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**Subcellular trafficking of VHL and O₂ homeostasis:
Discovery of a new nuclear export pathway**

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Subcellular trafficking of VHL and O₂ homeostasis:

Discovery of a new nuclear export pathway

Mireille Khacho

This thesis is submitted as a partial fulfillment of the
Ph.D. program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
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Abstract

Degradation of nuclear proteins by the ubiquitylation system often requires nuclear-cytoplasmic trafficking of E3 ubiquitin-ligases. The von Hippel-Lindau (VHL) tumor suppressor protein is the substrate recognition component of a Cullin-2-containing E3 ubiquitin-ligase that recruits the hypoxia-inducible factor (HIF) for oxygen-dependent degradation. The dynamic properties of VHL are essential for its ability to mediate efficient degradation of HIF. Interestingly, nuclear export of VHL requires ongoing transcription and is independent of the classical NES/CRM1 pathway. Examining this uncharacterized nuclear export pathway led to the identification of a discreet motif, “DxGx₂Dx₂L”, that directs transcription-dependent nuclear export of VHL. The “DxGx₂Dx₂L” motif is also found in other proteins, including Poly(A) Binding Protein (PABP1) to direct transcription-dependent nuclear export. The DxGx₂Dx₂L motif is denoted as TD-NEM (Transcription-Dependent Nuclear Export Motif) since inhibition of transcription by ActD or DRB abrogates its nuclear export activity. In VHL, TD-NEM is targeted by naturally-occurring mutations associated with renal carcinoma and polycythemia in humans. Disease-causing mutations of key residues of TD-NEM restrain the ability of VHL to efficiently mediate oxygen-dependent degradation of HIF by altering its nuclear export dynamics without affecting interaction with its substrate or core components of the E3 ubiquitin-ligase complex. Further studies aimed at understanding the mechanism of TD-NEM-mediated nuclear export led to the identification of a novel VHL and PABP1 interacting protein, the cytoplasmic translation elongation factor eEF1A. eEF1A, which has been implicated in the nuclear export of RNA species in lower eukaryotes, is involved in nuclear export of proteins encoding a TD-NEM in mammalian cells. eEF1A interacts specifically with TD-NEM and disrupting this interaction, by point mutations of the key residues within TD-NEM or siRNA-mediated knockdown of eEF1A, suppresses nuclear export. ActD suppresses eEF1A/TD-NEM interaction and abrogates eEF1A-mediated nuclear export of TD-NEM, providing a possible explanation for the inhibitory effect of ActD on nuclear export of TD-NEM-containing proteins. These results identify a novel and potentially ubiquitous, nuclear export motif, further highlight the role of nuclear-cytoplasmic shuttling of E3 ubiquitin-ligases in degradation of nuclear substrates and provide evidence that disease-causing mutations can target subcellular trafficking. Furthermore, these findings demonstrate that eEF1A, a mediator of RNA export in yeast, has an additional role in the nuclear export of proteins in mammalian cells.

Table of Contents

Abstract.....	ii
Table of Contents.....	iii
List of Figures.....	vii
List of Abbreviations.....	x
List of Publications.....	xiii
Authorizations for the Use of Published Materials.....	xv
Acknowledgments.....	xvi
1. INTRODUCTION.....	1
1.1. Cancer.....	1
1.1.1. Cancer: a complex and dynamic genetic disorder.....	1
1.1.2. The genes of cancer.....	1
1.1.3. Tumor suppressors and Knudson's two-hit model.....	2
1.2. THE VHL TUMOR SUPPRESSOR GENE AND ITS ROLE IN CANCER... 3	
1.2.1. VHL disease.....	3
1.2.2. The VHL tumor suppressor gene in hereditary and sporadic cancer.....	4
1.3. THE VHL TUMOR SUPPRESSOR PROTEIN IS A COMPONENT OF AN E3 UBIQUITIN-LIGASE COMPLEX.....	6
1.3.1. VHL: from gene to protein.....	6
1.3.2. E3 ligase complex links VHL to the ubiquitylation network.....	8
1.3.3. The Ubiquitylation system.....	10
1.3.4. Ubiquitin-mediated proteasomal degradation.....	11
1.3.5. E3 ubiquitin-ligases: key regulators of the ubiquitylation system.....	13
1.4. VHL LINKS THE UBIQUITYLATION PATHWAY TO OXYGEN HOMEOSTASIS.....	14
1.4.1. Oxygen homeostasis and hypoxia.....	14

1.4.2. HIF: a regulator of oxygen homeostasis.....	15
1.4.3. HIF: a substrate for the VBC-Cul2 E3 ubiquitin-ligase complex.....	16
1.5. VHL MEDIATES OXYGEN & pH-DEPENDENT REGULATION OF HIF.	19
1.5.1. VHL targets HIF α for O ₂ -dependent proteasomal degradation.....	19
1.5.2. Regulation of VHL/HIF interaction by proline hydroxylation.....	19
1.5.3. pH-dependent regulation of HIF.....	22
1.6. THE ROLE OF VHL IN CANCER.....	24
1.6.1. VHL loss abolishes O ₂ -mediated regulation of HIF.....	24
1.6.2. HIF regulation and cancer.....	24
1.6.3. Other VHL functions and their role in cancer.....	27
1.7. SUBCELLULAR DYNAMICS OF E3 UBIQUITIN-LIGASES:	
A REQUIREMENT FOR PROTEIN FUNCTION.....	28
1.7.1. Nuclear-cytoplasmic trafficking.....	28
1.7.2. Signal mediated nuclear import and export of proteins.....	29
1.7.3. Subcellular dynamics is essential for the function of proteins.....	30
1.7.4. Nuclear-cytoplasmic trafficking of E3 ubiquitin-ligases is required for degradation of nuclear substrates.....	33
1.8. SUBCELLULAR DYNAMICS OF VHL IS REQUIRED FOR ITS E3 UBIQUITIN-LIGASE AND TUMOR SUPPRESSOR FUNCTION.....	35
1.8.1. Subcellular localization of VHL: clues to subcellular trafficking properties.....	35
1.8.2. Transcription-dependent nuclear-cytoplasmic trafficking of VHL.....	36
1.8.3. Subcellular dynamics of VHL is regulated by extracellular H ⁺	39
1.8.4. VHL undergoes static detention by the nucleolar architecture.....	39
1.8.5. Transcription-dependent trafficking of VHL is required for HIF degradation.....	40

1.9. CLASSICAL VERSUS TRANSCRIPTION-DEPENDENT NUCLEAR EXPORT PATHWAYS.....	43
1.9.1. Nuclear pore complexes: A gateway through the nuclear envelope.....	43
1.9.2. Classical pathway for nuclear export of proteins.....	47
1.9.3. Other nuclear export pathways and the associated transporters.....	50
1.9.4. Transcription-dependent nuclear export: An undefined and CRM1-independent pathway.....	51
2. RATIONALE.....	53
3. HYPOTHESIS.....	54
4. AIMS.....	54
5. MATERIALS AND METHODS.....	55
6. RESULTS.....	64
6.1. VHL encodes a transcription-dependent nuclear export signal.....	64
6.2. Establishing a live cell nuclear export assay using photobleaching technology.....	67
6.3. Nuclear export of VHL is mediated by a discrete transcription-dependent motif.....	70
6.4. VHL and PABP1 share a common transcription-dependent nuclear export motif.....	72
6.5. Conserved residues of TD-NEM are essential for mediating efficient nuclear export of proteins.....	77
6.6. Cancer-causing mutations within TD-NEM abrogate nuclear export of VHL and oxygen-dependent degradation of HIF α	79
6.7. TD-NEM mediates efficient transcription-dependent nuclear export of proteins.....	82
6.8. The translation elongation factor eEF1A is a novel VHL and PABP1	

interacting protein.....	84
6.9. eEF1A is required for nuclear export of proteins encoding a TD-NEM.....	87
6.10. eEF1A mediates nuclear export from the cytoplasmic side of the nuclear envelope.....	96
6.11. eEF1A specifically interacts with the TD-NEM nuclear export sequence.....	99
6.12. eEF1A is required for nuclear export of TD-NEM.....	99
6.13. TD-NEM is present and functional in a wide spectrum of highly diverse proteins.....	101
7. DISCUSSION.....	105
8. CONCLUSIONS AND SIGNIFICANCE OF FINDINGS.....	120
9. FUTURE DIRECTIONS.....	122
10. REFERENCES.....	124
11. APPENDIX A- First author Publications	
12. APPENDIX B- Coauthor Publications and Published Highlights	

List of Figures

Figure 1. Inactivating mutations in the VHL tumor suppressor gene leads to disease...	5
Figure 2. VHL: from gene to protein.....	7
Figure 3. The VBC-Cul2 complex is similar to the yeast SCF complex.....	9
Figure 4. The ubiquitin-mediated degradation pathway.....	12
Figure 5. The HIF transcription factor is a key regulator of oxygen homeostasis.....	17
Figure 6. The VHL tumor suppressor protein is a component of an E3 ubiquitin-ligase complex that targets HIF α for proteasomal degradation.....	18
Figure 7. The VHL tumor suppressor protein targets HIF α for oxygen-dependent proteasomal degradation.....	21
Figure 8. pH-dependent regulation of HIF α	23
Figure 9. Loss of VHL function abolishes oxygen-dependent HIF α regulation.....	25
Figure 10. Nuclear import and export of proteins.....	31
Figure 11. Trafficking of E3 ubiquitin-ligases is required for degradation of nuclear proteins.....	34
Figure 12. Transcription-dependent subcellular trafficking of VHL.....	38
Figure 13. Nuclear-cytoplasmic trafficking of VHL is required for efficient degradation of HIF α	42
Figure 14. pH-dependent nucleolar detention of VHL prevents subcellular trafficking and HIF α degradation.....	44
Figure 15. The nuclear pore complex.....	45
Figure 16. The classical NES/CRM1 nuclear export pathway.....	48
Figure 17. Transcription-dependent nuclear export of VHL.....	65
Figure 18. The live cell FLIP nuclear export assay.....	68

<i>vitro</i>	95
Figure 33. The function of eEF1A in nuclear export of TD-NEM-containing proteins is Exp5-independent and exerted from the cytoplasmic side of the nuclear envelope.....	97
Figure 34. TD-NEM is sufficient for eEF1A interaction.....	100
Figure 35. eEF1A is required for TD-NEM-mediated nuclear export.....	102
Figure 36. TD-NEM is a functionally conserved nuclear export sequence.....	104
Figure 37. TD-NEM-mediated nuclear export of VHL is essential for efficient oxygen-dependent degradation of HIF α	109
Figure 38. The TD-NEM-mediated nuclear export pathway.....	119

List of Abbreviations

VHL	von Hippel-Lindau
RCC	renal clear cell carcinoma
Cul2	Cullin-2
VBC-Cul2	VHL-elongins B/C-Cullin-2 E3 ubiquitin-ligase complex
SCF	Skp1-Cdc53-F-box E3 ubiquitin-ligase complex
ATP	adenosine triphosphate
Mdm2	Murine double minute 2
HIF	Hypoxia inducible factor
EPO	erythropoietin
HRE	hypoxia response element
VEGF	Vascular endothelial growth factor
GLUT1	Glucose transporter 1
TGF α	Transforming growth factor alpha
ODDD	oxygen-dependent degradation domain
PHD	prolyl hydroxylase
EGFR	Epidermal growth factor receptor
ECM	extracellular matrix
NPC	Nuclear pore complex
NLS	Nuclear localization signal
NES	Nuclear export sequence
NTF2	nuclear transport factor 2
APC	adenomatous polyposis coli tumor suppressor protein

INI1	integrator interactor 1 tumor suppressor protein
RNA PolII	RNA Polymerase II
ActD	Actinomycin D
DRB	5,6-dichlorobenzimidazole
NoDS ^{H+}	Nucleolar Detention Signal mediated by H ⁺
TR	Transport receptor
FG	phenylalanine and glycine repeats
CRM1	Chromosome Region Maintenance or exportin 1
LMB	Leptomycin B
eEF1A	Eukaryotic translation elongation factor 1 alpha
PABP1	Poly(A)-binding protein 1
GFP	Green fluorescent protein
FLIP	Fluorescence loss in photobleaching
PK	Pyruvate kinase
TD-NEM	Transcription-dependent nuclear export motif
VSV	vesicular stomatitis virus
WCL	Whole cell lysate
PI	propidium iodide
siRNA	Small interfering RNA
CHX	Cycloheximide
Exp5	Exportin-5
SD	Standard media
AP	Acidosis permissive media

eEF2	Eukaryotic elongation factor 2
PR	Progesterone receptor
RanBP1	Ran Binding Protein 1

List of Publications

First Author Publications:

1. **Khacho M.**, Mekhail K., Pilon-Larose K. and Lee S. Cancer-causing mutations in a novel transcription-dependent nuclear export motif of VHL abrogate oxygen-dependent degradation of HIF. *Molecular and Cellular Biology*. 28(1):302-14 (2008).
2. **Khacho M.**, Mekhail K., Pilon-Larose K., Pause A., Cote J. and Lee S. eEF1A is a novel component of the mammalian nuclear protein export pathway. *Molecular Biology of the Cell*. 19(12):5296-308 (2008).
3. **Khacho M.** and Lee S. Subcellular dynamics of the VHL tumor suppressor: On the move for HIF degradation. *Future Oncology*. 5(1):85-95 (2009).

Collaborative Publications:

1. Mekhail K., **Khacho M.**, Gunaratnam L., and Lee S. Oxygen sensing by H⁺: implications for HIF and hypoxic cell memory. *Cell Cycle*. 8:1027-9 (2004).
2. Mekhail K., **Khacho M.**, Carrigan A., Hache RR., Gunaratnam L., and Lee S. Regulation of ubiquitin ligase dynamics by the nucleolus. *Journal of Cell Biology*. 170(5):733-44 (2005).
 - See "IN THIS ISSUE": Hiding proteins in the nucleolus. *Journal of Cell Biology*. 170:696 (2005).
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 - Paper is cited in the Faculty of 1000 where it was included in the Top Ten Hidden Jewels List (October 2005).

- Second most read citation in the Faculty of 1000 Neuroscience (October 2005)
- 3. Mekhail K., Rivero-Lopez L., **Khacho M.**, and Lee S. Restriction of rRNA synthesis by VHL maintains energy equilibrium under hypoxia. Cell Cycle. 5(20):2401-13 (2006).
- 4. Mekhail K., Rivero-Lopez L., Al-Masri A., Brandon C., **Khacho M.**, and Lee S. Identification of a common subnuclear localization signal. Molecular Biology of the Cell. 18(10): 3966-77 (2007).

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1. INTRODUCTION

1.1. CANCER

1.1.1. Cancer: a complex and dynamic genetic disorder.

Cancer is one of the leading causes of premature death in Canada, with an estimated 159, 900 new cases and 72, 700 deaths in 2007 (as reported by the Canadian Cancer Statistics). Cancer is a genetic disorder that arises through dynamic alterations of the genome which ultimately results in uncontrolled cell growth (Kinzler and Vogelstein, 1996; Nowell, 1976). Extensive research in this field has demonstrated that cancer is a multifactorial disease and the paths taken by cells on their route to becoming malignant are highly variable. To date there are over 100 genes implicated in tumor development such as those involved in cell cycle control, DNA repair, apoptosis and angiogenesis. As described by Hanahan and Weinberg, mutations in these cancer susceptibility genes manifest six fundamental changes in cell physiology that lead to malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis (new blood vessel formation) and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

1.1.2. The genes of cancer.

Of all the genes present in the human genome, there is a small subset of genes that appear to be of particular importance in the prevention, development and progression of cancer. These genes have been divided into two broad categories depending on their normal cellular function and are classified as follows; genes that promote growth are termed ‘oncogenes’ and those involved in growth suppression are known as ‘tumor suppressor

genes' (Fearon and Vogelstein, 1990). An extensive analysis of the functionality of these genes has demonstrated that oncogenes and tumor suppressor genes generally code for growth factors and growth factor receptors, transcription factors, cell cycle regulators, DNA repair proteins, apoptosis mediators and proteins involved in metastasis and invasion. Tumors generally develop and progress as a consequence of dominant gain of function mutations in oncogenes and/or recessive loss of function mutations in tumor suppressor genes. In fact the mutational alteration of oncogenes and tumor suppressor genes has been a tool in their identification in human and animal cancer cells.

1.1.3. Tumor suppressors and Knudson's two-hit model.

The existence of 'anti-cancer' genes, which were later named tumor suppressors, became evident in 1969 during a series of somatic cell hybrid fusion experiments (Harris et al., 1969). These experiments demonstrated that A9 cells were able to suppress the tumorigenic phenotype of malignant cells, indicating the likely existence of an anti-cancer factor in A9 cells which was lost in malignant cells. These experiments suggested that tumor suppressor genes were recessive in nature and must be completely inactivated for malignancy to arise. Subsequent work by Al Knudson led to the 'two-hit' model of tumorigenesis, which was first confirmed in retinoblastoma (Knudson, 1971; Knudson, 1985). According to Knudson, retinoblastoma occurs in retinal cells as a result of two genetic events, which lead to the inactivation of both copies of the retinoblastoma tumor suppressor gene (Knudson, 1985). He hypothesized that the inherited (familial) and the non-inherited (sporadic) forms of retinoblastoma are mechanistically linked. Basically, Knudson's two-hit model states that in the familial form of cancer, the affected individual inherits a mutated copy of the gene from one parent and a normal copy, present in all

cells, from the other parent. A subsequent somatic mutation in the target tissue inactivates the normal gene and leads to tumorigenesis (Knudson, 1971; Knudson, 1985). In sporadic cancers, however, both inactivating mutations must occur in the same somatic cell (Knudson, 1971). In this sense, the frequency of tumorigenesis is likely to be greater in individuals who carry a heterozygous mutation in their germline, which would serve as a predisposition to cancer. Although inheritance of one genetic defect predisposes individuals to cancer, this does not directly cause cancer since a second event is required.

1.2. THE VHL TUMOR SUPPRESSOR GENE AND ITS ROLE IN CANCER

1.2.1. VHL disease.

The von Hippel-Lindau (VHL) disease is a dominantly inherited cancer syndrome first described in the medical literature more than 100 years ago by a German ophthalmologist Eugen von Hippel, a Swedish neuropathologist Arvid Lindau, and a British surgeon E. Treacher Collins (Collins, 1894; von Hippel, 1904; Lindau, 1927). This disorder affects 1 in 36 000 individuals and has over 90% penetrance by the age of 65, with no ethnic, racial, cultural, or sexual gender bias. VHL disease is characterized by the presence of hypervascular tumors in multiple organs, including the central nervous system (cerebellum, brainstem and spinal cord), retina, pancreas, adrenal gland, endolymphatic sac of the inner ear, epididymis (male), broad ligament (female), and kidneys (Maher and Kaelin, 1997; Ohh and Kaelin, 2003; Richard et al., 1998). Although the majority of tumors associated with VHL disease are benign, kidney cancer is malignant and is the principle cause of morbidity and mortality for VHL patients (Ohh and Kaelin, 2003). Families afflicted with VHL disease have been classified into subcategories depending on

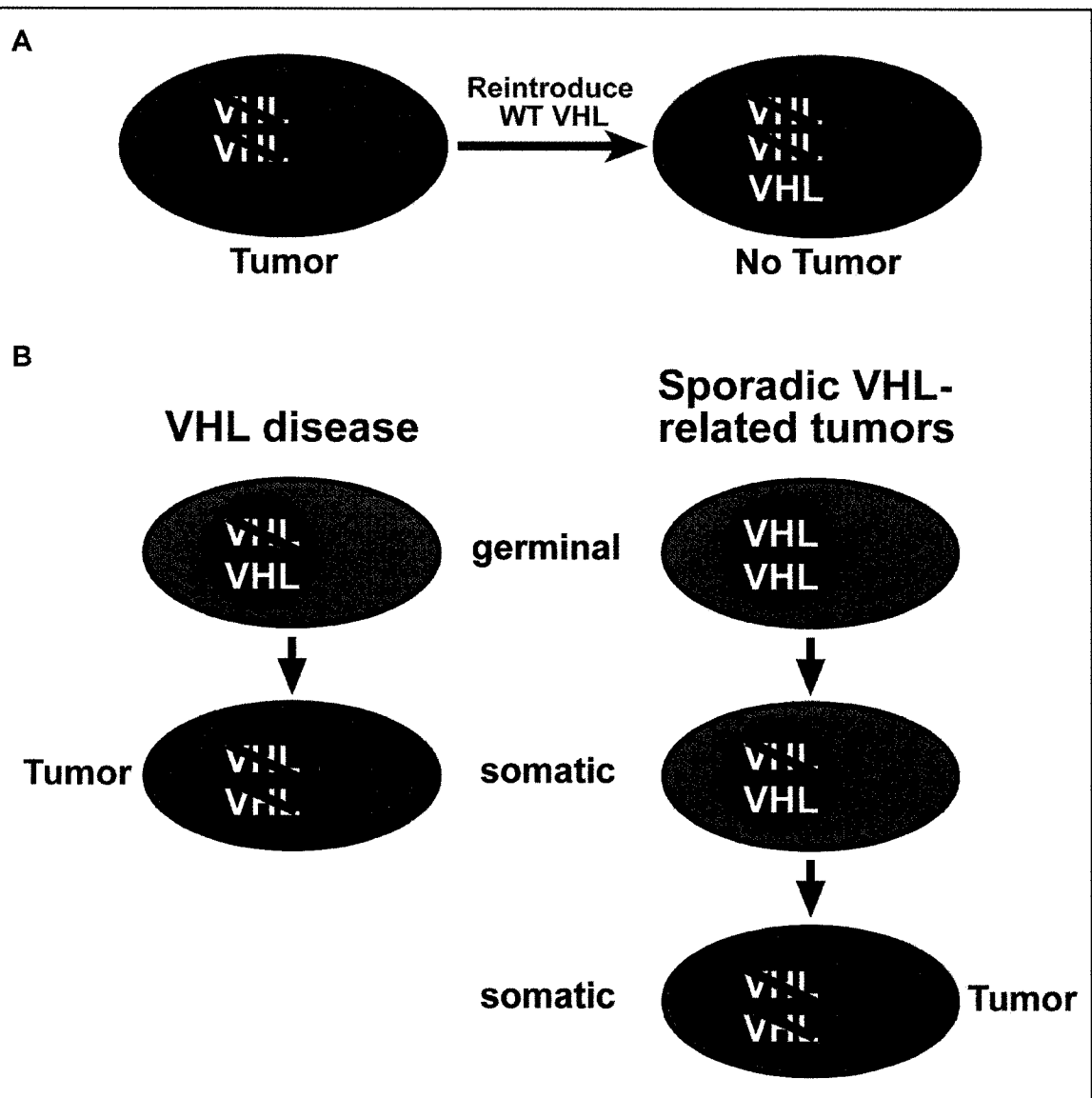
the patients' likelihood of developing pheochromocytoma, a neuroendocrine tumor arising from the adrenal medulla (Brauch et al., 1995; Chen et al., 1995; Maher and Kaelin, 1997). Type 1 VHL disease describes families with a low risk of pheochromocytoma while Type 2 VHL disease indicates those with a high risk of pheochromocytoma. Type 2 VHL disease is also further subdivided into low risk (Type 2A) or high risk (Type 2B) of kidney cancer. VHL families with a high risk of pheochromocytoma without the other classical characteristics of VHL disease are classified as Type 2C.

1.2.2. The VHL tumor suppressor gene in hereditary and sporadic cancer.

VHL disease is caused by inactivation of the VHL gene, which was identified by Latif *et al.* in 1993 (Latif et al., 1993). VHL was classified as a bona fide tumor suppressor after experiments showed that restoration of functional VHL in tumor cells that lacked the wild-type VHL gene suppressed tumorigenesis *in vivo* (Figure 1A) (Gnarra et al., 1996; Iliopoulos et al., 1995). Conforming to Knudson's 2-hit model, predisposition to VHL disease results from a germ-line mutation of the VHL tumor suppressor gene (Figure 1B). Tumors arise in VHL families when the remaining wild-type VHL allele is mutated or inactivated in a susceptible cell. In keeping with Knudson's 2-hit model, biallelic inactivation of VHL is commonly observed in sporadic renal cell carcinomas (RCC, kidney cancer) (Figure 1B) (Foster et al., 1994; Gallou et al., 1999; Gnarra et al., 1994; Kim and Kaelin, 2004; Ohh and Kaelin, 2003; Zhuang et al., 1996). Large studies have identified tumor-derived mutations throughout most of the VHL gene and observed striking correlations between certain mutations and their clinical manifestations. Such

Figure 1. Inactivating mutations in the VHL tumor suppressor gene leads to disease.

A) Reintroduction of VHL into VHL-deficient tumor cells prevents tumorigenesis *in vivo*, a characteristic shared by tumor suppressors. **B)** Following Knudson's 2-hit model, predisposition to VHL disease results from a germ-line mutation of the VHL tumor suppressor gene. A second hit to the remaining wild-type VHL allele gives rise to tumors. Sporadic VHL tumors arise through somatic biallelic inactivation of VHL.



genotype-phenotype correlations have revealed that mutations associated with Type 1 VHL disease are usually deletions, microinsertions, and nonsense mutations, whereas mutations associated with the Type 2 disease are commonly missense mutations (Chen et al., 1995; Neumann and Bender, 1998; Zbar et al., 1996). Various VHL mutations, not associated with VHL disease, have also been identified in congenital Chuvash polycythemia, an autosomal recessive disorder leading to the overproduction of red blood cells (Ang et al., 2002; Gordeuk et al., 2004; Pastore et al., 2003a; Pastore et al., 2003b).

1.3. THE VHL TUMOR SUPPRESSOR PROTEIN IS A COMPONENT OF AN E3 UBIQUITIN-LIGASE COMPLEX

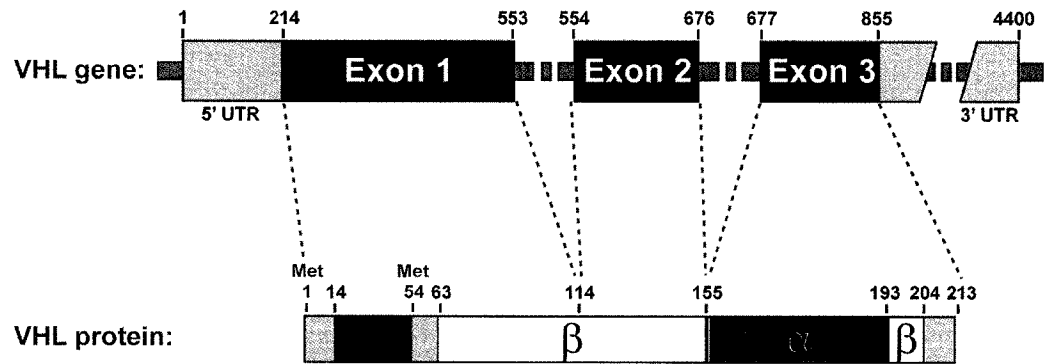
1.3.1. VHL: from gene to protein.

Linkage studies performed in the late 1980's showed that the gene encoding human VHL is located on chromosome 3p25-26 (Hosoe et al., 1990; Seizinger et al., 1988). The VHL gene is relatively small, consisting of three exons (Figure 2A), and is conserved among rodents, flies and worms. The human VHL gene is translated into a protein of 213 amino acids, which migrates at an apparent molecular weight of approximately 24-30 kDa (Figure 2A) (Iliopoulos et al., 1995). A second product of the VHL gene is also generated by an internal translation initiation from a methionine at codon 54 (Figure 2A). Several studies have demonstrated that both isoforms act similarly in biochemical and functional studies (Blankenship et al., 1999; Iliopoulos et al., 1998; Schoenfeld et al., 1998). Furthermore, both isoforms are capable of suppressing tumor development in mouse xenograft assays (Blankenship et al., 1999; Gnarra et al., 1996; Iliopoulos et al., 1995; Schoenfeld et al., 1998). For these reasons and for simplicity's sake, both of these

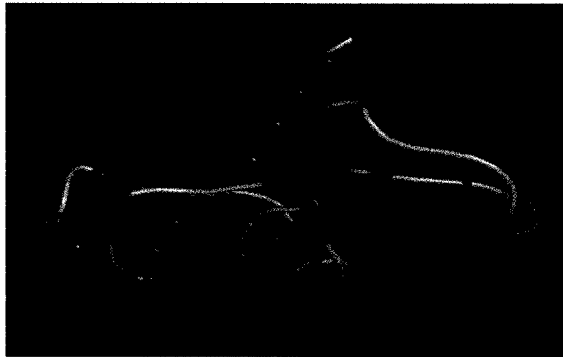
Figure 2. VHL: from gene to protein.

A) Schematic diagram representing the VHL gene and protein. VHL is a 213 amino acid protein encoded by three exons. Met1 and Met54 (in red) indicate the two in-frame methionines. **B)** A model of the VHL protein with colors representing different tertiary structures; red=helix, gray=coil, blue=strand, and green=turn (Min et al., 2002).

A



B



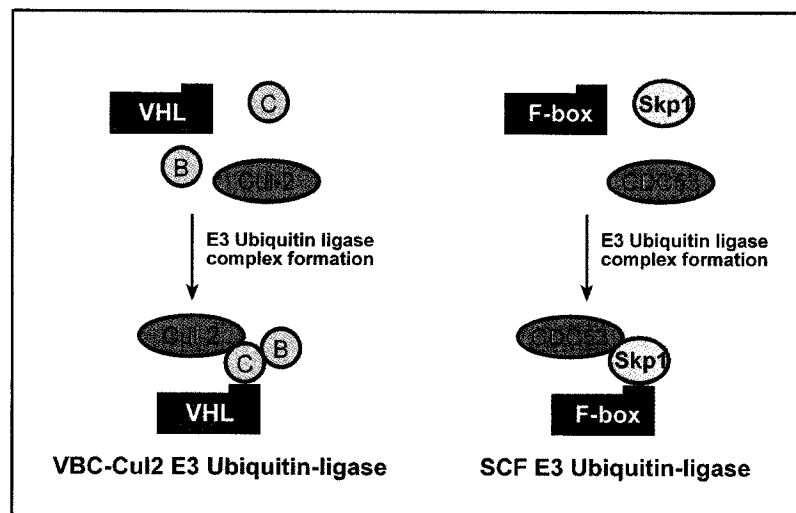
gene products will be referred to as VHL. Three-dimensional structural analysis of VHL identified two major structural domains, the α - and β -domains (Figure 2B) (Min et al., 2002; Stebbins et al., 1999). The β -domain consists of a seven-stranded β sandwich within amino acids 63-154 and one α -helix between amino acids 193-204. The smaller α -domain consists of three α -helices between residues 155-192. Interestingly, the α - and β -domains are considered 'hot spots' since VHL disease-associated mutations are frequently mapped to the surface residues on either domain (Richards et al., 1995). These observations suggested that these domains are critical for the tumor suppressor function of VHL.

1.3.2. E3 ligase complex links VHL to the ubiquitylation network.

Early on it was observed that VHL was not homologous, at the nucleotide or the amino acid levels, to any other known protein. Thus the identification of VHL-interacting proteins became essential in order to decipher its cellular function. Following a series of biochemical studies it was determined that VHL forms a multiprotein complex with elongin B, elongin C, Cullin-2 (Cul2), and Rbx1 (also known as ROC1/Hrt1) (Figure 3) (Duan et al., 1995b; Kamura et al., 2000; Kibel et al., 1995; Lonergan et al., 1998; Pause et al., 1997; Pause et al., 1999). Together, this multiprotein assembly is referred to as the VBC-Cul2 complex. Through mutagenic analysis and peptide competition experiments it has been shown that elongin C binds the alpha domain of VHL, within residues 157-171. In turn, elongin C serves as a docking site for Cul2 binding, where the interaction is stabilized in the presence of elongin B (Duan et al., 1995a; Duan et al., 1995b; Kibel et al., 1995; Kishida et al., 1995; Lonergan et al., 1998; Ohh et al., 1999; Pause et al., 1999). As mentioned previously, the α -domain of VHL is frequently altered in families with

Figure 3. The VBC-Cul2 complex is similar to the yeast SCF complex.

VHL assembles into a complex with elongins B, C and Cullin-2, known as VBC-Cul2, which strongly resembles the yeast SCF E3 ubiquitin-ligase complex.



VHL disease suggesting that formation of this complex is essential for the function of VHL. Interestingly, it was noticed that the primary sequences of elongin C and Cul2 resemble the yeast proteins Skp1 and Cdc53, respectively (Bai et al., 1996). These proteins interact with an F-box protein to form an SCF (Skp1-Cdc53-F-box) E3 ubiquitin-ligase complex, which targets specific proteins for polyubiquitylation (Figure 3) (Deshaies, 1999). Within the SCF complex it has been shown that Rbx1 recruits an E2 ubiquitin-conjugating enzyme while the F-box protein serves as the substrate recognition module to provide specificity to the ubiquitylation system. These observations led to the emergence of a model in which the VBC-Cul2 complex may act as an E3 ubiquitin-ligase and that VHL, specifically, might act as recognition motif in a manner that is analogous to an F-box protein. This view was strengthened by several lines of evidence which showed that: 1- the crystal structure of the VBC-Cul2 complex strongly resembled that of the SCF complex (Stebbins et al., 1999); 2- VHL immunoprecipitates contained ubiquitin-ligase activity in the presence of an E2 ubiquitin-conjugating enzyme (Iwai et al., 1999; Lisztwan et al., 1999); 3- Rbx1, a protein implicated in the SCF ubiquitin-ligase function, is also associated with VBC-Cul2 (Kamura et al., 1999). Together these findings indicated the involvement of VHL in the ubiquitylation network.

1.3.3. The Ubiquitylation system.

Ubiquitylation is a multiprotein pathway that destines marked proteins for degradation by the 26S proteasome (Hershko and Ciechanover, 1998; Weissman, 2001). This system of ubiquitin-mediated proteolysis is essential for the regulation of basic cellular processes by controlling the levels of many different proteins within the cell, such as cell-cycle regulators, transcription factors and signaling proteins. Proteins are marked for

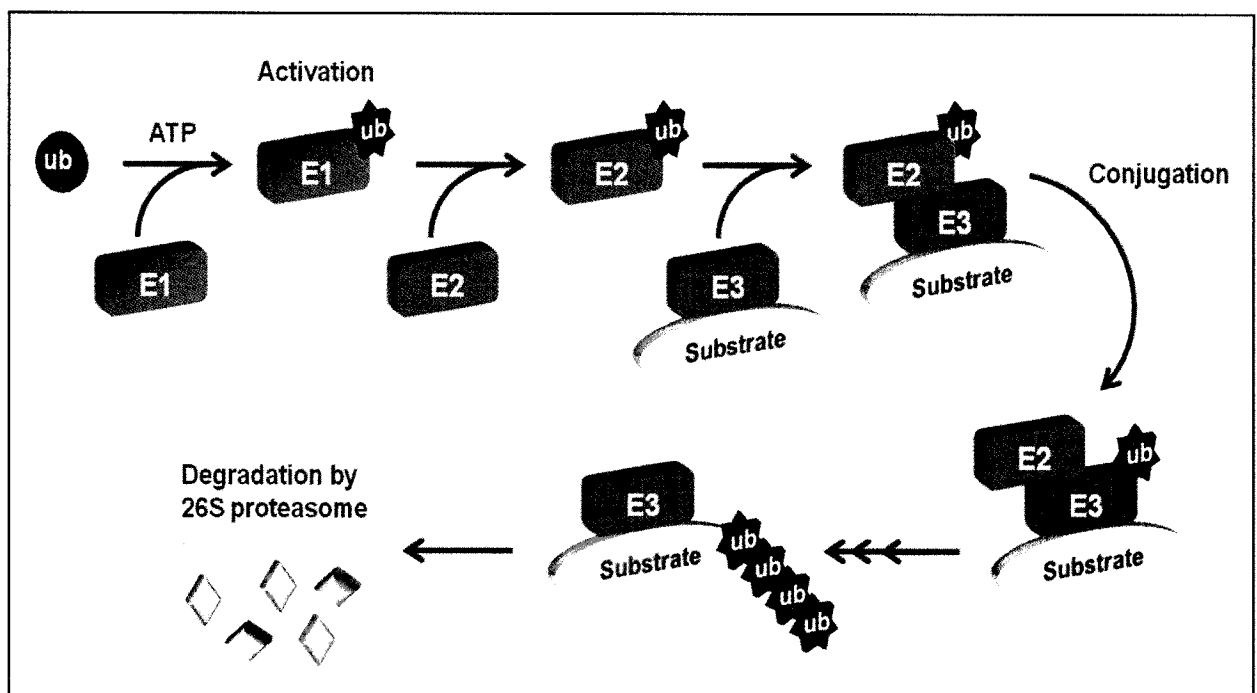
degradation following the covalent attachment of the small protein ubiquitin (Figure 4). The conjugation of ubiquitin to substrate proteins requires the action of three different enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligases (Weissman, 2001). The process of ubiquitylation begins when the ubiquitin molecule is activated by adenosine triphosphate (ATP) and loaded onto the E1 ubiquitin-activating enzyme through a high-energy thiol ester bond at the carboxy-terminal glycine of ubiquitin (Figure 4). This is followed by the transfer of activated ubiquitin from the E1 ubiquitin-activating enzyme to the E2 ubiquitin-conjugating enzyme by a transthioylation reaction involving the carboxy-terminus of ubiquitin (Figure 4). Finally, the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to the ϵ -amino group of a lysine residue in the target substrate is catalyzed by the E3 ubiquitin-ligase (Figure 4). The formation of polyubiquitin chains are generated through isopeptide bonds between the C-terminal glycine and lysine 48 of conjugated ubiquitin molecules.

1.3.4. Ubiquitin-mediated proteasomal degradation.

Ubiquitylation of a substrate protein marks it for degradation by a multisubunit, ATP-dependent protease known as the 26S proteasome (Coux et al., 1996; Klotzel, 2001). This multiprotein complex includes a catalytic component consisting of four stacked rings, made up of 28 subunits, to form a cylindrical chamber (the 20S core) (Groll et al., 1997). The two outer rings act as 'gates' to allow the entry of substrates and release of products, while the two inner rings harbor peptidase-like activities required for degradation of proteins. The 20S core is capped at either end by a multisubunit regulatory complex, the 19S cap, which is divided into two components, the 'base' and the 'lid'. The regulatory 19S cap is involved in recognition of polyubiquitin chains on

Figure 4. The ubiquitin-mediated degradation pathway.

Schematic diagram summarizing the ubiquitylation pathway that leads to proteasomal degradation of marked substrates. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-ligase.



the target proteins and its six ATPases are thought to possess chaperon-like activity utilized in the unfolding of protein substrates (Braun et al., 1999; Glickman et al., 1999; Strickland et al., 2000). After a substrate is tagged with a polyubiquitin chain it is recognized by the 19S regulatory cap (Deveraux et al., 1994; Glickman et al., 1998). The substrate is then unfolded and passed into the interior of the 20S core where proteolysis occurs (Chen and Hochstrasser, 1996; Fenteany et al., 1995).

1.3.5. E3 ubiquitin-ligases: key regulators of the ubiquitylation system.

Within a cell, the number of proteins involved in the ubiquitylation pathway increases with each step, such that there are many more E3 ubiquitin-ligases than there are E2 ubiquitin-conjugating enzymes or E1 ubiquitin-activating enzymes (Sun, 2006; Weissman, 2001). This increased number of participating proteins allows an equivocal increase in the specificity of the pathway. With this in mind, it can be understood how selectivity of this pathway relies heavily on E3 ubiquitin-ligases, which ultimately dictate substrate specificity. E3 ubiquitin-ligases can act individually or form a multisubunit complex that may include a member of the Cullin family of proteins to covalently modify a vast array of cellular proteins (Petroski and Deshaies, 2005; Weissman, 2001). Due to the fact that E3 ubiquitin-ligases serve as the substrate-recognition element their function is highly important for progression of the ubiquitin-mediated degradation cascade. In view of the essential role of E3 ubiquitin-ligases in regulation of many aspects of cellular functions and biological processes, there is mounting evidence that loss of function or deregulation of E3 ubiquitin-ligases contribute to the development of disease, including cancer (Chen et al., 2006; Mani and Gelmann, 2005; Nakayama and Nakayama, 2006; Sun, 2006). For example, the Mdm2 (Murine double minute 2) E3 ubiquitin-ligase,

which targets the p53 tumor suppressor protein for proteasomal degradation, has been found to be deregulated in a variety of human cancers, including breast carcinomas, soft tissue sarcomas, esophageal carcinomas, lung carcinoma, glioblastomas and malignant melanomas (Freedman et al., 1999; Momand et al., 1998; Momand et al., 1992; Zhang and Wang, 2000). Skp2, a component of the SCF ubiquitin-ligase complex, acts as an oncogene by targeting the p27 cell cycle regulator for ubiquitin-mediated degradation (Tsuetkov et al., 1999; Zheng et al., 2002). Evidence for overexpression of Skp2 has been documented in major human cancers targeting the breast, colon, lung, brain, prostate, and liver, among many others (Masuda et al., 2002; Shim et al., 2003; Signoretti et al., 2002; Yokoi et al., 2002).

1.4. VHL LINKS THE UBIQUITYLATION PATHWAY TO OXYGEN HOMEOSTASIS

1.4.1. Oxygen homeostasis and hypoxia.

The delicate balance of oxygen demand and supply, known as oxygen homeostasis, is a tightly regulated phenomenon and is critical for human development and physiology (Giaccia et al., 2004). In fact, one of the most fundamental adaptations imposed on biological organisms is the ability to respond to low oxygen levels (hypoxia) (Dang and Semenza, 1999). Hypoxia occurs due to an imbalance between the amount of oxygen supplied to the organism and the oxygen demand. Hypoxia is most often observed during ischemic diseases, such as cardiac arrest or stroke, muscle fatigue, and cancer (Harris, 2002; Thomlinson and Gray, 1955; Wagner, 2001). Under these circumstances, cells aim to reestablish equilibrium by activating pathways that lead to increased oxygen supply. Such pathways involve the transcriptional upregulation of a large group of genes that

encode proteins involved in oxygen homeostasis, angiogenesis and glucose metabolism, by the hypoxia inducible factors (HIFs) (Semenza, 1999; Semenza, 2003).

1.4.2. HIF: a regulator of oxygen homeostasis.

The quest for understanding the molecular mechanisms involved in oxygen homeostasis began with a striking observation- the induction of the erythropoietin (EPO) gene, which encodes a hormone that stimulates red blood cells production, in response to hypoxia (Safran and Kaelin, 2003; Weidemann and Johnson, 2008). The dramatic increase in EPO mRNA and protein levels, in response to low blood oxygen levels, activates erythropoietic responses that directly increase blood oxygen transport. Studies by Semenza and Wang that dissected DNA-protein interactions at the 3' enhancer of the EPO gene identified a protein complex bound only during hypoxia, which was named HIF-1 (Semenza and Wang, 1992). It was later determined that HIF is in fact a key regulator of oxygen homeostasis and involved in the alteration of over 100 genes in response to hypoxia (Maxwell et al., 2001). HIF is a heterodimeric complex composed of two basic helix-loop-helix proteins of the PAS family (Per/Arnt/SIM family); a constitutively expressed HIF β subunit and one of three hypoxia-inducible HIF α subunits (HIF1 α , HIF2 α or HIF3 α) (Semenza, 2000b; Wang et al., 1995). Together, these proteins act as a DNA-binding complex and bind to a core pentanucleotide sequence, composed of amino acids RCGTG, in the hypoxia response elements (HREs) of target genes, to induce transcriptional activity (Semenza and Wang, 1992; Wang et al., 1995). There are more than 60 genes which are directly targeted by HIF for transcriptional activation (Maxwell et al., 2001; Semenza, 2003). These genes generally encode proteins involved in angiogenesis, glucose metabolism, pH regulation, cell survival and cell proliferation,

such as the vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), and the transforming growth factor- α (TGF α) just to name a few (Figure 5).

1.4.3. HIF: a substrate for the VBC-Cul2 E3 ubiquitin-ligase complex.

One of the most noticeable characteristic of VHL tumors is their highly vascular nature. In fact, VHL tumors, such as haemangioblastomas and renal cell carcinomas, were observed to overproduce VEGF and EPO, both of which are hypoxia inducible genes (Krieg et al., 1998; Witzigmann-Voos et al., 1995). These observations prompted further studies that demonstrated an overproduction of hypoxia-inducible mRNAs, such as VEGF, in VHL-defective tumor cells, regardless of oxygen levels (Gnarra et al., 1996; Iliopoulos et al., 1996; Siemeister et al., 1996; Stratmann et al., 1997). Interestingly, the levels of such mRNAs could be restored to normalcy upon reintroduction of functional VHL (Iliopoulos et al., 1996). As mentioned previously, these mRNA species are normally under the control of the HIF transcription factor. In turn, HIF-1 α protein expression was shown to be stabilized in hypoxia, but undergo proteasomal degradation under normoxic conditions (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997; Wang et al., 1995). Several studies then showed that the HIF α subunit is polyubiquitylated in the presence of VHL but accumulates in VHL defective cells, leading to the upregulation of HIF downstream target genes (Cockman et al., 2000; Kamura et al., 2000; Maxwell et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000). This led to the identification of HIF α as a substrate of the VBC-Cul2 E3 ubiquitin-ligase (Figure 6).

Figure 5. The HIF transcription factor is a key regulator of oxygen homeostasis.

Schematic representation of the different cellular processes altered by the HIF transcription factor in response to hypoxia.

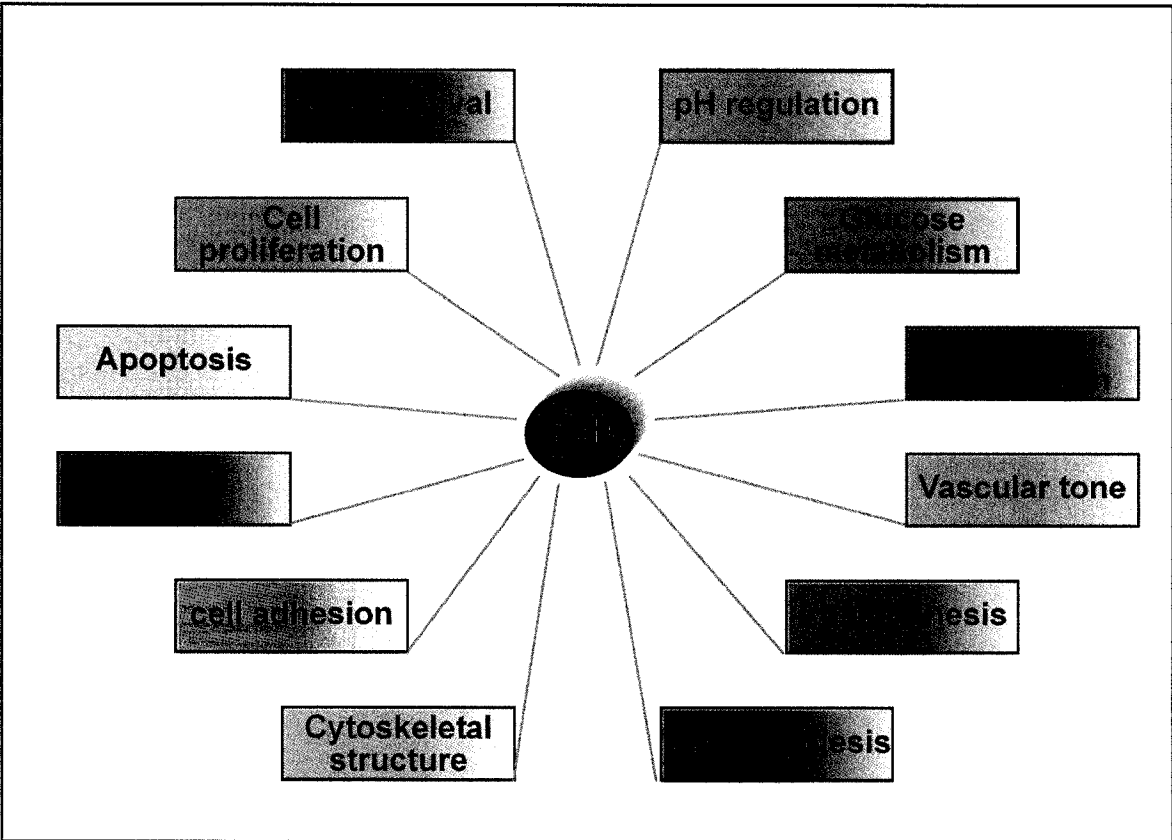
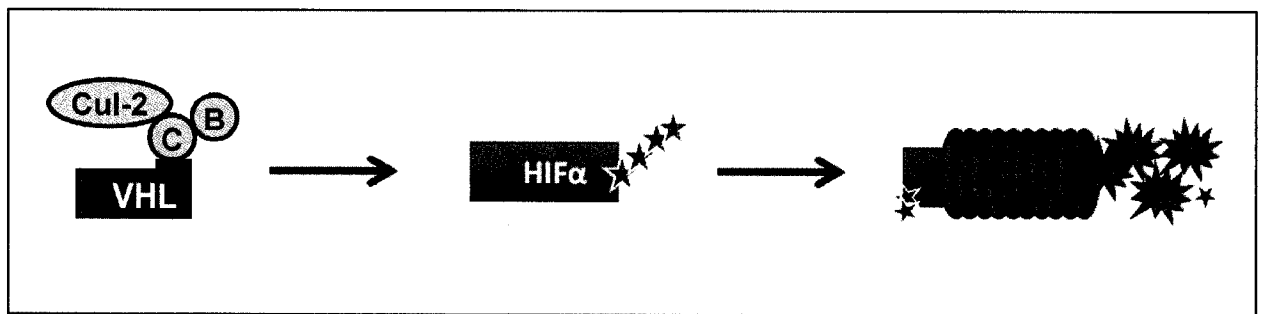


Figure 6. The VHL tumor suppressor protein is a component of an E3 ubiquitin-ligase complex that targets HIF α for proteasomal degradation.

Diagram depicting that the VBC-Cul2 E3 ubiquitin-ligase complex is responsible for ubiquitylation and proteasomal degradation of HIF α .



1.5. VHL MEDIATES OXYGEN and pH-DEPENDENT REGULATION OF HIF

1.5.1. VHL targets HIF α for O₂-dependent proteasomal degradation.

HIF α protein levels are regulated through proteasomal degradation via an O₂-dependent mechanism. In the early century, it was shown by many groups that ubiquitylation of HIF α protein was dependent on its interaction with the VHL tumor suppressor protein (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Binding experiments and structural analysis, using recombinant proteins, determined that the VBC-Cul2 complex interacts directly with HIF α through the VHL β -domain and the oxygen-dependent degradation (ODD) domain of HIF α (Ivan et al., 2001; Jaakkola et al., 2001; Ohh et al., 2000; Yu et al., 2001). Interaction of HIF α with VHL leads to its subsequent ubiquitylation, both *in vitro* and *in vivo*, through the VHL binding partners, elongins B, C and Cul-2, located at the α -domain (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Interestingly, *in vivo* experiments showed that this event occurred only in the presence of oxygen (Jaakkola et al., 2001; Yu et al., 2001). It is now widely accepted that the primary function of VHL as part of an E3 ubiquitin-ligase complex is to promote this oxygen-dependent degradation mechanism of HIF α (Ivan et al., 2001; Maxwell et al., 1999; Ohh et al., 2000).

