molecules (reviewed in (Haddad and Harb, 2005)).

Therefore, considering the primary role of HIF-1 in immune defense is not surprising that cells have likely evolved an auxiliary mechanism to rapidly sustain the stabilization of HIF-1α, for instance through the degradation of VHL protein in response to decreased availability of ElonginB/C heterodimer.

If verified, this will be an additional example of a cellular mechanism identified by the aid of viral proteins, further underlying their fundamental contribution in our understanding of cellular biology.

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