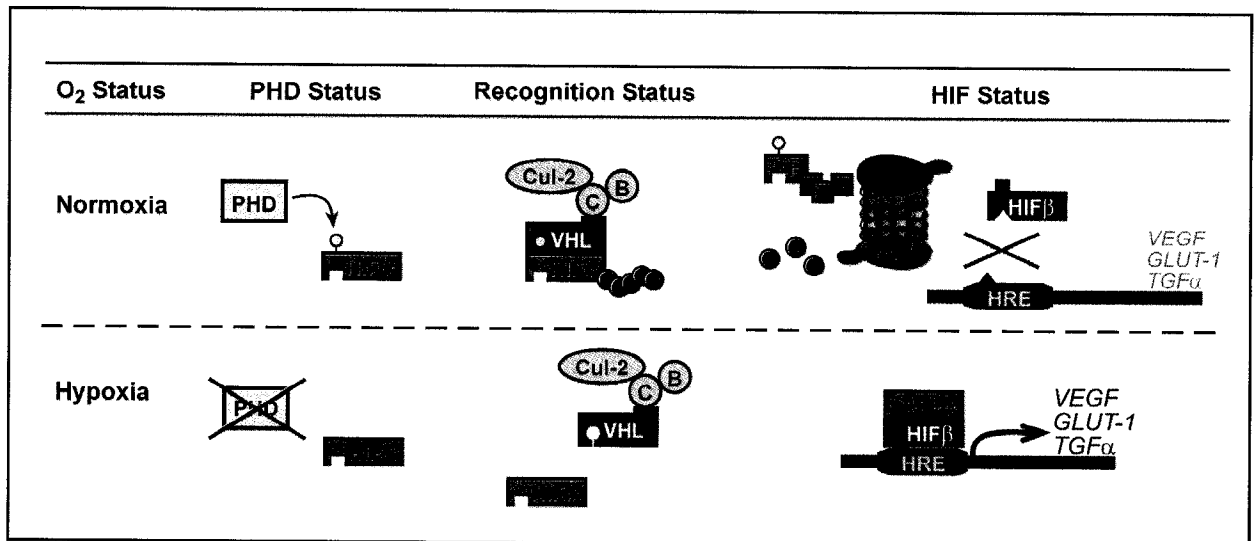
1.5.2. Regulation of VHL/HIF interaction by proline hydroxylation.

The observation that the VHL and HIF α interaction and subsequent HIF α degradation depended on the presence of oxygen inspired further study into this mechanism of regulation. It was soon discovered through analysis of post-translational modifications, that two specific prolyl residues in the ODD domain of HIF α were hydroxylated in the

presence of oxygen (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Studies conducted in *Caenorhabditis elegans* determined that this modification was accomplished by a novel EGL-9 dioxygenase (Epstein et al., 2001). This led to the identification of mammalian homologues of EGL-9, a series of non-heme, iron-dependent oxygenases known as prolyl hydroxylase domain-containing enzymes 1, 2 and 3 (PHDs), that performed an analogous function with respect to HIF α prolyl hydroxylation (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). PHDs consist of a tetramer containing two hydroxylase units and two protein disulphide isomerase subunits. Interestingly, the activity of such enzymes requires molecular oxygen, as well as ferrous iron and 2-oxoglutarate (Masson et al., 2001). These discoveries provided an explanation to the oxygen-dependent mechanism of HIF α degradation. Thus a model emerged, where under aerobic conditions, when oxygen is available; HIF α is hydroxylated at two key proline residues by PHDs (Figure 7). This post-translational modification allows VHL to recognize HIF α and target it for ubiquitylation and subsequent degradation by the 26S proteasome (Figure 7). A shift to low oxygen tension prevents hydroxylation of HIF α due to the requirement of molecular oxygen for the function of PHDs (Figure 7) (Ivan et al., 2001; Jaakkola et al., 2001). This suppresses the recognition and degradation of HIF α by the VBC-Cul2 complex and leads to HIF α stabilization (Figure 7). Once stabilized, the oxygen-regulated HIF α subunit heterodimerizes with its constitutively active partner HIF β to form the active HIF transcription factor, as described in section 1.4.2.

Figure 7. The VHL tumor suppressor protein targets HIF α for oxygen-dependent proteasomal degradation.

VHL is the recognition component of the VBC-Cul2 E3 ubiquitin-ligase complex that interacts with HIF α in an oxygen-dependent manner. Hydroxylation of HIF α at key proline residues by PHDs allows its recognition by VHL and its subsequent ubiquitylation and proteasomal degradation. However, during hypoxia PHDs are inactive leading to the stabilization of HIF α and formation of the active HIF transcription factor.

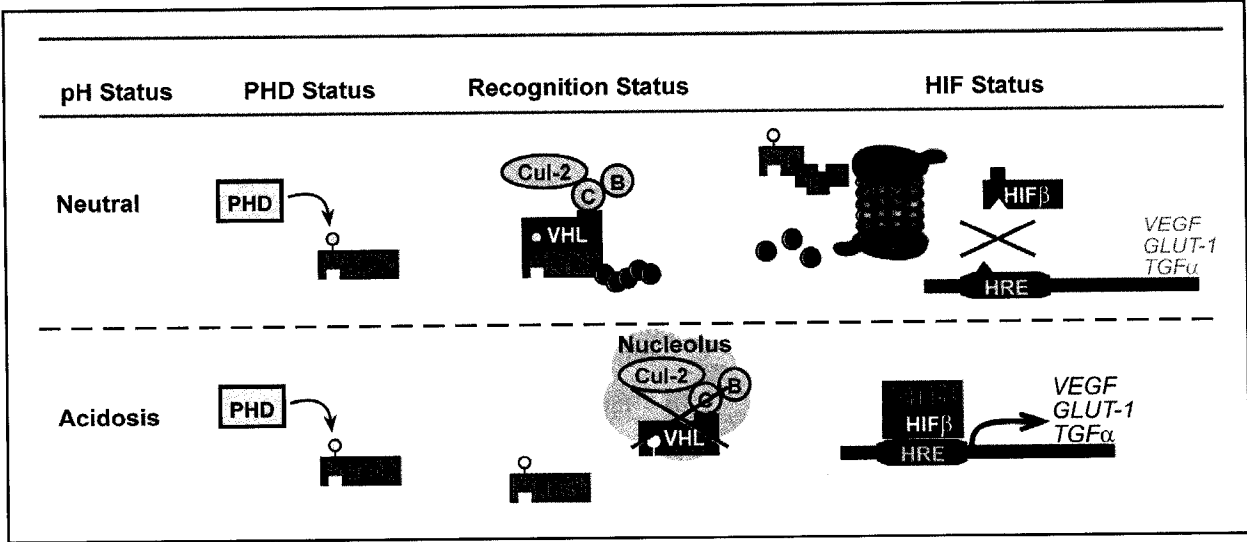


1.5.3. pH-dependent regulation of HIF.

Extracellular acidosis, as a consequence of anaerobic respiration or prolonged hypoxia, has been recently identified as a mechanism, apart from hypoxia, by which HIF α can escape VHL-mediated ubiquitylation and proteasomal degradation. A decrease in extracellular pH has been shown to trigger the confinement of VHL to subnuclear organelles known as nucleoli, where it is physically unable to interact with HIF α or the proteasome (Mekhail et al., 2004a; Mekhail et al., 2005). This pH-induced physical confinement of VHL to nucleoli allows HIF α to escape destruction in the presence of oxygen and activate its target genes (Figure 8) (Mekhail et al., 2005). Since anaerobic fermentation and acidification of the extracellular milieu are triggered once oxygen concentrations are reduced by 50-70% (Gladden, 2001) it can be argued that pH-mediated regulation of HIF α can occur during mild hypoxia (Mekhail et al., 2004b). In this regard, pH-induced stabilization of HIF α may occur prior to the inactivation of PHDs during acute hypoxia (Mekhail et al., 2004b). Inactivation of VHL under acidic conditions is insensitive to reoxygenation, allowing for prolonged HIF α activation following transient hypoxic stress, and can only be reversed by the reinstatement of neutral extracellular pH (Mekhail et al., 2004a). Neutralization of the extracellular pH allows VHL to resume its interaction with HIF α thereby mediating its ubiquitylation and proteasomal degradation (Mekhail et al., 2004a; Mekhail et al., 2004b). These recent findings have put forth a new model where pH-dependent regulation of VHL intersects with the previously identified oxygen-dependent control of HIF α .

Figure 8. pH-dependent regulation of HIF α .

An increase in extracellular acidosis triggers the nucleolar relocalization and inactivation of the VHL E3 ubiquitin-ligase function. This leads to the stabilization of HIF α regardless of oxygen tension.



1.6. THE ROLE OF VHL IN CANCER

1.6.1. VHL loss abolishes O₂-mediated regulation of HIF.

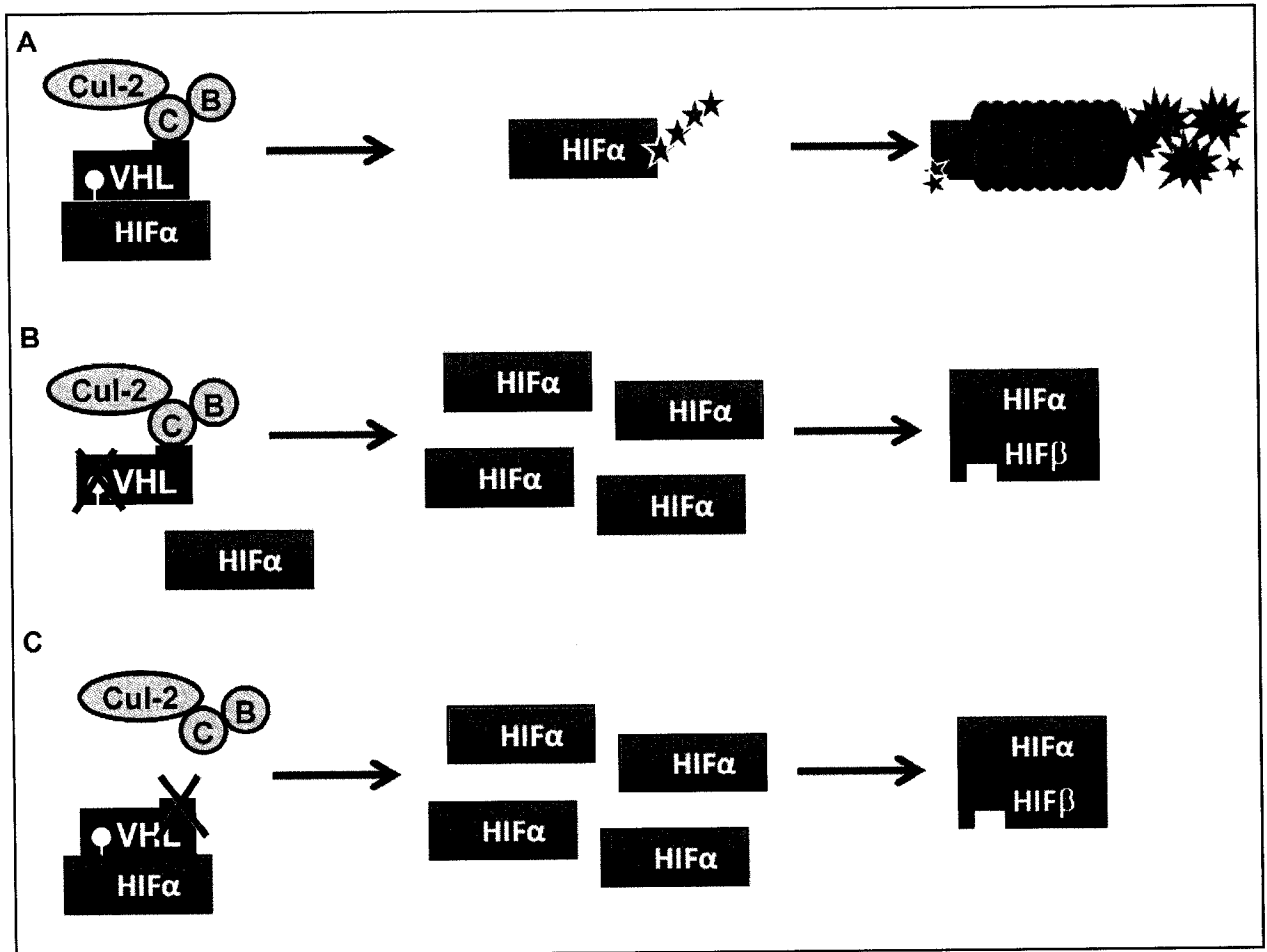
The majority of VHL inactivating mutations or deletions occur on the surface residues located at either the α - or β -domain, areas which are considered “hot spots” for VHL mutations (Stebbins et al., 1999). Mutations occurring in the β -domain prevent assembly of VHL with its substrate, HIF α , while those found in the α -domain disrupt E3 ubiquitin-ligase complex formation by preventing assembly with its core components elongins B, C and Cul2 (Figure 9). In either case such inactivating mutations and deletions lead to loss of VHL function and ultimately result in the constitutive activation of HIF α , regardless of oxygen status (Figure 9B and C) (Cockman et al., 2000; Ivan et al., 2001; Kaelin and Maher, 1998; Leroux and Hartl, 2000; Lonergan et al., 1998; Ohh et al., 2000). This in turn induces a transcriptional program that alters over 100 genes, almost all of which may be involved in promoting different aspects of tumorigenesis (Semenza, 2003). In this sense, the essential role of VHL as the master regulator of HIF α is warranted given that disruption of the VHL/HIF system ultimately leads to the VHL cancer syndrome or sporadic RCC (Harris, 2002). For this reason a great deal of effort has been invested into fully understanding the E3 ubiquitin-ligase properties of VHL and how they contribute to coordinating the efficient and rapid degradation of HIF α in order to suppress tumorigenesis.

1.6.2. HIF regulation and cancer.

HIF is a key element in the cellular response to hypoxia, a hallmark during the development of tumors, muscle stress and ischemic disorders. As mentioned earlier, the

Figure 9. Loss of VHL function abolishes oxygen-dependent HIF α regulation.

A) VHL promotes oxygen-dependent ubiquitylation and proteasomal degradation of HIF α . **B)** Mutations targeting the β -domain of VHL prevent assembly with HIF α and thus result in stabilization of HIF α . **C)** Mutations targeting the α -domain of VHL prevent VBC-Cul2 E3 ubiquitin-ligase complex formation and consequently the ubiquitylation and degradation of HIF α .



hypoxia-induced stabilization of HIF α and formation of the active HIF α /HIF β heterodimer results in the transcriptional induction of an array of genes, including VEGF, GLUT1 and TGF α , among others, implicated in angiogenesis, glycolysis and growth (Gunaratnam et al., 2003; Harris, 2002; Semenza, 2000a; Semenza, 2003). Since HIF is a key regulator of oxygen homeostasis, its dysregulation or untimely activation can be detrimental to the cell. In fact, the relative contribution of HIF1 α versus HIF2 α has become, in recent years, an emerging area of research. HIF1 α and HIF2 α have been reported to have common as well as unique target genes (Sowter et al., 2003). Due to these observations, HIF2 α is emerging as the oncogenic form of HIF α , at least in human RCC (Kondo et al., 2002; Maranchie et al., 2002; Seagroves and Johnson, 2002). For example, introduction of a HIF1 α mutant that escapes VHL recognition into RCC cells, containing wild-type VHL, does not induce a tumorigenic phenotype in SCID mice (Maranchie et al., 2002). However, a nondegradable form of HIF2 α is capable of inducing a tumorigenic phenotype in RCC cells expressing wild-type VHL (Kondo et al., 2002). This observation may be explained by recent findings demonstrating that HIF2 α , specifically, activates the TGF α and epidermal growth factor receptor (EGFR) pathway in VHL-negative RCC cells (Gunaratnam et al., 2003; Smith et al., 2005). These studies have shown that TGF α is a specific HIF2 α target and that HIF2 α , but not HIF1 α , is able to promote *in vitro* and *in vivo* tumorigenesis of RCC cells by constitutively activating the TGF α /EGFR oncogenic pathway (Gunaratnam et al., 2003; Smith et al., 2005). The importance of this pathway in the development of RCC was highlighted by the finding that transient and stable silencing of EGFR was sufficient to prevent HIF2 α -dependent tumorigenesis in several VHL-negative RCC cell lines (Smith et al., 2005). These data

suggest that activation of EGFR through HIF2 α -induced expression of TGF α provides a permanent self-sufficiency in growth signaling, which is a hallmark of cancer, to drive the growth autonomy of VHL-defective RCC cells (Hanahan and Weinberg, 2000; Smith et al., 2005).

1.6.3. Other VHL functions and their role in cancer.

Although much attention has been geared towards HIF regulation, VHL possesses several other HIF-independent as well as HIF-dependent functions that may contribute to tumor suppression. VHL associates with microtubules and has been shown to be involved in microtubule stabilization (Hergovich et al., 2003). VHL protects microtubule depolarization *in vivo* and thus maintains microtubule dynamics. The naturally-occurring VHL point mutants Y98H and Y112H, which are associated with Type 2C VHL disease that predispose individuals to development of adrenal-gland tumors and cerebellar haemangioblastomas, disrupt the microtubule stabilizing function of VHL (Hergovich et al., 2003). VHL is also required for deposition of the extracellular matrix (ECM) components, fibronectin and collagen IV. Loss of VHL results in cells with abnormal ECM deposition, which is the structural scaffolding surrounding cells, and may promote angiogenesis, as well as invasion and metastasis of tumor cells (Grosfeld et al., 2007; Kurban et al., 2008; Ohh et al., 1998). Recently, VHL has also been implicated in the HIF-dependent regulation of E-cadherin, a protein involved in cell adhesion. Reduction of E-cadherin showed a marked increase in the invasiveness of RCC cells that were restored with functional VHL, implying a possible involvement for E-cadherin in promoting malignancy in RCC (Esteban et al., 2006; Evans et al., 2007; Krishnamachary

et al., 2006). Together these findings establish that the proper functioning of VHL is essential for many aspects of cell integrity.

1.7. SUBCELLULAR DYNAMICS OF E3 UBIQUITIN-LIGASES: A REQUIREMENT FOR PROTEIN FUNCTION

1.7.1. Nuclear-cytoplasmic trafficking.

During evolution of eukaryotes, the formation of the nuclear envelope resulted in the segregation of the cell into two distinct cellular compartments, the nucleus and the cytoplasm (Copeland, 1938; Mans et al., 2004). This division necessitated the concomitant evolution of regulated nuclear-cytoplasmic transport pathways in order to maintain rapid and specific communication between these cellular compartments. The regulated and timely bidirectional trafficking of RNA and protein cargoes into and out of the nucleus is a prerequisite for basic biological processes, such as transcription and translation. Disruption of such nuclear-cytoplasmic transport pathways results in deregulation of cellular processes and may lead to various diseases (Smith and Koopman, 2004; Terry et al., 2007; Truant et al., 2007). Nuclear-cytoplasmic transport of all molecules, including proteins and RNA species, across the nuclear envelope occurs through channels formed by macromolecular structures known as nuclear pore complexes (NPCs) (Rout and Aitchison, 2001; Wentz, 2000). Although differences exist between protein and RNA transport the same fundamental sequence of events are conserved-essentially, cargoes bind to soluble transporters in the donor compartment, are transported through NPCs, and are released in the target compartment.

1.7.2. Signal-mediated nuclear import and export of proteins.

Nuclear-cytoplasmic trafficking of proteins across the nuclear envelope results from a consecutive cycle of protein entry into the nucleus (nuclear import) and exit from the nucleus to the cytoplasmic compartment (nuclear export). The translocation of proteins across the nuclear envelope is generally mediated by soluble transport receptors that recognize specific transport signals, such as nuclear localization signals (NLSs) and nuclear export signals (NESs), which mediate nuclear import and nuclear export respectively (Gorlich and Kutay, 1999). The directionality of transport across the NPCs is also mediated by an additional key component of nuclear-cytoplasmic transport, the small GTP-binding protein, Ran (Gorlich et al., 1996; Melchior et al., 1993; Moore and Blobel, 1994; Richards et al., 1997; Schlenstedt et al., 1995). A concentration gradient of Ran-GTP across the nuclear envelope controls the interaction of transporters with their cargo and determines the directionality of transport (Izaurralde et al., 1997). In the nucleus, Ran is in a GTP-bound state due to the nuclear localization of its nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991; Ohtsubo et al., 1989). On the other hand, the GTPase activating protein RanGAP1 and its stimulatory factor RanBP1, which are exclusively cytoplasmic proteins, are responsible for the hydrolysis of Ran-GTP to Ran-GDP in the cytoplasm (Bischoff et al., 1994; Bischoff et al., 1995a; Bischoff et al., 1995b; Coutavas et al., 1993; Mahajan et al., 1997; Matunis et al., 1996; Richards et al., 1996).

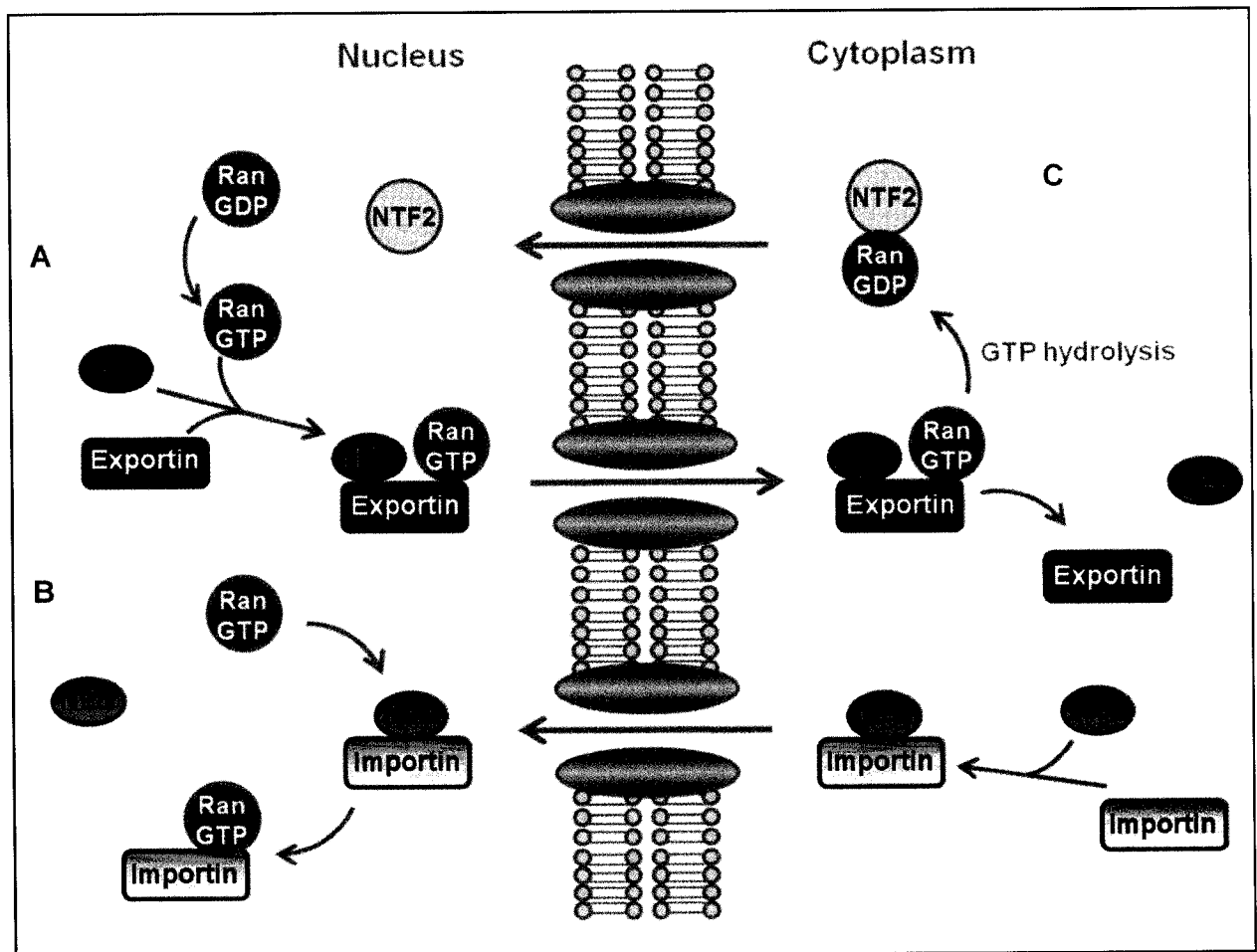
A general theme in the export of proteins from the nucleus to the cytoplasm is that specialized export receptors (exportins) recognize cargoes harboring specific export signals. Exportins form complexes with substrates in the nucleus with the aid of Ran

(Melchior and Gerace, 1998; Moore, 1998; Moore and Blobel, 1993). As mentioned above, the loading and release of substrates with exportins is dependent on a concentration gradient of RanGTP across the nuclear envelope (Izaurralde et al., 1997; Richards et al., 1997). Exportins preferentially bind their substrates at high nuclear RanGTP levels and exit the nucleus as exportin-cargo-RanGTP complexes (Figure 10) (Macara, 2001; Richards et al., 1997). Substrates are then released in the cytoplasm upon hydrolysis of RanGTP to RanGDP (Figure 10A). Exportins return to the nucleus for another round of export and Ran returns via the nuclear transport factor 2 (NTF2) (Figure 10A and C) (Ribbeck et al., 1998; Smith et al., 1998). Nuclear import receptors (importins), on the other hand, function in a reverse manner. Importins interact with NLS-containing substrates in the cytoplasm (Figure 10B). Importin-cargo complexes are then able to cross the NPC to enter the nuclear compartment. Once in the nucleus, the complex disassembles upon RanGTP binding.

1.7.3. Subcellular dynamics is essential for the function of proteins. Over the past decade a considerable amount of research has been geared towards understanding the intracellular transport of proteins and its importance for many biological processes, such as transcription and translation. This has led to an emerging concept that the functions of proteins are ultimately governed by their dynamic character and ability to transport to their proper destination. In order to perform a certain function proteins need to be directed to the proper cellular compartment, in a delicately regulated and coordinated fashion. For example, transcription factors need to be present in the nuclear compartment to effectively regulate gene expression. However, some may be kept in the cytoplasm until a signal triggers their import into the nucleus where they can perform their function

Figure 10. Nuclear import and export of proteins.

Schematic diagram depicting the general nuclear import and export pathways utilized by proteins to cross the nuclear envelope. **A)** In general, exportins bind to NES-containing proteins in the nucleus in the presence of RanGTP. Once across the nuclear envelope, GTP hydrolysis of Ran causes dissociation of the complex and release of cargo in the cytoplasm. **B)** Nuclear import of NLS-containing cargo is mediated by importins, which bind to cargo in the cytoplasm. RanGTP in the nucleus binds importin and induces cargo release. **C)** RanGDP is imported into the nucleus via NTF2.



(Hood and Silver, 1999). This represents a highly sophisticated level of regulation that requires efficient and specific transport pathways (Gorlich and Kutay, 1999). The knowledge acquired from studies involving subcellular trafficking of proteins has also aided in the understanding of different diseases, including cancer (Kau et al., 2004). It has been shown that the inability of a protein to localize to the “correct” subcellular compartment may result in disease due to loss of its normal function. For example, nuclear export of the adenomatous polyposis coli (APC) tumor suppressor protein is critical for its normal function (Rosin-Arbesfeld et al., 2000). Mutated or truncated APC in colorectal cancer, resulting in loss of NES function, leads to a mostly nuclear localization and functional inactivation (Rosin-Arbesfeld et al., 2003). The breast cancer-associated protein BARD1 must export from the nucleus for its proapoptotic function in the cytoplasm. However, dimerization of the breast and ovarian cancer susceptibility protein 1 (BRCA1) with BARD1 in the nucleus can alter its proapoptotic activity by blocking its nuclear export signal (Brzovic et al., 2001; Fabbro et al., 2002). The resultant nuclear localization of both proteins has been shown to promote cancer (Rodriguez et al., 2004). Also, the integrase interactor 1 (INI1) tumor suppressor forms a key component of the chromatin remodeling complex. Its nuclear localization, where it is associated with cell cycle arrest, is an important aspect of its tumor suppressor function (Craig et al., 2002). Interestingly, an INI1 cancer mutant, found in malignant rhabdoid tumors, is predominantly localized in the cytoplasm and is no longer able to induce cell cycle arrest (Craig et al., 2002).

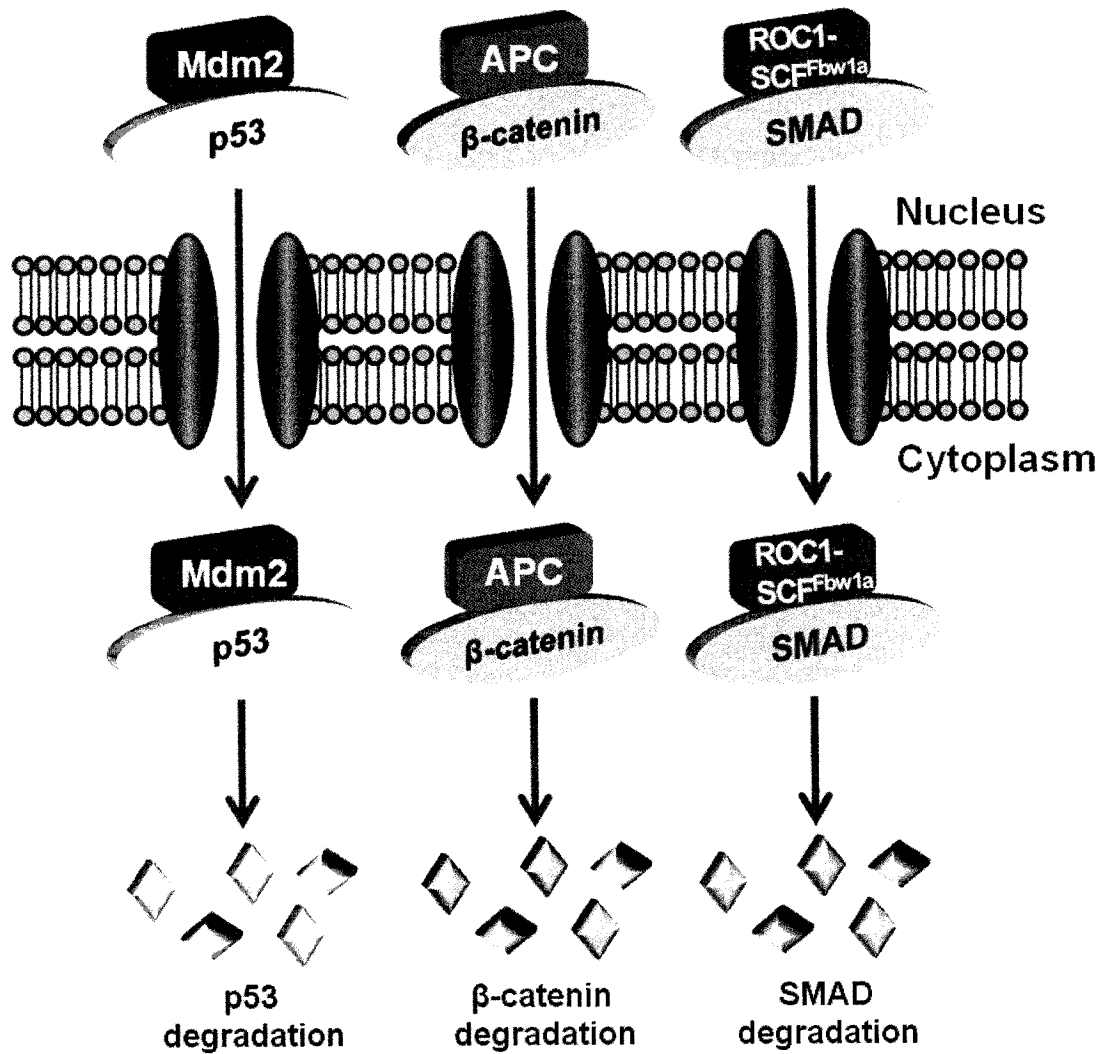
1.7.4. Nuclear-cytoplasmic trafficking of E3 ubiquitin-ligases is required for degradation of nuclear substrates.

Most molecules are highly mobile and participate in networks that often require dynamic movement within subcellular compartments and highly efficient transport between different subcellular compartments. This newly found appreciation for protein dynamics has emerged particularly for E3 ubiquitin-ligases. Due to the fact that E3 ubiquitin-ligases serve as the substrate-recognition element, which dictate substrate specificity, their function is important for progression of the ubiquitin-mediated degradation cascade. Considering the key role of E3 ubiquitin-ligases in regulation of many aspects of cellular functions and biological processes, it is not surprising that loss of function or deregulation of E3 ubiquitin-ligases can result in the development of disease, including cancer (Sun, 2006). Interestingly, subcellular trafficking is a prerequisite for the proper functioning of many E3 ubiquitin-ligases (Scheffner, 1999). It is now known that degradation of nuclear proteins by the ubiquitylation system often requires nuclear-cytoplasmic trafficking of E3 ubiquitin-ligases (Figure 11). One example is the ubiquitin-mediated degradation of the p53 tumor suppressor protein by the Mdm2 E3 ubiquitin-ligase (Momand et al., 1992; Oliner et al., 1993). Efficient proteasomal degradation of the nuclear p53 tumor suppressor requires the continuous nuclear-cytoplasmic shuttling of the Mdm2 E3 ubiquitin-ligase (Figure 11) (Freedman and Levine, 1998; Roth et al., 1998). Disrupting the nuclear export of Mdm2, such as by cancer-causing point mutations, impairs its ability to mediate degradation of p53 (Lindstrom et al., 2007). Also, nuclear export of the APC tumor suppressor is required for its E3 ubiquitin-ligase function. It has been shown that APC stimulates nuclear

Figure 11. Trafficking of E3 ubiquitin-ligases is required for degradation of nuclear proteins.

This schematic diagram provides examples where nuclear export is required for degradation by the ubiquitylation system. Degradation of p53, β -catenin and SMAD is dependent on the trafficking properties of the Mdm2, APC and ROC1-SCF^{Fbw1a} E3 ubiquitin-ligases, respectively.

E3 Ubiquitin-ligase/Substrate Complexes



export and degradation of the oncogenic β -catenin, and mutations in the APC NES signal reduces the rate of β -catenin degradation (Figure 11) (Fodde et al., 2001; Henderson, 2000; Neufeld et al., 2000). Another example is the ROC1-SCF^{Fbw1a} E3 ubiquitin-ligase, whose nuclear export is required for proteasomal degradation of the Smad3 transcription factor (Figure 11) (Fukuchi et al., 2001). The cyclin-dependent kinase inhibitor p27^{Kip1} requires nuclear export by Jab1 for proteasome-mediated degradation. A mutant form of p27^{Kip1} that fails to assemble with Jab1 cannot be exported from the nucleus and is not degraded by the proteasome (Scheffner, 1999; Tomoda et al., 1999).

1.8. SUBCELLULAR DYNAMICS OF VHL IS REQUIRED FOR ITS E3 UBIQUITIN-LIGASE AND TUMOR SUPPRESSOR FUNCTION

1.8.1. Subcellular localization of VHL: clues to subcellular trafficking properties.

Within the cell, the VHL tumor suppressor protein has been detected in several locations, including the mitochondria and endoplasmic reticulum (ER), however, the most striking and predominant subcellular localization is in the cytoplasmic compartment (Lee et al., 1996; Schoenfeld et al., 2001; Shiao et al., 2000). The VHL tumor suppressor protein exhibits a predominantly cytoplasmic localization for both endogenous VHL and exogenously expressed VHL fused to the green fluorescent protein (VHL-GFP), in all tested tissues and cell lines (Corless et al., 1997; Lee et al., 1996; Lee et al., 1999; Los et al., 1996; Ye et al., 1998). VHL-GFP was found to recapitulate the properties of endogenous VHL, not only for subcellular localization, but also in its ability to form the VBC-Cul2 complex and mediate E3 ubiquitin-ligase activity (Lee et al., 1999). Interestingly, the prominent cytoplasmic localization of VHL was shown to be altered by arrest of RNA Polymerase II (RNA PolII) mediated transcription (Lee et al., 1999).

Inhibition of RNA PolII-mediated transcription results in a nuclear shift in VHL localization. This observation demonstrated that VHL was able to mobilize between the nuclear and cytoplasmic compartments and supported the idea that VHL may be a nuclear-cytoplasmic shuttling protein.

1.8.2. Transcription-dependent nuclear-cytoplasmic trafficking of VHL.

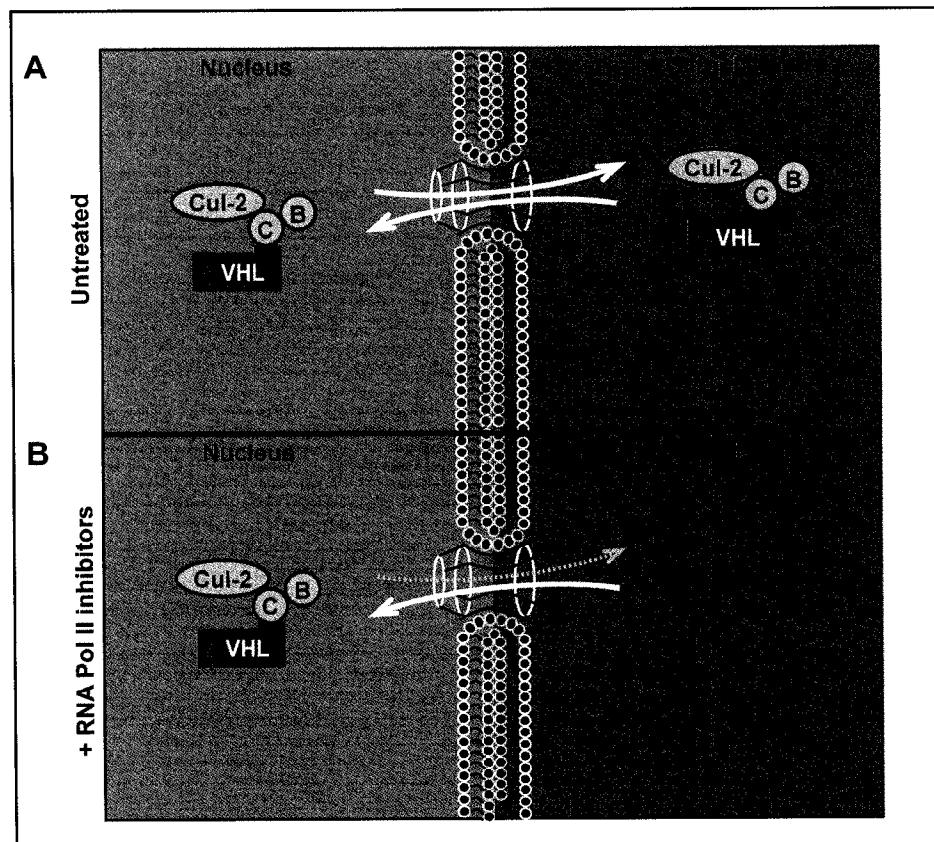
As discussed earlier, nuclear-cytoplasmic trafficking of proteins across the nuclear envelope results from a consecutive cycle of protein entry into the nucleus and exit from the nuclear to the cytoplasmic compartment. Thus the nuclear-cytoplasmic trafficking of VHL must be arranged by coordination of nuclear import and export pathways. The nuclear accumulation ensued by inhibitors of RNA PolII-dependent transcription was a key observation in the identification of the subcellular trafficking properties of VHL. Treatment of cells with RNA PolII transcriptional inhibitors, such as Actinomycin D (ActD), 5,6-dichlorobenzimidazole (DRB) or α -amanatin, results in a clear nuclear redistribution in the localization of a GFP-tagged VHL fusion protein (Groulx and Lee, 2002; Lee et al., 1999). This nuclear redistribution upon inhibition of transcription was observed for both transiently and stably expressed VHL-GFP, in multiple cell lines (Groulx and Lee, 2002; Lee et al., 1999). Quantification of the VHL-GFP fluorescence in the cell demonstrated that there was an approximate threefold increase in nuclear signal upon inhibition of transcription, without a change in total cellular fluorescence (Lee et al., 1999). The localization of VHL-GFP was not altered by the translational inhibitor, cycloheximide, demonstrating that ongoing protein synthesis is not required for the nuclear redistribution caused by inhibition of transcription. Interestingly, removal of DRB, a reversible inhibitor of RNA PolII-mediated transcription, restored the

predominantly cytoplasmic localization of VHL-GFP (Lee et al., 1999). Together these results demonstrated that VHL-GFP was able to enter the nucleus and, importantly, was also able to cross the nuclear envelope once again to reenter the cytoplasmic compartment.

In order to determine whether VHL traffics continuously between the nuclear and cytoplasmic compartments the dynamic profile of VHL was analyzed. Traditional methods for measuring trafficking properties of proteins were used, such as cellular fusion and nuclear export assays. These assays demonstrated that VHL-GFP is in fact a nuclear-cytoplasmic shuttling protein (Groulx et al., 2000; Lee et al., 1999). For example, nuclear export assays demonstrated that a VHL fusion protein expressed solely in the nucleus was able to export from the nucleus of digitonin permeabilized cells in an energy-dependent manner (Groulx et al., 2000). Also, in a cellular fusion assay, where transiently transfected VHL-GFP cells were fused with untransfected cells, the fluorescence signal representing VHL-GFP rapidly left the donor nucleus, with approximately 50% of the signal lost after less than 40 min (Lee et al., 1999). Interestingly, in the presence of DRB there was a marked decrease in the rate at which VHL-GFP left the donor nucleus, where it required more than 140 minutes to observe a 50% decrease in nuclear fluorescence of the donor cell (Lee et al., 1999). Furthermore, in the presence of DRB the fluorescence accumulated in the acceptor nuclei, whereas in the absence DRB the signal was distributed throughout the cytoplasm of the large polykaryon (Lee et al., 1999). Together these results suggested that VHL is a dynamic and nuclear-cytoplasmic shuttling protein that required ongoing RNA PolII-mediated transcription to sustain this trafficking profile (Figure 12). These observations also

Figure 12. Transcription-dependent subcellular trafficking of VHL.

A) VHL is a nuclear-cytoplasmic shuttling protein. **B)** The subcellular trafficking of VHL is sensitive to inhibitors of RNA PolII activity. In the presence of RNA PolII inhibitors VHL accumulates in the nucleus due to a decrease in nuclear export.



revealed that the nuclear accumulation brought upon by inhibition of transcription was due, at least in part, to the inhibition of nuclear export (Groulx et al., 2000; Lee et al., 1999).

1.8.3. Subcellular dynamics of VHL is regulated by extracellular H⁺.

As mentioned earlier, the predominantly cytoplasmic localization of VHL can be altered by changes in extracellular pH. A physiological decrease in the extracellular pH as a consequence of anaerobic respiration triggers a striking relocalization of endogenous VHL, and VHL-GFP, to the nucleolus of cells. This phenomenon was observed both in cultured cells and in the core of human tumors, where acidosis is commonly observed due to a hypoxic microenvironment (Mekhail et al., 2004a; Mekhail et al., 2005; Mekhail et al., 2006). Nucleolar relocalization of VHL also occurs in normoxia following extracellular acidification by metabolically active cells, known as the Warburg effect, which is often observed in cancer cells (Mekhail et al., 2004a). The extracellular pH required for relocalization of VHL to nucleoli is cell-type specific but typically ranges within 6.60 to 5.80, which is well within the range observed in physiological and pathological settings such as development, ischemia and tumorigenesis (Gladden, 2004; Mekhail et al., 2005; Smallbone et al., 2005).

1.8.4. VHL undergoes static detention by the nucleolar architecture.

Proteins often change their steady state subcellular localization while retaining their dynamic profiles. However, photobleaching experiments showed that the nucleolar architecture of acidotic cells captures and converts VHL to a static or immobile state (Mekhail et al., 2005). This is in stark contrast to nucleolar resident proteins, such as B23 and fibrillarin, which exchange between subcellular compartments and retain their

dynamic properties even in acidotic cells (Mekhail et al., 2005). The detention of VHL in the nucleolus results in a consequent loss of its mobility across the nuclear envelope and thus of nuclear-cytoplasmic trafficking. VHL remains sequestered in nucleoli, in a static state, even upon reoxygenation of hypoxic cells (Mekhail et al., 2004a). Nucleolar VHL can revert back to its original subcellular localization and resume its dynamic nuclear-cytoplasmic trafficking simply by neutralization of the extracellular milieu (Mekhail et al., 2004a). Mapping analysis revealed that a new type of protein localization sequence named NoDS^{H+} (nucleolar detention signal regulated by H⁺) is able to detain VHL in the nucleolus, along with several other proteins involved in basic cellular metabolism (Mekhail et al., 2005; Mekhail et al., 2007). The NoDS^{H+} is activated following an increase in extracellular hydrogen ion concentration and is inactivated after reversal to neutral pH conditions, causing rapid release of detained VHL into the nucleoplasm where the protein resumes its dynamic profile (Mekhail et al., 2005; Mekhail et al., 2007). This was the first reported evidence that cells have evolved a mechanism to regulate molecular processes by reversibly switching participants, such as VHL, between a mobile and a static state.

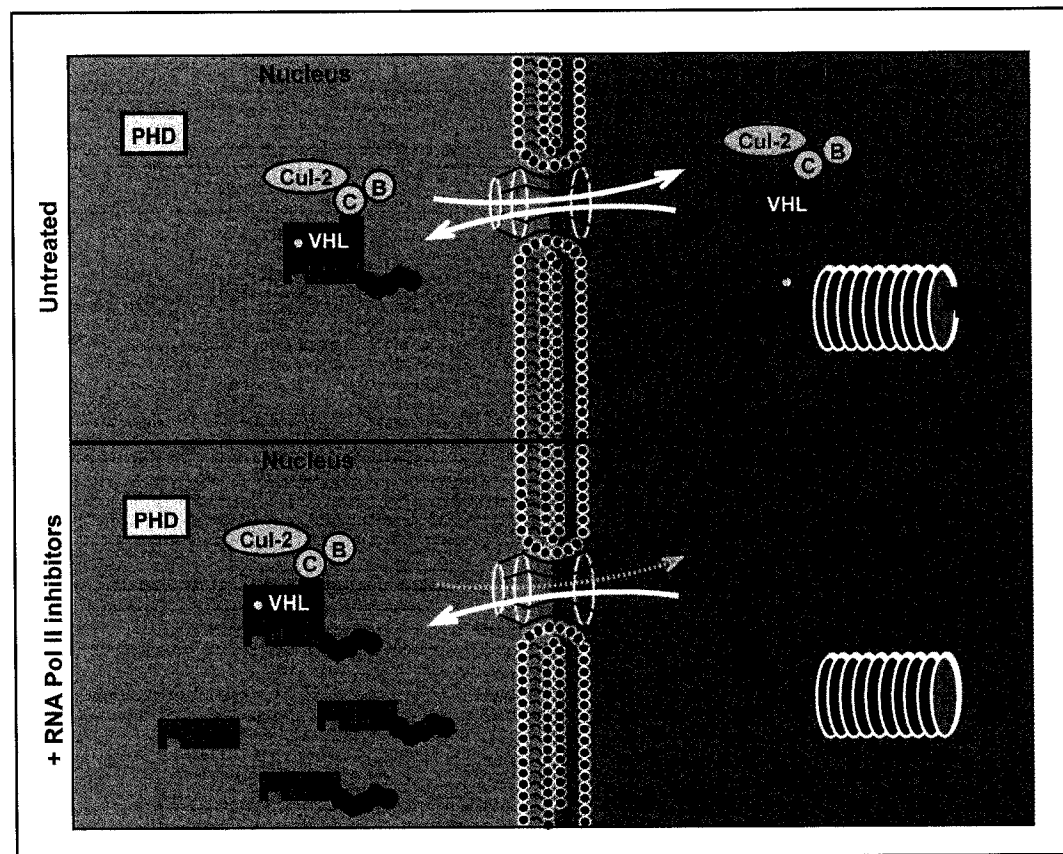
1.8.5. Transcription-dependent trafficking of VHL is required for HIF degradation.

Several nuclear substrates, such as p53, p27 and Smad3 are ubiquitinated in the nucleus but must undergo nuclear export for efficient ubiquitin-mediated degradation by the proteasome (Freedman and Levine, 1998; Fukuchi et al., 2001; Tomoda et al., 1999). HIF α is a predominantly nuclear protein when it is stabilized during hypoxic conditions (Groulx and Lee, 2002; Luo and Shibuya, 2001). The localization of HIF α is in stark contrast to the predominantly cytoplasmic steady-state localization of VHL, raising the

question of where VHL/HIF α interaction and HIF α ubiquitylation occurs. In order to address this question subcellular fractionation experiments were performed. Cells were placed in hypoxia, in order to stabilize HIF α , after which cells were reoxygenated to induce the oxygen-dependent ubiquitylation of HIF α . It was observed that immediately following reoxygenation of hypoxic cells ubiquitylated forms of HIF α were detectable in the nuclear fraction of digitonin fractionated cells, but not in the cytosolic fraction (Groulx and Lee, 2002). Nuclear ubiquitylation of HIF α is mediated by VHL since this event did not occur in VHL-negative cells (Groulx and Lee, 2002). Finally, the presence of VHL and ubiquitylated HIF α complexes in the nucleus confirmed that VHL mediates ubiquitylation of HIF α in the nuclear compartment (Groulx and Lee, 2002). Further studies revealed that after HIF α is ubiquitylated in the nucleus by the VBC-Cul2 complex a detectable cytoplasmic signal becomes apparent before its degradation (Groulx and Lee, 2002). The observation that HIF α ubiquitylation occurs in the nucleus while its degradation is a cytoplasmic event suggested that nuclear export is potentially required for this process. Interestingly, treatment with inhibitors of RNA PolII activity, which interfere with the dynamic profile of VHL, prevented the cytoplasmic accumulation of HIF α following reoxygenation of hypoxic cells. Consequently, this caused a marked increase in the half-life of HIF α after reoxygenation. Although inhibition of RNA PolII-mediated transcription altered the degradation of HIF α , there was no effect on HIF α nuclear ubiquitylation (Figure 13) (Groulx and Lee, 2002). Interestingly, altering the nuclear export pathway utilized by VHL by addition of a strong and classical NES, which has the ability to override the transcription-dependent trafficking of VHL, also caused a marked increase in the HIF α half-life in reoxygenated cells (Lee et al., 1999). Together,

Figure 13. Nuclear-cytoplasmic trafficking of VHL is required for efficient degradation of HIF α .

VHL mediates nuclear ubiquitylation and subsequent cytoplasmic degradation of HIF α . Inhibition of RNA PolIII-dependent transcription decreases the nuclear export activity of VHL and prevents efficient HIF α degradation.



these data strongly suggested a role for transcription-dependent nuclear-cytoplasmic trafficking of VHL for its E3 ubiquitin-ligase function and regulation of HIF α (Figure 13). Further proof of the importance of nuclear-cytoplasmic trafficking of VHL for its E3 ubiquitin-ligase function has been also recently demonstrated with the observation that nucleolar detention of VHL results in stabilization of HIF α . The pH-induced physical confinement of VHL to nucleoli, which results in a consequent loss of mobility of VHL across the nuclear envelope, allows HIF α to escape destruction in the presence of oxygen and activate its target genes (Figure 14) (Mekhail et al., 2004a; Mekhail et al., 2005). These data demonstrated that interfering with the dynamic profile of VHL inhibits its E3 ubiquitin-ligase function since it can no longer mediate HIF α ubiquitylation and degradation. The inactivation of VHL can only be reversed by the reinstatement of neutral extracellular pH (Mekhail et al., 2004a; Mekhail et al., 2004b). Thus, neutralization of the extracellular pH the nucleolus rapidly releases VHL from static detention, thereby restoring its mobility and ability to mediate HIF degradation (Mekhail et al., 2004a; Mekhail et al., 2005; Mekhail et al., 2004b). Together these studies highlight the importance of nuclear-cytoplasmic trafficking for the tumor suppressor function of VHL.

1.9. CLASSICAL VERSUS TRANSCRIPTION-DEPENDENT NUCLEAR EXPORT PATHWAYS

1.9.1. Nuclear pore complexes: A gateway through the nuclear envelope.

In the eukaryotic cell the nuclear and cytoplasmic compartments are physically segregated by the nuclear envelope, an impermeable double lipid bilayer. As described in section 1.7.1., any communication between these two compartments is mediated by the

Figure 14. pH-dependent nucleolar detention of VHL prevents subcellular trafficking and HIF α degradation.

A) Under normal oxygen and pH levels VHL shuttles continuously between the nuclear and cytoplasm in a transcription-dependent manner to mediate efficient degradation of HIF α . **B)** During hypoxia HIF α evades recognition by VHL, which leads to its stabilization. **C)** Hypoxia-induced acidosis triggers the static detention of VHL in the nucleolus, preventing its subcellular trafficking and ability to degrade HIF α . **D)** Acidosis can also be achieved in normoxic conditions by the Warburg effect.

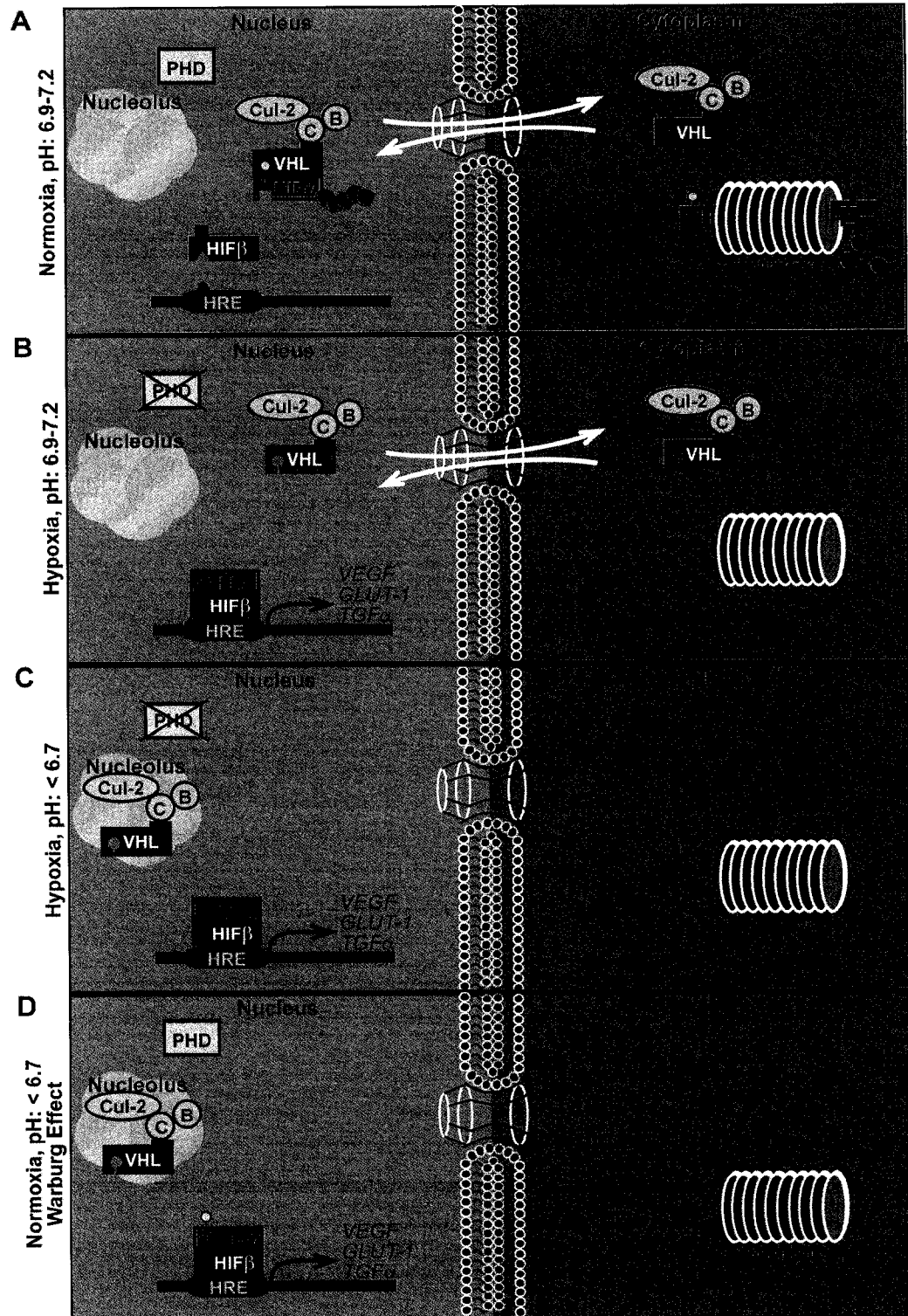
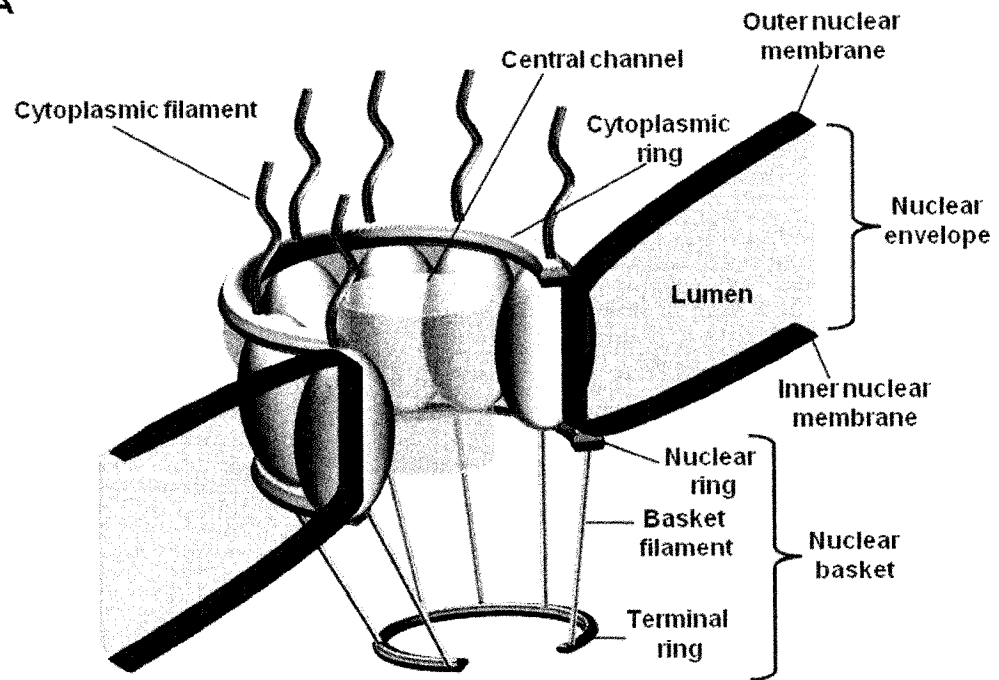


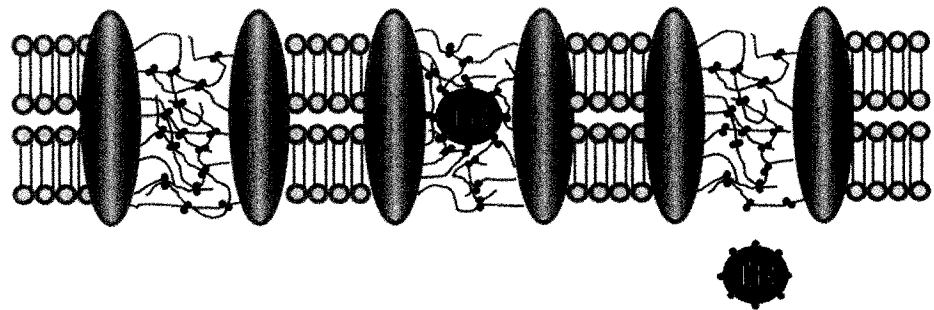
Figure 15. The nuclear pore complex.

A) A model of the nuclear pore complex. A cross-sectional view of the NPC embedded within the nuclear envelope, depicting the different components. **B)** The passage of transport receptors (TR) across the central channel of the NPC is facilitated by nucleoporins containing FG repeats. Only soluble transporters can cross the NPC by competing with FG interactions to transiently open the sieve.

A



B



NPCs, which are large macromolecular protein structures of about 125 MDa that perforate the nuclear envelope (Figure 15A) (Rout and Aitchison, 2001; Wentz, 2000). Typically, a somatic cell will contain between 1000 and 10,000 of these NPCs spanning the nuclear envelope (Cronshaw et al., 2002; Rout and Aitchison, 2001; Rout et al., 2000). The center of the NPC, perpendicular to the nuclear membrane, is the central channel through which transport of molecules occurs. The central channel is surrounded by eight spokes flanked by nuclear and cytoplasmic rings. Eight fibrils extend outward from the cytoplasmic ring, whereas at the nuclear ring exist eight fibrils joined at their distal ends to form a nuclear basket. Each NPC is composed of multiple copies of approximately 30 different protein subunits, known as nucleoporins (Cronshaw et al., 2002; Rout et al., 2000). Systematic analysis of NPCs in yeast has revealed that although the majority of nucleoporins are found on both sides of the NPC (symmetric nucleoporins) some are preferentially associated with either the nuclear or cytoplasmic face of the NPC (asymmetric nucleoporins). Several classes of nucleoporins exist within the NPC structure, including transmembrane proteins that anchor the NPC to the nuclear envelope, structural nucleoporins and FG (phenylalanine and glycine)-containing nucleoporins that provide the NPC-binding sites for transporters.

NPCs can accommodate a relatively large capacity of translocations, with a single NPC allowing a mass flow of about 100 MDa of proteins per second at rates in the order of 10^3 translocation events per second (Ribbeck and Gorlich, 2001). The central channel of the NPCs supports two types of transport; 1- passive diffusion of metabolites, ions and small proteins of up to 40 KDa (or 9 nm in diameter), 2- active transport of different RNA species and proteins larger than 40 KDa and up to 50 MDa (or 40 nm in diameter)

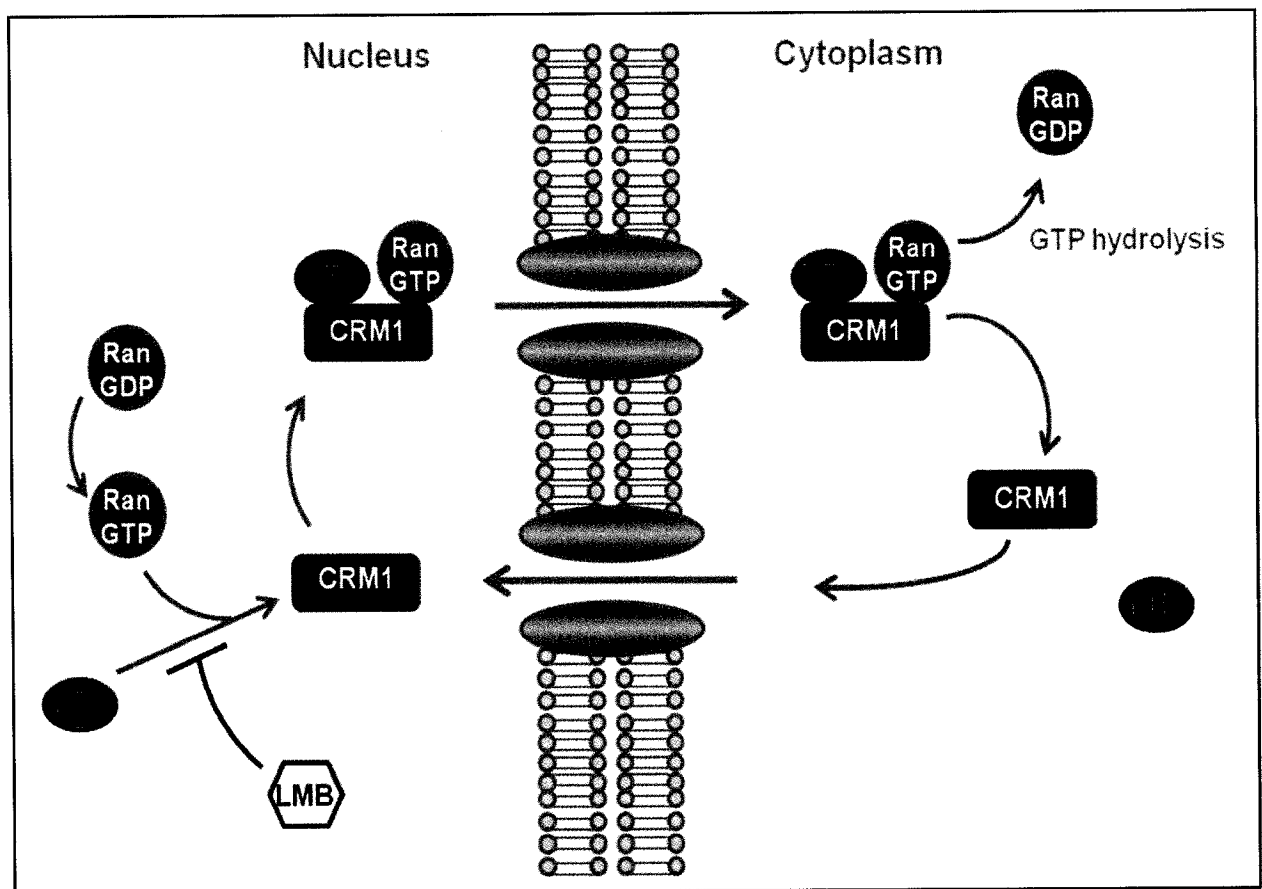
(Keminer and Peters, 1999; Paine, 1975; Paine et al., 1975; Pante and Kann, 2002). Although small proteins typically diffuse through the NPC, some proteins smaller than 40 KDa may require active transport (Breeuwer and Goldfarb, 1990). A general view of active transport is that proteins move across the nuclear pore by association with soluble transporters that can interact with FG repeats found on FG-containing nucleoporins (Bayliss et al., 1999; Bednenko et al., 2003; Lei and Silver, 2002; Radu et al., 1995; Weis, 2003). Although several models have been put forth, recently one of these models has been of particular interest. It has been postulated that the FG repeats on nucleoporins form an elastic and reversible sieve structure with hydrogel-like properties in the central channel of the nuclear pore (Frey and Gorlich, 2007; Frey et al., 2006; Radu et al., 1995). Only soluble transporters can cross such a hydrogel structure by competing with FG interactions to transiently open the sieve (Figure 15B) (Frey and Gorlich, 2007; Frey et al., 2006; Ribbeck and Gorlich, 2002).

1.9.2. Classical pathway for nuclear export of proteins.

Active nuclear export of proteins is mediated by specific amino acid sequences present within the primary structure of the protein. The first discovered and most widely studied nuclear export pathway involves the export of proteins containing a leucine-rich NES (Figure 16). The NES sequence was originally discovered in HIV-1 Rev protein and protein kinase inhibitor (PKI) (Fischer et al., 1995; Wen et al., 1995). NES is considered a nonconserved motif consisting of three or four hydrophobic residues (such as leucine or isoleucine) and follows the following consensus; $Lx_{2,3}(F/I/L/V/M)x_{1,2,3}Lx(I/V/L)$, where x represents any residue and the numbers indicate spacing. For example, the NES motif found in the HIV-1 Rev protein is LPPLERLTL (Fischer et al., 1995). To date, the NES

Figure 16. The classical NES/CRM1 nuclear export pathway.

Nuclear export of proteins generally occurs through the CRM1 exportin. Interaction of NES-containing proteins with CRM1 is inhibited by the drug LMB, which leads to inhibition of NES-mediated nuclear export.



sequence is found in all eukaryotes and is present in at least 75 proteins (la Cour et al., 2003). Although NES is not the only export signal, it represents, by far, the most widely used signal and for this reason is considered the classical signal for the export of proteins from the nucleus.

The transport of proteins across the nuclear envelope requires not only a specific transport signal but also a specialized transporter that can pass through the NPC. The majority of transporters, which includes importins and exportins, are of the β -karyopherin family of proteins. In humans there exists approximately 20 members of this family that together accommodate the transport of proteins, tRNAs, snRNAs (small nuclear RNA), miRNAs (miRNA) and ribosomal subunits. Members of the β -karyopherin family are large proteins of 90-130 KDa and are characterized by the presence of an N-terminal binding domain for the GTPase Ran (Gorlich et al., 1997). Export of proteins harboring the classical leucine-rich NES is facilitated by the CRM1 exportin (Chromosome Region Maintenance or exportin 1), the most studied member of the β -karyopherin family (Figure 16) (Fornerod et al., 1997; Fukuda et al., 1997; Stade et al., 1997). Studies leading to the discovery of CRM1 as a nuclear exporter were based on the inhibitory effect of the drug leptomycin B (LMB) on the export of NES-containing proteins (Wolff et al., 1997). In fact, LMB binds directly to CRM1 to inhibit its interaction with NES (Figure 16) (Kudo et al., 1999; Nishi et al., 1994). Since then, the number and functional diversity of NES-containing proteins has led CRM1-mediated transport to be viewed as the classical nuclear export pathway.

1.9.3. Other nuclear export pathways and the associated transporters.

In the years since discovery of the NES as a nuclear export sequence great advancements have been made in the field of nuclear export. Although nuclear export of the majority of proteins is thought to occur through the NES/CRM1 pathway, there also exist other CRM1-independent pathways. As mentioned in section 1.9.2., there are many members of the β -karyopherin family of transporters. Of these, several have been shown to mediate nuclear export of specific molecules. Some examples include: Exportin-4, which exports the eukaryotic initiation factor 5 alpha (eIF5A) (Lipowsky et al., 2000); Exportin-5, an exporter of t-RNA and the eukaryotic elongation factor 1 alpha (eEF1A) (Bohnsack et al., 2002; Calado et al., 2002); Exportin-6, which exports actin (Stuven et al., 2003) and Exportin-7, which transports p50RhoGAP and 14-3-3 σ from the nucleus (Mingot et al., 2004). While the list of exportins has grown over the years, these are all specialized to export only certain cargo proteins. Until now, there has not been an export pathway that matches the diversity of CRM1-dependent export. Furthermore, these exportins do not appear to recognize specific consensus sequences representing export signals, whether or not they exist, such as the ability of CRM1 to recognize NES.

In general, nuclear export of proteins and RNA species, such as tRNA, miRNA and snRNA, is mediated by exportins of the β -karyopherin family and involves the Ran cycle. However, several proteins unrelated to karyopherins have also been shown to mediate nuclear export of molecules in a manner that does not depend on the RanGTP-RanGDP gradient. This is particularly true for general mRNA export, whose key transport mediator is a heterodimer consisting of TAP and a small cofactor termed Nxt or p15 (Clouse et al., 2001; Gruter et al., 1998; Wiegand et al., 2002). Although this complex is

unrelated to karyopherins it maintains the ability to interact with FG-repeats of the FG nucleoporins, which is required to pass through the NPC (Fribourg et al., 2001; Levesque et al., 2001; Wiegand et al., 2002). The TAP-p15 complex connects mRNAs, which are found as messenger ribonucleoprotein particles (mRNPs) to the nuclear pores. Since TAP itself is not a RNA-binding protein, it interacts with adaptor proteins in the mRNPs, such as ALY/REF, in order to promote mRNA export (Rodrigues et al., 2001; Zhou et al., 2000). In addition, nuclear export of tRNA in yeast involves the translation elongation factor eEF1A and a newly characterized protein Cex1p (Grosshans et al., 2000a; McGuire and Mangroo, 2007). Interestingly, both of these proteins have been described as cytoplasmic components of the nuclear aminoacylation-dependent tRNA export pathway (Grosshans et al., 2000a; Grosshans et al., 2000b; Hopper and Shaheen, 2008; Kohler and Hurt, 2007; McGuire and Mangroo, 2007). eEF1A and Cex1p were found to copurify with one another and to interact directly with aminoacylated tRNA (Grosshans et al., 2000a; McGuire and Mangroo, 2007). Cex1p interacts with the NPC and it was postulated that Cex1p accepts aminoacyl-tRNAs from the nucleus at the cytoplasmic side of the NPC and delivers them to eEF1A through a channeling mechanism (McGuire and Mangroo, 2007). It remains unclear as to the mechanism by which these proteins cooperate from the cytoplasm to mediate tRNA export.

1.9.4. Transcription-dependent nuclear export: An undefined and CRM1-independent pathway.

The VHL tumor suppressor protein has been shown to engage in nuclear-cytoplasmic trafficking in a manner that requires ongoing RNA PolII-mediated transcription. Previous reports established that the sequence required for transcription-dependent

nuclear export required the exon2-encoded β -domain of VHL (Bonicalzi et al., 2001; Lee et al., 1999). However, residues within this region do not encode a classical leucine rich NES. Also, nuclear-cytoplasmic trafficking of VHL is not altered by treatment with LMB, the inhibitor of the classical NES nuclear export pathway mediated by the CRM1 transport receptor (Bonicalzi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999; Yoshida and Horinouchi, 1999). Together these data supported the idea that 1- VHL participates in dynamic nuclear-cytoplasmic trafficking, 2- nuclear export of VHL is independent of the classical CRM1/NES pathway, and 3- VHL exports from the nucleus utilizing a mechanism that requires ongoing RNA PolII-dependent transcription.

Although several studies have reported the dependency on ongoing RNA PolII activity for active nuclear-cytoplasmic shuttling, these have focused mostly on nuclear import of proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Siomi et al., 1997). The idea that transcription-dependent nuclear export of proteins may be a bona fide nuclear export pathway was substantiated by the discovery that another protein, the poly(A)-binding protein 1 (PABP1), also requires ongoing RNA PolII activity to export from the nucleus (Afonina et al., 1998). Similar to VHL, PABP1 is a predominantly cytoplasmic protein that shifts to a more nuclear localization upon arrest of RNA PolII-mediated transcription, using transcriptional inhibitors such as ActD and DRB (Afonina et al., 1998). The observation that nuclear export of VHL and PABP1 is transcription-dependent and independent of the classical NES/CRM1 pathway suggested that these proteins utilized a common, yet novel, nuclear export pathway.

2. RATIONALE

Nuclear-cytoplasmic trafficking is essential for the E3 ubiquitin-ligase function of VHL and oxygen-dependent degradation of HIF α (Groulx and Lee, 2002; Lee et al., 1999). Failure of VHL to continuously shuttle between the nuclear and cytoplasmic compartments leads to the stabilization of HIF α (Groulx and Lee, 2002; Lee et al., 1999; Mekhail et al., 2004a; Mekhail et al., 2005; Mekhail et al., 2004b). VHL engages in nuclear-cytoplasmic shuttling dynamics independently of the classical, leucine rich, nuclear export sequence NES (Fischer et al., 1995; Wen et al., 1995) since it is insensitive to LMB, a specific inhibitor of NES-mediated nuclear export by the CRM1 exporter. However, VHL accumulates in the nucleus upon addition of inhibitors of RNA PolII activity, such as ActD and DRB (Groulx and Lee, 2002; Lee et al., 1999). Interestingly, the general RNA metabolism and translation initiation factor PABP1 exhibits similar transcription-dependent trafficking dynamics as VHL since it also accumulates in the nucleus upon addition of inhibitors of RNA PolII activity and is insensitive to treatment with LMB (Afonina et al., 1998). This suggested the existence of a transcription-dependent nuclear export pathway that is employed by VHL, PABP1 and perhaps other proteins, that operates independently of the classical NES/CRM1 system. The goal of this study was to: 1- study the transcription-dependent nuclear-cytoplasmic trafficking pathway utilized by VHL; and 2- understand the role of nuclear-cytoplasmic trafficking on the tumor suppressor function of VHL.

3. *HYPOTHESIS*

VHL utilizes a novel transcription-dependent mechanism to export from the nucleus in order to promote its tumor suppressor function in mediating oxygen-dependent degradation of HIF α .

4. *AIMS*

- 4.1.** Identify a novel sequence that mediates transcription-dependent nuclear export of VHL.
- 4.2.** Identify cancer-causing mutations that disrupt nuclear export of VHL and its function in HIF α degradation.
- 4.3.** Study the mechanism utilized by VHL to engage in transcription-dependent nuclear export.

5. MATERIALS AND METHODS

5.1. Cell culture, transfections, drug treatments and hypoxia treatment.

786-0 (VHL-negative) renal carcinoma cells, MCF-7 cells, NIH 3T3 and A549 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Fisher) and 1% penicillin-streptomycin (P-S) in a 37°C and 5% CO₂ environment. Transient transfections in MCF-7 cells were conducted with Effective transfection reagent (QIAGEN). Transfected cells were incubated for 24 hrs before any manipulations or drug treatment. Stable cell lines were generated by stably transfecting 786-0 cells with the following Flag-tagged constructs; F-VHL-GFP, F-VHL(D121G)-GFP, F-VHL(D126Y)-GFP and F-VHL(G123A)-GFP, followed by G418 selection. Where indicated cells were treated at 37°C with a final concentration of 2 µM ActD, 10 µM LMB or 25 µg/ml DRB for 1 hour prior to photobleaching experiments and 8 µM ActD, 25 µg/ml DRB or 10 µM LMB for 3 hours prior to live cell fluorescence imaging or harvesting for immunoprecipitation. Proteasome inhibitor treatment was performed by using MG132 (Calbiochem) to a final concentration of 10 µM for 2 hours before the cells were harvested. Where indicated cells were treated with 100 µg/ml of cycloheximide for 2 hours.

Hypoxia treatment was performed in a hypoxic chamber at 37°C under a 1% O₂, 5% CO₂ and N₂-balanced atmosphere. Transient transfections in MCF-7 and A549 cells were conducted with Effective transfection reagent (QIAGEN). Transfected cells were incubated for 24 hrs before any manipulations or drug treatment. For SD or AP conditions a buffer-free medium (DME; Invitrogen) was freshly prepared and

supplemented with 5% (vol/vol) FBS and 1% P-S. 44 mM NaHCO₃ was added and the pH was adjusted to 7.2 (SD) or 6.6 (AP) with HCL. Air was bubbled into both types of media to stabilize the pH at 7.2. In hypoxia the SD media remained stable and the AP media reverted slowly to a pH of 6.6.

5.2. Expression vectors.

Human full length VHL, deletion, truncation or point mutants of VHL were cloned into pcDNA3.1 between an NH₂-terminal Flag-tag and a COOH-terminal GFP-tag, as previously described (Bonicalzi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999). F-GFP, F-GFP-NES and F-VHL-GFP-NLS, were previously described in Groulx et al., 2000 and Lee et al., 1999. The PK-GFP-NLS construct used for the *in vivo* FLIP nuclear export assay consists of pyruvate kinase (PK), which does not encode any localization determinant, GFP, and an NLS derived from the simian virus large T antigen SV40 as previously described (Groulx et al., 2000; Kalderon et al., 1984). Human full length VHL and deletion mutants, the strong nuclear export sequence (NES) from the human immunodeficiency virus REV were inserted into a F-PK-GFP-NLS construct that was previously described in Groulx et al., 2000, between the Flag-tag and PK using the ApaI and XhoI restriction sites. cDNAs corresponding to VHL(114-138), PABP1(296-317) and Cyclin C(158-179) that encode TD-NEM sequences and the full length Cyclin C were inserted into F-GFP or PK-GFP-NLS using ApaI and XhoI restriction sites. The human full length PABP1 and the deletion mutant Δ 296-317 were fused to GFP-F to produce the GFP-F-PABP1 and GFP-F-PABP1(Δ 296-317) fusion proteins. Human full length VHL and deletion mutants, the strong nuclear export sequence (NES) from the human immunodeficiency virus REV were inserted into a F-PK-GFP-NLS construct that

was previously described in Khacho et al., 2008 and Groulx et al., 2000. For F-eEF1A-GFP-NLS the human eEF1A cDNA was inserted between Flag and GFP-NLS using Apa1 and Xho1 restriction sites. The human PABP1 was fused to GFP-F to produce the GFP-F-PABP1 fusion protein. F-VHL-GFP, F-ΔC157-GFP and F-GFP adenoviruses were produced using the Cre-lox recombination system (Groulx and Lee, 2002; Lee et al., 1999). PK-GFP-NoDS^{H+} and PK-GFP-NLS-NoDS^{H+} were previously described in Mekhail et al., 2007. cDNAs corresponding to BAX(364-426), CycC(158-179), PR(101-121), RanBP1(64-83), TBP1(181-200) and TDRD3(583-607) that encode TD-NEM sequences were inserted into F-PK-GFP-NLS using Apa1 and Xho1 restriction sites.

5.3. siRNA-mediated knockdown.

For siRNA experiments cell were transfected with 100 nM of either of eEF1A siRNA (2991, 2804; Ambion), Exp5-1 siRNA (Lund et al., 2004), eEF2 siRNA (10791; Ambion), control siRNA (Ambion) or Effectene alone (mock) for 48 hours prior to photobleaching experiments or 72 hours prior to imaging. Where indicated siRNA transfected cells were treated with 8 μM ActD for 3 hours after a 72 hour incubation period with siRNA and prior to live cell fluorescence imaging.

5.4. Live cell fluorescence imaging.

Images of living cells transiently expressing GFP from experiments where photobleaching was not utilized were imaged with an Axiovert S100TV microscope (Carl Zeiss MicroImaging, Inc) equipped with a 40x/1.2 C-Apochromat water immersion objective using a digital charged-coupled device camera (Empix). Cell nuclei were stained with Hoechst 33342 (Sigma). Images were captured using the Northern Eclipse software package (Empix).

5.5. Photobleaching and microscopy.

Cells were cultured and transfected directly onto 35-mm dishes with coverslip bottoms (MatTek). Photobleaching and live cells microscopy was performed using a confocal microscope (LSM5 Pascal Laser Scanning Microscope, Carl Zeiss Canada). In all experiments cells were maintained at 37°C in an environmental chamber. A 63x plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to three rapid pulses of a 488-nm argon laser at 100% and image acquisition was at 1% of full laser power. A highly quantitative live cell nuclear export assay utilizing FLIP (Fluorescent loss in photobleaching) technology was used to measure nuclear export activity. For these cytoplasmic FLIP experiments a fusion protein consisting of the large and amorphous pyruvate kinase (PK) that does not encode localization determinants, GFP, and the nuclear localization signal (NLS) (Kalderson et al., 1984) derived from the simian virus large T antigen SV40 (PK-GFP-NLS) was used. Cells expressing a PK-GFP-NLS-tagged fusion protein were repeatedly bleached in a small cytoplasmic region and imaged at 30-second intervals. For cytoplasmic FLIP experiments of cells expressing a GFP-tagged fusion protein a large cytoplasmic region was initially bleached with three rapid pulses to eliminate the dominant cytoplasmic signal. This was followed by repetitive bleaching in a small region of the cytoplasm and imaged at 30-second intervals. Small bleached areas for cytoplasmic FLIPs were kept consistent in terms of size and distance from the nucleus. Fluorescence loss in the unbleached areas was quantified as previously described (Mekhail et al., 2005; Phair and Misteli, 2000) using the following equation $I_{rel} = (I_{(t)}/I_{(0)}) * (N_{(0)}/N_{(t)})$, where $I_{(t)}$ is the average intensity of the unbleached nucleus or cell at time point t, $I_{(0)}$ is the average

prebleached intensity of the nucleus or cell of interest, $N_{(0)}$ and $N_{(t)}$ are the average nuclear or cellular fluorescence intensity of a neighboring cell in the same field of vision at prebleach or at time t , respectively. This calculation accounts for any losses in fluorescence by normalizing the fluorescence of the cell of interest to that of a neighboring cell of approximate equal size and fluorescent intensity. Nuclear FLIPs were performed by repetitive bleaching of a small nuclear area and imaging at 15-second intervals. Loss of nuclear fluorescence was quantified using the above equation, however the value for $I_{(t)}$ in this case is the bleached nucleus. Pseudocolor images were generated to highlight differences in GFP fluorescence, red represents high fluorescent intensity and light blue represents low fluorescent intensity. The quantification graphic was generated by a FLIP/FRAP software. For all bleaching experiments 10 datasets were analyzed for each result. Pseudocoloring for bleaching experiments was achieved by applying the gradient map function of Photoshop (Adobe) to a montage of picture frames prepared with ImageJ software (National Institute of Health, Bethesda, MD). The Northern Eclipse (Empix), Excel (Microsoft) and FreeHand (Macromedia) software packages were also used to capture images, analyze the data and generate graphs.

5.6. Polykaryon assay.

MCF-7 cells were transfected to express fluorescent-labeled proteins and incubated under standard conditions for 24 hours. Usually 40 to 60% of cells presented strong fluorescence as observed by 488-nm fluorescence microscopy. Cells were trypsinized 24 hours after transfection and mixed with untransfected NIH 3T3 cells in a ratio of 1 to 10. The cell mixture was plated in 35-mm dishes with coverslip bottoms and incubated overnight in standard cell culture conditions. The confluent cell layer was visually

inspected for even distribution of fluorescent cells among untransfected cells. Cells were washed twice with prewarmed PBS and fused for 2 minutes by addition of a prewarmed 50% solution of polyethylene glycol (PEG) in PBS (Sigma-Aldrich). PEG was removed by four washes with prewarmed PBS and cells were then replenished with warmed standard cell culture media. Hoechst staining of DNA was used to identify donor and acceptor cells. Cells were observed under phase-contrast microscopy for fusion events and were monitored for the redistribution of nuclear expression of PK-GFP-NLS-tagged proteins.

5.7. In vitro nuclear export assay.

The in vitro export assay was performed as described in Groulx et al., 2000. Briefly, cells were plated and grown on a 35-mm coverslip plate. Cells were washed with transport buffer (TB) containing 20 mM HEPES pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM Mg(OAc)₂ and permeabilized at 4°C for 5 minutes with TB containing 50 µg/ml digitonin and a protease inhibitor mixture (Hoechst stain 33258 (Sigma) was used to monitor the permeabilization). After several washes with TB at 4°C, cells were incubated for 30-45 minutes at 20°C in the presence of a standard mixture that included MCF-7 cellular lysate, TB, 2 mM ATP, 2 mM GTP, and an ATP regenerating system (5 mM creatine phosphate and 20 units/ml creatine phosphokinase) to a final volume of 1 ml. To obtain the MCF-7 cellular lysate, first MCF-7 cells were incubated in RSB hypotonic buffer containing; 10 mM HEPES pH 6.2, 10 mM NaCl and 1.5 mM MgCl₂, for 15 min at 4°C. Cells were then homogenized in a tight pestle homogenizer. Protein concentrations were quantified using the BCA protocol.

For experiments using eEF1A or eEF2 depleted MCF-7 cellular lysates, cells were homogenized as described above. Lysate containing 0.5 mg of proteins was incubated in the presence of 10 µg of eEF1A antibody or 10 µg of eEF2 antibody and a cocktail of protease inhibitors for 1 hour while rotating at 4°C. Undepleted lysates were incubated with 10 µg of irrelevant anti-Flag antibody as a control followed by incubation with 20 µl protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 3 hours at 4°C. Cells were then spun to remove beads bound to immunoprecipitated eEF1A or eEF2. This was followed by another round of eEF1A or eEF2 depletion using 10 µg of eEF1A antibody or 10 µg of eEF2 antibody and 20 µl protein A/G PLUS-agarose beads; however the immunoprecipitation was performed overnight. The lysate was spun and an aliquot of the supernatant immunoblotted to verify depletion of eEF1A or eEF2. Actin was used as a control. Lysates that were successfully depleted of eEF1A or eEF2 were used in the export assay in the same manner as described above.

5.8. Immunofluorescence.

Cells were seeded onto coverslips and fixed with pre-chilled methanol for 10 min at -20 °C followed by prechilled acetone for 1 min at -20 °C. Anti-PABP1 (Upstate) monoclonal antibody was used. Cells were incubated for 1 hour with a primary antibody solution containing 10% FBS and 1% Triton-X-100 (v/v) at room temperature in a humidified chamber. Cells were then washed several times in PBS before a 1 hour incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch) at room temperature in a dark humidified chamber. Hoechst stain 33342 (Sigma) was added to visualize nuclei and coverslips were mounted using Fluoromount G (EMS).

5.9. Bioinformatic analysis.

Proteins containing a TD-NEM motif were identified using the motif and My Genomics Resource Center softwares.

5.10. Immunoprecipitation and immunoblotting.

Cells were lysed in lysis buffer containing 0.5% Igepal CA630, 100mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, and 1 mM sodium orthovanadate with 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin. Cell lysates were incubated with anti-Flag M2 beads (Sigma) over night while tumbling at 4°C. Beads were washed several times and eluted with Flag peptides (Sigma). For total cell lysates, cells were washed several times in phosphate-buffered saline (PBS), lysed with 4% SDS in PBS, boiled for 5 minutes and the DNA was sheared by passage through a 19-gauge needle. Protein concentration was quantified using the bicinchoninic acid (BCA) method (Pierce). Samples were separated on denaturing polyacrylamide gels, transferred onto PVDF membranes and blocked in skimmed milk powder in PBS containing 0.2% Tween 20 before incubation with Flag-M2 (Sigma), HIF2- α (Novus biologicals), actin (sigma), GFP (AVES lab inc.), eEF1A (Santa Cruz), eEF2 (Cell Signaling technology) or Expotin-5 (a kind gift from Ian G. Macara) antibodies. Membranes were washed with 0.2% Tween-PBS and blotted with a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) and detected by western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

5.11. Silver staining.

Cells were lysed in lysis buffer containing 0.5% Igepal CA630, 100mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, and 1 mM sodium orthovanadate with 2 µg/ml

leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin. Cell lysates were incubated with anti-Flag M2 beads (Sigma) overnight while tumbling at 4°C. Beads were washed several times and eluted with Flag peptides (Sigma). For total cell lysates, cells were washed several times in phosphate-buffered saline (PBS), lysed with 4% SDS in PBS, boiled for 5 minutes and the DNA was sheared by passage through a 19-gauge needle. Protein concentration was quantified using the bicinchoninic acid (BCA) method (Pierce). Samples were separated on denaturing polyacrylamide gels. Silver stained gels were performed according to manufacturer's protocol (Bio-Rad laboratories).

5.12. Radioisotope Labeling.

Cells were plated on 35-mm dishes after which they were transfected with 100 nM eEF1A, eEF2 or scrambled siRNA (mock) for 48 and 72 hours. At the indicated times cells were incubated for 30 min in glutamine-, methionine, and cysteine-free DMEM and then labeled with 10 µCi/ml of [³⁵S]Met for 30 minutes. Cells were harvested and lysed for 30 minutes at 4°C with modified RIPA lysis buffer containing 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA and a cocktail of protease inhibitors. Total cellular lysates were separated on a denaturing polyacrylamide gel. The gel was transferred onto a PVDF membrane and [³⁵S]Met labeling was visualized by autoradiography. The membrane was washed three times in PBST, blocked in skimmed milk powder in PBST before incubation with eEF1A and eEF2 antibodies.

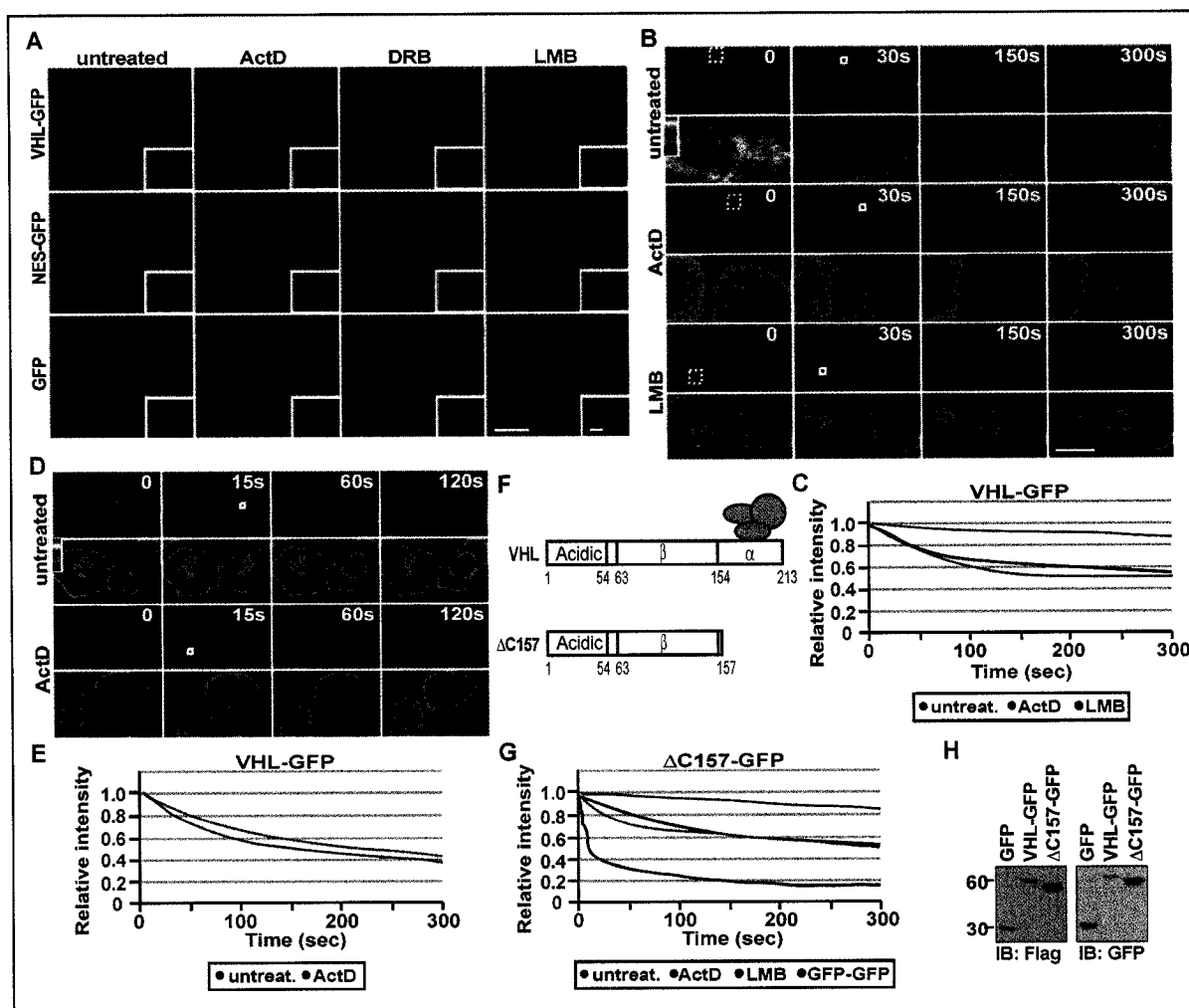
6. RESULTS

6.1. VHL encodes a transcription-dependent nuclear export signal.

VHL engages in a nuclear export pathway that requires ongoing RNA PolIII activity (Groulx and Lee, 2002; Lee et al., 1999). As previously reported, addition of the RNA PolIII inhibitors ActD or DRB resulted in nuclear accumulation of the highly cytoplasmic VHL-GFP fusion protein (Figure 17A) (Groulx and Lee, 2002; Lee et al., 1999). These RNA PolIII inhibitors did not have an effect on GFP alone or GFP fused to a strong, classical, leucine rich nuclear export signal (GFP-NES) (Figure 17A). In contrast, LMB, a compound that abolishes CRM1-dependent nuclear export of NES-containing proteins (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1999; Stade et al., 1997), had no effect on the steady-state localization of VHL-GFP but caused nuclear accumulation of NES-GFP (Figure 17A). It has been previously demonstrated, through cellular fusion experiments, that the nuclear accumulation of VHL-GFP following inhibition of RNA PolIII activity is a result of decreased nuclear export (Lee et al., 1999). To further verify this, nuclear export of VHL-GFP was studied by FLIP (fluorescence loss in photobleaching) analysis. In such an experiment, the cytoplasmic region of cells transiently expressing VHL-GFP is repetitively photobleached (white boxes shown in Figure 17B) and the loss of nuclear fluorescence is monitored over time. A decrease in nuclear GFP fluorescence, relative to the level of GFP fluorescence at $t=0$, indicates that the GFP-tagged fusion protein has been exported from the nucleus to the cytoplasm. Through cytoplasmic FLIP experiments, it was determined that nuclear accumulation by ActD was due to a considerable decrease in the rate of nuclear export of VHL-GFP (Figure 17B and refer to quantification in C). In contrast, LMB, which does not alter the

Figure 17. Transcription-dependent nuclear export of VHL.

(A) ActD alters the steady state localization of VHL. MCF-7 cells transiently expressing VHL-GFP, NES-GFP or GFP were either untreated or treated with LMB (10 μ M), ActD (8 μ M) or DRB (25 μ g/ml) for 3 hours. Insets are the corresponding Hoechst staining of the cells and scale bars represent 10 μ m. (B and C) Cytoplasmic FLIP reveals that ActD decreases nuclear export of VHL. MCF-7 cells transiently expressing VHL-GFP were treated with ActD (2 μ M) or LMB (10 μ M) for 1 hour. Cells were initially bleached in a large cytoplasmic region (dashed squares) to reduce cytoplasmic signal and then submitted to repetitive bleaching in a small cytoplasmic region (white squares). Cells were imaged between pulses and the corresponding kinetics for loss of nuclear fluorescence was calculated and plotted on a graph (C and see Materials and Methods). In (B) pseudocolored panels are included to better illustrate subtle changes in fluorescence intensity. Scale bar represents 10 μ m. (D and E) Nuclear FLIP analysis was performed on cells treated as in (B) by repetitive bleaching in a small area in the nucleus. The loss of nuclear fluorescence was graphed. (F and G) Δ C157 exports from the nucleus in a transcription-dependent manner. Δ C157-GFP transiently expressing cells were treated and analyzed as described in (B). (H) Western blot analysis using anti-Flag and anti-GFP antibodies verifies that the Flag and GFP-tagged VHL, Δ C157 and GFP fusion proteins used for photobleaching experiments are not fragmented.



steady-state localization of VHL-GFP, also did not affect its nuclear export activity (Figure 17B and C). In order to eliminate the possibility that the observed decrease in nuclear export was due to nuclear retention, the intra-nuclear dynamics of VHL-GFP were analyzed. Nuclear FLIP experiments, where a small nuclear region was bleached repetitively, revealed similar intra-nuclear dynamics between ActD-treated and untreated cells demonstrating that ActD (or DRB) did not cause nuclear retention of VHL-GFP (Figure 17D and E). Also, loss of GFP fluorescence in all the FLIP experiments was not due to fragmentation of the GFP-fusion proteins as demonstrated by immunoblot analysis (Figure 17H).

Next it was important to determine whether the signal mediating nuclear export was encoded by VHL or was present within the E3 ubiquitin-ligase complex. To decipher this we tested a C-terminal deletion of the α -domain of VHL (Δ C157), which is required for assembly with core E3 ubiquitin-ligase components elongins B, C and Cullin-2 (Bonicalzi et al., 2001; Ohh et al., 2000). Δ C157-GFP, which does not form an E3 ubiquitin-ligase complex (Figure 17F), retained the ability to engage in transcription-dependent nuclear export since it accumulated in the nucleus upon addition of ActD or DRB (data not shown). Furthermore, Δ C157-GFP displayed reduced nuclear export dynamics in the presence of RNA PolII inhibitors as demonstrated by cytoplasmic FLIP analysis (Figure 17G). These data indicate the presence of a transcription-dependent nuclear export signal between residues 1 and 157 of VHL and suggest that formation of the E3 ubiquitin-ligase complex is not required for export of VHL.

6.2. Establishing a live cell nuclear export assay using photobleaching technology.

In order to define the region encoding a nuclear export signal, larger truncation mutants of VHL were constructed. However, these GFP-tagged truncation mutants failed to provide insight on transcription-dependent nuclear export of VHL since they exhibited export rates similar to GFP-GFP, which diffuses across the nuclear envelope (Figure 17G). To further examine the nuclear export properties of VHL, we established a quantitative live cell nuclear export assay utilizing FLIP technology (Figure 18), which is less abrasive than traditional *in vitro* export assays. A fusion protein consisting of the large and amorphous pyruvate kinase (PK) that does not encode localization determinants, GFP, and a nuclear localization signal (NLS) (Kalderon et al., 1984) derived from the simian virus large T antigen SV40 (PK-GFP-NLS) was used to ensure that any movement across the nuclear envelope is not due to diffusion (Figure 19A and see Methods). Also, the presence of such a strong NLS would override an intrinsic nuclear import signal ensuring that all fusion proteins being tested exhibit similar rates of nuclear import. PK-GFP-NLS strictly localized in the nucleus at steady state owing to the strong NLS activity (Figure 19B). PK-GFP-NLS did not undergo a significant loss of nuclear GFP fluorescence after cytoplasmic FLIP, as expected, since this protein fails to export from the nucleus (Figure 19B and E). Addition of a strong, classical and LMB-sensitive NES of the human immunodeficiency virus REV (Fischer et al., 1995) to the reporter protein (NES-PK-GFP-NLS) resulted in a rapid loss of nuclear GFP fluorescence during cytoplasmic FLIP demonstrating that this fusion protein engages in nuclear export activity (Figure 19B and E). The inability of PK-GFP-NLS to export from the nucleus

Figure 18. The live cell FLIP nuclear export assay.

A protein consisting of the PK-GFP-NLS reporter fused to a protein or peptide of interest is expressed in nuclei of living cells. If the fusion protein can export from the nucleus to the cytoplasm then repetitive photobleaching in a small cytoplasmic region will result in a decrease in nuclear fluorescence. Conversely, a fusion protein that does not export from the nucleus will not be photobleached and the nuclear fluorescence will remain unchanged.

Figure 19. A transcription-dependent nuclear export sequence is encoded within the exon-2-encoded β -domain of VHL. (A) Schematic diagram of the PK-GFP-NLS fusion protein used for the live cell FLIP nuclear export assay describing the region where protein or peptide sequences were fused. (B-E) MCF-7 cells transiently expressing the indicated constructs were submitted to cytoplasmic FLIP analysis where a small cytoplasmic region (white squares) within specific cells (dashed circle outlines the cell nucleus) was repeatedly bleached. Kinetics for the loss of nuclear fluorescence from images obtained in B, C and D were calculated and plotted on a graph (E). PK refers to the PK-GFP-NLS reporter construct and NES, VHL and $\Delta 114-254$ indicate the sequences fused to PK-GFP-NLS. (F) MCF-7 cells were transiently transfected with the indicated fusion constructs. Cells were lysed, immunoprecipitated with anti-Flag beads and submitted to immunoblot analysis using an anti-Flag antibody. All fusion proteins ran at the appropriate molecular weights indicating that the fusion proteins were not fragmented. (G and H) MCF-7 cells transiently transfected with PK-GFP-NLS or NES-PK-GFP-NLS were subjected to nuclear FLIP by repetitively bleaching a small area (white square) within the nucleus (dotted outline). Cells were imaged between pulses and kinetics for the loss of nuclear fluorescence were calculated and plotted on a graph. Scale bar represents 10 μm .

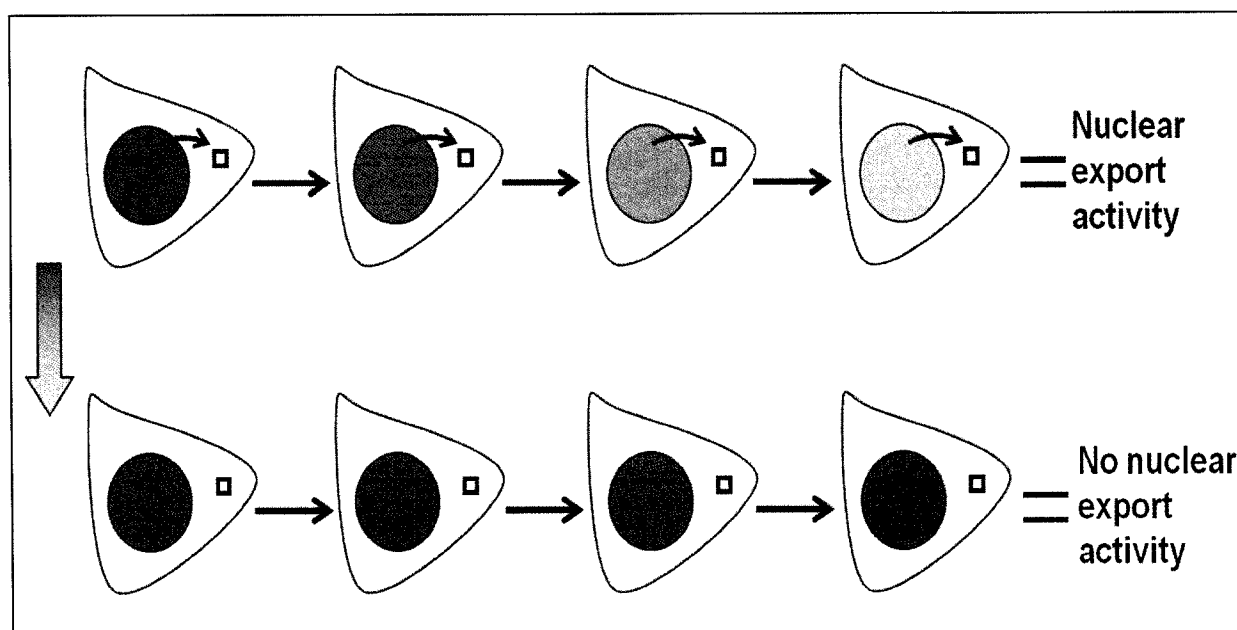
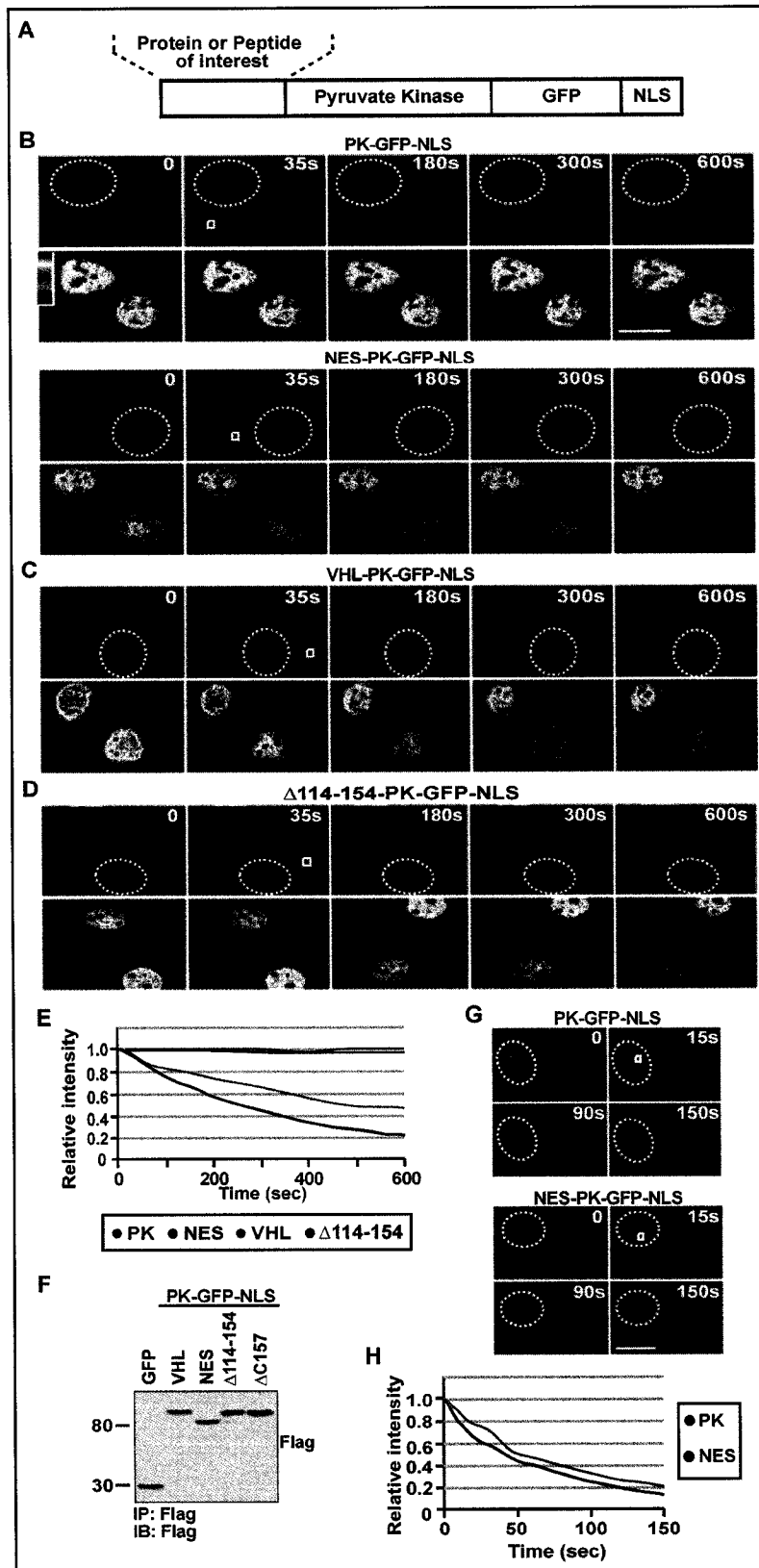


Figure 19. A transcription-dependent nuclear export sequence is encoded within the exon-2-encoded β -domain of VHL. (A) Schematic diagram of the PK-GFP-NLS fusion protein used for the live cell FLIP nuclear export assay describing the region where protein or peptide sequences were fused. (B-E) MCF-7 cells transiently expressing the indicated constructs were submitted to cytoplasmic FLIP analysis where a small cytoplasmic region (white squares) within specific cells (dashed circle outlines the cell nucleus) was repeatedly bleached. Kinetics for the loss of nuclear fluorescence from images obtained in B, C and D were calculated and plotted on a graph (E). PK refers to the PK-GFP-NLS reporter construct and NES, VHL and $\Delta 114-254$ indicate the sequences fused to PK-GFP-NLS. (F) MCF-7 cells were transiently transfected with the indicated fusion constructs. Cells were lysed, immunoprecipitated with anti-Flag beads and submitted to immunoblot analysis using an anti-Flag antibody. All fusion proteins ran at the appropriate molecular weights indicating that the fusion proteins were not fragmented. (G and H) MCF-7 cells transiently transfected with PK-GFP-NLS or NES-PK-GFP-NLS were subjected to nuclear FLIP by repetitively bleaching a small area (white square) within the nucleus (dotted outline). Cells were imaged between pulses and kinetics for the loss of nuclear fluorescence were calculated and plotted on a graph. Scale bar represents 10 μm .



compared to its NES-containing counterpart is not a consequence of unforeseen nuclear retention or significant difference in nuclear dynamics between both molecules as revealed by nuclear FLIP (Figure 19G and H). Nuclear fluorescence was also similar for both fusion proteins indicating that the difference in nuclear export capacity is not due to a large difference in expression levels (Figure 19B).

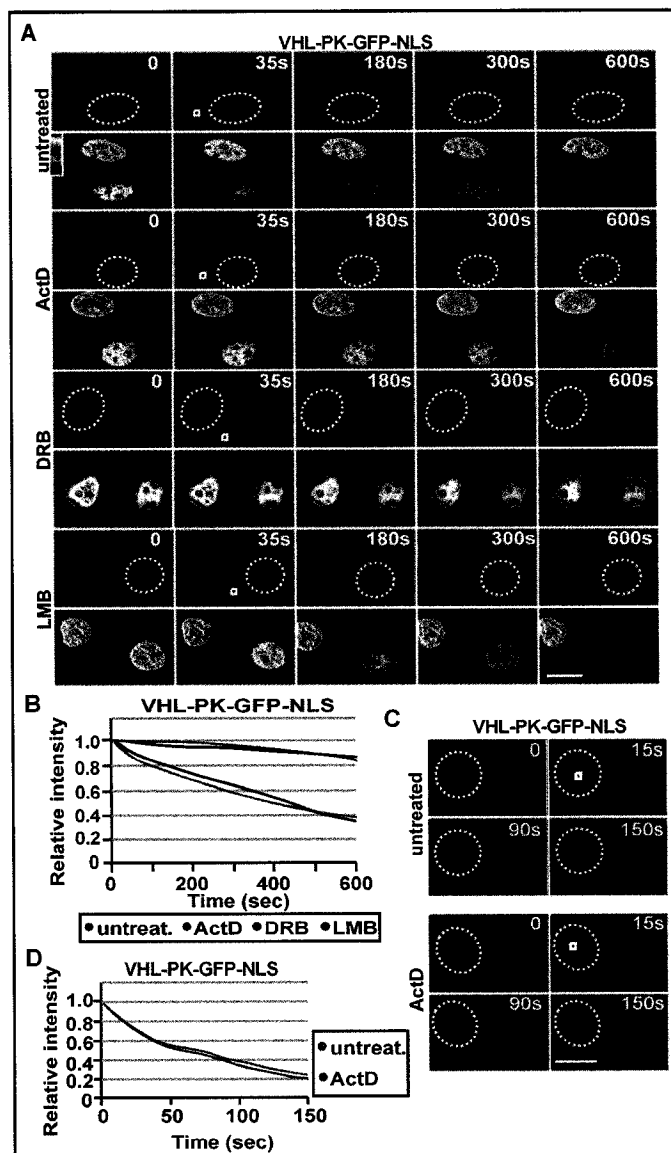
Full length VHL was able to confer nuclear export activity to the PK-GFP-NLS reporter confirming that this protein encodes nuclear export activity (Figure 19C and E). The kinetics for the loss of nuclear signal of VHL-PK-GFP-NLS was very different compared to molecules that are able to passively diffuse suggesting that the loss of nuclear signal is not a consequence of fragmentation of the fusion protein (Figure 19F). Removal of the exon-2-encoded β -domain (Δ 114-154-PK-GFP-NLS) abrogated the ability of VHL to export the reporter protein from the nucleus (Figure 19D and E). This observation is consistent with previous reports demonstrating that nuclear accumulation of VHL upon treatment with RNA PolII inhibitors requires the exon-2-encoded β -domain of VHL (Bonicalzi et al., 2001; Lee et al., 1999). ActD or DRB, but not LMB, inhibited nuclear export of VHL-PK-GFP-NLS (Figure 20A and B) without affecting intra-nuclear dynamics (Figure 20C and D) similar to what was observed with VHL-GFP (Figure 17) confirming that inhibitors of RNA PolII-mediated transcription significantly alter nuclear export of VHL. Furthermore, these results verify that the transcription-dependent nuclear export property of VHL was maintained in the VHL-PK-GFP-NLS fusion protein.

6.3. Nuclear export of VHL is mediated by a discrete transcription-dependent motif.

We used this *in vivo* FLIP nuclear export assay to map the region within the exon-2-encoded β -domain that mediates transcription-dependent nuclear export of VHL. Further

Figure 20. Nuclear export of VHL-PK-GFP-NLS is inhibited by RNA PolII inhibitors.

(A and B) Cells were transiently transfected with VHL-PK-GFP-NLS and were either treated with ActD (2 μ M), DRB (25 μ g/ml) or LMB (10 μ M) for 1 hour or left untreated. Cytoplasmic FLIP was performed as described above to verify nuclear export activity. **(C and D)** Nuclear FLIP was performed on cells transiently expressing VHL-PK-GFP-NLS and treated as described in (A). Scale bars represent 10 μ m.



mutagenesis analysis revealed that a discrete domain between residues 114-131 of VHL is required for efficient nuclear export of the PK-GFP-NLS reporter (Figure 21A, B and C). The deletion mutant Δ 114-131-GFP (without PK and NLS) also displayed a markedly reduced rate of nuclear export compared to wild type VHL-GFP (Figure 21D and E). The subcellular localization and the nuclear export dynamics of Δ 114-131-GFP was unaffected by treatment with ActD or DRB (Figure 21D), but rather exhibited a similar export rate to Δ C157-GFP after ActD treatment (Figure 17G). More importantly, residues 114-138 alone were sufficient to confer efficient nuclear export properties to PK-GFP-NLS, which was abolished upon addition of ActD (Figure 22A and B) or DRB (data not shown). The rate of export observed with residues 114-138 was similar to that of Δ C157 (Figure 17G), indicating that these residues alone confer nuclear export activity to the full length protein. These results were also confirmed using traditional assays for measuring nuclear export activity. In a cellular fusion assay, this sequence was able to efficiently export the PK-GFP-NLS reporter protein from the donor nucleus to the acceptor nuclei compared to the PK-GFP-NLS control (Figure 22C). Residues 114-138 also stimulated nuclear export of PK-GFP-NLS in an *in vitro* nuclear export assay (Figure 22D and E). We have, therefore, identified a novel and discrete motif that mediates transcription-dependent nuclear export of VHL.

6.4. VHL and PABP1 share a common transcription-dependent nuclear export motif.

VHL and PABP1 are implicated in distinct molecular networks but share the characteristic of engaging in a nuclear export pathway that requires ongoing RNA PolII

Figure 21. Residues 114-131 are required for nuclear export of VHL. (A) Mapping the nuclear export domain of VHL. Schematic diagram indicates deletion mutants of VHL that were submitted to cytoplasmic FLIP to assess the nuclear export activity. (+) and (-) indicates the ability or inability of the fusion protein to engage in nuclear export. (B and C) MCF-7 cells transiently transfected with PK-GFP-NLS, Δ C157-PK-GFP-NLS or Δ 114-131-PK-GFP-NLS were submitted to cytoplasmic FLIP where a small cytoplasmic region of a cell was bleached repetitively. The loss of nuclear GFP fluorescence was monitored over time and plotted on a graph (C). (D and E) Cells transiently expressing Δ 114-131-GFP were initially bleached in a large cytoplasmic region followed by repetitive bleaching in a small cytoplasmic region after being treated for 1 hour with 2 μ M ActD or left untreated. Kinetics for the loss of nuclear fluorescence were calculated and plotted on a graph (E).

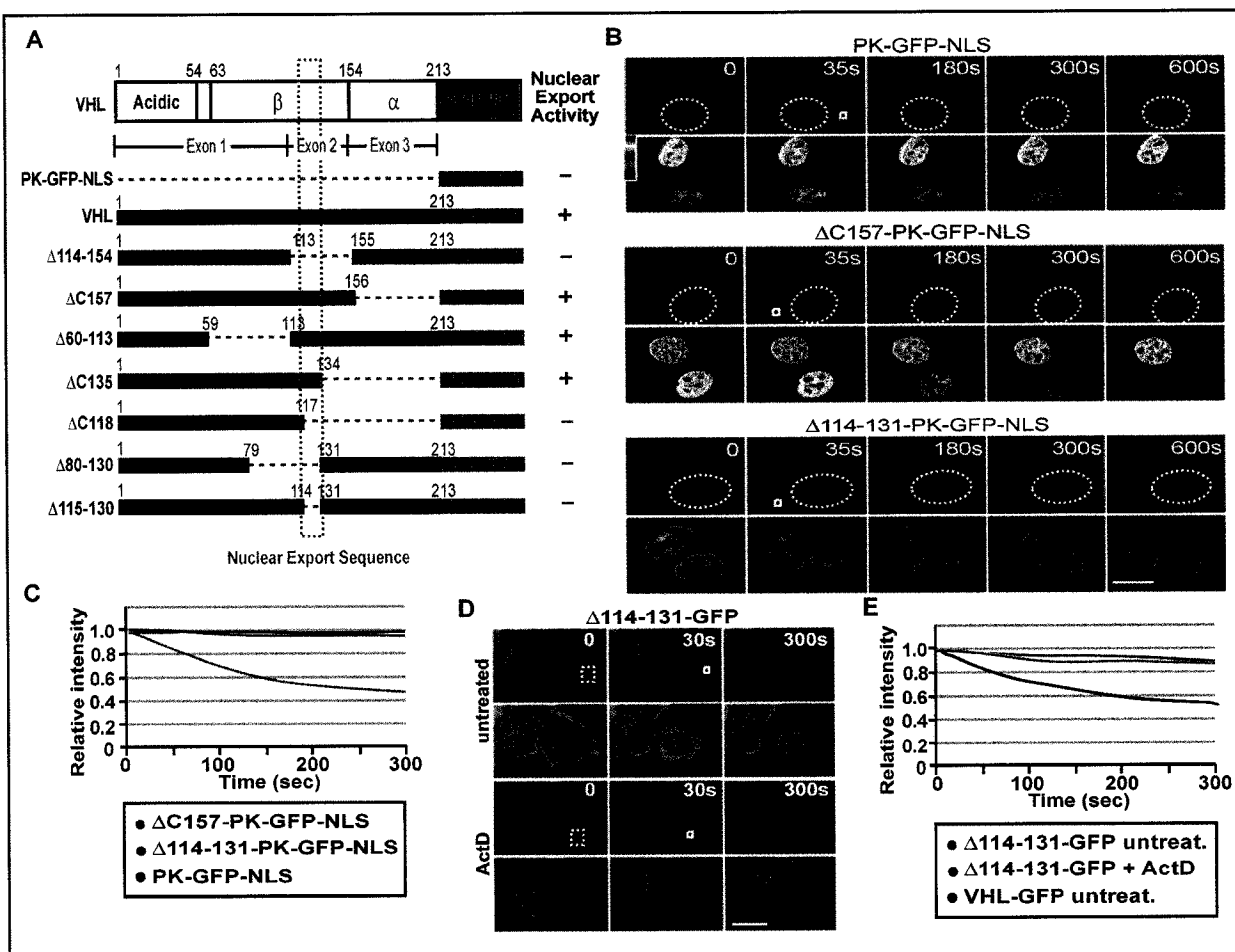
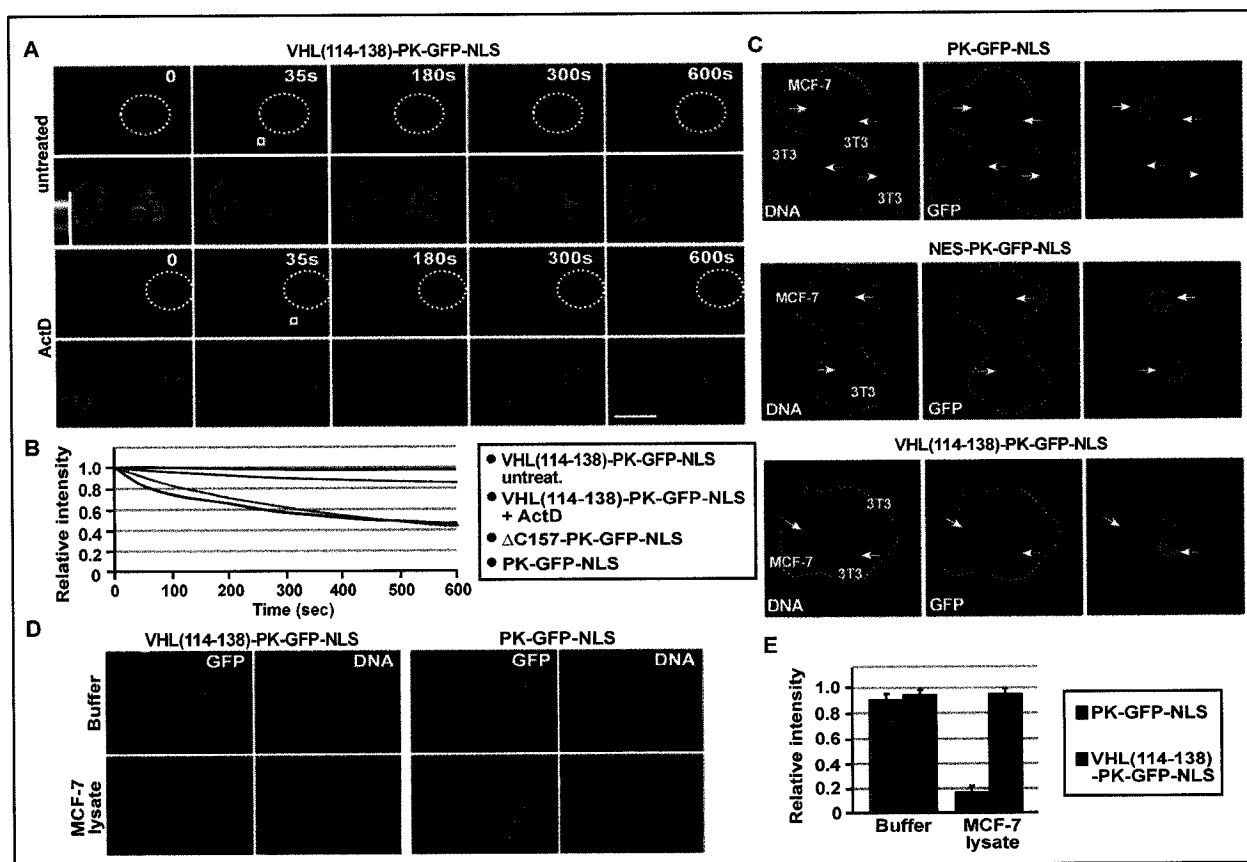


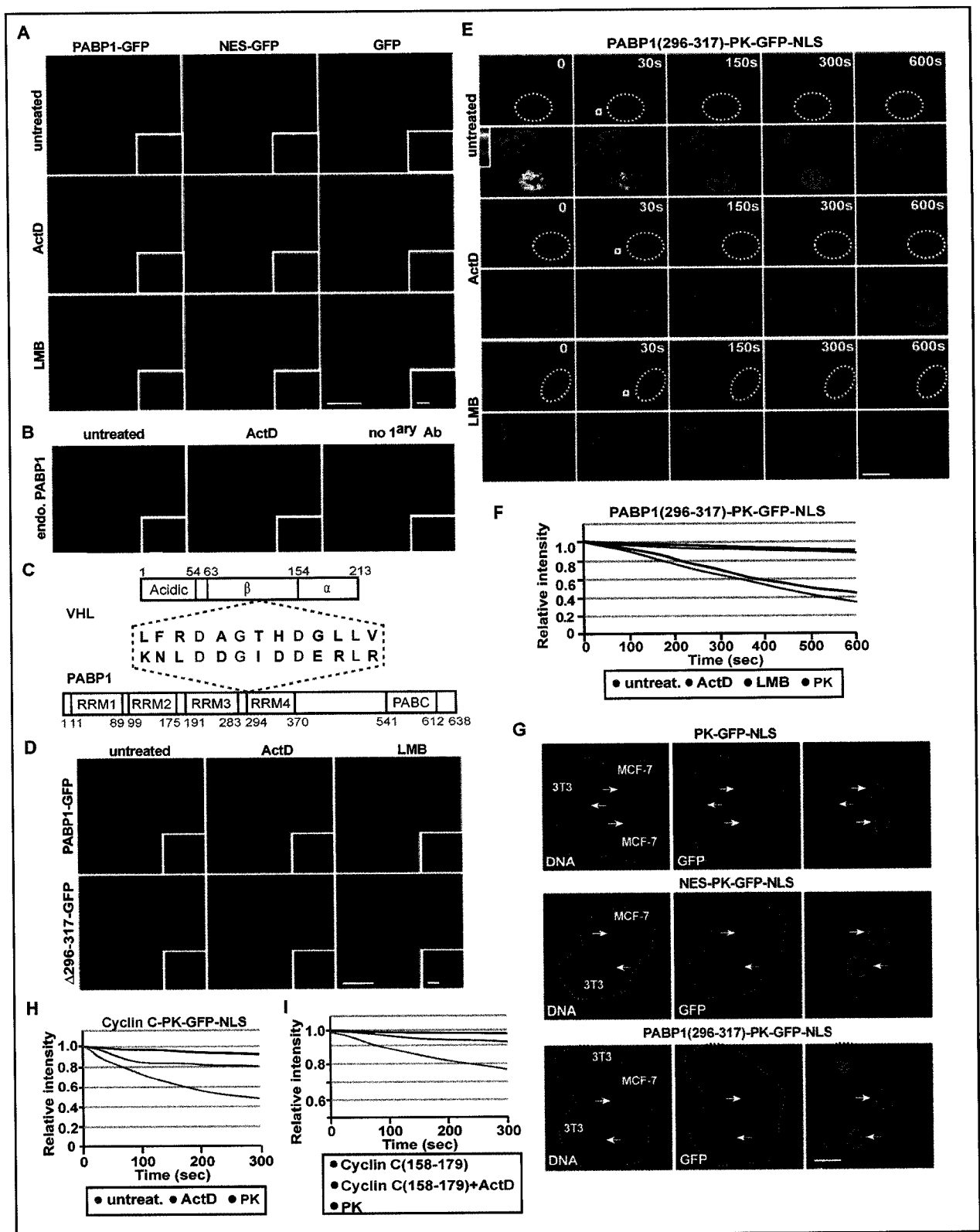
Figure 22. Residues 114-138 mediate transcription-dependent nuclear export of VHL. (A and B) MCF-7 cells were transiently transfected to express VHL(114-138)-PK-GFP-NLS. Cells were treated with a final concentration of 8 μ M ActD or left untreated for 1hr before being subjected to photobleaching. Cytoplasmic FLIP was performed by repetitively bleaching a small cytoplasmic region (white squares) of a cell (dotted circle outlines cell nucleus). Cells were imaged between pulses and the corresponding kinetics for the loss of nuclear fluorescence was calculated and graphed (B). In (B) the nuclear export kinetics of Δ C157-PK-GFP-NLS revealed by a cytoplasmic FLIP analysis was plotted. Scale bar represents 10 μ m. (C) VHL(114-138) can mediate export in a polykaryon fusion assay. MCF-7 cells (donors) were fused with NIH 3T3 cells (acceptors) using PEG and the transfer of nuclear fluorescence from donor to acceptor cells was monitored. Donor and acceptor cells were differentiated by a cell specific Hoechst staining pattern. White arrows indicate donor cells and yellow arrows indicate acceptor cells. Scale bar represents 10 μ m. (D and E) VHL(114-138) can mediate export in an *in vitro* nuclear export assay. MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin after which they were incubated with transport buffer containing ATP, GTP and ATP-regenerating system in the presence of buffer or MCF-7 cellular lysate. Relative loss in nuclear fluorescence was calculated and plotted on a graph (E).



activity (Afonina et al., 1998; Lee et al., 1999). Similar to VHL, the subcellular trafficking dynamics of the highly cytoplasmic PABP1 are affected by treatment with ActD, such that both exogenously expressed PABP1-GFP and endogenous PABP1 accumulate in the nucleus following inhibition of RNA PolII activity (Figure 23A and B) (Afonina et al., 1998). PABP1 is also unaffected by treatment with LMB (Figure 23A). Sequence alignment revealed a striking similarity between residues 114-138 of VHL and 296-317 of PABP1 that consisted of DxGx₂Dx₂L (Figure 23C). Consistent with a possible role in nuclear export, removal of residues 296-317 from full length PABP1 caused a drastic shift in the steady-state localization from exclusively cytoplasmic to highly nuclear (Figure 23D). This deletion mutant of PABP1 was also insensitive to ActD treatment compared to wild type (Figure 23D). Residues 296-317 of PABP1 were sufficient to mediate nuclear export of the PK-GFP-NLS reporter as efficiently as residues 114-138 of VHL in our FLIP nuclear export assay (Figure 23E and F). In addition, ActD or DRB, but not LMB, abolished the ability of residues 296-317 of PABP1 to mediate nuclear export of the PK-GFP-NLS reporter (Figure 23E and F). The ability of residues 296-317 of PABP1 to mediate nuclear export was also tested using a traditional cellular fusion assay. Residues 296-317 of PABP1 were able to efficiently export the reporter protein from the donor nucleus to the acceptor nuclei (Figure 23G), similar to results obtained with residues 114-138 of VHL (Figure 22C). This led us to postulate that the DxGx₂Dx₂L consensus sequence might act as a common motif for transcription-dependent nuclear export of proteins.

A search of sequence data banks identified several proteins that contains a DxGx₂Dx₂L consensus motif. We randomly selected the cell cycle regulator Cyclin C, whose nuclear-

Figure 23. VHL and PABP1 share a common transcription-dependent nuclear export motif. (A) PABP1 exports by a transcription-dependent mechanism. MCF-7 cells transiently expressing PABP1-GFP, NES-GFP or GFP alone were either untreated or treated with 8 μ M ActD or 10 μ M LMB for 3 hours. Insets are the corresponding Hoechst staining of the cells. (B) Endogenous PABP1 is also sensitive to ActD treatment. MCF-7 cells were treated like in (A) and subjected to immunofluorescence using an anti-PABP1 antibody. Insets show nuclei stained with Hoechst. No 1ary Ab is a primary antibody exclusion control. (C) Schematic diagram depicting a region of alignment between the nuclear export sequence of VHL and PABP1. Conserved residues are in red. (D) MCF-7 cells transiently expressing PABP1(Δ 296-317)-GFP or PABP1-GFP were treated as described in (A). Insets show Hoechst staining. (E and F) Residues 296-317 of PABP1 encode a transcription-dependent nuclear export sequence. Transfected MCF-7 cells were treated with 2 μ M ActD or 10 μ M LMB for 1 hour before cytoplasmic FLIP. Loss of nuclear fluorescence was plotted on a graph. (G) PABP1(296-317) can mediate export in a polykaryon fusion assay. MCF-7 cells (donors) were fused with NIH 3T3 cells (acceptors) using PEG and the transfer of nuclear fluorescence from donor to acceptor cells was monitored. Donor and acceptor cells were differentiated by a cell specific Hoechst staining pattern. White arrows=donor and yellow arrows=acceptor cells. (H) Full length Cyclin C can export the PK-GFP-NLS reporter in a transcription-dependent manner. Cells were treated like (E and F). (I) Residues 158-179 of Cyclin C encode a transcription-dependent nuclear export motif. MCF-7 cells were treated the same as (G). Cells were subjected to cytoplasmic FLIP.



cytoplasmic shuttling profile has not been previously investigated, to test the activity of its potential nuclear export sequence. Full length Cyclin C was fused to PK-GFP-NLS to prevent any passive diffusion through the nuclear pores. Cyclin C was able to confer nuclear export activity to the PK-GFP-NLS reporter in a process that was inhibited by addition of ActD (Figure 23H). Furthermore, residues 158-179 of Cyclin C, which encode the DxGx₂Dx₂L motif, were able to export the reporter PK-GFP-NLS from the nucleus in an ActD-sensitive manner (Figure 23I). Further characterization of Cyclin C, and other proteins, will reveal whether the DxGx₂Dx₂L motif is involved in their subcellular trafficking profiles. Nonetheless, we have identified a novel motif that mediates efficient transcription-dependent nuclear export of VHL, PABP1 and perhaps Cyclin C. For the sake of simplicity, the DxGx₂Dx₂L motif will be hereafter referred to as TD-NEM for Transcription-Dependent Nuclear Export Motif.

6.5. Conserved residues of TD-NEM are essential for mediating efficient nuclear export of proteins

Analysis of the TD-NEM sequence from VHL, PABP1 and Cyclin C failed to identify additional conserved residues surrounding the basal DxGx₂Dx₂L motif (Figure 24A) suggesting that these four amino acids may encode transcription-dependent nuclear export activity. Substitution of the four key residues to alanines abolished nuclear export activity of residues 114-138 from VHL (Figure 24A-C). Substitution of the aspartic acid, leucine, or glycine residues to alanine reduced nuclear export activity by approximately 50-70% compared to the basal sequence (Figure 24A, D and E). In addition, substituting the glycine 123 to alanine in full-length VHL, tagged to GFP not PK-GFP-NLS, shifted the steady-state localization towards more predominantly nuclear (Figure 24F) and

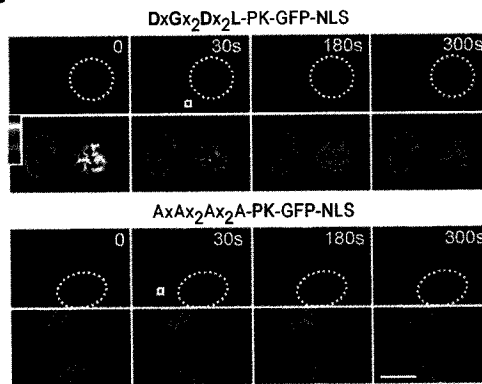
Figure 24. Identification of key residues that mediate transcription-dependent nuclear export. (A) Sequence alignment depicting conserved residues (blue) in the transcription-dependent nuclear export motifs of VHL, PABP1 and Cyclin C. Residues in red indicate alanine substitutions at key residues in the TD-NEM sequence of VHL. (B and C) Conserved residues in the DxGx₂Dx₂L consensus sequence are essential for nuclear export. MCF-7 cells were transiently transfected with VHL(115-130)-PK-GFP-NLS (DxGx₂Dx₂L) or VHL(115-130AAAA)-PK-GFP-NLS (AxAx₂Ax₂A) where the four key residues were substituted to alanines. Cytoplasmic FLIP was performed and the loss of nuclear fluorescence was plotted on a graph (C). (D and E) Single alanine substitution of key residues within the DxGx₂Dx₂L consensus sequence has a differential effect on nuclear export activity. MCF-7 cells transiently expressing the indicated PK-GFP-NLS-tagged constructs were subjected to cytoplasmic FLIP. Graph represents the loss of nuclear fluorescence (E). (F and G) Single amino acid substitution of G123A in full length VHL affects steady-state localization and nuclear export activity. Steady-state localization of VHL(G123A)-GFP expressed in MCF-7 cells compared to wild type VHL-GFP (F). Insets are the corresponding Hoechst staining of the cells. (G) Cells expressing VHL-GFP or VHL(G123A)-GFP were subjected to cytoplasmic FLIP and the loss of nuclear fluorescence was monitored and graphed.

A

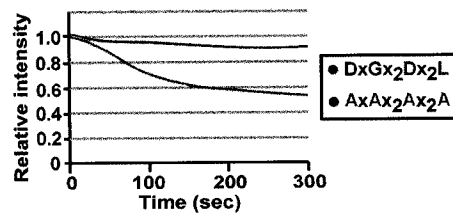
VHL: LFR **D** A G T H **D** G L L V N Q
PABP1: KNL **D** D G I D D E R L R K E
Cyclin C: Y V Q **D** M G Q E D M L L P L A
..... DxGx₂Dx₂L

LFR **D** A G T H D G L L V N Q → DxGx₂Dx₂L
LFR A A A T H A G L A V N Q → AxAx₂Ax₂A
LFR A A G T H D G L L V N Q → D121A
LFR **D** A A T H **D** G L L V N Q → G123A
LFR **D** A G T H A G L L V N Q → D126A
LFR **D** A G T H D G L A V N Q → L129A

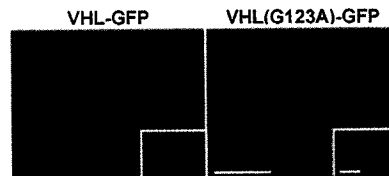
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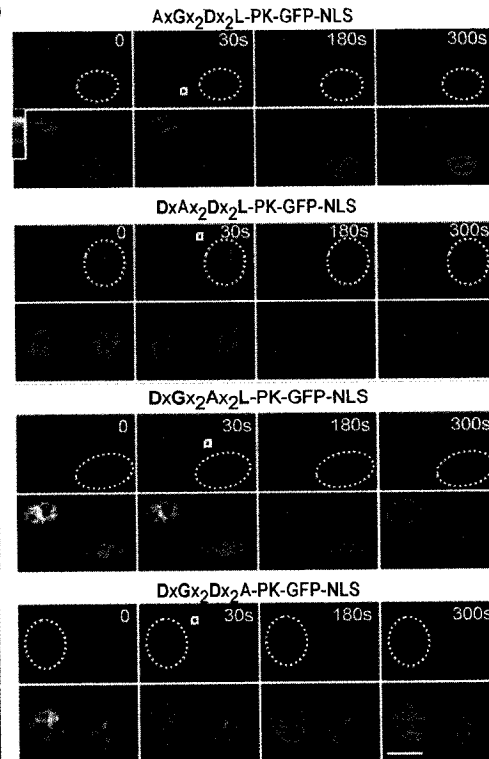
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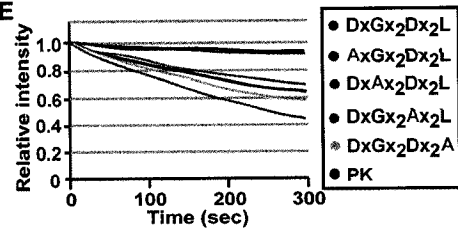
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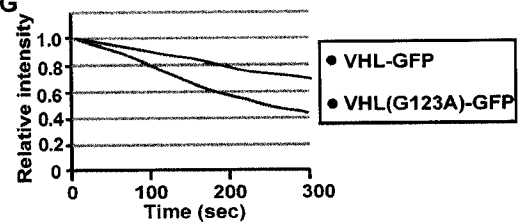
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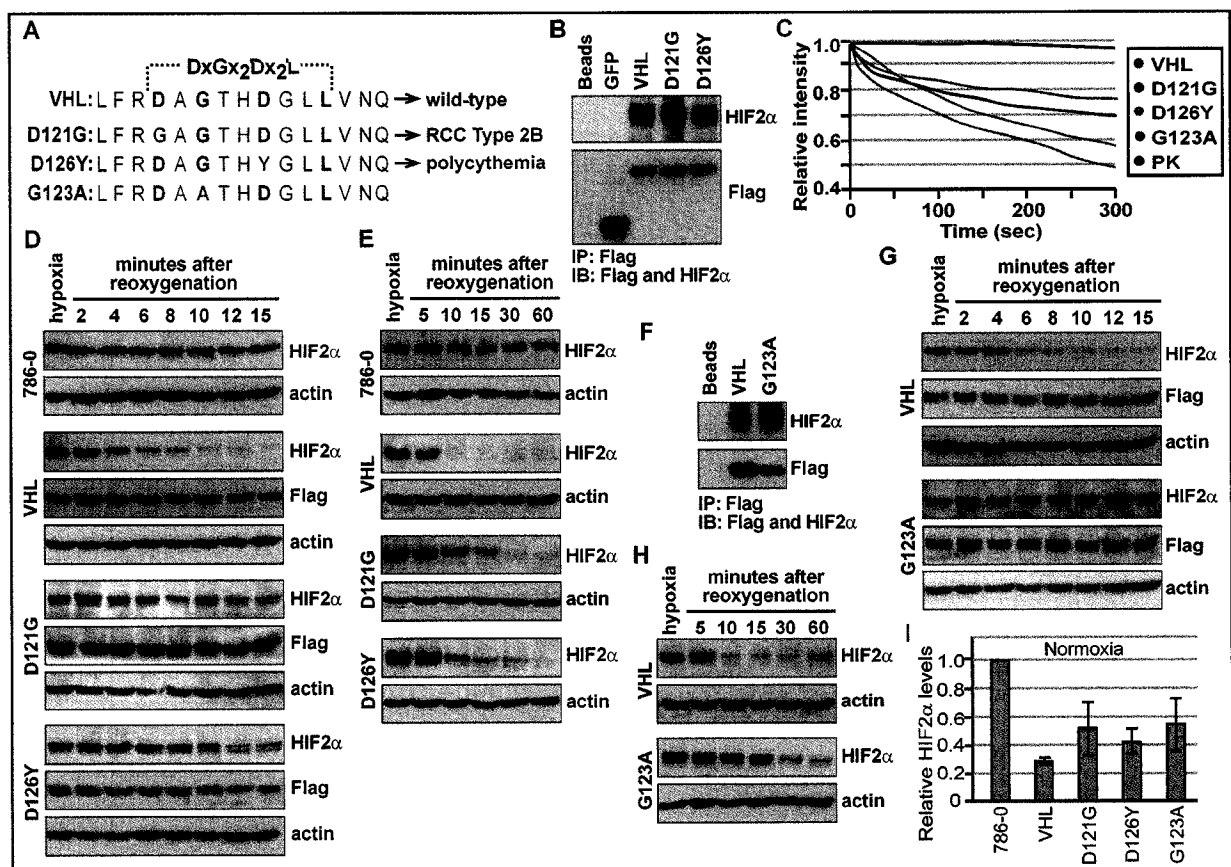


reduced its ability to export from the nucleus (Figure 24G) further demonstrating that the DxGx₂Dx₂L is a nuclear export motif. These data demonstrate that all four conserved residues in TD-NEM play a central role in mediating nuclear export.

6.6. Cancer-causing mutations within TD-NEM abrogate nuclear export of VHL and oxygen-dependent degradation of HIF α .

To study the functional consequence of TD-NEM mutations in the context of a full length VHL protein we searched for naturally occurring point mutations within the key TD-NEM residues that are associated with VHL disease. A substitution of the first aspartic acid residue of the consensus, DxGx₂Dx₂L, to glycine (D121G) is a germline mutation associated with Type 2B VHL disease, characterized by high risk of RCC (Figure 25A) (Gallou et al., 2004; Rasmussen et al., 2006; Whaley et al., 1994). This mutant is of particular interest since previous studies have reported that it maintains its ability to form an E3 ubiquitin-ligase complex and to bind and ubiquitylate HIF α *in vitro* (Hansen et al., 2002). In addition, the second aspartic acid residue of the VHL TD-NEM has been reported to be substituted to tyrosine (D126Y) in individuals afflicted with polycythemia (Figure 25A) (Pastore et al., 2003a; Pastore et al., 2003b). Stable cell lines of D121G-GFP, D126Y-GFP and G123A-GFP (not PK-GFP-NLS) were generated in VHL-defective 786-0 RCC cells to further study the effect of these point mutants on the ability of VHL to export from the nucleus and mediate oxygen-dependent degradation of HIF α . As previously reported, immunoprecipitation analysis revealed that the VHL mutant D121G is able to bind as efficiently to HIF2 α as wild type VHL (Figure 25B). Likewise, VHL D126Y was also able to efficiently bind HIF2 α (Figure 25B). Cytoplasmic FLIP

Figure 25. Cancer-causing mutations within TD-NEM of VHL abrogate nuclear export and oxygen-dependent degradation of HIF2 α . (A) Sequence alignment depicting cancer-causing mutations (red) in the key TD-NEM residues of VHL that lead to renal cell carcinoma (RCC) type 2B (D121G) and polycythemia (D126Y) in humans. G123A, which exhibits a defect in nuclear export was also used. (B) D121G and D126Y retain HIF binding. Cells stably expressing Flag-tagged constructs were placed under hypoxic conditions and treated with 10 μ M MG132 for 2 hours before harvesting. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag and anti-HIF2 α antibodies. (C) Cancer-causing mutants D121G and D126Y decrease the nuclear export activity of VHL. Cells stably expressing VHL-GFP, D121G-GFP, D126Y-GFP, G123A-GFP or transiently expressing PK-GFP-NLS were submitted to cytoplasmic FLIP. (D and E) Cells expressing D121G or D126Y exhibit a deficiency in HIF2 α degradation. Stable cell lines of VHL-GFP, D121G-GFP, D126Y-GFP and the VHL-defective cell line 786-0 were incubated for 20 hours in hypoxia before being reoxygenated for the indicated time. Western blot analysis was performed using anti-HIF2 α antibody. Levels of VHL or its mutant counterparts were monitored using anti-Flag antibody and actin was used to ensure equal loading. (F) G123A retains HIF α binding. Cells were treated the same as in (C). (G and H) Cells expressing G123A exhibit a deficiency in HIF2 α degradation. G123A stably expressing cells were treated as in (D) and (E). (I) D121G, D126Y and G123A stable cells express higher normoxic HIF2 α levels compared to wild type VHL. Stable cell lines were analyzed by western blot using anti-HIF2 α and anti-actin antibodies. HIF2 α levels were normalized to actin and values, calculated as relative to HIF2 α levels in 786-0 cells, were plotted on a graph.



experiments revealed a marked decreased rate of nuclear export of D121G and D126Y, of approximately 40% and 20% respectively, compared to wild type VHL (Figure 25C). The function of VHL in oxygen-dependent degradation of HIF α has been linked to its ability to export from the nucleus (Groulx and Lee, 2002). Thus, we decided to examine the effect of nuclear export defective VHL mutants on oxygen-dependent degradation of HIF α . Cells were exposed to hypoxia to promote HIF α accumulation followed by reoxygenation (Figure 25D). VHL defective RCC 786-0 cells did not display a decrease in HIF2 α (786-0 cells express HIF2 α but not HIF1 α) levels following reoxygenation of hypoxic cells, as expected, since these cells do not express wild type VHL (Figure 25D). Reintroduction of wild type VHL caused rapid degradation of HIF2 α upon reoxygenation of hypoxic cells (Figure 25D). In contrast, both the D121G and D126Y mutants were not as efficient in degrading HIF2 α in reoxygenated cells (Figure 25D). Mutants D121G and D126Y were eventually capable of degrading HIF2 α after long periods of reoxygenation consistent with their ability to assemble with HIF α and mediate ubiquitylation (Figure 25E). These data support the hypothesis that VHL mutants that are defective in nuclear export display a reduced ability to degrade HIF α even though they are able to bind to and ubiquitylate HIF α .

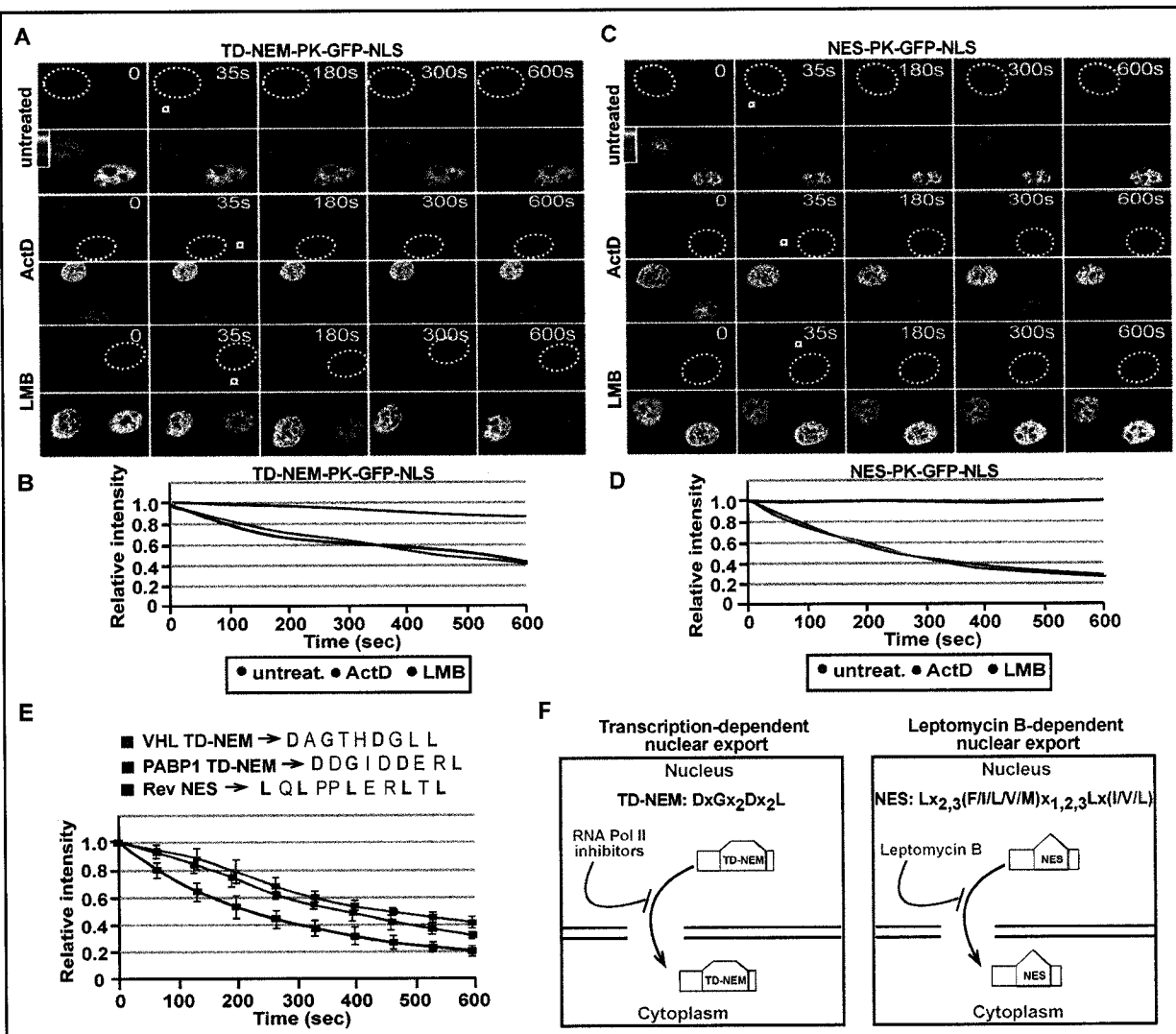
In light of these results we were interested in testing if the nuclear export defective G123A, although not a known disease-causing mutation, would also exhibit a decreased efficiency to degrade HIF α (Figure 25A). Stably expressed G123A maintained its ability to interact with HIF2 α (Figure 25F) but displayed a reduced nuclear export activity (Figure 25C). The G123A mutant displayed a striking defect in degradation of HIF2 α upon reoxygenation of hypoxic cells (Figure 25G and H). These results were consistent

with the idea that decreased nuclear export alters the ability of VHL to mediate efficient degradation of HIF α . Furthermore, we noticed that 786-0 cells expressing the nuclear export defective mutants, D121G, D126Y and G123A, generally displayed higher levels of HIF2 α than wild type VHL, but still lower than the VHL defective 786-0 (Figure 25I). This may be explained by the fact that the nuclear export defective mutants are still able to bind and ubiquitylate HIF α and partially able to export from the nucleus. None the less, the data shown in Figure 25 suggests that nuclear export of VHL is required for efficient oxygen-dependent degradation of HIF α .

6.7. TD-NEM mediates efficient transcription-dependent nuclear export of proteins

Efficient nuclear export of proteins is required for cellular homeostasis and survival. The classical NES/CRM1 pathway was the first general and discreet motif identified which mediates nuclear export of a wide array of different proteins (Fischer et al., 1995; Wen et al., 1995). The herein described TD-NEM is a discreet motif that is sensitive to drugs that inhibit RNA PolII activity but operates independently of the classical NES pathway since it is insensitive to LMB as demonstrated in our live cell nuclear export assay (Figure 26A and B). This is in contrast to what is observed with the classical NES whose activity is inhibited by LMB but not by RNA PolII inhibitors (Figure 26C and D). VHL and PABP1 TD-NEM display nuclear export activities nearly as efficient as the Rev NES, which is thought to be the strongest NES yet characterized (Figure 26E) (Henderson and Eleftheriou, 2000). Based on these results we suggest that TD-NEM is an efficient nuclear export motif that operates independently of NES (Figure 26F).

Figure 26. TD-NEM is a novel and efficient transcription-dependent nuclear export motif. (A-D) TD-NEM, contrary to the classical NES, mediates nuclear export in a ActD-sensitive but LMB-insensitive manner. Cells transiently expressing PK-GFP-NLS-tagged TD-NEM of VHL or NES of the Rev protein were either untreated or treated with ActD or LMB as previously described before being submitted to cytoplasmic FLIP. White squares indicate the bleached area and the dotted circles outline nucleus of the cell of interest. The loss nuclear fluorescence was monitored, calculated and plotted on a graph (B and D). (E) TD-NEM is an efficient nuclear export motif. Cells transiently expressing PK-GFP-NLS-tagged TD-NEM of VHL or PABP1, or the Rev NES, one of the strongest export signals, were submitted to cytoplasmic FLIP in order to compare the efficiency of nuclear export. (F) Model of nuclear export mediated by TD-NEM consensus sequence compared to the classical NES. Nuclear export of TD-NEM is abrogated by RNA PolIII inhibitors, such as ActD and DRB, whereas NES activity is abrogated by LMB.



6.8. The translation elongation factor eEF1A is a novel VHL and PABP1 interacting protein.

As previously shown, addition of RNA PolII inhibitors, such as Actinomycin D (ActD), inhibits nuclear export of both VHL and PABP1. In contrast, nuclear export of VHL and PABP1 is not affected by leptomycin B (LMB), a compound that abolishes CRM1-dependent nuclear export of NES-containing proteins (Afonina et al., 1998; Kudo et al., 1999; Lee et al., 1999). This observation indicates that nuclear export of these proteins is independent of the classical CRM1/NES nuclear export pathway. Since nuclear export of VHL and PABP1 requires ongoing RNA PolII-dependent transcription we considered the possibility that TD-NEM-mediated nuclear export occurs via an mRNA export pathway. To test this possibility we made use of the vesicular stomatitis virus (VSV) and the influenza virus, both of which have the innate property of shutting down host cell gene expression through inhibition of mRNA nuclear export (Faria et al., 2005; Satterly et al., 2007; von Kobbe et al., 2000). We reasoned that if VHL were utilizing an mRNA export pathway to exit from the nucleus then inhibiting general mRNA export should, in principle, abrogate VHL export as well. However, inhibiting mRNA export using either VSV or the influenza virus did not affect nuclear export of VHL (data not shown). Also, we were not able to identify any mRNA or tRNA species associated with VHL (data not shown) suggesting that nuclear export of VHL does not occur through an mRNA or tRNA export pathway.

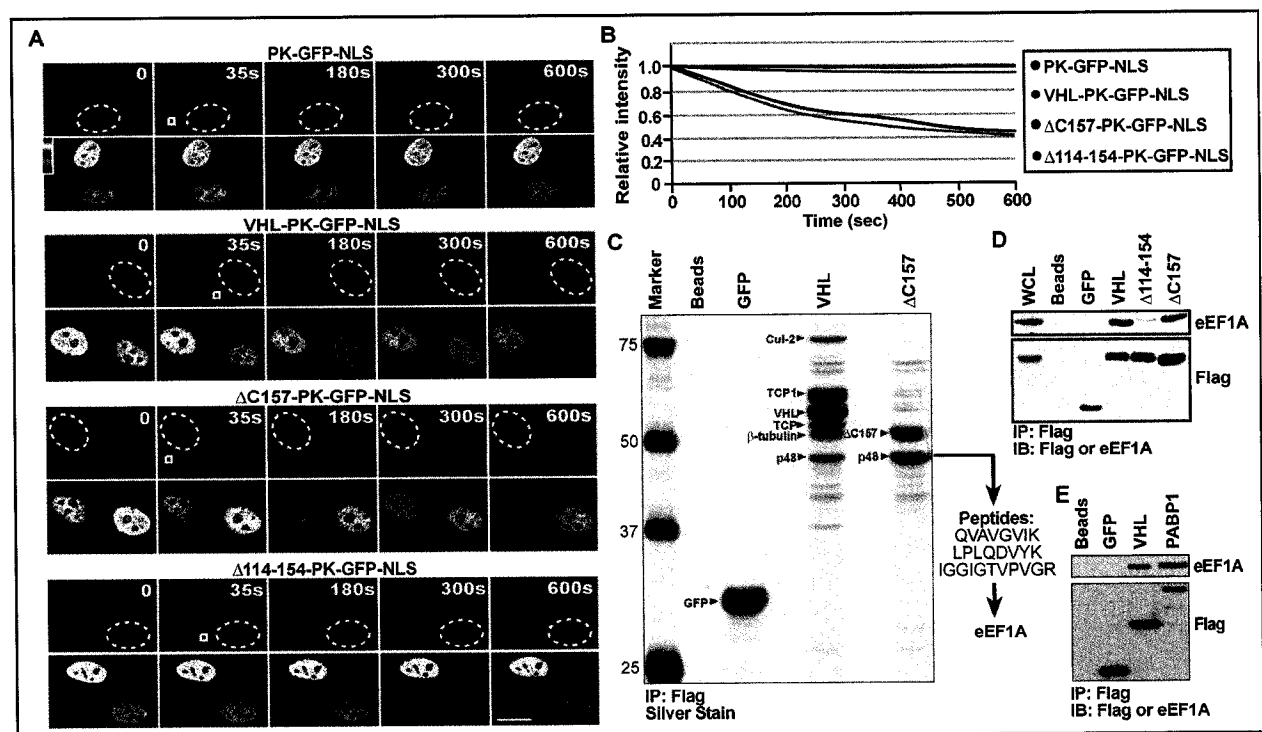
To further investigate the mechanism involved in nuclear export of VHL and PABP1, we decided to search for novel interacting proteins that are common to both proteins. To do so, we produced adenoviruses that express Flag-tagged VHL and Δ C157, a C-terminal

truncation mutant of VHL that fails to bind to core E3 ubiquitin-ligase components. As discussed and shown previously, $\Delta C157$ maintains sensitivity to ActD (Bonicalzi et al., 2001; Lee et al., 1999) and retains the ability to mediate nuclear export of the PK-GFP-NLS reporter in a FLIP nuclear export assay, which measures whether or not a protein can exit from the nucleus, as compared to PK-GFP-NLS alone that does not encode any nuclear export signals (Figure 27A). Immunoprecipitation of VHL followed by silver staining of the gel revealed several previously identified associated proteins including the core ubiquitin-ligase component Cul-2 and elongins B and C (Figure 27C and data not shown) (Feldman et al., 1999; Hergovich et al., 2003; Pause et al., 1997). In addition, we observed a previously unidentified but highly abundant band that migrated at 48 kDa (Figure 27C). $\Delta C157$ only assembled with the 48 kDa protein but not with the core ubiquitin-ligase component Cul-2 or other associated proteins, as expected (Bonicalzi et al., 2001; Ohh et al., 2000). The p48 kDa band was the only major protein found associated with $\Delta C157$ even in higher or lower percentage gels.

Mass spectrometry peptide sequence analysis identified p48 as the eukaryotic elongation factor 1A (eEF1A), a key component of the translational machinery (Andersen et al., 2003). Association of VHL or $\Delta C157$ with endogenous eEF1A was confirmed by immunoprecipitation and western blot analysis using an anti-eEF1A antibody (Figure 27D). Interestingly, endogenous eEF1A was not able to interact with a deletion mutant of VHL, $\Delta 114-154$, that does not encode a TD-NEM and fails to engage in nuclear export (Figure 27D) (Bonicalzi et al., 2001; Lee et al., 1999) suggesting that eEF1A may play a role in nuclear export of VHL. More importantly, PABP1 also interacted with endogenous eEF1A (Figure 27E). Since $\Delta C157$ retains transcription-dependent nuclear

Figure 27. Identification of a novel VHL and PABP1 interacting protein. (A and B)

Full length VHL and Δ C157 mediate nuclear export of a PK-GFP-NLS reporter protein, but not Δ 114-154. MCF-7 cells transiently expressing VHL, Δ C157 or Δ 114-154 fused to the PK-GFP-NLS reporter protein were subjected to the live cell FLIP nuclear export assay. A small region of the cytoplasm (white squares) within specific cells (dashed circles outline cell nuclei) was repeatedly photobleached. Kinetics for the loss of nuclear fluorescence, which indicates nuclear export activity, were calculated and plotted on a graph (B). In (A) pseudocolored panels are included to better illustrate subtle changes in fluorescence intensity. Scale bar represents 10 μ m. (C) Identification of a novel VHL-interacting protein. VHL-/- 786-0 cells were infected with Flag-tagged GFP, VHL-GFP or Δ C157-GFP adenoviruses. Anti-Flag beads were incubated with lysis buffer (Beads lane) or lysates from infected cells. Immunoprecipitates were run on a 10% SDS-PAGE gel and subjected to silver staining. Excised bands were analyzed by mass spectrometry. VHL associated with known interacting proteins, such as Cul2, the cytosolic chaperonin TCP-1 and β -tubulin. p48 was identified as the eukaryotic elongation factor 1 alpha (eEF1A). Lanes referred to as "Beads" were incubated with lysis buffer alone. (D) eEF1A can interact with VHL and Δ C157 but not with Δ 114-154. 786-0 cells infected with adenoviruses to express Flag and GFP-tagged VHL, Δ C157, Δ 114-154 or GFP alone were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. Whole cell lysates (WCL) are from 786-0 cells infected with F-VHL-GFP. (E) eEF1A is also a novel PABP1-interacting protein. MCF-7 cells were transiently transfected with Flag and GFP-tagged VHL, PABP1 or GFP alone. Immunoprecipitation and western blot analysis was the same as in (D).

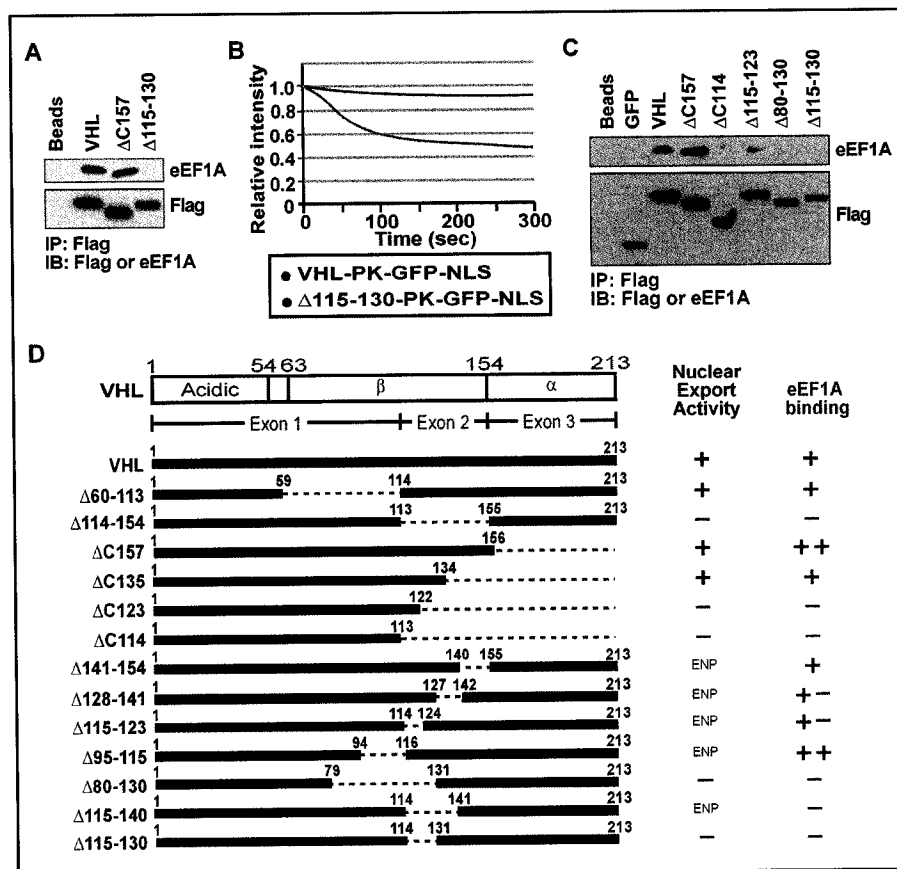


export activity and only binds to eEF1A, we decided to further characterize this new VHL and PABP1-associated protein for its potential involvement in nuclear export. We reasoned that if eEF1A is involved in transcription-dependent nuclear export, its interaction with VHL would be dependent on the presence of TD-NEM, which is encoded within residues 115-130. We found that the nuclear export-defective VHL Δ 115-130, which lacks the DxGx₂Dx₂L motif that encodes TD-NEM, fails to bind to eEF1A (Figure 28A and B). Truncation and deletion analysis revealed a clear correlation between VHL mutants that are able to export the PK-GFP-NLS reporter from the nucleus, in the *in vivo* nuclear export assay, and interact with endogenous eEF1A (Figure 28C and D). These results pointed to a possible role for eEF1A in TD-NEM-mediated nuclear export of proteins.

6.9. eEF1A is required for nuclear export of proteins encoding a TD-NEM.

The requirement for eEF1A to mediate nuclear export of TD-NEM-containing proteins was assessed using different approaches. First, we tested the effect of inhibitors of RNA PolIII-dependent transcription, such ActD and DRB, which cause nuclear accumulation of VHL and PABP1 (Bonicalzi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999) and abrogate TD-NEM mediated nuclear export. We wanted to test the possibility that ActD alters nuclear export by interfering with the TD-NEM/eEF1A interaction. Since ActD and DRB cause a nuclear shift in the localization of VHL, we fused VHL-GFP to a strong nuclear localization signal (NLS) in order to start with equal nuclear levels of protein in treated versus untreated cells. Addition of ActD or DRB, which inhibit TD-NEM activity (Figure 29B and C) and cause nuclear accumulation of VHL and PABP1 (Groulx and Lee, 2002; Lee et al., 1999), partially prevented assembly between VHL-GFP-NLS and

Figure 28. TD-NEM of VHL is required for interaction with eEF1A. (A and B) The transcription-dependent nuclear export sequence of VHL, encoded within residues 115-130, is required to interact with eEF1A and mediate nuclear export. (A) Cellular lysates from cells transiently expressing Flag-tagged VHL-GFP, Δ 157-GFP or Δ 115-130-GFP were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag and anti-eEF1A antibodies. Lanes referred to as “Beads” indicate that the Flag-beads were incubated with lysis buffer alone. (B) MCF-7 cells transiently expressing PK-GFP-NLS tagged VHL or Δ 115-130 were submitted to the live cell FLIP nuclear export assay. A small cytoplasmic region of the cell was photobleached repetitively and the loss of nuclear fluorescence, which is indicative of nuclear export activity, was monitored over time. The graph represents the relative loss of nuclear fluorescence over time. (C and D) Mapping the eEF1A binding region within VHL. (C) Cellular lysates from cells transiently expressing the indicated constructs were immunoprecipitated and immunoblotted the same as in (A). (D) Schematic diagram indicates deletion and truncation mutants of VHL that were submitted to immunoprecipitation with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. These mutants of VHL were also fused to PK-GFP-NLS and submitted to the live cell FLIP nuclear export assay. (+), (+-), (-+) and (-) indicate, in a decreasing order, the ability of the fusion proteins to interact with eEF1A or export from the nucleus. (ENP) indicates that the experiment was not performed.



eEF1A (Figure 29A). Since this experiment most likely captures an *in vitro* interaction it provides a link between transcription-dependent nuclear export and eEF1A. These data demonstrate a correlation between inhibition of TD-NEM nuclear export and eEF1A binding and suggest the possibility that ActD and DRB may block nuclear export of VHL by altering its interaction with eEF1A.

Second, we tested the effect of silencing endogenous eEF1A using RNA interference. Silencing of eEF1A with two independent siRNAs markedly reduced the levels of eEF1A protein (Figure 30A) but did not result in measurable cell death as quantified by propidium iodide (PI) exclusion (data not shown). We hypothesized that if eEF1A is required for transcription-dependent nuclear export then silencing it should alter the steady state localization of TD-NEM-containing proteins in a fashion that is reminiscent to the nuclear accumulation induced by ActD or DRB. Silencing eEF1A led to nuclear accumulation of endogenous PABP1 and transiently expressed VHL-GFP and Δ C157-GFP, which was similar to that induced by ActD treatment, when compared to untreated cells or those transfected with a scrambled siRNA (Figure 29B-D). However, localization of the soluble cytoplasmic fusion protein, GFP-NES, which is altered by LMB, was not affected by eEF1A knockdown or ActD treatment (Figure 30E). Since eEF1A is highly abundant it is difficult to achieve a complete knockdown of its protein levels, most likely resulting in residual eEF1A protein. We hypothesized that silencing eEF1A and treating with ActD, which partially inhibits interaction between eEF1A and VHL (Figure 29A), should result in an additive effect since the residual eEF1A would be blocked by ActD. Indeed, transfecting cells with eEF1A siRNA followed by ActD

Figure 29. Inhibitors of RNA PolII-dependent transcription block the interaction between VHL and eEF1A. (A) ActD and DRB hinder the interaction between eEF1A and VHL. MCF-7 cells transiently expressing F-GFP or F-VHL-GFP-NLS were untreated or treated with 8 μ M ActD or 25 μ g/ml DRB for 3 hrs before harvesting cells. Lysates were immunoprecipitated with anti-Flag beads and immunoblotted with Flag or eEF1A antibodies. (B and C) ActD inhibits TD-NEM-mediated nuclear export of VHL. MCF-7 cells transiently expressing VHL-PK-GFP-NLS were either untreated or treated with 2 μ M ActD for 1 hour before being submitted to cytoplasmic FLIP analysis. Cells were repeatedly bleached in a small cytoplasmic region (white squares) within cells (dotted circles outline the nuclei of bleached cells). The loss of nuclear fluorescence was monitored (C), calculated and plotted on a graph (B). Scale bar represents 10 μ m.

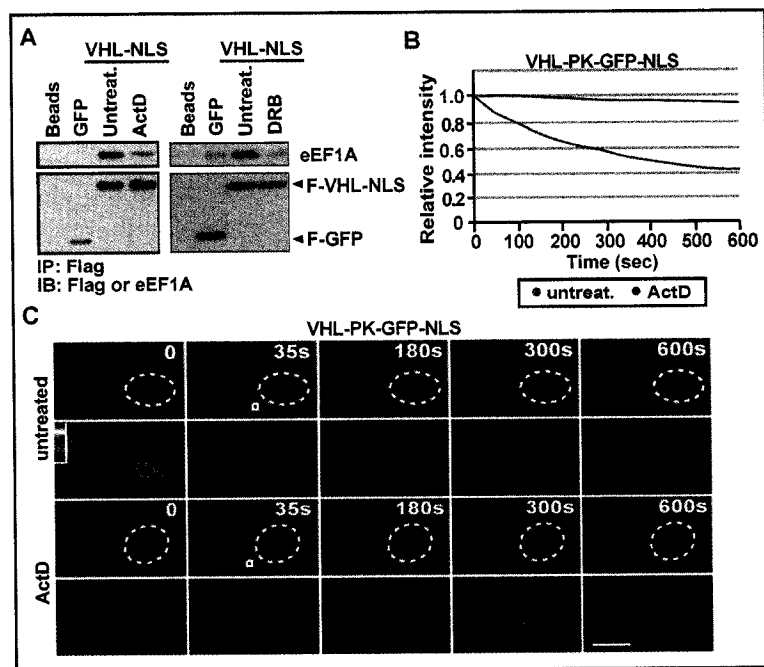
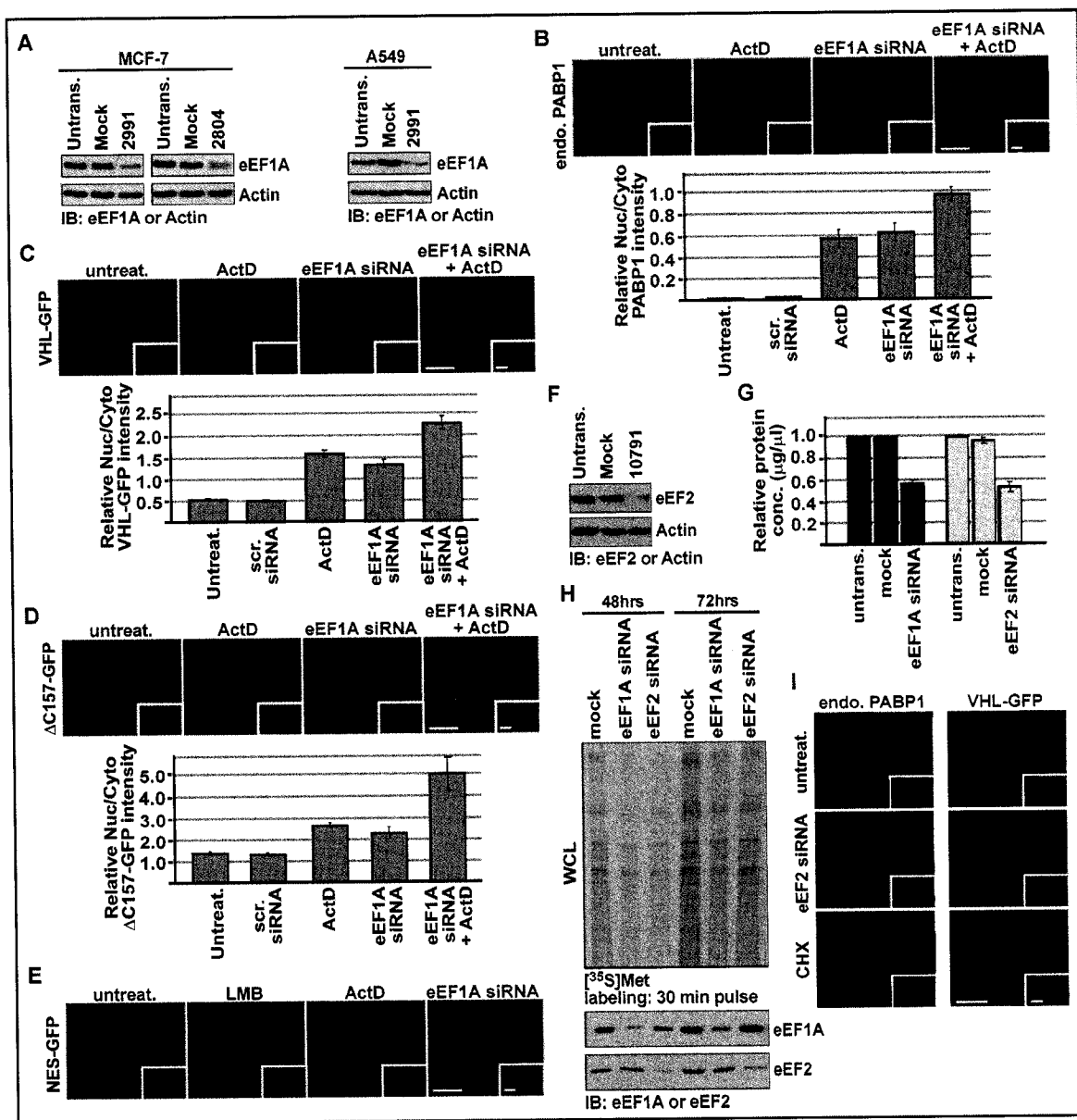


Figure 30. siRNA-mediated silencing of eEF1A causes nuclear accumulation of TD-NEM-containing proteins. (A) MCF-7 and A549 cells were either mock transfected or transfected with 100 nM eEF1A siRNA (2991 or 2804) for 72 hours and then subjected to western blot analysis using anti-eEF1A antibody. Actin was used as a loading control. (B-E) eEF1A knockdown and ActD treatment cause nuclear accumulation of endogenous PABP1 and exogenous VHL-GFP and Δ C157-GFP. MCF-7 cells were treated the same as in (A) or cotransfected with VHL-GFP, Δ C157-GFP or GFP-NES. Where indicated, cells were treatment with 8 μ M ActD for 3 hours or 10 μ M LMB for 3 hours before microscopy. Endogenous PABP1 was detected by immunofluorescence using a PABP1 specific antibody. In (B-E) insets show Hoechst staining of DNA and scale bars represent 10 μ m. Graphs represent relative nuclear:cytoplasmic ratios of fluorescence intensity. (F-I) Nuclear accumulation of TD-NEM-containing proteins in the absence of eEF1A is not due to an overall decrease in protein translation. (F) A549 cells were either mock transfected or transfected with 100 nM eEF2 siRNA (10791) for 72 hours and analyzed using anti-eEF2 antibody. (G and H) A549 cells were untransfected, mock transfected or transfected with eEF1A or eEF2 siRNA for (H) 48 hours and/or (G and H) 72 hours. (G) Total cellular protein levels were measured using a standard protein quantification method. Protein levels were calculated relative to levels in untransfected cells. (H) At the indicated times, cells were pulse-labeled for 30 minutes with [35 S]Met. Bottom immunoblots are from the same membrane. (I) Cells were transfected with 100 nM eEF2 siRNA alone or cotransfected with VHL-GFP for 72 hours. Endogenous PABP1 was detected the same as (B). Cells were treated with 100 μ g/ml cycloheximide for 2 hours.

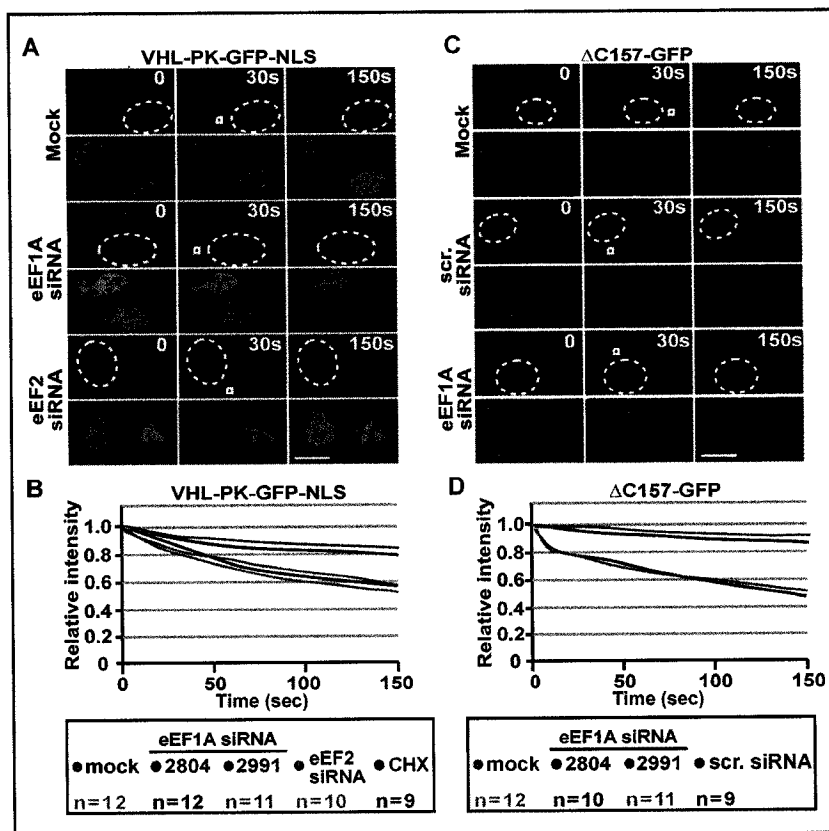


treatment resulted in a significant increase in the nuclear/cytoplasmic ratio of endogenous PABP1 and transiently expressed VHL-GFP and Δ C157-GFP (Figure 30B-D). In fact, we observed a complete nuclear shift of Δ C157-GFP (Figure 30D), a protein that fails to interact with most VHL-binding proteins (Figure 17C).

In order to eliminate the possibility that nuclear accumulation is due to a block in translation elongation we silenced the elongation factor eEF2 (Figure 30F) using RNA interference. eEF2 silencing was performed in a manner to achieve a comparable decrease in protein synthesis and translational activity as that obtained with eEF1A silencing, which was measured by protein quantification and [³⁵S]-methionine incorporation, respectively (Figure 30G and H). Silencing eEF2 did not have an effect on the steady-state localization of either endogenous PABP1 or transiently expressed VHL-GFP (Figure 30I). Also, treatment with cycloheximide, an inhibitor of protein translation, also had no effect (Figure 30I) confirming that the change in steady-state localization was not due to a requirement for ongoing protein synthesis.

Next, we examined the effect of silencing eEF1A on nuclear export activity. Using the *in vivo* nuclear export assay, it was observed that siRNA-mediated reduction of eEF1A protein decreased the nuclear export rate of VHL-PK-GFP-NLS and Δ C157-GFP to levels similar to those observed upon treatment with ActD when compared to mock or control scrambled siRNA (Figure 31A-D). However, silencing of eEF1A did not have an effect on the nuclear export rate of a classical nuclear export sequence (NES-PK-GFP-NLS) (refer to Figure 35C). This indicates that silencing this translational factor does not alter nuclear-cytoplasmic trafficking in general. Likewise, silencing eEF2 or treatment with cycloheximide confirmed that the reduced rate of nuclear export of VHL-PK-GFP-

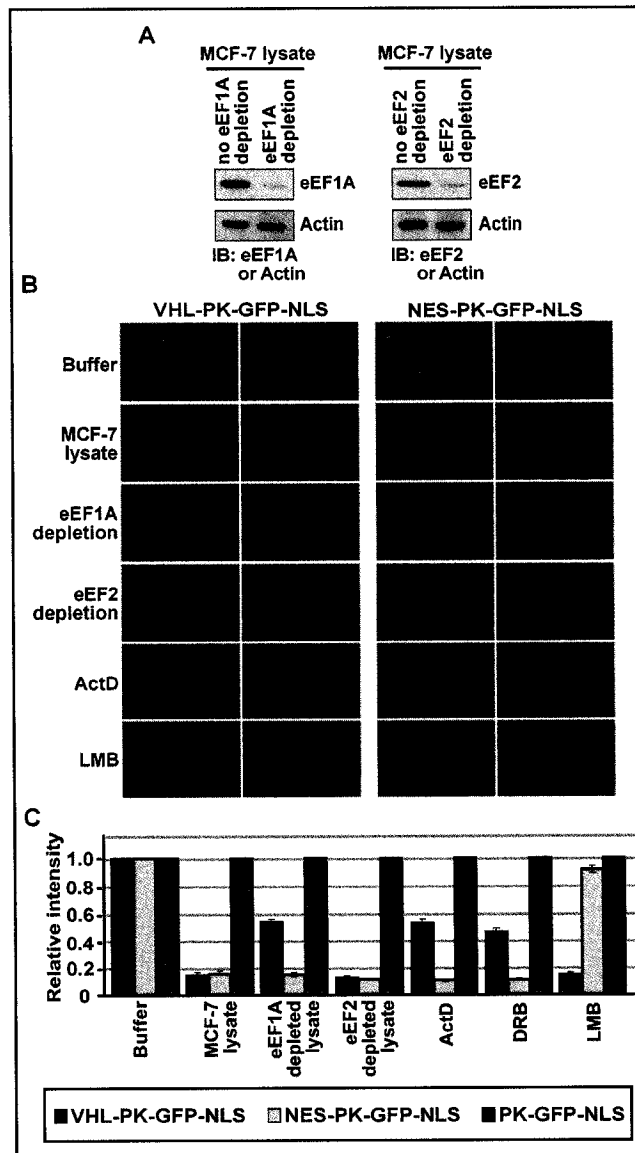
Figure 31. eEF1A is required for nuclear export of TD-NEM-containing proteins in living cells. (A-D) Silencing of eEF1A alters nuclear export of TD-NEM-containing proteins. MCF-7 cells were either mock transfected or cotransfected with 100 nM eEF1A siRNA (2991 or 2804) or control siRNA and VHL-PK-GFP-NLS (A and B) or Δ C157-GFP (C and D) for 48 hours before being submitted to the live cell FLIP nuclear export assay. In (B) cells were also treated with cycloheximide (CHX) for 1 hour before cytoplasmic FLIP analysis. Cells were repetitively photobleached in a small cytoplasmic area (white box) within a cell of interest (dotted circle outlines the cell nucleus). The loss of nuclear fluorescence was monitored and plotted on a graph. The line graphs represent mean values obtained from the indicated number of cells used (n=) per condition. Scale bars represent 10 μ m.



NLS was not due to the requirement for ongoing protein synthesis (Figure 30A and B) but specifically from the decreased levels of endogenous eEF1A.

To further uncouple the function of eEF1A in translation from its role in nuclear export and to provide additional evidence for a role in TD-NEM-mediated nuclear export, we performed *in vitro* nuclear export assays. This assay serves to: 1- uncouple ongoing translation with nuclear export activity of eEF1A since there is negligible ongoing translation in the *in vitro* export assay and; 2- eliminate the possibility for the loss of unknown factor(s) due to the partial arrest in translation in siRNA treatment for 48 and 72 hours. Addition of cellular lysate depleted of eEF1A, using eEF1A specific antibodies (Figure 32A and refer to Materials and Methods), resulted in a significant decrease in nuclear export of VHL-PK-GFP-NLS in digitonin-permeabilized cells when compared to nondepleted lysate or lysate incubated with an irrelevant Flag antibody (Figure 32B and C). Addition of ActD or DRB to living cells prior to digitonin treatment, followed by addition of cellular lysate containing ActD or DRB, resulted in a similar decrease in nuclear export of VHL-PK-GFP-NLS (Figure 32B and C). However, addition of cellular lysate depleted of eEF2 (Figure 32A) did not have an effect (Figure 32B and C). *In vitro* nuclear export of NES-PK-GFP-NLS, which is sensitive to LMB (Figure 32B and C), was not affected by either eEF1A or eEF2 depletion, or ActD and DRB treatment (Figure 32B and C). These observations verify the results obtained with siRNA-mediated silencing of eEF1A and demonstrate that indeed eEF1A is required for nuclear export of TD-NEM. These results demonstrate that eEF1A binds, to and is involved in nuclear export of, TD-NEM-containing proteins, and are consistent with a previous report

Figure 32. eEF1A is required for nuclear export of TD-NEM-containing proteins *in vitro*. (A) MCF-7 lysate containing 0.5 mg of protein (refer to Figure 35D) was depleted of eEF1A or eEF2 using eEF1A or eEF2 specific antibodies (refer to Materials and Methods for details). The depletion of eEF1A and eEF2 was assessed by western blot analysis. Actin was used as a control. (B and C) MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin and incubated with transport buffer alone, MCF-7 lysate, or MCF-7 lysate that was depleted of eEF1A or eEF2. Where indicated cells were pretreated with 8 μ M ActD, 25 μ g/ml DRB or 10 μ M LMB for 3 hrs before permeabilization with digitonin and the nuclear export assay was performed using undepleted MCF-7 lysate containing the same concentrations of drugs as was used in the pretreatment. All conditions were performed in the presence of ATP, GTP and ATP-regenerating system. Nuclear export was monitored by loss of nuclear fluorescence (B). Relative loss in nuclear fluorescence was calculated and plotted on a graph (C).



showing that eEF1A is required for export of molecules in a yeast genetic system (Grosshans et al., 2000a)

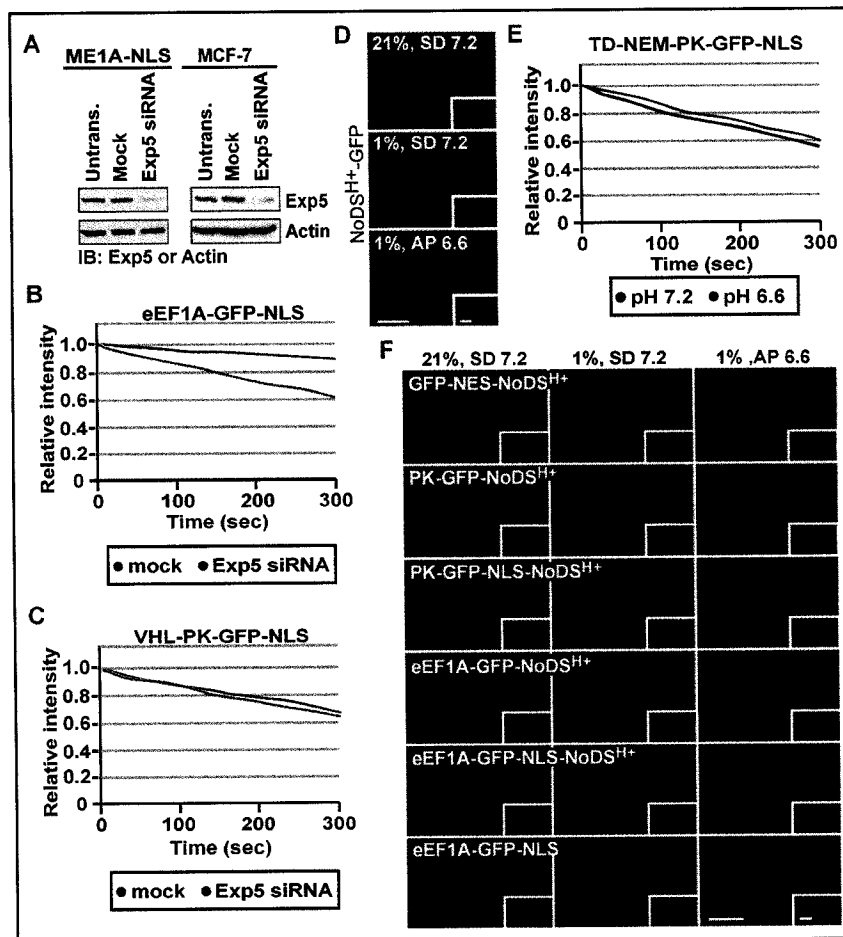
6.10. eEF1A mediates nuclear export from the cytoplasmic side of the nuclear envelope.

eEF1A is known to be an exclusively cytoplasmic protein, however, nuclear export of eEF1A utilizing Exportin-5 (Exp5) has been previously reported, as a nuclear exclusion mechanism. Thus, we examined the role of Exp5 in TD-NEM-mediated nuclear export since this exportin plays a role in nuclear export of eEF1A. siRNA-mediated silencing of endogenous Exp5 (Figure 33A) essentially abolished the ability of an eEF1A-GFP-NLS fusion protein to engage in nuclear export (Figure 33B) as previously shown by other groups (Bohnsack et al., 2002; Calado et al., 2002), though, in our cellular system, the steady-state localization of this fusion protein was mainly nuclear (see Figure 33F). In contrast, silencing of Exp5 did not disturb the nuclear export rate of VHL-PK-GFP-NLS (Figure 33C). These data suggest that the export pathway utilized by TD-NEM is independent of Exp5 and, consistent with data obtained with ActD and DRB, argues that tRNA may play a more minor role in this process.

These data raised the possibility that eEF1A may promote nuclear export of TD-NEM-containing proteins without having to enter the nucleoplasm. This would be consistent with a model proposed by Grosshans *et al.* (2000) and McGuire *et al.* (2007) that eEF1A can stimulate nuclear export of tRNA from the cytoplasmic side of the nuclear envelope and that eEF1A ongoing nuclear import rate, if any, is very slow (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). To test this, we designed an assay based on the nucleolar detention

Figure 33. The function of eEF1A in nuclear export of TD-NEM-containing proteins is Exp5-independent and exerted from the cytoplasmic side of the nuclear envelope.

(A-C) Nuclear export by TD-NEM is independent of Exp5. **(A)** ME1A-NLS (MCF-7 cells stably expressing eEF1A-GFP-NLS) and MCF-7 cells were transfected with 100 nM Exp5 siRNA for 48 hours and then lysed and subjected to western blot analysis using an anti-Exp5 antibody. Actin was used as a loading control. ME1A-NLS cells **(B)** or MCF-7 cells transiently expressing VHL-PK-GFP-NLS **(C)** were mock transfected or transfected with 100 nM Exp5 siRNA and incubated for 48 hours after which nuclear export was analyzed by the live cell FLIP nuclear export assay, as previously described. Loss of nuclear fluorescence was plotted on a graph **(B and C)**. **(D-F)** eEF1A is not targeted to the nucleolus by the NoDS^{H+}. **(D)** MCF7 cells were cultured in standard media (SD) and transiently transfected to express the indicated GFP-tagged constructs. Cells were replenished with either fresh standard media (SD, pH 7.2) or acidification permissive media (AP, initial pH 7.2) that allows maximal extracellular acidification to pH 6.6 (see Materials and methods). Cells either remained in normoxia (21% O₂) or transferred to hypoxia (1% O₂) for 18 h. Extracellular pH at endpoint is indicated on each panel. **(E)** MCF-7 cells transiently expressing TD-NEM fused to PK-GFP-NLS were treated the same as in **(D)**. Cytoplasmic FLIP was performed to assess nuclear export activity. Loss of nuclear fluorescence was monitored and plotted on a graph. **(F)** MCF-7 cells transiently expressing the indicated constructs were incubated in the same conditions as in **(D)**. The localization of GFP-tagged fusion proteins was assessed by fluorescent microscopy. In **(D)** and **(F)** insets show Hoechst staining of DNA and scale bars represent 10 μ m.



properties of the previously identified NoDS^{H+}, which targets proteins for static detention by the nucleolar architecture of acidotic cells (Mekhail et al., 2005; Mekhail et al., 2007; Mekhail et al., 2006). The NoDS^{H+} signal has been shown to be functional in targeting proteins to the nucleolus only if such proteins can enter the nucleus. We reasoned that, upon NoDS^{H+} activation, an eEF1A-NoDS^{H+} fusion protein would be captured and detained by the nucleolus only if eEF1A is able to enter the nucleoplasm. NoDS^{H+}-GFP displays a diffuse nucleocytoplasmic localization pattern similar to GFP alone in neutral pH but accumulated in the nucleolus in acidotic cells (Figure 33D), as previously described (Mekhail et al., 2005; Mekhail et al., 2006). Similarly, NES-NoDS^{H+}-GFP, which can diffuse into the nucleus but has a steady-state cytoplasmic localization due the presence of a strong and classical NES, is targeted to the nucleolus during acidosis (Figure 33F). PK-GFP-NoDS^{H+} failed to accumulate in the nucleolus of acidotic cells since it cannot import into the nucleoplasm, in contrast to the same fusion protein with an NLS (Figure 33F). eEF1A-GFP-NoDS^{H+} did not accumulate in the nucleolus suggesting that eEF1A does not import into the nucleoplasm (Figure 33F). Addition of a NLS caused the complete nucleolar sequestration of eEF1A-NoDS^{H+} in acidosis indicating that this protein can be captured by the nucleolus once imported into the nucleus (Figure 33F). NoDS^{H+}-containing proteins that display a similar cytoplasmic distribution at steady state to eEF1A, but can import into the nucleoplasm, are captured by the nucleolus in acidic pH (Figure 33F and see Mekhail *et al.*, 2005). The rate of nuclear export of TD-NEM-containing proteins is similar regardless of the extracellular pH (Figure 33E) providing evidence that eEF1A can function in nuclear export of TD-NEM-containing proteins without having to enter the nucleus.

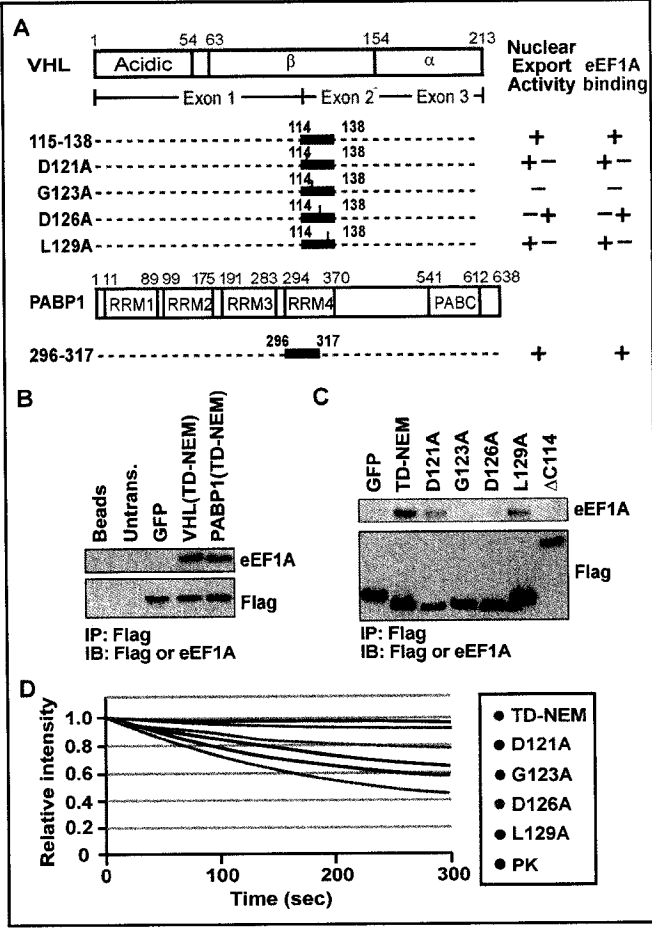
6.11. eEF1A specifically interacts with the TD-NEM nuclear export sequence.

Next, we tested the ability of eEF1A to interact with the minimal TD-NEM sequence. As expected, we found that both residues 114-138 of VHL and residues 296-317 of PABP1 alone, which encode TD-NEM and mediate transcription-dependent nuclear export activity, were sufficient to bind to eEF1A (Figure 34A and B). Single amino acid substitutions of key residues in the DxGx₂Dx₂L motif of VHL markedly reduced binding to eEF1A and nuclear export activity (Figure 34A, C and D). These data further demonstrate a clear correlation between nuclear export activity of TD-NEM and its ability to interact with eEF1A.

6.12. eEF1A is required for nuclear export of TD-NEM.

In order to verify the involvement of eEF1A during transcription-dependent nuclear export our last criterion was to test the requirement for eEF1A in the nuclear export of the minimal TD-NEM sequence. We have previously shown that TD-NEM of both VHL and PABP1 mediate nuclear export of a PK-GFP-NLS reporter protein in a manner that is sensitive to ActD, but insensitive to LMB, an inhibitor of the classical NES/CRM1 nuclear export pathway. In contrast, nuclear export of the classical NES is inhibited by LMB but is unaffected by ActD. This demonstrates that nuclear export through TD-NEM operates independently of the classical CRM1/NES-mediated nuclear export pathway. siRNA-mediated silencing of eEF1A results in a decreased nuclear export rate of TD-NEM from both VHL and PABP1 to levels similar to those observed upon treatment with ActD (Figure 35A and B). However, silencing of eEF1A did not have an effect on nuclear export of NES (Figure 35C) further demonstrating that the classical NES/CRM1 nuclear export pathway operates independently of eEF1A. These results

Figure 34. TD-NEM is sufficient for eEF1A interaction. (A) Schematic diagram depicts the TD-NEM of VHL encoded within residues 115-138 and alanine substitution of the key residues of TD-NEM. Schematic diagram also depicting the TD-NEM of PABP1 encoded between residues 296-317. (+), (+-), (-+) and (-) indicate, in a decreasing order, the ability of the fusion proteins to interact with eEF1A or export from the nucleus. (B) The transcription-dependent nuclear export sequence, TD-NEM, of VHL and PABP1 is sufficient to interact with endogenous eEF1A. MCF-7 cells transiently expressing GFP alone or the TD-NEM sequences of VHL or PABP1 tagged with Flag and GFP were submitted to immunoprecipitation with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. TD-NEM refers to residues 114-138 of VHL and residues 298-317 of PABP1. (C) Conserved residues in TD-NEM are required for eEF1A interaction. TD-NEM refers to residues 114-138 of VHL. Flag and GFP-tagged D121A, G123A, D126A and L129A indicate the amino acid that was substituted to alanine within residues 114-138 of VHL. Immunoprecipitation and western blot was as described above. (D) Conserved residues in TD-NEM are required for efficient nuclear export. MCF-7 cells transiently expressing TD-NEM, D121A, G123A, D126A or L129A fused to PK-GFP-NLS or expressing PK-GFP-NLS alone were subjected to the live cell FLIP nuclear export assay. The loss of nuclear fluorescence during cytoplasmic FLIP was monitored and graphed.



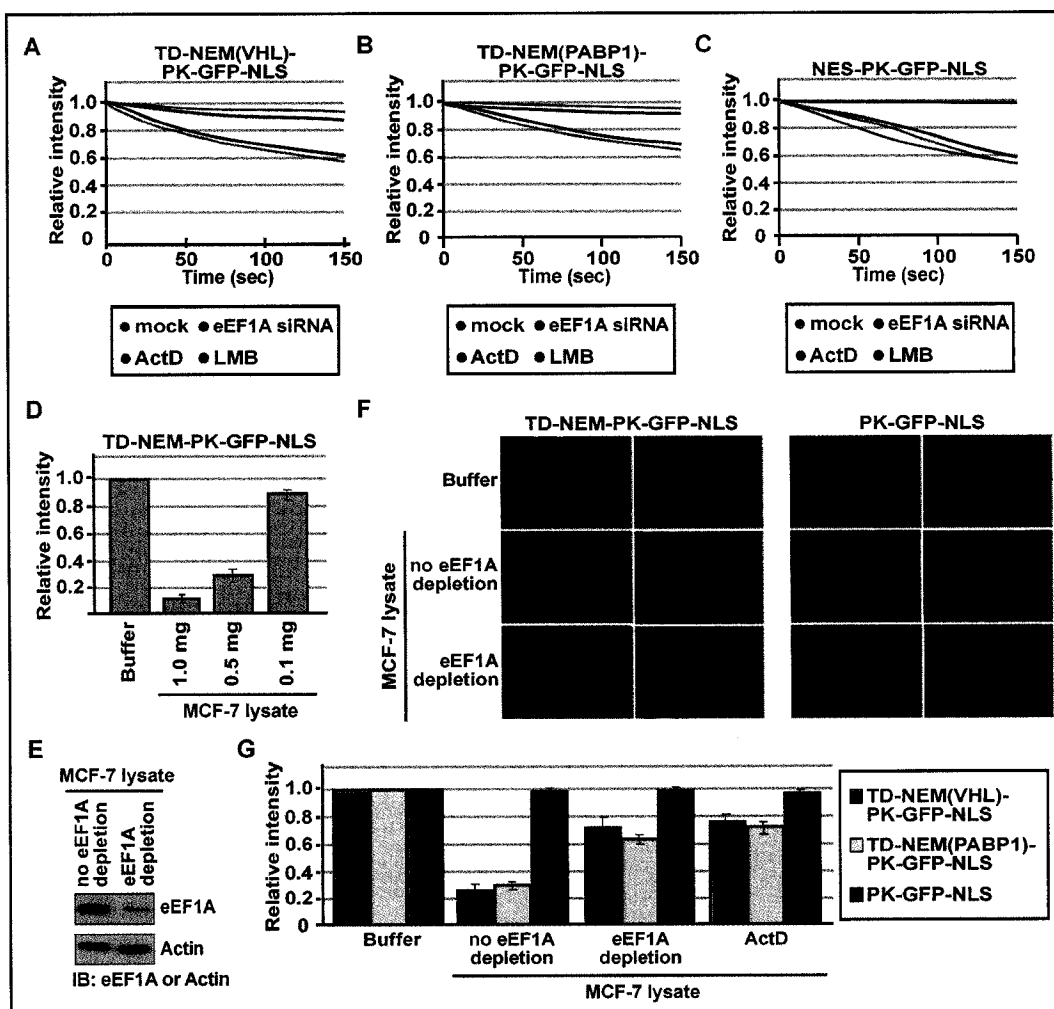
also support the involvement of eEF1A in TD-NEM-mediated nuclear export since the decreased export of TD-NEM upon silencing of eEF1A is not due to a decrease in protein synthesis or a general effect on nuclear-cytoplasmic trafficking.

Next we tested the requirement of eEF1A to mediate nuclear export of TD-NEM in an *in vitro* nuclear export assay. Addition of decreasing amounts of cellular lysate to digitonin-permeabilized cells expressing TD-NEM-PK-GFP-NLS demonstrated the presence of a limiting factor in the lysate, which is required for mediating TD-NEM nuclear export (Figure 35D). Addition of cellular lysate depleted from eEF1A, using eEF1A specific antibodies (Figure 35E), resulted in a significant decrease in nuclear export of TD-NEM of VHL and PABP1 in digitonin-permeabilized cells when compared to nondepleted lysate or lysates incubated with an irrelevant Flag antibody (Figure 35F and G). Addition of ActD to living cells prior to digitonin treatment, followed by addition of cellular lysate in the presence of ActD, resulted in a similar decrease in nuclear export (Figure 35G). These observations verify the results obtained with siRNA-mediated silencing of eEF1A and demonstrate that indeed eEF1A is required for nuclear export of TD-NEM.

6.13. TD-NEM is present and functional in a wide spectrum of highly diverse proteins.

The identification of a TD-NEM sequence in functionally divergent proteins, such as VHL, PABP1 and Cyclin C, suggested the possibility that this may be a general signal for nuclear export of proteins. To investigate this further, a search of protein sequence data banks was performed using the DxGx₂Dx₂L consensus sequence. This bioinformatics search revealed that many proteins encode a DxGx₂Dx₂L motif, some of which are shown

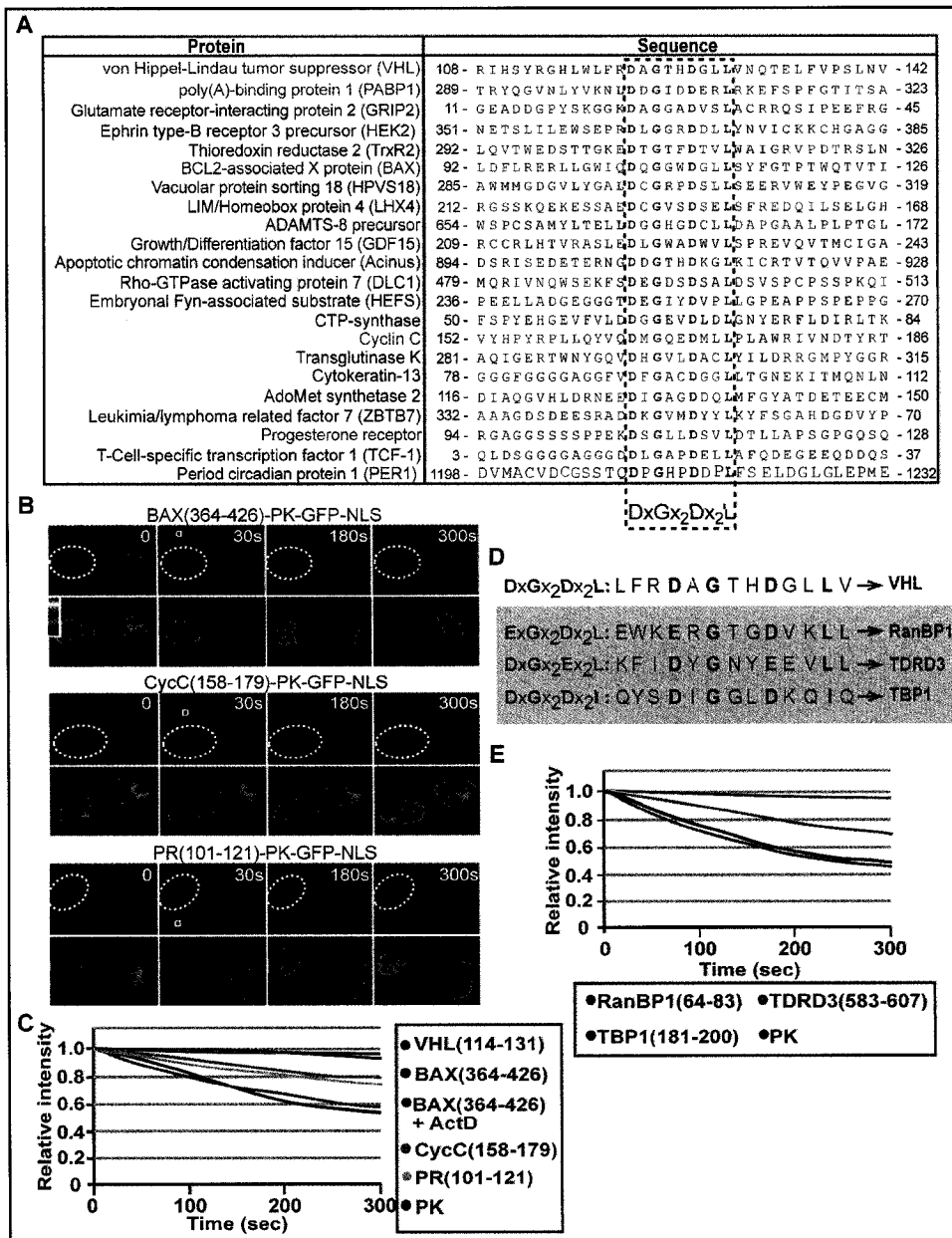
Figure 35. eEF1A is required for TD-NEM-mediated nuclear export. (A-C) TD-NEM nuclear export requires eEF1A and is independent of the CRM1/NES nuclear export pathway. MCF-7 cells were transiently cotransfected with the indicated constructs and 100 nM eEF1A siRNA for 48 hours prior to being assessed by the live cell FLIP nuclear export assay. Where indicated, cells were transfected with the indicated constructs then treated with either 2 μ M ActD or 10 μ M LMB for 1 hour prior to cytoplasmic FLIP. The loss of nuclear fluorescence was monitored and graphed. (D-G) eEF1A is required for nuclear export of TD-NEM in an *in vitro* export assay. (D) Protein levels were quantified using standard protein quantification methods. Buffer alone or cellular lysate containing different protein amounts in the presence of ATP, GTP and ATP-regenerating system were added to digitonin-permeabilized cells transiently expressing TD-NEM-PK-GFP-NLS. Relative loss in nuclear fluorescence was calculated and plotted on a graph. (E) MCF-7 lysate containing 0.5 mg of protein was depleted of eEF1A using eEF1A specific antibody (refer to Materials and Methods for details). The depletion of eEF1A was assessed by western blot analysis. Actin was used as a control. (F and G) MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin and incubated with transport buffer alone, MCF-7 lysate or MCF-7 lysate that was depleted of eEF1A. Where indicated cells were pretreated with 8 μ M ActD for 3 hrs before permeabilization with digitonin and the nuclear export assay was performed using undepleted MCF-7 lysate containing 8 μ M ActD. All conditions were performed in the presence of ATP, GTP and ATP-regenerating system. Nuclear export was monitored by loss of nuclear fluorescence (F). Relative loss in nuclear fluorescence was calculated and plotted on a graph (G).



in Figure 36A. The potential TD-NEM nuclear export sequences from randomly selected proteins such as the pro-apoptotic BAX and the Progesterone hormone Receptor (PR) were tested for their ability to mediate nuclear export of the PK-GFP-NLS reporter protein in the in vivo nuclear export assay. The minimal TD-NEM sequences from BAX and PR were able to export the reporter PK-GFP-NLS from the nucleus albeit with different efficiencies than that observed for other TD-NEM sequences (Figure 36B, C). Furthermore, the nuclear export activity of DxGx₂Dx₂L from BAX (Figure 36C) was reduced considerably in the presence of ActD. In addition, a search for DxGx₂Dx₂L variants with naturally-occurring conservative substitutions of aspartic acids to glutamic acid or the leucine to isoleucine was performed. This revealed many other proteins, including the Ran Binding Protein 1 (RanBP1), tudor-containing protein TDRD3 and the 26S proteasome subunit ATPase TBP-1 (Figure 36E and data not shown). These variants displayed nuclear export rates comparable to levels observed for the DxGx₂Dx₂L sequence found in VHL, PABP1, BAX and others (Figure 36C and E). So far, all of the DxGx₂Dx₂L motifs tested, regardless of the protein of origin, have shown strong, transcription-dependent nuclear export activity. We suggest that DxGx₂Dx₂L is a general nuclear export sequence present in a wide spectrum of proteins to mediate their transcription-dependent nuclear export.

Figure 36. TD-NEM is a functionally conserved nuclear export sequence.

(A) The DxGx₂Dx₂L consensus sequence was used to search the motif and My Genomics Resource Center databases for sequence alignments. **(B and C)** BAX and PR contain functional TD-NEMs. The indicated constructs were transiently expressed in MCF-7 cells. BAX(364-426)-PK-GFP-NLS was also treated with ActD (2 μ M) for 1 hour. Specific cells (dotted circles) were subjected to cytoplasmic FLIP (white squares). The corresponding kinetics for loss of nuclear fluorescence was plotted on a graph (C). **(D and E)** Active nuclear export of TD-NEM variants with aspartic to glutamic acid or leucine to isoleucine substitutions. MCF-7 cells were transiently transfected with the indicated constructs and subjected to cytoplasmic FLIP. Kinetics for the loss of nuclear fluorescence was monitored over time and graphed (E).



7. DISCUSSION

The advent of new technologies such as photobleaching in the field of cell biology has lead to major conceptual changes in our understanding of protein dynamics and subcellular trafficking (Lippincott-Schwartz et al., 2003). Previously, proteins were thought to be relatively immobile entities localizing and functioning in distinct compartments. In recent years it has become apparent that the function of many proteins is dependent on their dynamic profile. In keeping with this vision, the VHL tumor suppressor serves as an excellent model for this emerging concept.

It has become increasingly evident that degradation of nuclear proteins by the ubiquitylation pathway requires nuclear-cytoplasmic trafficking of the E3 ubiquitin-ligase as well as the substrate protein. Efficient degradation of nuclear proteins, such as p53, Smad3 and HIF α , is tightly linked to the ability of the E3 ubiquitin-ligase to engage in nuclear export (Fukuchi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999; Lindstrom et al., 2007). Thus VHL, the ubiquitylation component of an E3 ubiquitin-ligase complex that mediates oxygen-dependent degradation of HIF α , was an ideal subject for studying the functional role of nuclear-cytoplasmic trafficking of E3 ubiquitin-ligases. Although the majority of proteins that export from the nucleus utilize the classical NES/CRM1 pathway, we have previously shown that nuclear-cytoplasmic trafficking of VHL is independent of this nuclear export pathway and requires ongoing RNA PolII-mediated transcription (Groulx and Lee, 2002; Lee et al., 1999). The initial goal of this study was to identify the sequence that mediates transcription-dependent nuclear export of VHL. In doing so, we stumbled upon a sequence that may potentially mediate nuclear export of several proteins, including the mRNA nuclear export factor PABP1.

VHL and PABP1 share the distinct ability to engage in constitutive and highly dynamic nuclear-cytoplasmic trafficking utilizing a pathway that requires ongoing RNA PolII activity. This study has led to the identification of a new functional domain, TD-NEM, which is present in both VHL and PABP1 and mediates their transcription-dependent export from the nucleus. The rate of nuclear export of TD-NEM is approximately 70-80% of that observed for the Rev NES, perhaps the strongest LMB-sensitive NES so far identified, suggesting that this motif is highly efficient in mediating nuclear egression. Several lines of evidence support a role for TD-NEM as a nuclear export motif. First, removal of TD-NEM from full-length VHL and PABP1 altered the steady-state distribution of these predominantly cytoplasmic proteins to mostly nuclear. Also, a single amino acid substitution of glycine to alanine in full-length VHL altered the steady-state distribution from mostly cytoplasmic to nuclear accompanied by a reduction in sensitivity to ActD or DRB treatment and rate of nuclear export. Second, TD-NEM alone from VHL and PABP1 was sufficient to confer transcription-dependent nuclear export properties to a large reporter protein (i.e. PK-GFP) in multiple living cells and in vitro assays. Based on these data, TD-NEM represents a new, and possibly ubiquitous, nuclear export motif that operates independently of the known CRM1-NES pathway.

In this study, we show that transcription-dependent nuclear export of VHL and PABP1 is mediated by a simple and linear sequence, DxGx₂Dx₂L. Each of the four conserved residues of TD-NEM was found to be required for full activity of the export signal. It is not surprising that a mere four residues can act as a transport signal considering that the classical NES relies on a consensus sequence of only a few conserved residues (Fischer et al., 1995; Wen et al., 1995). There were no other apparent conserved residues between

the core TD-NEM of VHL, PABP1 and Cyclin C, however, these three independent TD-NEMs displayed slightly different nuclear export activities suggesting a functional role of the non-conserved residues in modulating the activity of TD-NEM. These observations are similar to the classical NES that can display different export activities dependent on the surrounding amino acid context (Kutay and Guttinger, 2005). Whether other substitutions or subtle differences of this TD-NEM, such as different spacing between the key residues, retain activity also remains to be tested. If functional combinations are found, this would simply provide additional evidence to support a ubiquitous nature of TD-NEM.

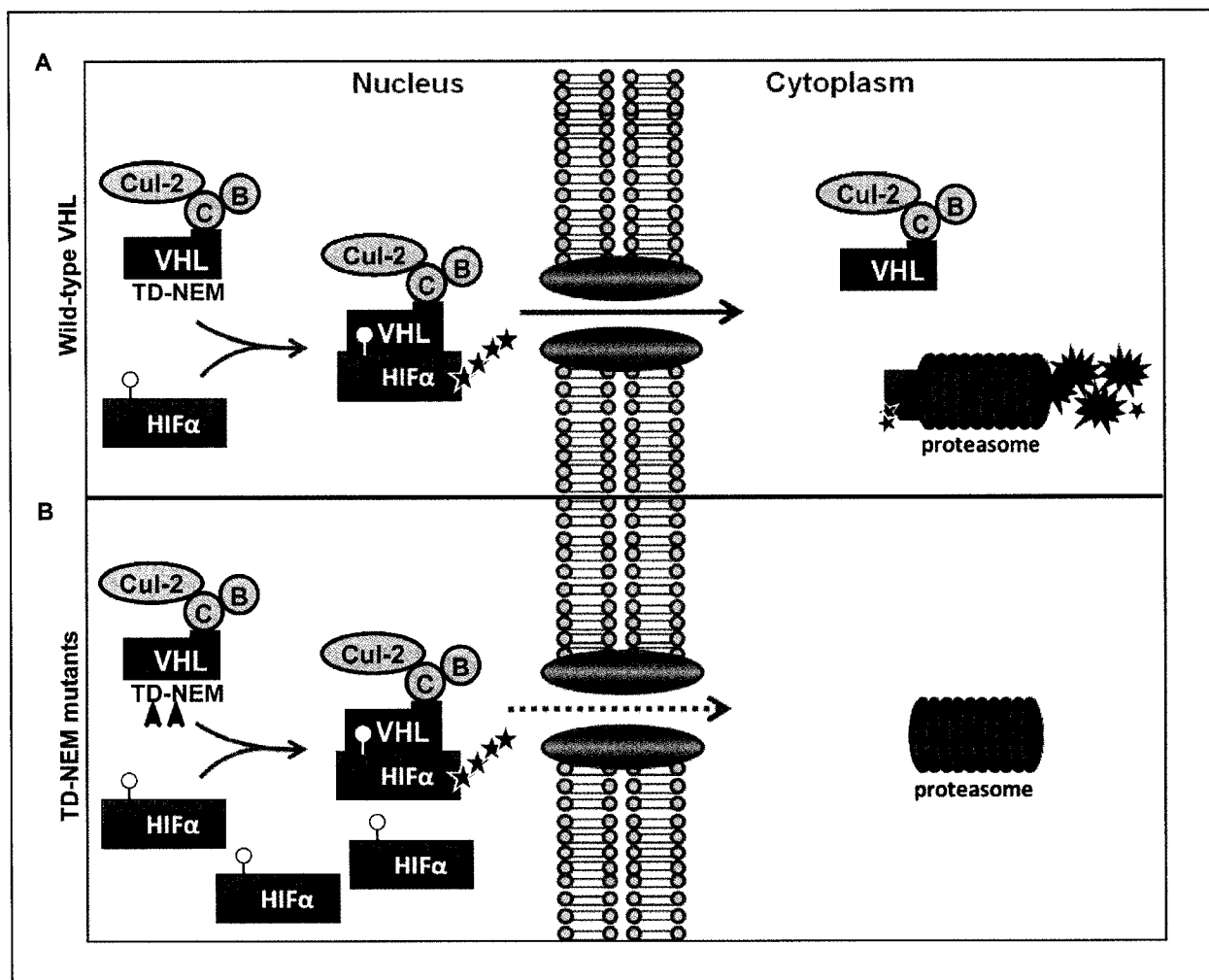
We have previously shown that perturbing the nuclear-cytoplasmic trafficking profile of VHL is detrimental to its ability to mediate oxygen-dependent degradation of HIF α . Discovery of the TD-NEM sequence of VHL allowed for the further investigation and dissection of the importance of VHL dynamics for degradation of HIF α . Data presented in this study substantiate the vital role of nuclear-cytoplasmic trafficking for the E3 ubiquitin-ligase function of VHL. Here we show that the naturally-occurring TD-NEM mutations, D121G and D126Y, which lead to RCC and polycythemia respectively, abrogate nuclear export of VHL. It has been puzzling as to how VHL cancer-causing mutations, such as D121G, retain the ability to bind and ubiquitylate HIF α *in vitro* yet are able to develop classical tumors associated with VHL disease (Hansen et al., 2002). We show that D121G and D126Y maintain the ability to bind to HIF2 α , consistent with previously published data. Expression of these mutants leads to an extended HIF2 α stability following reoxygenation of hypoxic cells, providing a correlation between the efficiency to mediate degradation of HIF α and nuclear export activity. We cannot

exclude the possibility that other aspects of the ubiquitylation pathway may be affected *in vivo*, however, we did predict a defect in HIF2 α degradation with G123A, a mutant that is restrained in its ability to export from the nucleus but retains its ability to bind to HIF2 α . The G123A mutant further supports the concept that the TD-NEM motif of the β -domain of VHL is not involved in HIF α binding or E3 ubiquitin-ligase complex formation but plays an essential role in HIF α degradation by mediating nuclear export. We achieved low expressing stable cell lines of the nuclear export defective mutants though expression was still higher than endogenous VHL. This raises the possibility that the nuclear-export mutants may be partially rescued by overproduction and may have more pronounced defects at physiological levels. The differences in HIF α stability observed with the nuclear export defective mutants may translate into different types of VHL disease as observed with Type 2A and Type 2B mutants with respect to HIF α binding (Li et al., 2007). Whether the prolonged presence and activity of HIF α provides an explanation as to how patients with these mutations develop tumors remains to be tested.

It is now known that the transcription-dependent nuclear-cytoplasmic trafficking of VHL is essential for nuclear ubiquitylation and subsequent cytoplasmic degradation of HIF α (Figure 37). Our data illustrates that the dynamic profile of a protein is instrumental for functional integrity and provides evidence that mutations targeting subcellular trafficking can abrogate protein function. It was previously thought that VHL inactivating mutations most often prevented assembly with its substrate, HIF α , or core components of the E3 ubiquitin-ligase, elongins B, C and Cul-2. Either of these outcomes would result in

Figure 37. TD-NEM-mediated nuclear export of VHL is essential for efficient oxygen-dependent degradation of HIF α .

A) Nuclear export of VHL is mediated by TD-NEM and is required for efficient degradation of HIF α . **B)** Mutations targeting the key residues of TD-NEM in VHL decrease its nuclear export activity. This in turn abrogates the ability of VHL to mediate efficient oxygen-dependent degradation of HIF α . Thus disease mutations targeting subcellular trafficking of VHL abrogate its function in mediating efficient degradation of HIF α without affecting interaction with its substrate.



stabilization of HIF α protein levels. Here we show that disease mutations targeting subcellular trafficking of VHL abrogate its function in mediating efficient degradation of HIF α without affecting interaction with its substrate (Figure 37). Together, these recent findings provide evidence that the oxygen-regulated shuttling dynamics of VHL are instrumental for functional integrity and are essential in the control of HIF activation, oxygen homeostasis, and suppression of tumorigenesis.

Regulated and signal-mediated transport of proteins between the nucleus and the cytoplasm is critical for many cellular processes. Several studies have reported the need for ongoing RNA PolII activity to actively shuttle proteins between the nuclear and cytoplasmic compartments. Most studies have focused on the role of ongoing transcription in nuclear import of proteins such as hnRNPs (Pinol-Roma and Dreyfuss, 1991; Pinol-Roma and Dreyfuss, 1992; Pinol-Roma and Dreyfuss, 1993; Siomi et al., 1997). However, there are also proteins that necessitate RNA PolII-mediated transcription to efficiently export from the nucleus, including VHL and PABP1 (Afonina et al., 1998; Griffis et al., 2004; Groulx and Lee, 2002; Lee et al., 1999; Zhang et al., 2005). In an attempt to understand the mechanism of transcription-dependent nuclear export mediated by TD-NEM, we set out to identify components involved in this nuclear export pathway. In doing so, we have identified a member of the translational machinery, eEF1A, as a factor involved in the transcription-dependent nuclear export pathway.

The correlation between binding and nuclear export activity argues that the interaction between TD-NEM and eEF1A is biochemically relevant. Removal of TD-NEM from full-length VHL totally abolished binding to eEF1A. Also, TD-NEM alone from both

VHL and PABP1, which consists of only a few residues, bound to eEF1A. However, mutations of key residues within the DxGx₂Dx₂L consensus sequence of TD-NEM, particularly G123A, measurably hindered the ability of TD-NEM to bind to eEF1A and mediate export of reporter proteins. Yet, it is the data obtained with the functional assays that substantiates a role for eEF1A in TD-NEM-dependent nuclear export. Silencing of endogenous eEF1A alters the steady-state localization of full-length endogenous PABP1 and transiently expressed VHL, and the nuclear export competent VHL truncation mutant Δ C157. The observed nuclear accumulation of these TD-NEM-containing proteins suggested the possibility that eEF1A is involved in nuclear export, however, the possibility of a potential role for eEF1A in cytoplasmic retention could not be formally excluded. It is the reduction of nuclear export rate obtained in a FLIP nuclear export assay of cells treated with siRNA against eEF1A that argued for a direct role for eEF1A in the nuclear export activity of TD-NEM in living cells. Furthermore, depletion of endogenous eEF1A from a cellular lysate resulted in a decrease in nuclear export activity of TD-NEM-containing proteins in an *in vitro* nuclear export assay arguing that eEF1A is directly involved in nuclear export. Put together, these data, in addition to the binding results, support a role for eEF1A in TD-NEM-dependent nuclear export.

Several lines of evidence suggest that eEF1A itself is a key element in the coordination of TD-NEM nuclear export, rather than its contribution to protein translation as a member of the translational machinery. First, acute inhibition of protein translation by cycloheximide did not have an effect on TD-NEM-mediated nuclear export. Second, chronic inhibition of translation was tested by silencing of another translation elongation factor, eEF2, such that a comparable decrease in steady-state protein levels and

translational activity, as in eEF1A silencing, was achieved. Knockdown of eEF2 did not have an effect on the localization or the nuclear export activity of TD-NEM-containing proteins compared to silencing of eEF1A. In addition, these experiments suggest that blockage of protein synthesis during the period of the experiments did not deplete a protein whose synthesis would have been required for nuclear export of TD-NEM-containing proteins. Finally, the role of eEF1A in TD-NEM-mediated nuclear export was addressed using *in vitro* nuclear export assays. This assay uncouples ongoing translation with nuclear export activity of eEF1A and it eliminates the possibility for the loss of unknown factor(s) required for nuclear export. Together these results aid to uncouple the role of eEF1A in TD-NEM-mediated nuclear export and protein synthesis.

In general, nuclear export of proteins requires the action of exportins, which typically shuttle between the nucleus and cytoplasm to export nuclear cargo. However, eEF1A does not appear to engage in efficient nuclear/cytoplasmic trafficking. Subcellular trafficking studies have revealed that eEF1A that does import into the nucleus is immediately exported back to the cytoplasm by Exp5 explaining its highly cytoplasmic distribution at steady state (Bohnsack et al., 2002; Calado et al., 2002). Work conducted by several groups, including the present study, have failed to detect endogenous or exogenous eEF1A in the nuclear compartment. In fact, Calado *et al.* (2002) proposed the Exp5 pathway as a way to exclude eEF1A from nuclei if it were ever to enter, such as after cell division. In this study we demonstrate that TD-NEM-mediated nuclear export is independent of the Exp5 pathway. Also, the nucleolar retention experiments shown here, in addition to data from other groups, suggest that eEF1A may exert its function in TD-NEM-dependent nuclear export from the cytoplasmic side of the nuclear envelope.

In recent years it has become apparent that several proteins, unrelated to exportins, play important roles in the nuclear export of proteins and RNAs by exerting their effects from the cytoplasmic side of the nuclear envelope. Current advances in understanding RNA export have revealed a crucial role for several cytoplasmic proteins in the export of mRNA. The DEAD-box protein Dbp5, an ATPase that binds to the cytoplasmic filaments of the NPC, was found to be essential for mRNA export and postulated to act as a molecular motor to pull mRNAs through the nuclear pore (Cole and Scarcelli, 2006; Hodge et al., 1999; Schmitt et al., 1999; Tseng et al., 1998; Weirich et al., 2004). Mutations of the *DBP5* gene or altering the interaction between Dbp5 and Nup159, a nucleoporin of the cytoplasmic filament of the NPC, lead to a dramatic and rapid block of mRNA export (Hodge et al., 1999; Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998). In addition, the ATPase activity of Dbp5 is stimulated by Gle1, an essential mRNA export factor that is also primarily located at the cytoplasmic face of the NPC (Alcazar-Roman et al., 2006; Weirich et al., 2006). Gle1 mutant strains have been previously shown to exhibit strong defects in mRNA export. In accordance with these observations it has been recently shown that a mutant variant of Gle1 that fails to interact with cytoplasmic Dbp5 considerably reduced the ability of mRNAs to export from the nucleus (Alcazar-Roman et al., 2006; Weirich et al., 2006). Also, the GTPase activating protein RanGAP1 and its stimulatory factor RanBP1, which are crucial for the hydrolysis of RanGTP to RanGDP, are exclusively cytoplasmic proteins (Bischoff et al., 1994; Bischoff et al., 1995a; Bischoff et al., 1995b; Coutavas et al., 1993; Mahajan et al., 1997; Matunis et al., 1996; Richards et al., 1996). Mislocalization of either RanGAP1 or RanBP1 to the nuclear compartment through microinjection experiments inhibited major

nuclear export of proteins and certain RNA species (Izaurre et al., 1997). These examples demonstrate that proteins residing in the cytoplasm can orchestrate events on the cytoplasmic side of the nuclear envelope that are equally as important as those occurring in the nucleus during nuclear export of proteins and RNA species.

Consistent with a possible cytoplasmic role for eEF1A is the work by Grosshans *et al.* (2000) that reported a role for cytoplasmic eEF1A in nuclear export of tRNAs in yeast. In a yeast genetic system, strains with reduced levels of, or mutated, eEF1A exhibited strong accumulation of mature tRNAs in the nuclear compartment (Grosshans et al., 2000a; McGuire and Mangroo, 2007). Since eEF1A was undetectable in the nuclear compartment, Grosshans *et al.* postulated that eEF1A may function at the cytoplasmic face of the nuclear pore to facilitate the release of charged tRNAs from the aminoacyl-tRNA synthase. Interestingly, another cytoplasmic protein, Cexp1, has been recently identified as a component of the tRNA export pathway. Both eEF1A and the newly characterized protein Cexp1 have been described as cytoplasmic components of the nuclear aminoacylation-dependent tRNA export pathway (Grosshans et al., 2000a; Grosshans et al., 2000b; Hopper and Shaheen, 2008; Kohler and Hurt, 2007; McGuire and Mangroo, 2007). eEF1A and Cexp1 were found to copurify with one another and to interact directly with aminoacylated tRNA (Grosshans et al., 2000a; McGuire and Mangroo, 2007). Depletion of Cexp1 also reduced the efficiency of nuclear tRNA export (McGuire and Mangroo, 2007). However, cells with reduced levels of both eEF1A and Cexp1 showed a significantly increased level of nuclear tRNA retention. These observations support the idea that eEF1A and Cexp1 function in the same pathway, as cytoplasmic export factors involved in tRNA export. In fact, given that Cexp1 associates

with the NPC by interacting with Nup116p, it was proposed that Cexp1 collects aminoacyl-tRNAs from nuclear export receptors at the cytoplasmic side of the NPC and transfers them to eEF1A using a channeling mechanism (McGuire and Mangroo, 2007). Now we have evidence to support a role for eEF1A in nuclear export of TD-NEM-containing proteins.

These findings raise several interesting questions as to the mechanisms that could be involved in this process. Since a common theme for cytoplasmic nuclear export factors is their involvement in the final steps of transport, it is possible that eEF1A functions in receiving proteins as they pass through the NPC. TD-NEM could interact with a yet unidentified transporter or large ribonucleoprotein complexes in the nucleus followed by exchange of the cargo with eEF1A on the cytoplasmic side of the nuclear pore. In this sense, eEF1A may utilize its GDP/GTP binding properties to release proteins in the cytoplasm, similar to the manner by which RanGTP hydrolysis to RanGDP results in the release of exportin-cargo complexes. A current view of subcellular trafficking is that import or export of proteins across the nuclear pore occurs by association with soluble transporters that can interact with phenylalanine-glycine (FG) repeats found on many nucleoporins (Bayliss et al., 1999; Bednenko et al., 2003; Lei and Silver, 2002; Radu et al., 1995; Weis, 2003). One possibility is that eEF1A can open up the nuclear pore from the cytoplasmic side engaging nuclear TD-NEM-containing proteins. This model is supported by the recent observation that the DEAD-box protein Dbp5, which binds to the cytoplasmic filaments of the NPC, is essential for mRNA export and postulated to act as a molecular motor to pull mRNAs through the nuclear pore (Cole and Scarcelli, 2006; Hodge et al., 1999; Schmitt et al., 1999; Weirich et al., 2006). It is tempting to speculate

that cytoplasmic eEF1A may act in a similar fashion to facilitate nuclear export of TD-NEM-containing proteins. Alternatively, since the nucleolar retention experiments were performed using exogenous eEF1A we cannot formally exclude the possibility that a small population of endogenous eEF1A is capable of entering the nuclear compartment. In fact, this is an intriguing possibility that would easily explain the role of eEF1A in nuclear export of RNA species and TD-NEM proteins. eEF1A may engage in slow, Exp5-independent, nuclear-cytoplasmic shuttling. If this were true, the ability of eEF1A to export TD-NEM-containing proteins would rely more on its sheer quantity, considering it is one of the most abundant proteins in the cell, rather than high dynamics. We have shown that the dynamics of proteins containing a TD-NEM sequence are affected by inhibitors of RNA PolII activity, such as ActD and DRB, which ultimately lead to nuclear accumulation and abrogation of nuclear export activity. In trying to understand how RNA PolII activity is linked to TD-NEM-mediated nuclear export we found that treatment with ActD or DRB partially abrogated binding of eEF1A with TD-NEM. This provides a possible explanation for the ability of ActD and DRB to block TD-NEM activity and nuclear export of VHL and PABP1. It is still unclear as to the exact mechanism by which these drugs inhibit the eEF1A/TD-NEM interaction and thus nuclear export of TD-NEM-containing proteins. Logically, these inhibitors most likely act through direct or indirect effects on either primary or secondary components of this pathway. It is well known that LMB directly interacts with and inhibits the action of CRM1, the exporter of NES-containing proteins (Kudo et al., 1999). Since two different transcriptional inhibitors, which possess unique structural properties, both affect nuclear export and the interaction between eEF1A and TD-NEM it is unlikely that they act

directly on eEF1A, although we cannot formally exclude this possibility. The more plausible explanation, however, is that these inhibitors cause a secondary effect through transcriptional inhibition. This would imply that the activity of RNA PolII is required for the interaction of eEF1A with TD-NEM. While TD-NEM-mediated nuclear export does not appear to occur via mRNA export, we cannot exclude the possibility that other RNA PolII-dependent RNA species may be involved. Elucidating the mechanism by which RNA PolII inhibitors abolish TD-NEM binding to eEF1A and its nuclear export activity should yield interesting information as the processes involved in TD-NEM-mediated nuclear export.

Discovery of LMB as an inhibitor of NES-mediated nuclear export has aided in both deciphering the components of this pathway and identification of many proteins that export from the nucleus (Fornerod et al., 1997; Fukuda et al., 1997). Similarly, our use of transcriptional inhibitors, such as ActD and DRB, has led to the discovery of a nuclear export pathway that relies on the TD-NEM nuclear export signal. In the midst of uncovering the nuclear export pathway of VHL, we envisioned that the discovery of TD-NEM through inhibitors of RNA PolII-dependent transcription will help uncover another class of proteins that undergo TD-NEM-mediated nuclear export. A bioinformatics search of protein databases using the Dx₂Gx₂DxL consensus sequence revealed many proteins that encode this motif. The TD-NEM sequences from randomly chosen proteins, such as the cell cycle regulator Cyclin C, the proapoptotic BAX and the progesterone hormone receptor, were able to confer nuclear export activity to a reporter protein. Furthermore, the TD-NEM sequences from Cyclin C and BAX were sensitive to ActD treatment. More importantly, the full length Cyclin C conferred transcription-dependent

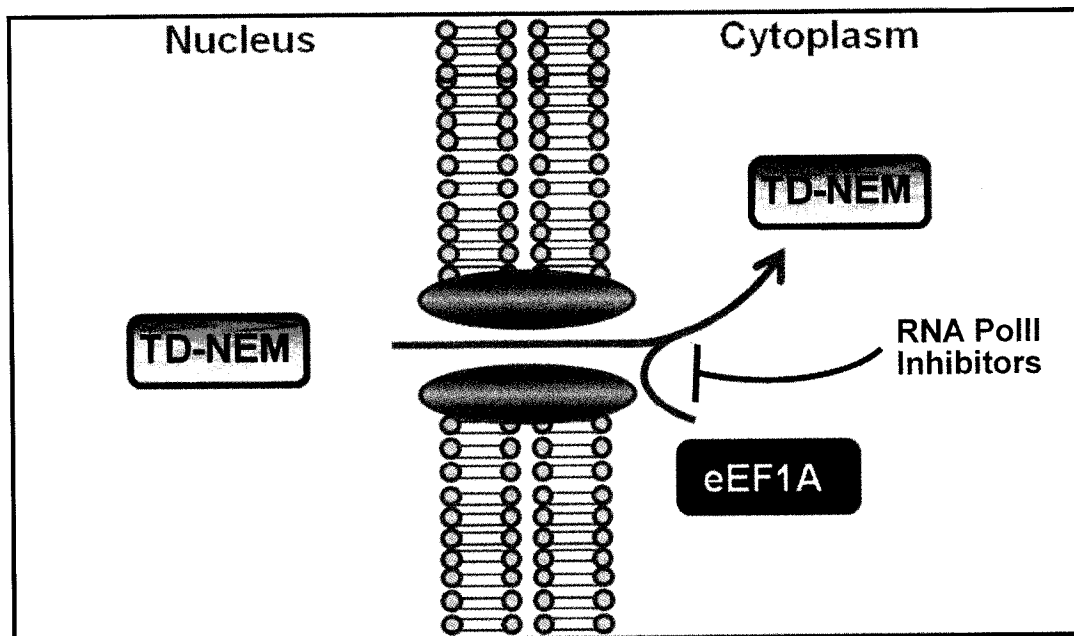
nuclear export activity to the reporter protein. Since the conserved residues of the classical NES can be substituted with similar amino acids, such as leucine to isoleucine, we were interested in testing the flexibility of the TD-NEM sequence. We examined the expandability of this sequence by testing if naturally-occurring conservative permutations of its key residues, such as aspartic acid to glutamic acid or leucine to isoleucine, retain activity. A protein database search using variants of the standard TD-NEM sequence revealed even more proteins that may potentially utilize this sequence to export from the nucleus, such as RanBP1, the tudor-containing protein TDRD3 and the 26S proteasome subunit ATPase TBP-1. In total, eight TD-NEM and variants isolated from independent proteins were able to mediate nuclear export of the PK-GFP-NLS reporter in our live cell FLIP nuclear export assay. Obviously, it remains to be determined if the TD-NEM sequence from various proteins can mediate their respective nuclear export and, if so, in what biochemical and physiological settings this occurs. Further work will be required to decipher the respective role and the interplay of these subcellular trafficking determinants on the function of individual proteins. Nonetheless, it must be appreciated that we have yet to stumble on an inactive form of TD-NEM supporting the idea that this is a highly active nuclear export motif present in a vast array of proteins.

Based on data presented in this study, we suggest that TD-NEM represents a new, and potentially ubiquitous, nuclear export motif that operates independently of the known NES/CRM1 pathway. We propose that TD-NEM acts as a common motif that drives nuclear export of proteins by utilizing a highly abundant and cytoplasmic component of the translational machinery, eEF1A (Figure 38). We envision that cells have evolved a general nuclear export pathway that may act in parallel to the classical NES system.

Figure 37. TD-NEM-mediated nuclear export of VHL is essential for efficient oxygen-dependent degradation of HIF α .

A) Nuclear export of VHL is mediated by TD-NEM and is required for efficient degradation of HIF α . **B)** Mutations targeting the key residues of TD-NEM in VHL decrease its nuclear export activity. This in turn abrogates the ability of VHL to mediate efficient oxygen-dependent degradation of HIF α . Thus disease mutations targeting subcellular trafficking of VHL abrogate its function in mediating efficient degradation of HIF α without affecting interaction with its substrate.

TD-NEM-mediated nuclear export pathway



8. CONCLUSIONS AND SIGNIFICANCE OF FINDINGS

The initial aim of this study was to understand the nuclear-cytoplasmic trafficking properties of the VHL tumor suppressor protein. In doing so, we have identified a novel and discrete motif, DxGx₂Dx₂L, referred to as TD-NEM, which mediates efficient transcription-dependent nuclear export of VHL. This motif was also common among other proteins, including PABP1, where it mediated its respective transcription-dependent export from the nucleus. Nuclear export by TD-NEM requires ongoing RNA PolII-mediated transcription and operates independently of the classical CRM1/NES-mediated export pathway. We have shown that key residues within TD-NEM of VHL are targeted by naturally-occurring point mutations associated with renal carcinoma and polycythemia in humans. These disease-causing mutations, which alter the dynamic profile of VHL, restrain oxygen-dependent degradation of HIF α without affecting interaction between substrate and E3 ubiquitin-ligase (Figure 37). Our results highlight the essential role of nuclear-cytoplasmic dynamics in protein function and provide evidence that mutations targeting subcellular trafficking can lead to disease.

Exploring the mechanism utilized for VHL and PABP1 transport led to the discovery that TD-NEM-mediated nuclear export involves an essential member of the translational machinery, the translation elongation factor eEF1A (Figure 38). eEF1A is a novel VHL and PABP1 interacting protein that binds specifically to the TD-NEM sequence. siRNA-induced silencing or antibody-mediated depletion of eEF1A decreases TD-NEM nuclear export kinetics. Inhibition of RNA PolII-dependent transcription impairs the TD-NEM/eEF1A interaction thereby decreasing TD-NEM-mediated nuclear export. Since eEF1A exhibits a predominantly cytoplasmic localization we propose that its functional

role is orchestrated from the cytoplasmic side of the nuclear envelope. The results presented here highlight the essential role of cytoplasmic export factors in the coordination of events that lead to export from the nuclear to the cytoplasmic compartment. Meanwhile, these results also provide a link between the translational apparatus and the subcellular trafficking machinery demonstrating that these two central pathways in basic metabolism can cooperate to sustain the highly dynamic nature of cellular processes.

In an attempt to understand the nuclear export of VHL we have, in fact, identified a novel nuclear export pathway. The newly identified TD-NEM sequence is present in multiple proteins with various biological functions. The ubiquitous nature of this signal suggests that it may represent a general nuclear export pathway. Interestingly, the TD-NEM-mediated nuclear export pathway utilizes eEF1A, which entails a novel function for this translation factor. Previously, cytoplasmic eEF1A has been implicated in the nuclear export pathway of tRNA in yeast. Here we propose that eEF1A is a cytoplasmic component of the TD-NEM-dependent nuclear export pathway in mammalian cells. The fact that; 1- the TD-NEM sequence is present in many proteins and 2- eEF1A is a highly abundant protein, leads us to speculate that this is a ubiquitous pathway. Since inhibition of eEF1A did not affect NES-mediated nuclear export of proteins this suggests that eEF1A and CRM1 operate independently. Thus TD-NEM-mediated nuclear export through eEF1A may represent a general pathway that operates in parallel to the classical NES.

9. FUTURE DIRECTIONS

The presence of the novel TD-NEM sequence in many proteins, some of which were investigated in this study, suggests this may act as a general nuclear export pathway. The challenge will be to demonstrate the functional role of TD-NEM in these proteins, particularly for those that have not yet been shown to participate in nuclear-cytoplasmic trafficking. There is emerging evidence that a large class of proteins is able to export utilizing an NES-independent pathway. It would be interesting to test whether these proteins encode a TD-NEM and if they engage in transcription-dependent nuclear export. The TD-NEM nuclear export motif utilizes a pathway that requires RNA PolII-mediated transcription. Inhibition of RNA PolII activity abrogates TD-NEM-mediated nuclear export and the interaction with eEF1A. Although these results suggest the requirement of RNA PolII transcribed RNA species, we were not able to identify any mRNA associated with VHL. It would be interesting to test if other RNA PolII-dependent RNA species, such as noncoding RNAs, are involved in this pathway. Future work aiming at elucidating the mechanism by which RNA PolII inhibitors abolish the nuclear export activity of TD-NEM and interaction with eEF1A should yield interesting information as to the process of transcription-dependent nuclear export.

eEF1A is reported to be an exclusively cytoplasmic protein, suggesting that its function in TD-NEM-mediated nuclear export may be exerted from the cytoplasmic side of the nuclear envelope. Future work should be aimed at solidifying this hypothesis. If eEF1A does indeed exert its function from the cytoplasm it would be crucial to examine the exact role of eEF1A in this process. An essential step in the nuclear export of proteins is the dissociation of the export complex in the cytoplasm. For example, during NES-

mediated classical nuclear export, the small GTPase Ran, which can switch between GDP- and GTP-bound states, mediates the dissociation of the cargo-exporter complex. Interestingly, eEF1A is a GTP-binding protein and cycles between GTP-bound and GDP-bound states. Thus it should be tested whether cytoplasmic eEF1A is involved in the final steps of TD-NEM nuclear export where it may function similar to Ran in the release of cargoes in the cytoplasm. A second possibility is that cytoplasmic eEF1A may interact with the NPC and function in pulling cargoes through the pores, similar to the role of Dbp1 in mRNA export. Identifying any interaction of eEF1A with nuclear pore components should provide further insight. Alternatively, a small population of endogenous eEF1A may be capable of entering the nuclear compartment to promote nuclear export. In this case, it would be important to test the possibility that endogenous eEF1A can enter the nucleus.

The fact that eEF1A may function only from the cytoplasmic side of the nuclear envelope implies the existence of a yet unidentified nuclear exporter that would interact with TD-NEM-containing cargo in the nucleus to facilitating its passage through the NPC. However we cannot rule out the possibility that the TD-NEM/eEF1A system operates independently of known classical nuclear export pathways. Future work in unraveling other components of this export pathway will further uncover the mechanism of transcription-dependent nuclear export of proteins and clarify the precise role of eEF1A during this process.

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APPENDIX A- First Author Publications

Cancer-Causing Mutations in a Novel Transcription-Dependent Nuclear Export Motif of VHL Abrogate Oxygen-Dependent Degradation of Hypoxia-Inducible Factor^{∇†}

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It is thought that degradation of nuclear proteins by the ubiquitylation system requires nuclear-cytoplasmic trafficking of E3 ubiquitin ligases. The von Hippel-Lindau (VHL) tumor suppressor protein is the substrate recognition component of a Cullin-2-containing E3 ubiquitin ligase that recruits hypoxia-inducible factor (HIF) for oxygen-dependent degradation. We demonstrated that VHL engages in nuclear-cytoplasmic trafficking that requires ongoing transcription to promote efficient HIF degradation. Here, we report the identification of a discreet motif, DXGX₂DX₂L, that directs transcription-dependent nuclear export of VHL and which is targeted by naturally occurring mutations associated with renal carcinoma and polycythemia in humans. The DXGX₂DX₂L motif is also found in other proteins, including poly(A)-binding protein 1, to direct its transcription-dependent nuclear export. We define DXGX₂DX₂L as TD-NEM (transcription-dependent nuclear export motif), since inhibition of transcription by actinomycin D or 5,6-dichlorobenzimidazole abrogates its nuclear export activity. Disease-causing mutations of key residues of TD-NEM restrain the ability of VHL to efficiently mediate oxygen-dependent degradation of HIF by altering its nuclear export dynamics without affecting interaction with its substrate. These results identify a novel nuclear export motif, further highlight the role of nuclear-cytoplasmic shuttling of E3 ligases in degradation of nuclear substrates, and provide evidence that disease-causing mutations can target subcellular trafficking.

Ubiquitylation is a multiprotein pathway that destines marked proteins for degradation by the 26S proteasome (22, 59). The conjugation of ubiquitin to proteins requires the action of three different enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. The process of ubiquitylation begins with the loading of a ubiquitin molecule onto the E1 ubiquitin-activating enzyme. This is followed by the transfer of ubiquitin from the E1 to the E2 ubiquitin-conjugating enzyme. Finally, transfer of ubiquitin from the E2 to the lysine residue of a target substrate is catalyzed by the E3 ubiquitin ligase. Selectivity of this pathway relies heavily on E3 ubiquitin ligases, which ultimately dictate substrate specificity. E3 ubiquitin ligases can act individually or form a multisubunit complex that may include a member of the Cullin family of proteins to covalently modify a vast array of cellular proteins. In view of the essential role of E3 ubiquitin ligases in regulation of many aspects of cellular functions and biological processes, there is mounting evidence that loss of function or deregulation of E3 ligases contributes to the development of disease.

Degradation of nuclear substrates by the ubiquitylation system often requires nuclear-cytoplasmic trafficking of both the E3 ubiquitin ligase and the substrate protein (2, 54). One

example is the ubiquitin-mediated degradation of the p53 tumor suppressor protein by the Mdm2 (murine double minute 2) E3 ubiquitin ligase (45, 47). Mdm2 shuttles continuously between the nucleus and the cytoplasm in order to efficiently degrade nuclear p53 (12, 53). Cancer-causing point mutations that disrupt nuclear export of Mdm2 are impaired in mediating proteasomal degradation of p53 (37). Nuclear export of the ROC1-SCF^{Fbw1a} E3 ubiquitin ligase is also required for the proteasomal degradation of the Smad3 transcription factor (13). Another example is the cyclin-dependent kinase inhibitor p27^{Kip1}, which requires nuclear export by Jab1 for proteasome-mediated degradation. A mutant form of p27^{Kip1} that fails to assemble with Jab1 cannot be exported from the nucleus and is not degraded by the proteasome (54, 58).

The von Hippel-Lindau tumor suppressor protein (VHL) is a vital component of the VBC-Cul2 E3 ubiquitin ligase complex, as it acts as the substrate recognition protein to provide specificity to the degradation process (25, 27, 30, 38, 39, 50). VHL promotes the recruitment, ubiquitylation, and subsequent proteasomal degradation of the alpha subunit of hypoxia-inducible factor (HIF) in an oxygen-dependent manner (26, 41). Under conditions of normal oxygen tension (normoxia), HIF α is hydroxylated at key prolyl residues within the oxygen-dependent degradation domain by prolyl hydroxylases (5, 8, 24, 26). This posttranslational modification promotes the interaction between HIF α and VHL and leads to ubiquitin-mediated degradation of HIF α (62). Under conditions of low oxygen tension (hypoxia), prolyl hydroxylation does not occur, leading to the stabilization of HIF α , since it fails to assemble with VHL (26, 46, 62). Stabilization of HIF α results in increased transcription of an array of hypoxia-inducible genes, including vascular endothelial growth factor, glucose trans-

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porter 1, and transforming growth factor α , among others, that modulate angiogenesis, glycolysis, and growth (11, 18, 20, 55, 56). Numerous inactivating mutations of the VHL gene lead to the stabilization of HIF α and are associated with the VHL cancer syndrome, in which afflicted individuals develop different tumors, such as renal clear cell carcinoma (RCC), retinal angioma, nervous system hemangioblastoma, and pheochromocytoma (6, 23, 33, 40). Inactivating mutations of VHL often prevent assembly with its substrate, HIF, or core components of the E3 ubiquitin ligase, elongins B and C, and Cullin 2, resulting in constitutive activation of HIF α targets (7, 24, 28, 35, 39, 46).

Nuclear-cytoplasmic trafficking is essential for the E3 ubiquitin ligase function of VHL and oxygen-dependent degradation of HIF α (17, 34). Failure of VHL to continuously shuttle between the nuclear and cytoplasmic compartments leads to the stabilization of HIF α (17, 34, 42–44). VHL engages in nuclear-cytoplasmic shuttling dynamics independently of the classical, leucine-rich, nuclear export sequence (NES) (9, 60) but accumulates in the nucleus upon addition of inhibitors of RNA polymerase II (Pol II) activity (17, 34). Interestingly, the general RNA metabolism and translation initiation factor poly(A)-binding protein 1 (PABP1) exhibits similar transcription-dependent trafficking dynamics as VHL, since it also accumulates in the nucleus upon addition of inhibitors of RNA Pol II activity (1). These results suggest the existence of a transcription-dependent nuclear export pathway that is employed by VHL, PABP1, and perhaps other proteins and that operates independently of the classical NES/CRM1 system.

Here we report the identification of a novel and discreet nuclear export motif, DXGX₂DX₂L. We define this motif as TD-NEM (transcription-dependent nuclear export motif), since it mediates nuclear export of proteins in a manner that requires ongoing RNA Pol II-dependent transcription but operates independently of the classical NES pathway. Disease mutations of TD-NEM of VHL alter its ability to be exported from the nucleus and to mediate oxygen-dependent degradation of HIF α without affecting interaction with its substrate. These results highlight the requirement of nuclear-cytoplasmic trafficking of E3 ubiquitin ligases for degradation of their nuclear substrates, provide evidence that mutations targeting subcellular trafficking can lead to disease, and identify a novel motif that mediates efficient nuclear export of proteins.

MATERIALS AND METHODS

Cell culture, transfections, drug treatments, and hypoxia treatment. 786-0 (VHL-negative) renal carcinoma cells, MCF-7 cells, and NIH 3T3 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin in a 37°C and 5% CO₂ environment. Transient transfections in MCF-7 cells were conducted with Effective transfection reagent (Qiagen). Transfected cells were incubated for 24 h before any manipulations or drug treatment. Stable cell lines were generated by stably transfecting 786-0 cells with the following Flag-tagged constructs: F-VHL-GFP, F-VHL(D121G)-GFP, F-VHL(D126Y)-GFP, and F-VHL(G123A)-GFP, followed by G418 selection. Where indicated, cells were treated at 37°C with a final concentration of 2 μ M actinomycin D (ActD), 10 μ M leptomycin B (LMB), or 25 μ g/ml 5,6-dichlorobenzimidazole (DRB) for 1 h prior to photobleaching experiments and 10 μ M ActD or 10 μ M LMB for 3 h prior to live cell fluorescence imaging. Proteasome inhibitor treatment was performed with MG132 (Calbiochem) at a final concentration of 10 μ M for 2 h before the cells were harvested. Hypoxia treatment was

performed in a hypoxic chamber at 37°C under a 1% O₂, 5% CO₂, and N₂-balanced atmosphere.

Expression vectors. Human full-length VHL and deletion, truncation, or point mutants of VHL were cloned into pcDNA3.1 between an NH₂-terminal Flag tag and a COOH-terminal green fluorescent protein (GFP) tag, as previously described (4, 17, 34). F-GFP and F-GFP-NES were previously described by Groulx et al. (16) and Lee et al. (34). The PK-GFP-NLS construct used for the in vivo fluorescence loss in photobleaching (FLIP) nuclear export assay consisted of pyruvate kinase (PK), which does not encode any localization determinant, GFP, and a nuclear localization signal (NLS) derived from the simian virus 40 large T antigen as previously described (16, 29). Human full-length VHL and deletion mutants and the strong NES from the human immunodeficiency virus Rev were inserted into a F-PK-GFP-NLS construct that was previously described by Groulx et al. (16), between the Flag tag and PK using the ApaI and XhoI restriction sites. cDNAs corresponding to VHL residues 114 to 138 [VHL(114–138)], PABP1(296–317), and cyclin C(158–179), which encode TD-NEM sequences and the full-length cyclin C, were inserted into PK-GFP-NLS using ApaI and XhoI restriction sites. The human full-length PABP1 and the deletion mutant Δ 296–317 were fused to GFP-F to produce the GFP-F-PABP1 and GFP-F-PABP1(Δ 296–317) fusion proteins.

Live cell fluorescence imaging. Images of living cells transiently expressing GFP from experiments where photobleaching was not utilized were imaged with an Axiovert S100TV microscope (Carl Zeiss MicroImaging, Inc.) equipped with a 40 $\times$, 1.2 C-Apochromat water immersion objective using a digital charged-coupled-device camera (Empix). Cell nuclei were stained with Hoechst 33342 (Sigma). Images were captured using the Northern Eclipse software package (Empix).

Photobleaching and microscopy. Cells were cultured and transfected directly onto 35-mm dishes with coverslip bottoms (MatTek). Photobleaching and live cell microscopy were performed using a confocal microscope (LSM5 Pa laser scanning microscope; Carl Zeiss Canada). In all experiments cells were maintained at 37°C in an environmental chamber. A 63 $\times$ plan Apo oil immersion lens with a 1.4 numerical aperture was used for bleaching and imaging. Indicated areas were exposed to three rapid pulses of a 488-nm argon laser at 100%, and image acquisition was at 1% of full laser power. For cytoplasmic FLIP experiments of cells expressing a GFP-tagged fusion protein, a large cytoplasmic region was initially bleached with three rapid pulses to eliminate the dominant cytoplasmic signal. This was followed by repetitive bleaching in a small region of the cytoplasm and imaged at 30-second intervals. For cytoplasmic FLIP experiments of cells expressing a PK-GFP-NLS-tagged fusion protein, cells were repeatedly bleached in a small cytoplasmic region and imaged at 30-second intervals. Small bleached areas for cytoplasmic FLIPs were kept consistent in terms of size and distance from the nucleus. Fluorescence loss in the unbleached areas was quantified as previously described (43, 51) using the following equation: $I_{rel} = (I_t/I_0) \cdot (N_0/N_t)$, where I_t is the average intensity of the unbleached nucleus or cell at time point t , I_0 is the average prebleached intensity of the nucleus or cell of interest, and N_0 and N_t are the average nuclear or cellular fluorescence intensity of a neighboring cell in the same field of vision prebleach or at time t , respectively. This calculation accounts for any losses in fluorescence by normalizing the fluorescence of the cell of interest to that of a neighboring cell of approximately equal size and fluorescent intensity. Nuclear FLIPs were performed by repetitive bleaching of a small nuclear area and imaging at 15-second intervals. Loss of nuclear fluorescence was quantified using the above equation; however, the value for I_t in this case was that of the bleached nucleus. For fluorescence recovery after photobleaching (FRAP) experiments, a large nuclear region was photobleached once with three rapid pulses and images were collected every 5 seconds. Recovery of the fluorescent signal within the bleached region was calculated as described by Phair and Misteli (51) and the following equation: $I_{rel} = (I_t/I_0) \cdot (T_0/T_t)$, where T_t is the total cellular intensity at time t , T_0 is the total cellular intensity before bleaching, I_0 is the intensity in the bleached area before bleaching, and I_t is the intensity of the previously bleached region at time t . Pseudocolor images were generated to highlight differences in GFP fluorescence, with red representing high fluorescent intensity and light blue representing low fluorescent intensity. The quantification graphic was generated by using FLIP/FRAP software. For all bleaching experiments, 10 data sets were analyzed for each result. Pseudocoloring for bleaching experiments was achieved by applying the gradient map function of Photoshop (Adobe) to a montage of picture frames prepared with ImageJ software (National Institutes of Health, Bethesda, MD). The Northern Eclipse (Empix), Excel (Microsoft), and FreeHand (Macromedia) software packages were also used to capture images, analyze the data, and generate graphs.

Polykaryon assay. MCF-7 cells were transfected to express fluorescently labeled proteins and incubated under standard conditions for 24 h. Usually, 40 to 60% of cells presented strong fluorescence as observed by 488-nm fluorescence

microscopy. Cells were trypsinized 24 h after transfection and mixed with untransfected NIH 3T3 cells in a ratio of 1 to 10. The cell mixture was plated in 35-mm dishes with coverslip bottoms and incubated overnight under standard cell culture conditions. The confluent cell layer was visually inspected for even distribution of fluorescent cells among untransfected cells. Cells were washed twice with prewarmed phosphate-buffered saline (PBS) and fused for 2 min by addition of a prewarmed 50% solution of polyethylene glycol (PEG) in PBS (Sigma-Aldrich). PEG was removed thoroughly by four washes with prewarmed PBS, and cells were then replenished with warmed standard cell culture medium. Hoechst staining of DNA was used to identify donor and acceptor cells. Cells were observed under phase-contrast microscopy for fusion events and were monitored for the redistribution of nuclear expression of PK-GFP-NLS-tagged proteins.

In vitro nuclear export assay. The in vitro export assay was performed as described by Groulx et al. (16). Briefly, cells were plated and grown on a 35-mm coverslip plate. Cells were washed with transport buffer (TB) containing 20 mM HEPES pH 7.3, 110 mM KO-acetate (KOAc), 5 mM NaOAc, 2 mM Mg(OAc)₂ and permeabilized at 4°C for 5 min with TB containing 50 µg/ml digitonin and a protease inhibitor mixture (Hoechst stain 33258 [Sigma] was used to monitor the permeabilization). After several washes with TB at 4°C, cells were incubated for 30 to 45 min at 20°C in the presence of a standard mixture that included TB, 2 mM ATP, 2 mM GTP, and an ATP-regenerating system (5 mM creatine phosphate and 20 units/ml creatine phosphokinase). Where indicated, MCF-7 total cell lysate was added to the standard mixture.

Immunofluorescence. Cells were seeded onto coverslips and fixed with prechilled methanol for 10 min at -20°C followed by prechilled acetone for 1 min at -20°C. Anti-PABP1 monoclonal antibody was used (Upstate). Cells were incubated for 1 h with a primary antibody solution containing 10% (vol/vol) fetal bovine serum and 1% (vol/vol) Triton X-100 at room temperature in a humidified chamber. Cells were then washed several times in PBS before a 1-h incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch) at room temperature in a dark humidified chamber. Hoechst stain 33342 (Sigma) was added to visualize nuclei, and coverslips were mounted using Fluoromount G (EMS).

Bioinformatic analysis. Proteins containing a TD-NEM were identified using the emotif and My Genomics Resource Center software.

Immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer containing 0.5% Igepal CA630, 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, and 1 mM sodium orthovanadate with 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 µg/ml pepstatin. Cell lysates were incubated with anti-Flag M2 beads (Sigma) overnight while tumbling at 4°C. Beads were washed several times and eluted with Flag peptides (Sigma). For total cell lysates, cells were washed several times in PBS, lysed with 4% sodium dodecyl sulfate (SDS) in PBS, and boiled for 5 min, and the DNA was sheared by passage through a 19-gauge needle. The protein concentration was quantified using the bicinchoninic acid method (Pierce). Samples were separated on denaturing polyacrylamide gels. Western blots gels were transferred onto polyvinylidene difluoride membranes and blocked in skimmed milk powder in PBS containing 0.2% Tween 20 before incubation with Flag-M2 (Sigma), HIF2α (Novus Biologicals), actin (Sigma), and GFP (AVES Lab Inc.) antibodies. Membranes were washed with 0.2% Tween-PBS and blotted with a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) and detected by using Western Lightening chemiluminescence reagent plus (Perkin-Elmer).

RESULTS

Nuclear export of VHL is mediated by a discreet transcription-dependent motif. VHL engages in a nuclear export pathway that requires ongoing RNA Pol II activity (17, 34). As previously reported, addition of the RNA Pol II inhibitors ActD or DRB resulted in nuclear accumulation of the highly cytoplasmic VHL-GFP but had no effect on GFP alone or when fused to a strong, classical, leucine-rich nuclear export signal (see Materials and Methods) (Fig. 1A) (17, 34). In contrast, LMB, a compound that abolishes CRM1-dependent nuclear export of NES-containing proteins (10, 14, 31, 57), had no effect on the steady-state localization of VHL-GFP but caused nuclear accumulation of NES-GFP (Fig. 1A). Nuclear accumulation by ActD was due to a considerable decrease in the rate of nuclear export of VHL-GFP, as demonstrated by cytoplasmic FLIP experiments (Fig. 1B; see also the quantifi-

cation in panel C) and by cellular fusion experiments (data not shown) (34). In the cytoplasmic FLIP experiments, a small region of the cytoplasm is repetitively bleached (Fig. 1B), and a decrease in nuclear GFP fluorescence indicates that the GFP-tagged fusion protein has been exported from the nucleus to the cytoplasm. Furthermore, nuclear FLIP revealed similar intranuclear dynamics between ActD-treated and untreated cells, demonstrating that ActD (or DRB) did not cause nuclear retention of VHL-GFP (Fig. 1D; see also Fig. S1 in the supplemental material). Loss of GFP fluorescence in the FLIP experiments was not due to fragmentation of the GFP-fusion proteins, as demonstrated by immunoblot analysis (Fig. 1F). Next, it was important to determine whether the signal mediating nuclear export was encoded by VHL or was present within the E3 ubiquitin ligase complex. We also tested a C-terminal deletion of the α-domain of VHL (ΔC157), which is required for assembly with core E3 ubiquitin ligase components elongins B and C and Cullin 2 (4, 46). ΔC157 retained the ability to engage in transcription-dependent nuclear export, since it accumulated in the nucleus upon addition of ActD or DRB (data not shown) and displayed reduced nuclear export dynamics in the presence of RNA Pol II inhibitors as demonstrated by cytoplasmic FLIP analysis (Fig. 1E). These data indicate the presence of a transcription-dependent nuclear export signal between residues 1 and 157 of VHL and suggest that formation of the E3 ubiquitin ligase complex is not required for export of VHL.

Larger GFP-tagged truncation mutants of VHL failed to provide insight on transcription-dependent nuclear export of VHL, since they exhibited export rates similar to GFP-GFP, which diffuses across the nuclear envelope (Fig. 1F). To further examine the nuclear export properties of VHL, we established a quantitative live cell nuclear export assay utilizing FLIP technology. A fusion protein consisting of the large and amorphous PK, GFP, and an NLS (29) derived from the simian virus 40 large T antigen (PK-GFP-NLS) was used to ensure that any movement across the nuclear envelope was not due to diffusion (Fig. 2A) (see Materials and Methods). Also, the presence of such a strong NLS would override an intrinsic nuclear import signal, ensuring that all fusion proteins being tested exhibited similar rates of nuclear import. PK-GFP-NLS strictly localized in the nucleus at steady state, owing to the strong NLS activity (Fig. 2B). PK-GFP-NLS did not undergo a significant loss of nuclear GFP fluorescence after cytoplasmic FLIP, as expected, since this protein fails to export from the nucleus (Fig. 2B and E). Addition of a strong, classical, and LMB-sensitive NES of the human immunodeficiency virus Rev (9) to the reporter protein (NES-PK-GFP-NLS) resulted in a rapid loss of nuclear GFP fluorescence during cytoplasmic FLIP, demonstrating that this fusion protein engages in nuclear export (Fig. 2B and E). The inability of PK-GFP-NLS to export from the nucleus compared to its NES-containing counterpart is not a consequence of unforeseen nuclear retention or a significant difference in nuclear dynamics between the two molecules as revealed by nuclear FLIP (see Fig. S2 in the supplemental material). Nuclear fluorescence was also similar for both fusion proteins, indicating that the difference in nuclear export capacity is not due to a large difference in expression levels (see Fig. S2 in the supplemental material). Full-length VHL was able to confer nuclear export activity to the PK-GFP-NLS reporter,

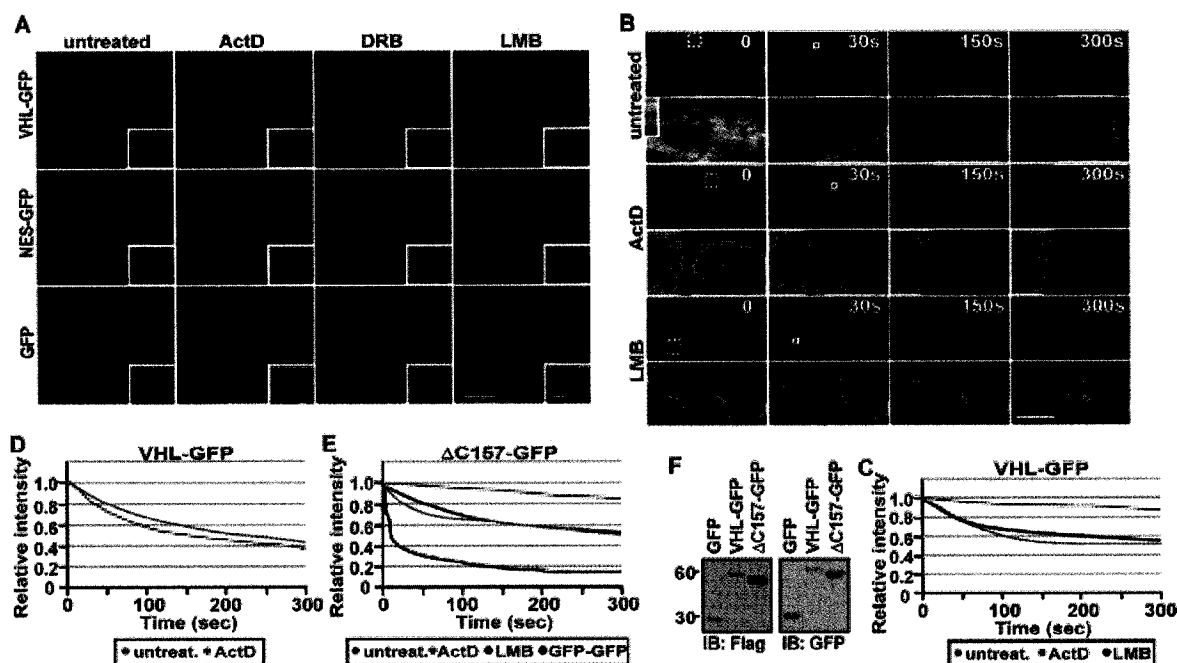


FIG. 1. Transcription-dependent nuclear export of VHL. (A) ActD alters the steady-state localization of VHL. MCF-7 cells transiently expressing VHL-GFP, NES-GFP, or GFP were either untreated or treated with LMB (10 μ M), ActD (8 μ M), or DRB (25 μ g/ml). Insets are the corresponding Hoechst staining of the cells. Bars, 10 μ m. (B and C) Cytoplasmic FLIP reveals that ActD decreases nuclear export of VHL. MCF-7 cells transiently expressing VHL-GFP were treated with ActD (2 μ M) or LMB (10 μ M) for 1 h. Cells were initially bleached in a large cytoplasmic region (dashed squares) to reduce cytoplasmic signal and then submitted to repetitive bleaching in a small cytoplasmic region (white squares). Cells were imaged between pulses, and the corresponding kinetics for loss of nuclear fluorescence were calculated and plotted on a graph (C) (see Materials and Methods). Bar, 10 μ m. (D) Nuclear FLIP analysis was performed on cells treated as for panel B by repetitive bleaching in a small area in the nucleus. The loss of nuclear fluorescence was graphed. (E) Δ C157 exports from the nucleus in a transcription-dependent manner. Δ C157-GFP-transiently expressing cells were treated and analyzed as described for panel B. (F) Western blot analysis using anti-Flag and anti-GFP antibodies verified that the Flag- and GFP-tagged VHL, Δ C157, and GFP fusion proteins used for photobleaching experiments were not fragmented.

confirming that this protein encodes nuclear export activity (Fig. 2C and E). The kinetics for the loss of nuclear signal of VHL-PK-GFP-NLS was very different compared to molecules that are able to passively diffuse, suggesting that the loss of nuclear signal is not a consequence of fragmentation of the fusion protein (see Fig. S2 in the supplemental material). ActD or DRB, but not LMB, inhibited nuclear export of VHL-PK-GFP-NLS (Fig. 2F; see also Fig. S2 in the supplemental material) without affecting intranuclear dynamics (see Fig. S2 in the supplemental material), similar to what was observed with VHL-GFP (Fig. 1) and confirming that inhibitors of RNA Pol II-mediated transcription significantly alter nuclear export of VHL. Furthermore, these results verify that the transcription-dependent nuclear export property of VHL was maintained in the fusion protein. Removal of the exon-2-encoded β -domain (Δ 114-154-PK-GFP-NLS) abrogated the ability of VHL to export the reporter protein from the nucleus (Fig. 2D and E). This observation is consistent with previous reports demonstrating that nuclear accumulation of VHL upon treatment with RNA Pol II inhibitors requires the exon-2-encoded β -domain of VHL (4, 34).

We used this *in vivo* FLIP nuclear export assay to map the region within the exon-2-encoded β -domain that mediates transcription-dependent nuclear export of VHL. Further mutagenesis analysis revealed that a discrete domain between residues 114 and 131 of VHL is required for efficient nuclear export of the PK-GFP-NLS reporter (Fig. 3A and B; see also

Fig. S3 in the supplemental material). The deletion mutant Δ 114-131-GFP (without PK and NLS) also displayed a markedly reduced rate of nuclear export compared to wild-type VHL-GFP (Fig. 3C; see also Fig. S3 in the supplemental material). The subcellular localization and the nuclear export dynamics of Δ 114-131-GFP were unaffected by treatment with ActD or DRB (Fig. 3C and data not shown; see also Fig. S3 in the supplemental material) but rather exhibited a similar export rate to Δ C157-GFP after ActD treatment (Fig. 1E). More importantly, residues 114 to 138 alone were sufficient to confer efficient nuclear export properties to PK-GFP-NLS, which was abolished upon addition of ActD (Fig. 4A and B) or DRB (data not shown). The rate of export observed with residues 114 to 138 was similar to that of Δ C157, indicating that these residues alone confer nuclear export activity to the full-length protein (Fig. 4B). In a cellular fusion assay, this sequence was able to efficiently export the PK-GFP-NLS reporter protein from the donor nucleus to the acceptor nuclei compared to the PK-GFP-NLS control (Fig. 4C). Residues 114 to 138 also stimulated nuclear export of PK-GFP-NLS in an *in vitro* nuclear export assay (Fig. 4D and E). We have, therefore, identified a novel and discreet motif that mediates transcription-dependent nuclear export of VHL.

VHL and PABP1 share a common transcription-dependent nuclear export motif. VHL and PABP1 are implicated in distinct molecular networks but share the characteristic of engag-

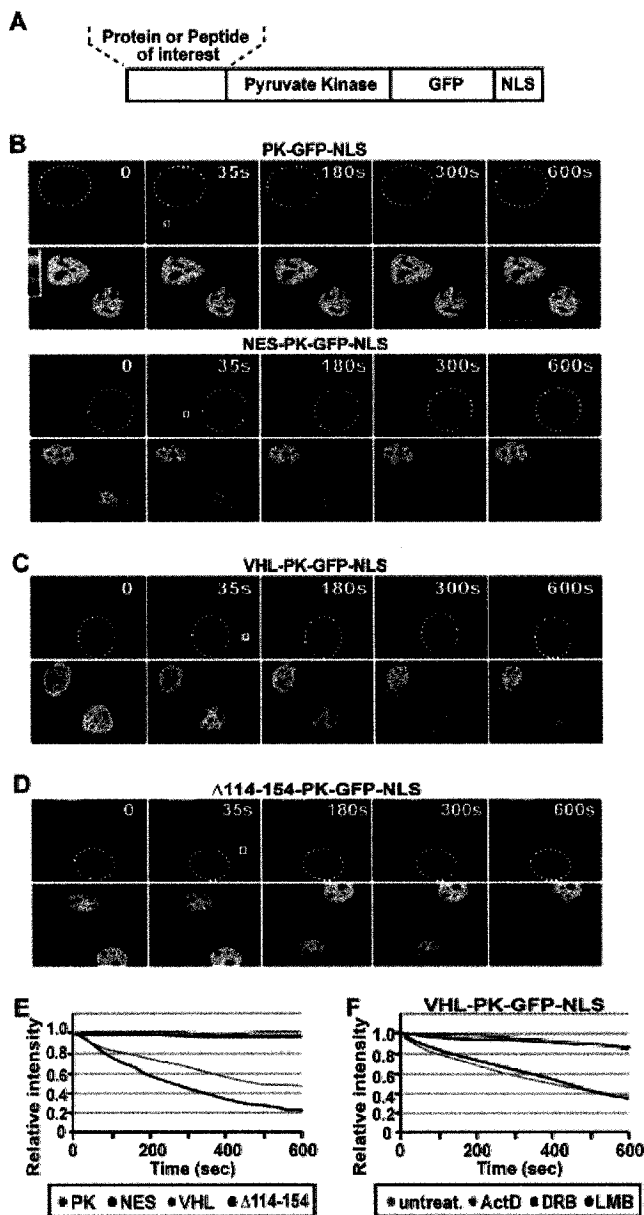


FIG. 2. A transcription-dependent nuclear export sequence is encoded within the exon-2-encoded β -domain of VHL. (A) Schematic diagram of the PK-GFP-NLS fusion protein used for the live cell FLIP nuclear export assay, describing the region where protein or peptide sequences were fused. (B to E) MCF-7 cells transiently expressing the indicated constructs were submitted to cytoplasmic FLIP analysis, in which a small cytoplasmic region (white squares) within specific cells (a dashed circle outlines the cell nucleus) was repeatedly bleached. Kinetics for the loss of nuclear fluorescence from images obtained in panels B, C, and D were calculated and plotted on a graph (E). PK refers to the PK-GFP-NLS reporter construct, and NES, VHL, and $\Delta 114-254$ indicate the sequences fused to PK-GFP-NLS. Bar, 10 μ m. (F) Cells were transiently transfected with VHL-PK-GFP-NLS and were either treated with ActD (2 μ M), DRB (25 μ g/ml), or LMB (10 μ M) for 1 h or left untreated. Cytoplasmic FLIP was performed as described above to verify nuclear export activity.

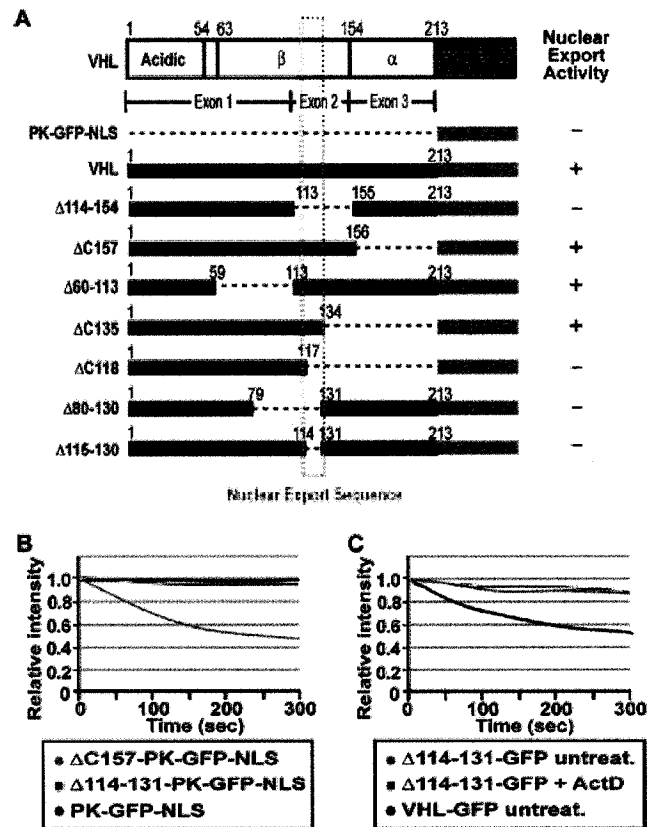


FIG. 3. Residues 114 to 131 are required for nuclear export of VHL. (A) Map of the nuclear export domain of VHL. The schematic diagram indicates deletion mutants of VHL that were submitted to cytoplasmic FLIP to assess the nuclear export activity. + and - indicate the ability or inability of the fusion protein to engage in nuclear export. (B) MCF-7 cells transiently transfected with PK-GFP-NLS, $\Delta C157$ -PK-GFP-NLS, or $\Delta 114-131$ -PK-GFP-NLS were submitted to cytoplasmic FLIP, in which a small cytoplasmic region of a cell was bleached repetitively. The loss of nuclear GFP fluorescence was monitored over time and plotted on a graph. (C) Cells transiently expressing $\Delta 114-131$ -GFP were initially bleached in a large cytoplasmic region followed by repetitive bleaching in a small cytoplasmic region after being treated for 1 h with 2 μ M ActD or left untreated. Kinetics for the loss of nuclear fluorescence were calculated and plotted on a graph.

ing in a nuclear export pathway that requires ongoing RNA Pol II activity (1, 34). Similar to VHL, the subcellular trafficking dynamics of the highly cytoplasmic PABP1 are affected by treatment with ActD (Fig. 5A and B), but not LMB (Fig. 5A) (1). Sequence alignment revealed a striking similarity between residues 114 to 138 of VHL and 296 to 317 of PABP1 that consisted of DXGX₂DX₂L (Fig. 5C). Consistent with a possible role in nuclear export, removal of residues 296 to 317 from full-length PABP1 caused a drastic shift in the steady-state localization from exclusively cytoplasmic to highly nuclear (Fig. 5D). This deletion mutant of PABP1 was also insensitive to ActD treatment compared to wild type (Fig. 5D). Residues 296 to 317 of PABP1 were sufficient to mediate nuclear export of the PK-GFP-NLS reporter as efficiently as residues 114 to 138 of VHL in our FLIP nuclear export assay (Fig. 5E; see also Fig. S4 in the supplemental material). In addition, ActD or DRB, but not LMB, abolished the ability of residues 296 to 317 of

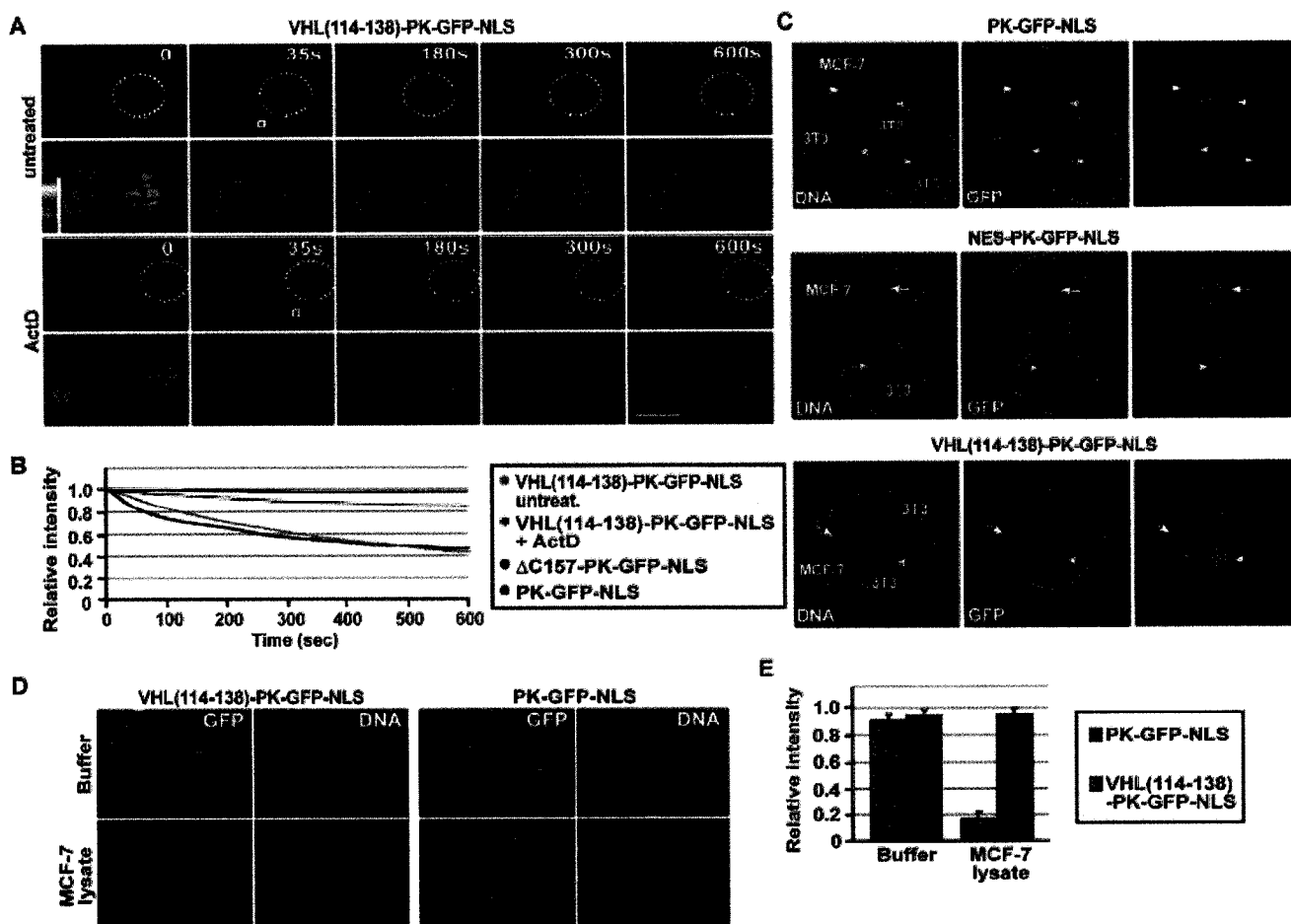


FIG. 4. Residues 114 to 138 mediate transcription-dependent nuclear export of VHL. (A and B) MCF-7 cells were transiently transfected to express VHL(114-138)-PK-GFP-NLS. Cells were treated with a final concentration of 8 μ M ActD or left untreated for 1 h before being subjected to photobleaching. Cytoplasmic FLIP was performed by repetitively bleaching a small cytoplasmic region (white squares) of a cell (the dotted circle outlines the cell nucleus). Cells were imaged between pulses, and the corresponding kinetics for the loss of nuclear fluorescence were calculated and are graphed (B). The nuclear export kinetics of Δ C157-PK-GFP-NLS revealed by a cytoplasmic FLIP analysis were plotted (B). Bar, 10 μ m. (C) VHL(114-138) can mediate export in a polykaryon fusion assay. MCF-7 cells (donors) were fused with NIH 3T3 cells (acceptors) using PEG, and the transfer of nuclear fluorescence from donor to acceptor cells was monitored. Donor and acceptor cells were differentiated by a cell-specific Hoechst staining pattern. White arrows indicate donor cells, and yellow arrows indicate acceptor cells. Bar, 10 μ m. (D and E) VHL(114-138) can mediate export in an in vitro nuclear export assay. MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin, after which they were incubated with transport buffer containing ATP, GTP, and an ATP-regenerating system in the presence of buffer or MCF-7 cell lysate. Relative loss in nuclear fluorescence was calculated and plotted on a graph (E).

PABP1 to mediate nuclear export of the PK-GFP-NLS reporter (Fig. 5E; see also Fig. S4 in the supplemental material). Residues 296 to 317 of PABP1 were able to efficiently export the reporter protein from the donor nucleus to the acceptor nuclei in a cellular fusion assay (Fig. 5F), similar to results obtained with residues 114 to 138 of VHL (Fig. 4C). This led us to postulate that the DXGX₂DX₂L consensus sequence might act as a common motif for transcription-dependent nuclear export of proteins. A search of sequence data banks identified several proteins that contain a DXGX₂DX₂L motif. We randomly selected the cell cycle regulator, cyclin C, whose nuclear-cytoplasmic shuttling profile had not been previously investigated, to test the activity of its potential nuclear export sequence. Full-length cyclin C was fused to PK-GFP-NLS to prevent any passive diffusion through nuclear pores. Cyclin C was able to confer nuclear export activity to the PK-GFP-NLS

reporter in a process that was inhibited by addition of ActD (Fig. 5G). Furthermore, residues 158 to 179 of cyclin C, which encode the DXGX₂DX₂L motif, were able to export the reporter PK-GFP-NLS from the nucleus in an ActD-sensitive manner (Fig. 5H). Further characterization of cyclin C, and other proteins, will reveal whether the DXGX₂DX₂L motif is involved in their subcellular trafficking profiles. Nonetheless, we have identified a novel motif that mediates efficient transcription-dependent nuclear export of VHL, PABP1, and perhaps cyclin C. For the sake of simplicity, the DXGX₂DX₂L motif is referred to as a TD-NEM.

Conserved residues of TD-NEM are essential for mediating efficient nuclear export of proteins. Analysis of the TD-NEM sequences from VHL, PABP1, and cyclin C failed to identify additional conserved residues surrounding the basal DXGX₂DX₂L motif (Fig. 6A), suggesting that these four amino

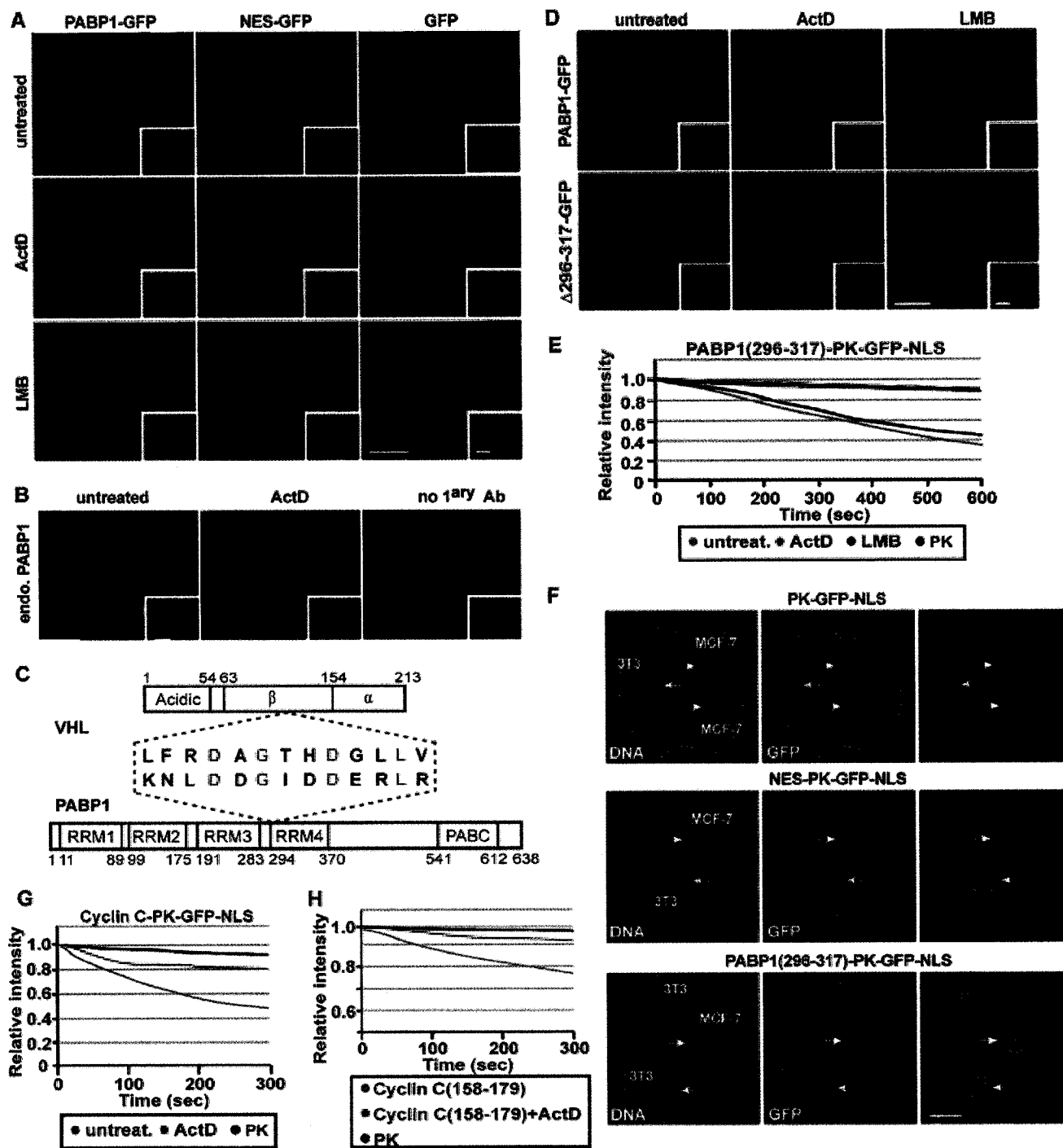


FIG. 5. VHL and PABP1 share a common transcription-dependent nuclear export motif. (A) PABP1 exports by a transcription-dependent mechanism. MCF-7 cells transiently expressing PABP1-GFP, NES-GFP, or GFP alone were either untreated or treated with 8 μ M ActD or 10 μ M LMB for 3 h. Insets are the corresponding Hoechst staining of the cells. Bars, 10 μ m. (B) Endogenous PABP1 is also sensitive to ActD treatment. MCF-7 cells were either left untreated or were treated with 8 μ M ActD. Endogenous PABP1 was detected by immunofluorescence using an anti-PABP1 antibody. Insets show nuclei stained with Hoechst stain. A primary antibody exclusion (no 1^{ary} Ab) control is also shown. Bar, 10 μ m. (C) Schematic diagram depicting a region of alignment between the nuclear export sequence of VHL and PABP1. Conserved residues are indicated in red. (D) MCF-7 cells transiently expressing PABP1(Δ 296-317)-GFP or PABP1-GFP were treated as described for panel A. Insets are the corresponding Hoechst staining of the cells. Bar, 10 μ m. (E) Residues 296 to 317 of PABP1 encode a transcription-dependent nuclear export sequence. Transfected MCF-7 cells were treated with 2 μ M ActD or 10 μ M LMB for 1 h before being submitted to cytoplasmic FLIP. The corresponding loss of nuclear fluorescence was monitored, measured, and plotted on a graph. (F) PABP1(296-317) can mediate export in a polykaryon fusion assay. MCF-7 cells (donors) were fused with NIH 3T3 cells (acceptors) using PEG, and the transfer of nuclear fluorescence from donor to acceptor cells was monitored. Donor and acceptor cells were differentiated by a cell-specific Hoechst staining pattern. White arrows indicate donor cells, and yellow arrows indicate acceptor cells. Bar, 10 μ m. (G) Full-length cyclin C can export the PK-GFP-NLS reporter in a transcription-dependent manner. Cells were treated with 2 μ M ActD for 1 h or untreated, and the loss of nuclear fluorescence after cytoplasmic FLIP was plotted on a graph. (H) Residues 158 to 179 of cyclin C encode a transcription-dependent nuclear export motif. The indicated constructs were transiently expressed in MCF-7 cells and treated the same as for panel G. Cells were subjected to cytoplasmic FLIP, and the corresponding kinetics for loss of nuclear fluorescence were plotted on a graph.

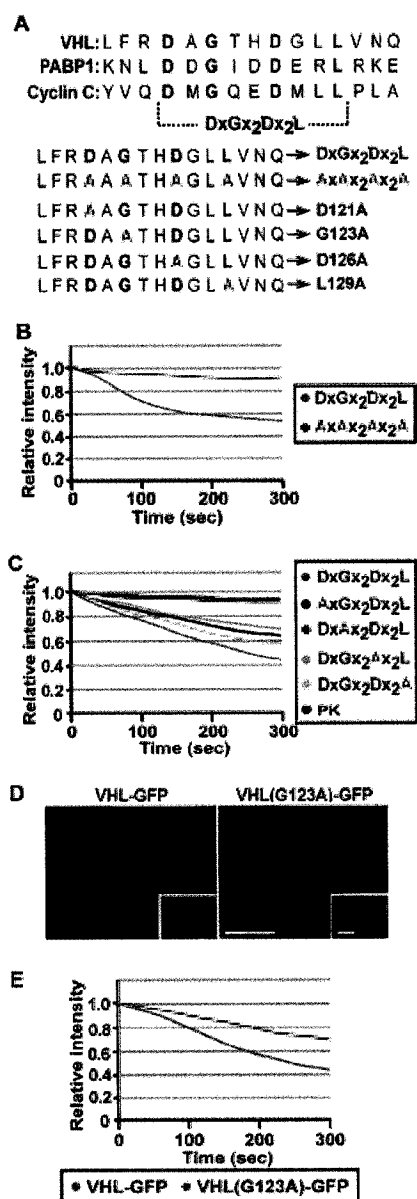


FIG. 6. Identification of key residues that mediate transcription-dependent nuclear export. (A) Sequence alignment depicting conserved residues (blue) in the transcription-dependent nuclear export motifs of VHL, PABP1, and cyclin C. Residues in red indicate alanine substitutions at key residues in the TD-NEM sequence of VHL. (B) Conserved residues in the DXGX₂DX₂L consensus sequence are essential for nuclear export. MCF-7 cells were transiently transfected with VHL(115-130)-PK-GFP-NLS (DxGx₂Dx₂L) or VHL(115-130AAAA)-PK-GFP-NLS (AxAx₂Ax₂A), where the four key residues were replaced with alanines. Cytoplasmic FLIP was performed, and the loss of nuclear fluorescence was plotted on a graph. (C) Single alanine substitutions of key residues within the DXGX₂DX₂L consensus sequence have a differential effect on nuclear export activity. MCF-7 cells transiently expressing the indicated PK-GFP-NLS-tagged constructs were subjected to cytoplasmic FLIP. The graph represents the loss of nuclear fluorescence. (D and E) A single amino acid substitution of G123A in full-length VHL affects steady-state localization and nuclear export activity. Steady-state localization of VHL(G123A)-GFP expressed in MCF-7 cells compared to wild-type VHL-GFP is shown (D). Insets are the corresponding Hoechst staining of the cells. Cells expressing VHL-GFP or VHL(G123A)-GFP were subjected to cytoplasmic FLIP, and the loss of nuclear fluorescence was monitored and graphed (E).

acids may encode transcription-dependent nuclear export activity. Replacement of the four key residues with alanines abolished nuclear export activity of residues 114 to 138 from VHL (Fig. 6A and B; see also Fig. S5 in the supplemental material). Replacement of the aspartic acid, leucine, or glycine residues with alanine reduced nuclear export activity by approximately 50 to 70% compared to the basal sequence (Fig. 6A and C; see also Fig. S5 in the supplemental material). In addition, replacing glycine 123 with alanine in full-length VHL, tagged to GFP and not PK-GFP-NLS, shifted the steady-state localization towards a more predominantly nuclear localization (Fig. 6D) and reduced its ability to export from the nucleus (Fig. 6E), further demonstrating that DxGx₂Dx₂L is a nuclear export motif. These data demonstrate that all four conserved residues in TD-NEM play a central role in mediating nuclear export.

Cancer-causing mutations within TD-NEM abrogate nuclear export of VHL and oxygen-dependent degradation of HIF α . To study the functional consequence of TD-NEM mutations in the context of a full-length VHL protein, we searched for naturally occurring point mutations within the key TD-NEM residues that are associated with VHL disease. Replacement of the first aspartic acid residue of the consensus, DXGX₂DX₂L, with glycine (D121G) is a germ line mutation associated with type 2B VHL disease, characterized by a high risk of RCC (Fig. 7A) (15, 52, 61). This mutant is of particular interest, since previous studies have reported that it maintains its ability to form an E3 ubiquitin ligase complex and to bind and ubiquitylate HIF α in vitro (19). In addition, the second aspartic acid residue of the VHL TD-NEM has been reported to be replaced with tyrosine (D126Y) in individuals afflicted with polycythemia (Fig. 7A) (48, 49). Stable cell lines of D121G-GFP, D126Y-GFP, and G123A-GFP (not PK-GFP-NLS) were generated in VHL-defective 786-0 RCC to further study the effect of these point mutants on the ability of VHL to be exported from the nucleus and mediate oxygen-dependent degradation of HIF α . As previously reported, immunoprecipitation analysis revealed that the VHL mutant D121G is able to bind as efficiently to HIF2 α as wild-type VHL (Fig. 7B). Likewise, VHL D126Y was also able to efficiently bind HIF2 α (Fig. 7B). Cytoplasmic FLIP experiments revealed a markedly decreased rate of nuclear export of D121G and D126Y, of approximately 40% and 20%, respectively, compared to wild-type VHL (Fig. 7C). The function of VHL in oxygen-dependent degradation of HIF α has been linked to its ability to export from the nucleus (17). Thus, we decided to examine the effect of nuclear export-defective VHL mutants on oxygen-dependent degradation of HIF α . Cells were exposed to hypoxia to promote HIF α accumulation, followed by reoxygenation (Fig. 7D). VHL-defective RCC 786-0 cells did not display a decrease in HIF2 α levels (786-0 cells express HIF2 α but not HIF1 α) following reoxygenation of hypoxic cells, as expected, since these cells do not express wild-type VHL (Fig. 7D). Reintroduction of wild-type VHL caused rapid degradation of HIF2 α upon reoxygenation of hypoxic cells (Fig. 7D). In contrast, both the D121G and D126Y mutants were not as efficient in degrading HIF2 α in reoxygenated cells (Fig. 7D). Mutants D121G and D126Y were eventually capable of degrading HIF2 α after long periods of reoxygenation, consistent with their ability to assemble with HIF α and mediate ubiquitylation (Fig. 7E). These data support the hypothesis that VHL mu-

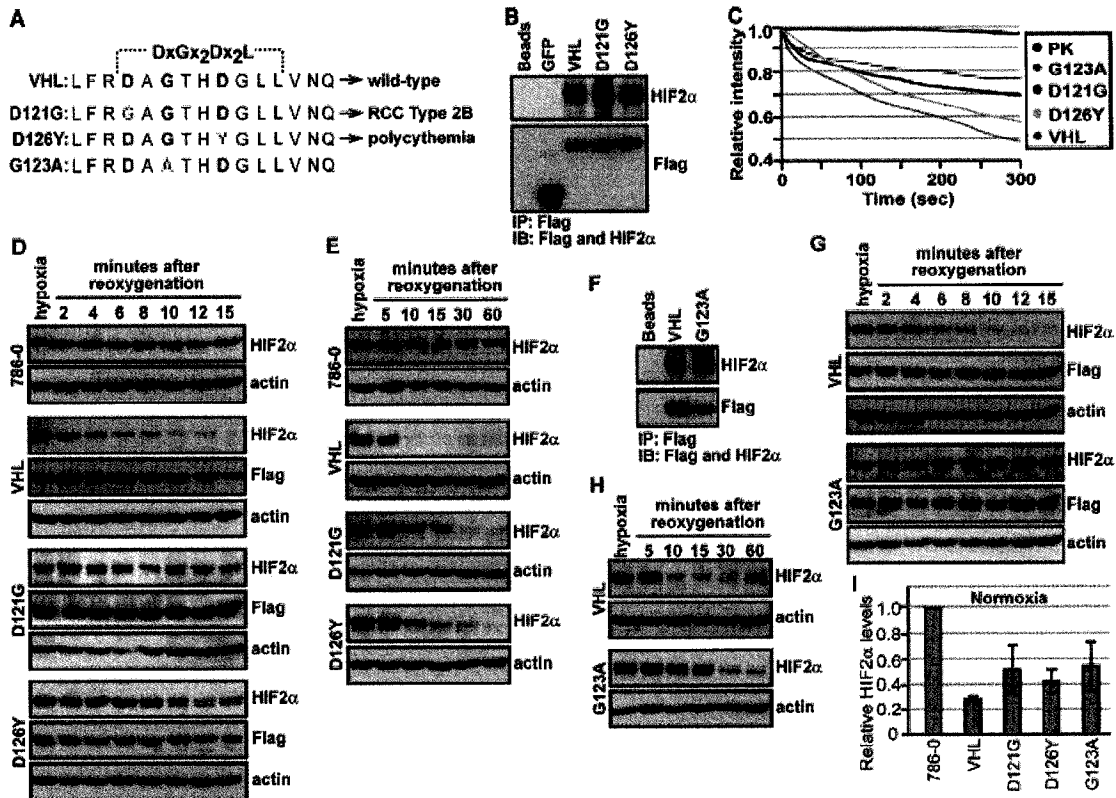


FIG. 7. Cancer-causing mutations within TD-NEM of VHL abrogate nuclear export and oxygen-dependent degradation of HIF2 α . (A) Sequence alignment depicting cancer-causing mutations (red) in the key TD-NEM residues of VHL that lead to RCC type 2B (D121G) and polycythemia (D126Y) in humans. G123A, which exhibits a defect in nuclear export as shown in Fig. 6, was also used to test its ability to mediate HIF degradation. (B) D121G and D126Y retain the ability to bind to HIF. Cells stably expressing Flag-tagged VHL-GFP, D121G-GFP, D126Y-GFP, and GFP were placed under hypoxic conditions and treated with 10 μ M MG132 for 2 h before being harvested. Cell lysates were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag and anti-HIF2 α antibodies. (C) Cancer-causing mutants D121G and D126Y in TD-NEM decrease the nuclear export activity of VHL. Cells stably expressing VHL-GFP, D121G-GFP, D126Y-GFP, or G123A-GFP or transiently expressing PK-GFP-NLS were submitted to cytoplasmic FLIP as described previously. The loss of nuclear fluorescence was monitored and plotted on a graph. (D and E) Cells expressing D121G or D126Y exhibit a deficiency in HIF degradation. Stable cell lines of VHL-GFP, D121G-GFP, D126Y-GFP, and the VHL-defective cell line 786-0 were incubated for 20 h under hypoxic conditions before being reoxygenated by placing them in a normoxic environment, for the indicated time. Cells were lysed with 4% SDS and submitted to Western blot analysis using an anti-HIF2 α antibody. Levels of VHL or its mutant counterparts were monitored using anti-Flag antibody, and actin was used to ensure equal loading of lysates. (F) G123A retains the ability to bind to HIF. Cells stably expressing the VHL point mutant, G123A, were treated the same as described for panel B. (G and H) Cells expressing G123A exhibit a deficiency in HIF degradation. G123A stably expressing cells were treated in the same manner as described for panels D and E. (I) D121G, D126Y, and G123A stable cells express higher normoxic HIF levels compared to wild-type VHL. Stable cell lines incubated under normoxic conditions were lysed with 4% SDS and submitted to Western blot analysis using anti-HIF2 α and antiactin antibodies. HIF2 α levels were normalized to actin and values, calculated relative to HIF2 α levels in 786-0 cells, were plotted on a graph.

tants that are defective in nuclear export display a reduced ability to degrade HIF α even though they are able to bind to and ubiquitylate HIF α .

In light of these results, we were interested in testing if the nuclear export-defective G123A, although not a known disease-causing mutation, would also exhibit a decreased efficiency in degrading HIF α (Fig. 7A). Stably expressed G123A maintained its ability to interact with HIF2 α (Fig. 7F) but displayed a reduced nuclear export activity (Fig. 7C). The G123A mutant displayed a striking defect in degradation of HIF2 α upon reoxygenation of hypoxic cells (Fig. 7G and H). We noticed that 786-0 cells expressing the nuclear export-defective mutants D121G, D126Y, and G123A generally displayed higher levels of HIF2 α than wild-type VHL, but levels were still lower than with the VHL-defective 786-0 cells (Fig.

7I). This may be explained by the fact that the nuclear export-defective mutants are still able to bind and ubiquitylate HIF α and partially able to be exported from the nucleus. Nonetheless, the data shown in Fig. 7 suggest that nuclear export of VHL is required for efficient oxygen-dependent degradation of HIF α .

TD-NEM mediates efficient transcription-dependent nuclear export of proteins. Efficient nuclear export of proteins is required for cellular homeostasis and survival. The classical NES/CRM1 pathway was the first general and discreet motif identified which mediates nuclear export of a wide array of different proteins (9, 60). The herein-described TD-NEM is a discreet motif that is sensitive to drugs that inhibit RNA Pol II activity but operates independently of the classical NES pathway, since it is insensitive to LMB, as demonstrated in our live

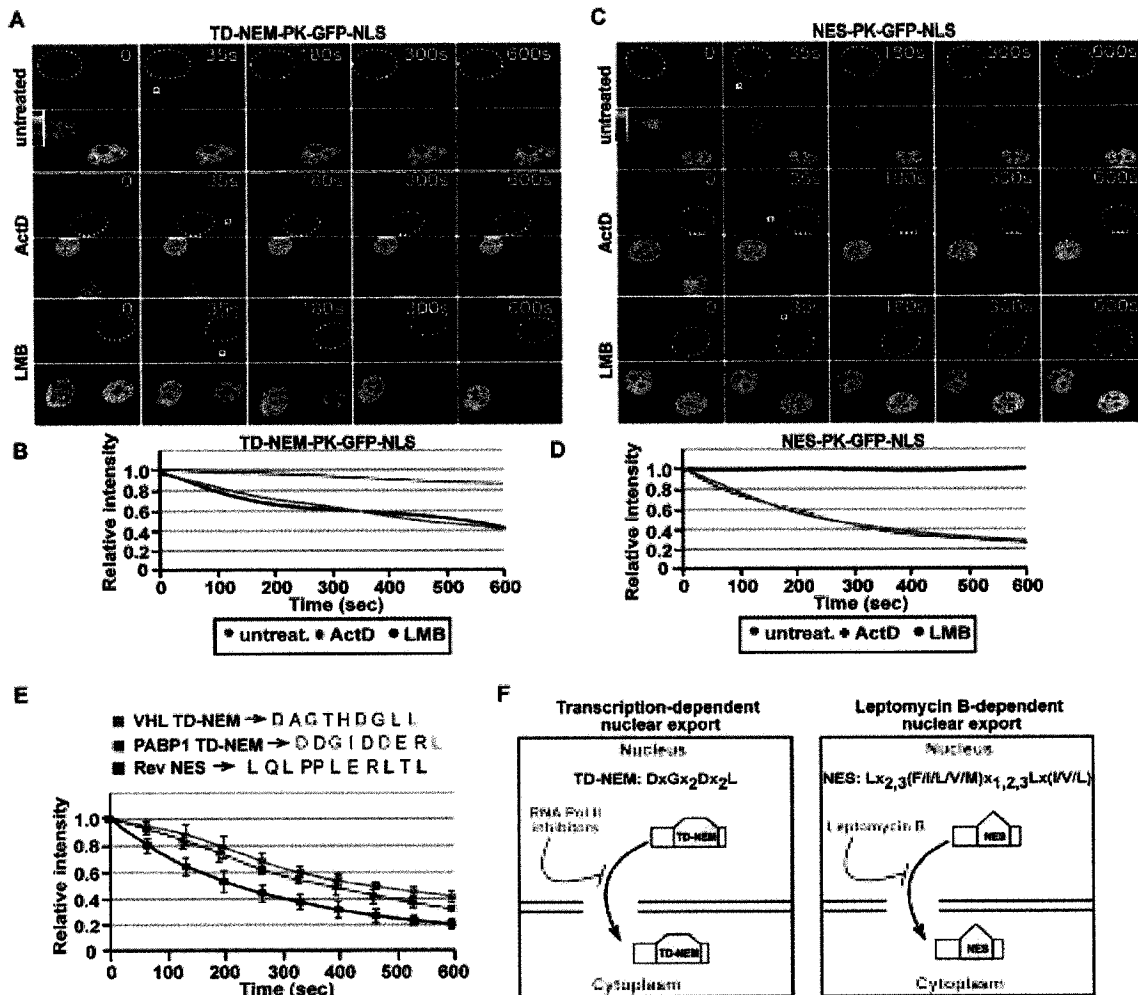


FIG. 8. TD-NEM is a novel and efficient transcription-dependent nuclear export motif. (A to D) TD-NEM, contrary to the classical NES, mediates nuclear export in an ActD-sensitive but LMB-insensitive manner. Cells transiently expressing PK-GFP-NLS-tagged TD-NEM of VHL or NES of the Rev protein were either untreated or treated with ActD or LMB as previously described before being submitted to cytoplasmic FLIP. White squares indicate the bleached area, and the dotted circles outline the nucleus of the cell of interest. The loss of nuclear fluorescence was monitored, calculated, and plotted on a graph (B and D). (E) TD-NEM is an efficient nuclear export motif. Cells transiently expressing PK-GFP-NLS-tagged TD-NEM of VHL or PABP1, or the Rev NES, one of the strongest export signals, were submitted to cytoplasmic FLIP in order to compare the efficiency of nuclear export. (F) Model of nuclear export mediated by the TD-NEM consensus sequence compared to the classical NES (3). Nuclear export of TD-NEM is abrogated by RNA Pol II inhibitors, such as ActD and DRB, whereas NES activity is abrogated by LMB.

cell nuclear export assay (Fig. 8A and B). This is in contrast to what is observed with the classical NES, whose activity is inhibited by LMB but not by RNA Pol II inhibitors (Fig. 8C and D). VHL and PABP1 TD-NEM display nuclear export activities nearly as efficient as the Rev NES, which is thought to be the strongest NES yet characterized (Fig. 8E) (21). Based on these results, we suggest that TD-NEM is an efficient nuclear export motif.

DISCUSSION

We report the identification of a novel and discreet motif, DXGX₂DX₂L, referred to as TD-NEM, which mediates efficient transcription-dependent nuclear export of VHL. This motif was also common among other proteins, including

PABP1, where it mediated its respective transcription-dependent export from the nucleus. Nuclear export by TD-NEM requires ongoing RNA Pol II-mediated transcription and operates independently of the classical CRM1/NES-mediated export pathway (Fig. 8F). Key residues within TD-NEM of VHL are targeted by naturally occurring point mutations associated with renal carcinoma and polycythemia in humans. These disease-causing mutations, which alter the dynamic profile of VHL, restrain oxygen-dependent degradation of HIF α without affecting interaction between substrate and E3 ubiquitin ligase. Our results highlight the essential role of nuclear-cytoplasmic dynamics in protein function and provide evidence that mutations targeting subcellular trafficking can lead to disease.

It has become increasingly evident that degradation of nuclear proteins by the ubiquitylation pathway requires nuclear-

cytoplasmic trafficking of the E3 ubiquitin-ligase as well as the substrate protein. Efficient degradation of nuclear proteins, such as p53, Smad3, and HIF α , is tightly linked to the ability of the E3 ligase to engage in nuclear export (13, 17, 34, 37). Thus, VHL, the ubiquitylation component of an E3 ubiquitin ligase complex that mediates oxygen-dependent degradation of HIF α , was an ideal subject for studying the functional role of nuclear-cytoplasmic trafficking of E3 ligases. We have previously shown that nuclear-cytoplasmic trafficking of VHL requires ongoing RNA Pol II-mediated transcription (17, 34). The initial goal of this study was to identify the sequence that mediates transcription-dependent nuclear export of VHL. We stumbled upon a sequence that may potentially mediate nuclear export of several proteins, including the mRNA nuclear export factor PABP1. VHL and PABP1 share the distinct ability to engage in constitutive and highly dynamic nuclear-cytoplasmic trafficking, utilizing a pathway that requires ongoing RNA Pol II activity. We have identified a new functional domain, TD-NEM, which is present in both VHL and PABP1 and mediates their transcription-dependent export from the nucleus. The rate of nuclear export of TD-NEM is approximately 70 to 80% of that observed for the Rev NES, perhaps the strongest LMB-sensitive NES so far identified, suggesting that this motif is highly efficient in mediating nuclear egression of molecules. Several lines of evidence support a role for TD-NEM as a nuclear export motif. First, removal of TD-NEM from full-length VHL and PABP1, or a single amino acid substitution of glycine to alanine, altered the steady-state distribution from mostly cytoplasmic to nuclear, accompanied by a reduction in sensitivity to ActD or DRB treatment and the rate of nuclear export. Also, TD-NEM alone from VHL and PABP1 was sufficient to confer transcription-dependent nuclear export properties to a large reporter protein (i.e., PK-GFP) in multiple living cells and in vitro assays. Based on these data, we suggest that TD-NEM represents a new, and possibly ubiquitous, nuclear export motif that operates independently of the known CRM1-NES pathway.

In this study we have shown that transcription-dependent nuclear export of VHL and PABP1 is mediated by a simple and linear sequence, DXGX₂DX₂L. Each of the four conserved residues of TD-NEM was found to be required for full activity of the export signal. It is not surprising that a mere four residues can act as a transport signal, considering that the classical NES, among others, relies on a consensus sequence of only a few conserved residues (9, 60). It would be of interest to examine the flexibility of this sequence by testing if naturally occurring conservative permutations of its key residues, such as aspartic acid to glutamic acid or leucine to isoleucine, retain activity. There were no other apparent conserved residues between the core TD-NEM of VHL, PABP1, and the putative TD-NEM of cyclin C; however, these three independent TD-NEMs displayed slightly different nuclear export activities, suggesting a functional role of the nonconserved residues in modulating the activity of TD-NEM. These observations are similar to the classical NES, which can display different export activities dependent on the surrounding amino acid context (32). Whether other substitutions or subtle differences of this TD-NEM, such as different spacing between the key residues, retain activity also remains to be tested. If functional combina-

tions are found, this would simply provide additional evidence to support a ubiquitous nature of TD-NEM.

Discovery of LMB as an inhibitor of NES-mediated nuclear export has aided in both deciphering the components of this pathway and identifying many proteins that are exported from the nucleus (10, 14). Similarly, our use of transcriptional inhibitors, such as ActD and DRB, has led to the discovery of a nuclear export pathway that relies on the TD-NEM export signal. We envision that the discovery of TD-NEM through inhibitors of RNA Pol II-dependent transcription will help uncover another class of proteins that undergo nuclear export and the mechanism by which this occurs. It is still unclear as to how these drugs inhibit nuclear export of TD-NEM-containing proteins, but it is most likely through direct or indirect effects on either primary or secondary components of this pathway. It is well known that LMB directly interacts with and inhibits the action of CRM1, the exporter of NES-containing proteins (31). It will be interesting to see if ActD and DRB have a direct effect on a possible TD-NEM exporter or if they cause a secondary effect through transcriptional inhibition.

We have previously shown that perturbing the nuclear-cytoplasmic trafficking profile of VHL is detrimental to its ability to mediate oxygen-dependent degradation of HIF α . Here we report that naturally occurring TD-NEM mutations D121G and D126Y, which lead to RCC and polycythemia, respectively, abrogate nuclear export of VHL. It has been puzzling as to how VHL cancer-causing mutations, such as D121G, retain the ability to bind and ubiquitylate HIF α in vitro yet are able to develop classical tumors associated with VHL disease (19). We showed that D121G and D126Y maintain the ability to bind to HIF2 α , consistent with previously published data. Expression of these mutants leads to an extended HIF2 α stability following reoxygenation of hypoxic cells, providing a correlation between the efficiency to mediate degradation of HIF α and nuclear export activity. We cannot exclude the possibility that other aspects of the ubiquitylation pathway may be affected in vivo; however, we did predict a defect in HIF2 α degradation with G123A, a mutant that is restrained in its ability to export from the nucleus but retains its ability to bind to HIF2 α . The G123A mutant further supports that the TD-NEM of the β -domain of VHL is not involved in HIF α binding or E3 ubiquitin ligase complex formation but plays an essential role in HIF α degradation by mediating nuclear export. We achieved low-expressing stable cell lines of the nuclear export-defective mutants, though expression was still higher than endogenous VHL. This raises the possibility that the nuclear export mutants may be partially rescued by overproduction and may have more pronounced defects in physiological settings. The differences in HIF α stability observed with the nuclear export-defective mutants may translate into different types of VHL disease, as observed with type 2A and type 2B mutants with respect to HIF α binding (36). Whether the prolonged presence and activity of HIF α provide an explanation for how patients with these mutations develop tumors remains to be tested. Nonetheless, our data illustrate that the dynamic profile of a protein is instrumental for functional integrity and provide evidence that mutations targeting subcellular trafficking can abrogate protein function.

In conclusion, we propose that TD-NEM is a novel nuclear export motif which utilizes a pathway that requires RNA Pol

II-mediated transcription. There is emerging evidence that a large class of proteins is able to export by utilizing an NES-independent pathway. It would be interesting to test whether these proteins encode a TD-NEM and if they engage in transcription-dependent nuclear export. Future work aimed at elucidating the mechanism by which RNA Pol II inhibitors abolish the nuclear export activity of TD-NEM should yield interesting information as to the process involved in TD-NEM-mediated nuclear export and perhaps uncover another general nuclear export pathway.

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eEF1A Is a Novel Component of the Mammalian Nuclear Protein Export Machinery

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The cytoplasmic translation factor eEF1A has been implicated in the nuclear export of tRNA species in lower eukaryotes. Here we demonstrate that eEF1A plays a central role in nuclear export of proteins in mammalian cells. TD-NEM (transcription-dependent nuclear export motif), a newly characterized nuclear export signal, mediates efficient nuclear export of several proteins including the von Hippel-Lindau (VHL) tumor suppressor and the poly(A)-binding protein (PABP1) in a manner that is dependent on ongoing RNA polymerase II (RNA PolII)-dependent transcription. eEF1A interacts specifically with TD-NEM of VHL and PABP1 and disrupting this interaction, by point mutations of key TD-NEM residues or treatment with actinomycin D, an inhibitor of RNA PolII-dependent transcription, prevents assembly and nuclear export. siRNA-induced knockdown or antibody-mediated depletion of eEF1A prevents *in vivo* and *in vitro* nuclear export of TD-NEM-containing proteins. Nuclear retention experiments and inhibition of the Exportin-5 pathway suggest that eEF1A stimulates nuclear export of proteins from the cytoplasmic side of the nuclear envelope, without entering the nucleus. Together, these data identify a role for eEF1A, a cytoplasmic mediator of tRNA export in yeast, in the nuclear export of proteins in mammalian cells. These results also provide a link between the translational apparatus and subcellular trafficking machinery demonstrating that these two central pathways in basic metabolism can act cooperatively.

INTRODUCTION

In eukaryotes, the formation of the nuclear envelope resulted in the segregation of the cell into two distinct cellular compartments, the nucleus and the cytoplasm. This division necessitated the concomitant evolution of regulated nuclear-cytoplasmic transport pathways in order to maintain rapid and specific communication between these cellular compartments. The regulated and timely bidirectional trafficking of RNA and protein cargoes into and out of the nucleus is a prerequisite for basic biological processes. Disruption of such nuclear-cytoplasmic transport pathways results in deregulation of cellular processes and may lead to various

diseases (Smith and Koopman, 2004; Terry *et al.*, 2007; Truant *et al.*, 2007).

Nuclear-cytoplasmic transport of all molecules, such as proteins and RNA species, across the nuclear envelope occurs through channels formed by macromolecular structures known as nuclear pore complexes (NPCs; Wentz, 2000; Rout and Aitchison, 2001). Although differences exist between protein and RNA transport, the same fundamental sequence of events are conserved; essentially, cargoes bind to soluble transporters in the donor compartment, are transported through NPCs, and are released in the target compartment. A general theme in the export of proteins from the nucleus to the cytoplasm is that specialized export receptors (exportins) of the β -karyopherin family of transporters recognize cargoes harboring specific export signals, such as the recognition of the classical leucine-rich nuclear export signal (NES) by the CRM1 exportin (Fischer *et al.*, 1995; Wen *et al.*, 1995; Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Stade *et al.*, 1997; Gorlich and Kutay, 1999). Exportins form complexes with substrates in the nucleus with the aid of a small GTPase, Ran (Moore and Blobel, 1993; Melchior and Gerace, 1998; Moore, 1998). The loading and release of substrates with exportins is dependent on a concentration gradient of RanGTP across the nuclear envelope (Izaurralde *et al.*, 1997; Richards *et al.*, 1997). Exportins preferentially bind their substrate at high nuclear RanGTP levels and exit the nucleus as exportin-cargo-RanGTP complexes (Richards *et al.*, 1997; Macara, 2001). Substrates are then released in the cytoplasm upon hydrolysis of RanGTP to RanGDP and exportins return to the nucleus for another round of export.

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Abbreviations used: ActD, actinomycin D; CRM1, Chromosome Region Maintenance (exportin 1); Dbp5, the DEAD box RNA helicase Rat8p/Dbp5p; eEF1A, eukaryotic translation elongation factor 1 α ; FLIP, fluorescence loss in photobleaching; LMB, leptomycin B; NES, nuclear export sequence; NLS, nuclear localization signal; NoDS^{H+}, nucleolar detention signal regulated by [H⁺]; PABP1, poly(A)-binding protein 1; PK, pyruvate kinase; TD-NEM, transcription-dependent nuclear export motif; VHL, von Hippel-Lindau.

In recent years it has become apparent that several proteins, unrelated to exportins, play important roles in the nuclear export of proteins and RNAs by exerting their effects from the cytoplasmic side of the nuclear envelope. Current advances in understanding RNA export have revealed a crucial role for several cytoplasmic proteins in the export of mRNA. The DEAD-box protein Dbp5, an ATPase that binds to the cytoplasmic filaments of the NPC, was found to be essential for mRNA export and was postulated to act as a molecular motor to pull mRNAs through the nuclear pore (Tseng *et al.*, 1998; Hodge *et al.*, 1999; Schmitt *et al.*, 1999; Weirich *et al.*, 2004; Cole and Scarcelli, 2006). Mutations of the DBP5 gene or altering the interaction between Dbp5 and Nup159, a nucleoporin of the cytoplasmic filament of the NPC, lead to a dramatic and rapid block of mRNA export (Snay-Hodge *et al.*, 1998; Tseng *et al.*, 1998; Hodge *et al.*, 1999; Schmitt *et al.*, 1999). In addition, the ATPase activity of Dbp5 is stimulated by Gle1, an essential mRNA export factor that is also primarily located at the cytoplasmic face of the NPC (Alcazar-Roman *et al.*, 2006; Weirich *et al.*, 2006). Gle1 mutant strains have been previously shown to exhibit strong defects in mRNA export. In accordance with these observations it has been recently shown that a mutant variant of Gle1 that fails to interact with cytoplasmic Dbp5 considerably reduced the ability of mRNAs to export from the nucleus (Alcazar-Roman *et al.*, 2006; Weirich *et al.*, 2006). Also, the GTPase-activating protein RanGAP1 and its stimulatory factor RanBP1, which are crucial for the hydrolysis of RanGTP to RanGDP, are exclusively cytoplasmic proteins (Coutavas *et al.*, 1993; Bischoff *et al.*, 1994; Bischoff *et al.*, 1995a,b; Matunis *et al.*, 1996; Richards *et al.*, 1996; Mahajan *et al.*, 1997). Mislocalization of either RanGAP1 or RanBP1 to the nuclear compartment through microinjection experiments inhibited major nuclear export of proteins and certain RNA species (Izaurrealde *et al.*, 1997).

In yeast, the involvement of cytoplasmic factors has also been demonstrated for nuclear export of tRNA. Both the translation elongation factor eEF1A and the newly characterized protein Cex1p have been described as cytoplasmic components of the nuclear aminoacylation-dependent tRNA export pathway (Grosshans *et al.*, 2000a,b; Kohler and Hurt, 2007; McGuire and Mangroo, 2007; Hopper and Shaheen, 2008). eEF1A and Cex1p were found to copurify with one another and to interact directly with aminoacylated tRNA (Grosshans *et al.*, 2000a; McGuire and Mangroo, 2007). In a yeast genetic system, strains with reduced levels of, or mutated, eEF1A exhibited strong accumulation of mature tRNAs in the nuclear compartment (Grosshans *et al.*, 2000a; McGuire and Mangroo, 2007). Depletion of Cex1p also reduced the efficiency of nuclear tRNA export (McGuire and Mangroo, 2007). However, cells with reduced levels of both eEF1A and Cex1p showed a significantly increased level of nuclear tRNA retention. These observations support the idea that eEF1A and Cex1p function in the same pathway, as cytoplasmic export factors involved in tRNA export. In fact, because Cex1p interacts with the NPC it was postulated that Cex1p accepts aminoacyl-tRNAs from the nucleus at the cytoplasmic side of the NPC and delivers them to eEF1A through a channeling mechanism (McGuire and Mangroo, 2007). Altogether, these examples provide evidence that proteins residing in the cytoplasm orchestrate events on the cytoplasmic side of the nuclear envelope that are equally as important as those occurring in the nucleus during nuclear export of proteins and RNA species.

We have recently identified a novel nuclear export sequence, TD-NEM (transcription-dependent nuclear export motif), that mediates efficient nuclear export of proteins such

as the von Hippel-Lindau (VHL) tumor suppressor and the poly(A)-binding protein PABP1 (Khacho *et al.*, 2008). Nuclear export through TD-NEM, which is encoded by the consensus sequence DxGx₂Dx₂L, requires ongoing RNA Polymerase II (RNA PolII)-mediated transcription and operates independently of the classical CRM1/NES-mediated nuclear export pathway (Khacho *et al.*, 2008). Here we report the identification of the translation elongation factor eEF1A as a cytoplasmic factor involved in TD-NEM-mediated nuclear export. These results suggest that eEF1A, a mediator of tRNA export in yeast, is also involved in the nuclear export of proteins in mammalian cells. These findings further argue that factors limited to the cytoplasmic compartment can be essential mediators of the nuclear export pathway.

MATERIALS AND METHODS

Cell Culture, Transfections, Drug Treatments, and Hypoxia Treatment

786-0 (VHL-negative) renal carcinoma cells, MCF-7 cells and A549 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P-S) in a 37°C and 5% CO₂ environment. Transient transfections in MCF-7 and A549 cells were conducted with Effective transfection reagent (QIAGEN, Chatsworth, CA). Transfected cells were incubated for 24 h before any manipulations or drug treatment. Where indicated cells were treated at 37°C with a final concentration of 2 μ M actinomycin D (ActD) or 10 μ M leptomycin B (LMB) for 1 h before photobleaching experiments and 8 μ M ActD, 25 μ g/ml DRB (5,6-dichloro-1- β -D-ribofuranosylbenzimidazole), or 10 μ M LMB for 3 h before live cell fluorescence imaging or harvesting for immunoprecipitation. Where indicated, cells were treated with 100 μ g/ml cycloheximide for 2 h. For SD or AP conditions a buffer-free medium (DME; Invitrogen, Carlsbad, CA) was freshly prepared and supplemented with 5% (vol/vol) FBS and 1% P-S. NaHCO₃, 44 mM, was added and the pH was adjusted to 7.2 (SD) or 6.6 (AP) with HCL. Air was bubbled into both types of media to stabilize the pH at 7.2. In hypoxia the SD media remained stable and the AP media reverted slowly to a pH of 6.6.

Plasmids and Adenoviruses

VHL and deletion mutants were cloned into pcDNA3.1 between an NH₂-terminal Flag-tag and a COOH-terminal green fluorescent protein (GFP) tag, as previously described (Lee *et al.*, 1999; Bonicalzi *et al.*, 2001; Groulx and Lee, 2002). F-VHL-GFP-NLS and F-GFP were previously described in Groulx *et al.* (2000) and Lee *et al.* (1999). Human full-length VHL and deletion mutants, the strong nuclear export sequence (NES) from the human immunodeficiency virus REV were inserted into a F-PK-GFP-NLS construct that was previously described in Khacho *et al.* (2008) and Groulx *et al.* (2000). cDNAs corresponding to VHL(114–138) and PABP1(296–317) that encode TD-NEM sequences were inserted into F-GFP or F-PK-GFP-NLS. For F-eEF1A-GFP-NLS the human eEF1A cDNA was inserted between Flag and GFP-NLS using ApaI and XhoI restriction sites. The human PABP1 was fused to GFP-F to produce the GFP-F-PABP1 fusion protein. F-VHL-GFP, F- Δ C157-GFP, and F-GFP adenoviruses were produced using the Cre-lox recombination system (Lee *et al.*, 1999; Groulx and Lee, 2002). PK-GFP-NoDS^{HT} and PK-GFP-NLS-NoDS^{HT} were previously described in Mekhail *et al.* (2007).

Small Interfering RNA

For small interfering RNA (siRNA), experiments cell were transfected with 100 nM of either of eEF1A siRNA (2991, 2804; Ambion, Austin, TX), Exp5-1 siRNA (Lund *et al.*, 2004), eEF2 siRNA (10791; Ambion), control siRNA (Ambion), or Effectene alone (mock) for 48 h before photobleaching experiments or 72 h before. Where indicated siRNA-transfected cells were treated with 8 μ M ActD for 3 h after a 72-h incubation period with siRNA and before live cell fluorescence imaging. For photobleaching experiments, siRNA-transfected cells were treated with 2 μ M ActD for 1 h after a 48-h incubation period with siRNA and before FLIP (fluorescent loss in photobleaching) analysis.

Live Cell Fluorescence Imaging

Images of living cells transiently expressing GFP from experiments where photobleaching was not utilized were imaged with an Axiovert S100TV microscope (Carl Zeiss MicroImaging, Thornwood, NY) equipped with a 40 $\times$ /1.2 C-Apochromat water immersion objective using a digital charged-coupled device camera (Empix Imaging, Mississauga, Ontario, Canada). Cell nuclei were stained with Hoechst 33342 (Sigma, St. Louis, MO). Images were captured using the Northern Eclipse software package (Empix Imaging).

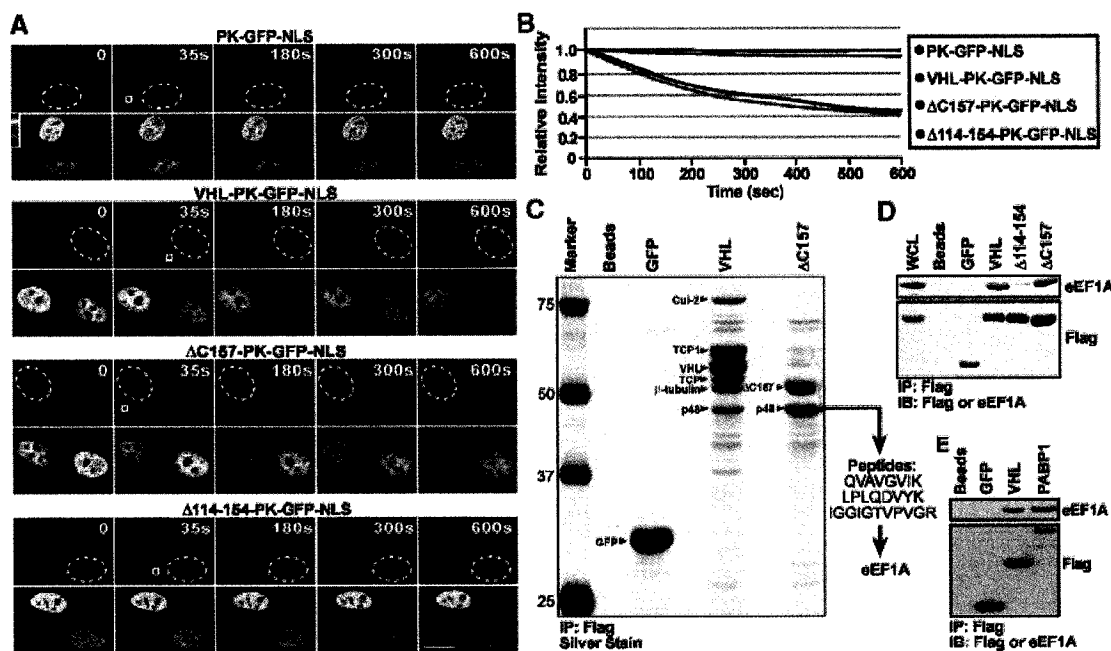


Figure 1. Identification of a novel VHL and PABP1 interacting protein. (A and B) Full-length VHL and the truncation mutant Δ C157 mediate nuclear export of a PK-GFP-NLS reporter protein; however Δ 114–154 is nuclear export deficient. MCF-7 cells transiently expressing VHL, Δ C157 or Δ 114–154 fused to the PK-GFP-NLS reporter protein were subjected to the live cell FLIP nuclear export assay. The PK-GFP-NLS reporter consists of the large and amorphous pyruvate kinase (PK) to ensure that movement across the NPC is not due to diffusion, GFP to provide fluorescence, and a strong nuclear localization signal (NLS) to ensure all fusion proteins exhibit similar rates of nuclear import and to obtain a strong steady-state nuclear localization. In this assay a small region of the cytoplasm (white squares) within specific cells (dashed circles outline cell nuclei) was repeatedly photobleached. Kinetics for the loss of nuclear fluorescence, which indicates nuclear export activity, from images in A were calculated and plotted on a graph (B). (A) Pseudocolored panels are included to better illustrate subtle changes in fluorescence intensity (red represents highest intensity; blue represents lowest intensity). Scale bar, 10 μ m. (C) Identification of a novel VHL-interacting protein. VHL-/- 786-0 cells were infected with Flag-tagged GFP, VHL-GFP, or Δ C157-GFP adenoviruses. Anti-Flag beads were incubated with lysis buffer (Beads lane) or lysates from infected cells. Immunoprecipitates were run on a 10% SDS-PAGE gel and subjected to silver staining. Bands were excised and analyzed by mass spectrometry. VHL associated with known interacting proteins, such as Cullin-2 (Cul-2), the cytosolic chaperonin TCP-1 and β -tubulin. p48 was identified as the eukaryotic elongation factor 1 alpha (eEF1A). Lanes referred to as “Beads” indicate that the Flag-beads were incubated with lysis buffer alone. (D) eEF1A can interact with VHL and Δ C157 but not with Δ 114–154. 786-0 cells infected with adenoviruses to express Flag and GFP-tagged VHL, Δ C157, Δ 114–154, or GFP alone were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. Whole cell lysates (WCL) were obtained from 786-0 cells infected with F-VHL-GFP. (E) eEF1A is also a novel PABP1-interacting protein. MCF-7 cells were transiently transfected with Flag and GFP-tagged VHL, PABP1, or GFP alone. Immunoprecipitation and Western blot analysis was the same as in D.

Photobleaching and Microscopy

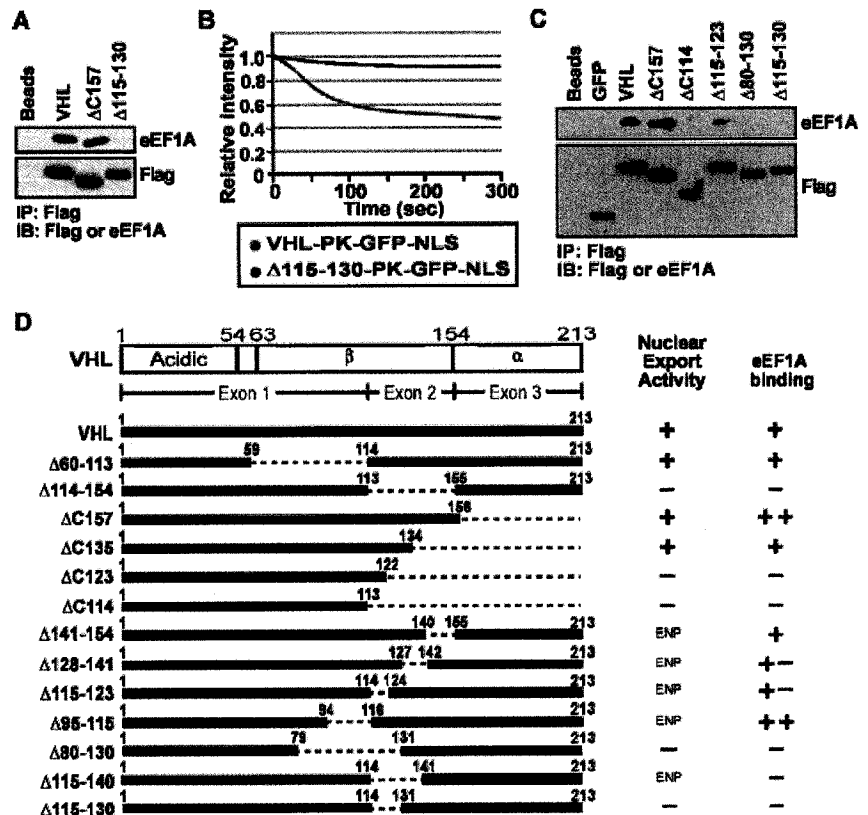
Cells were cultured and transfected directly onto 35-mm dishes with coverslip bottoms (MatTek, Ashland, MA). Photobleaching and live cells microscopy was performed using a confocal microscope (LSM5 Pascal Laser Scanning Microscope, Carl Zeiss, Toronto, Ontario, Canada). In all experiments cells were maintained at 37°C in an environmental chamber. A 63 $\times$ plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to three rapid pulses of a 488-nm argon laser at 100%, and image acquisition was at 1% of full laser power. A highly quantitative live cell nuclear export assay utilizing FLIP (fluorescent loss in photobleaching) technology was used to measure nuclear export activity. For these cytoplasmic FLIP experiments, a fusion protein consisting of the large and amorphous pyruvate kinase (PK), which does not encode localization determinants, GFP, and the nuclear localization signal (NLS; Kalderon *et al.*, 1984) derived from the simian virus large T antigen SV40 (PK-GFP-NLS) was used. Cells expressing a PK-GFP-NLS-tagged fusion protein were repeatedly bleached in a small cytoplasmic region and imaged at 30-s intervals. Small bleached areas for cytoplasmic FLIPs were kept consistent in terms of size and distance from the nucleus. Fluorescence loss in the unbleached areas (nuclei) was quantified as previously described (Phair and Misteli, 2000; Mekhail *et al.*, 2005) using the following equation: $I_{rel} = (I_{(t)}/I_{(0)}) \times (N_{(0)}/N_{(t)})$, where $I_{(t)}$ is the average intensity of the unbleached nucleus or cell at time point t , $I_{(0)}$ is the average prebleached intensity of the nucleus or cell of interest, and $N_{(0)}$ and $N_{(t)}$ are the average nuclear or cellular fluorescence intensity of a neighboring cell in the same field of vision at prebleach or at time t , respectively. This calculation accounts for any losses in fluorescence by normalizing the fluorescence of the cell of interest to that of a neighboring cell of approximate equal size and fluorescent intensity. Pseudocolor images were generated to highlight differences in GFP fluorescence: red represents high fluorescent intensity and light

blue represents low fluorescent intensity. The quantification graphic was generated by a FLIP software. For all bleaching experiments ~10 datasets were analyzed for each result. Pseudocoloring for bleaching experiments was achieved by applying the gradient map function of Photoshop (Adobe, San Jose, CA) to a montage of picture frames prepared with ImageJ software (<http://rsb.info.nih.gov/ij/>; National Institutes of Health, Bethesda, MD). The Northern Eclipse (Empix Imaging), Excel (Microsoft, Redmond, WA), and FreeHand (Macromedia, San Francisco, CA) software packages were also used to capture images, analyze the data, and generate graphs.

Immunoprecipitation, Silver Staining, and Immunoblotting

Cells were lysed in lysis buffer containing 0.5% Igepal CA630, 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, and 1 mM sodium orthovanadate with 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 μ g/ml pepstatin. Cell lysates were incubated with anti-Flag M2 beads (Sigma) overnight while tumbling at 4°C. Beads were washed several times and eluted with Flag peptides (Sigma). For total cell lysates, cells were washed several times in phosphate-buffered saline (PBS), lysed with 4% SDS in PBS, and boiled for 5 min, and the DNA was sheared by passage through a 19-gauge needle. Protein concentration was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Samples were separated on denaturing polyacrylamide gels. Silver-stained gels were performed according to manufacturer's protocol (Bio-Rad Laboratories, Richmond, CA). Western blot gels were transferred onto PVDF membranes and blocked in skimmed milk powder in PBS containing 0.2% Tween 20 (PBST) before incubation with Flag-M2 (Sigma), eEF1A (Santa Cruz Biotechnology, Santa Cruz, CA), actin (Sigma), eEF2 (Cell Signaling Technology, Beverly, MA) or Expotin-5 (a kind gift from

Figure 2. TD-NEM of VHL is required for interaction with eEF1A. (A and B) The transcription-dependent nuclear export sequence of VHL, encoded within residues 115–130, is required to interact with eEF1A and mediate nuclear export. (A) Cellular lysates from cells transiently expressing Flag-tagged VHL-GFP, Δ 157-GFP, or Δ 115–130-GFP were immunoprecipitated with anti-Flag beads and immunoblotted with anti-Flag and anti-eEF1A antibodies. Lanes referred to as “Beads” indicate that the Flag-beads were incubated with lysis buffer alone. (B) MCF-7 cells transiently expressing PK-GFP-NLS-tagged VHL or Δ 115–130 were submitted to the live cell FLIP nuclear export assay. A small cytoplasmic region of the cell was photobleached repetitively and the loss of nuclear fluorescence, which is indicative of nuclear export activity, was monitored over time. The graph represents the relative loss of nuclear fluorescence over time. (C and D) Mapping the eEF1A-binding region within VHL. (C) Cellular lysates from cells transiently expressing the indicated constructs were immunoprecipitated and immunoblotted the same as in A. (D) Schematic diagram indicates deletion and truncation mutants of VHL that were submitted to immunoprecipitation with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. These mutants of VHL were also fused to PK-GFP-NLS and submitted to the live cell FLIP nuclear export assay. (+), (+ –), (– +), and (–) indicate, in a decreasing order, the ability of the fusion proteins to interact with eEF1A or export from the nucleus. ENP, experiment was not performed.



Ian Macara, University of Virginia, Charlottesville, VA) antibodies. Membranes were washed with 0.2% Tween-PBS and blotted with a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Waltham, MA).

Immunofluorescence

Cells were seeded onto coverslips and fixed with prechilled methanol for 10 min at -20°C followed by prechilled acetone for 1 min at -20°C . Anti-PABP1 mAb was used (Upstate Biotechnology, Lake Placid, NY). Cells were incubated for 1 h with a primary antibody solution containing 10% FBS and 1% Triton-X-100 (vol/vol) at room temperature in a humidified chamber. Cells were then washed several times in PBS before a 1-h incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch) at room temperature in a dark humidified chamber. Hoechst stain 33342 (Sigma) was added to visualize nuclei and coverslips were mounted using Fluoromount G (EMS, Hatfield, PA).

Radioisotope Labeling

Cells were plated on 35-mm dishes after which they were transfected with 100 nM eEF1A, eEF2, or scrambled siRNA (mock) for 48 and 72 h. At the indicated times cells were incubated for 30 min in glutamine-, methionine-, and cysteine-free DMEM and then labeled with 10 $\mu\text{Ci}/\text{ml}$ [^{35}S]Met for 30 min. Cells were harvested and lysed for 30 min at 4°C with modified RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, and a cocktail of protease inhibitors. Total cellular lysates were separated on a denaturing polyacrylamide gel. The gel was transferred onto a PVDF membrane, and [^{35}S]Met labeling was visualized by autoradiography. The membrane was washed three times in PBST and blocked in skimmed milk powder in PBST before incubation with eEF1A and eEF2 antibodies.

In Vitro Nuclear Export Assay

The in vitro export assay was performed as described in Groulx *et al.* (2000). Briefly, cells were plated and grown on a 35-mm coverslip plate. Cells were washed with transport buffer (TB) containing 20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $\text{Mg}(\text{OAc})_2$ and permeabilized at 4°C for 5 min

with TB containing 50 $\mu\text{g}/\text{ml}$ digitonin and a protease inhibitor mixture (Hoechst stain 33258; Sigma) was used to monitor the permeabilization). After several washes with TB at 4°C , cells were incubated for 30–45 min at 20°C in the presence of a standard mixture that included MCF-7 cellular lysate, TB, 2 mM ATP, 2 mM GTP, and an ATP-regenerating system (5 mM creatine phosphate and 20 U/ml creatine phosphokinase) to a final volume of 1 ml. To obtain the MCF-7 cellular lysate, first MCF-7 cells were incubated in RSB hypotonic buffer containing: 10 mM HEPES, pH 6.2, 10 mM NaCl and 1.5 mM MgCl_2 , for 15 min at 4°C . Cells were then homogenized in a tight pestle homogenizer. Protein concentrations were quantified using the BCA protocol. For experiments using eEF1A- or eEF2-depleted MCF-7 cellular lysates, cells were homogenized as described above. Lysate containing 0.5 mg of proteins was incubated in the presence of 10 μg of eEF1A antibody or 10 μg of eEF2 antibody and a cocktail of protease inhibitors for 1 h while rotating at 4°C . Undepleted lysates were incubated with 10 μg of irrelevant Flag antibody as a control. This was followed by incubation with 20 μl protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 3 h at 4°C . Cells were then spun to remove beads bound to immunoprecipitated eEF1A or eEF2. This was followed by another round of immunoprecipitation using 10 μg of eEF1A antibody or 10 μg of eEF2 antibody and 20 μl protein A/G PLUS-agarose beads; however the immunoprecipitation was performed overnight. The lysate was spun and an aliquot of the supernatant immunoblotted to verify depletion of eEF1A or eEF2. Actin was used as a control. Lysates that were successfully depleted of eEF1A or eEF2 were used in the export assay in the same manner as described above.

RESULTS

The Translation Elongation Factor eEF1A Is a Novel VHL and PABP1-interacting Protein

The VHL tumor suppressor, a recognition motif of a Cullin-2-containing E3 ubiquitin ligase complex, and PABP1, a general RNA metabolism and translation initiation factor, engage in a nuclear export pathway that is dependent on ongoing RNA PolIII activity and a novel nuclear export

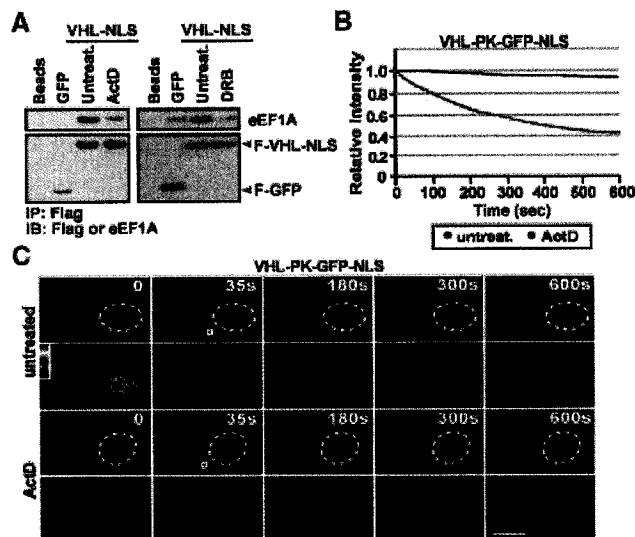


Figure 3. Inhibitors of RNA PolII-dependent transcription block the interaction between VHL and eEF1A. (A) ActD and DRB hinder the interaction between eEF1A and VHL. MCF-7 cells transiently expressing F-GFP or F-VHL-GFP-NLS were untreated or treated with 8 μ M ActD or 25 μ g/ml DRB for 3 h before harvesting cells. Lysates were immunoprecipitated with anti-Flag beads and immunoblotted with Flag or eEF1A antibodies. (B and C) ActD inhibits TD-NEM-mediated nuclear export of VHL. MCF-7 cells transiently expressing VHL-PK-GFP-NLS were either untreated or treated with 2 μ M ActD for 1 h before being submitted to cytoplasmic FLIP analysis. Cells were repeatedly bleached in a small cytoplasmic region (white squares) within cells (dotted circles outline the nuclei of bleached cells). The loss of nuclear fluorescence was monitored (C), calculated, and plotted on a graph (B). Scale bar, 10 μ m.

motif, TD-NEM (Afonina *et al.*, 1998; Lee *et al.*, 1999; Groulx and Lee, 2002; Khacho *et al.*, 2008). As previously reported, addition of RNA PolII inhibitors, such as ActD, inhibits nuclear export of both VHL and PABP1. In contrast, nuclear export of VHL and PABP1 is not affected by leptomycin B (LMB), a compound that abolishes CRM1-dependent nuclear export of NES-containing proteins (Afonina *et al.*, 1998; Kudo *et al.*, 1999; Lee *et al.*, 1999; Khacho *et al.*, 2008). This observation indicates that nuclear export of these proteins is independent of the classical CRM1/NES nuclear export pathway. Because nuclear export of VHL and PABP1 requires ongoing RNA PolII-dependent transcription, we considered the possibility that TD-NEM-mediated nuclear export occurs via an mRNA export pathway. However, inhibiting mRNA export using either the vesicular stomatitis virus (VSV; von Kobbe *et al.*, 2000; Faria *et al.*, 2005) or the influenza virus (Satterly *et al.*, 2007), did not affect nuclear export of VHL (data not shown). Also, we were not able to identify any mRNA or tRNA species associated with VHL (data not shown), suggesting that nuclear export of VHL does not occur through an mRNA or tRNA export pathway. To further investigate the mechanism involved in nuclear export of VHL and PABP1, we produced adenoviruses that express Flag-tagged VHL and Δ C157, a C-terminal truncation mutant of VHL. Δ C157 fails to bind to core E3 ubiquitin ligase components but retains the ability to mediate nuclear export of the PK-GFP-NLS reporter in a FLIP nuclear export assay, which measures whether or not a protein can exit from the nucleus (see *Materials and Methods*), compared with PK-GFP-NLS alone that does not encode any nuclear export signals (Figure 1, A and B), and maintains sensitivity to

ActD (Lee *et al.*, 1999; Bonicalzi *et al.*, 2001; Khacho *et al.*, 2008). Immunoprecipitation of VHL followed by silver staining of the gel revealed several previously identified associated proteins including the core ubiquitin ligase component Cullin-2 (Cul-2) and elongins B and C (Figure 1C and data not shown; Pause *et al.*, 1997; Feldman *et al.*, 1999; Hergovich *et al.*, 2003). In addition, we observed a previously unidentified but highly abundant band that migrated at 48 kDa (Figure 1C). Δ C157 only assembled with the 48-kDa protein but not with the core ubiquitin ligase component Cullin-2 or other associated proteins, as expected (Figure 1C and Ohh *et al.*, 2000; Bonicalzi *et al.*, 2001). The p48 kDa band was the only major protein found associated with Δ C157 even in higher or lower percentage gels. Peptide sequence analysis identified p48 as the eukaryotic elongation factor 1A (eEF1A), a key component of the translational machinery (Andersen *et al.*, 2003).

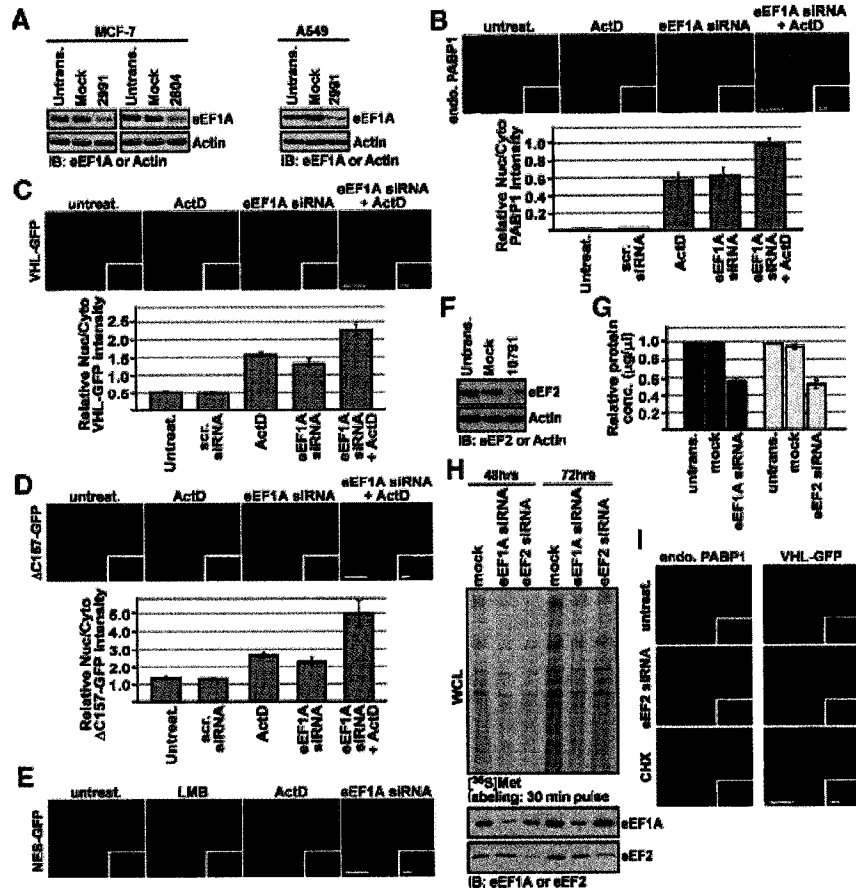
Assembly of VHL and Δ C157 with endogenous eEF1A was confirmed by immunoprecipitation and Western blot analysis using anti-eEF1A antibodies (Figure 1D). Interestingly, endogenous eEF1A was not able to interact with a deletion mutant of VHL, Δ 114–154, that does not encode a TD-NEM and fails to engage in nuclear export (Lee *et al.*, 1999; Bonicalzi *et al.*, 2001; Khacho *et al.*, 2008), suggesting that eEF1A may play a role in nuclear export of VHL. More importantly PABP1 also interacted with endogenous eEF1A (Figure 1E). Because Δ C157 retains transcription-dependent nuclear export activity and only binds to eEF1A, we decided to further characterize this new VHL and PABP1-associated protein for its potential involvement in nuclear export.

We reasoned that if eEF1A is involved in transcription-dependent nuclear export, its interaction with VHL would be dependent on the presence of TD-NEM, which is encoded within residues 115–130. We found that the nuclear export-defective VHL Δ 115–130 (Khacho *et al.*, 2008), which lacks the DxxGxxDxxL motif that encodes TD-NEM, fails to bind to eEF1A (Figure 2, A and B). Truncation and deletion analysis revealed a clear correlation between VHL mutants that are able to export the PK-GFP-NLS reporter from the nucleus, in an *in vivo* nuclear export assay, and interact with endogenous eEF1A (Figure 2, C and D). These results point to a possible role for eEF1A in TD-NEM-mediated nuclear export of proteins.

eEF1A Is Required for Nuclear Export of Proteins Encoding a TD-NEM

The requirement for eEF1A to mediate nuclear export of TD-NEM-containing proteins was assessed using different approaches. First, we tested the effect of inhibitors of RNA PolII-dependent transcription, such as ActD and DRB, which cause nuclear accumulation of VHL and PABP1 (Lee *et al.*, 1999; Bonicalzi *et al.*, 2001; Groulx and Lee, 2002; Khacho *et al.*, 2008) and abrogate TD-NEM-mediated nuclear export (Khacho *et al.*, 2008). We wanted to test the possibility that ActD alters nuclear export by interfering with the TD-NEM/eEF1A interaction. Because ActD and DRB cause a nuclear shift in the localization of VHL, we fused VHL-GFP to a strong nuclear localization signal (NLS) in order to start with equal nuclear levels of protein in treated versus untreated cells. Addition of ActD or DRB, which inhibit TD-NEM activity (Figure 3, B and C, and Khacho *et al.*, 2008) and cause nuclear accumulation of VHL and PABP1 (Lee *et al.*, 1999; Groulx and Lee, 2002; Khacho *et al.*, 2008), partially prevented assembly between VHL-GFP-NLS and eEF1A (Figure 3A). Because this experiment most likely captures an *in vitro* interaction it provides a link between transcription-dependent nuclear export and eEF1A. These data demon-

Figure 4. siRNA-mediated silencing of eEF1A causes nuclear accumulation of TD-NEM-containing proteins. (A) eEF1A-specific siRNA reduces eEF1A protein level. MCF-7 and A549 cells were either mock-transfected or transfected with 100 nM eEF1A siRNA (2991 or 2804) for 72 h and then subjected to Western blot analysis using anti-eEF1A antibody. Actin was used as a loading control. (B) eEF1A knockdown and ActD treatment cause nuclear accumulation of endogenous PABP1. MCF-7 cells were transiently transfected with 100 nM control scrambled siRNA, 100 nM eEF1A siRNA (2991 or 2804), or untransfected for 72 h. Where indicated cells were transfected with 100 nM eEF1A siRNA for 72 h followed by treatment with 8 μ M ActD for 3 h. Localization of endogenous PABP1 was determined by immunofluorescence using a PABP1-specific antibody. Scale bar, 10 μ m. (C–E) eEF1A knockdown and ActD treatment results in nuclear accumulation of transiently expressed VHL-GFP and Δ C157-GFP, but does not affect GFP-NES. MCF-7 cells were cotransfected with 100 nM eEF1A siRNA (2991 or 2804) or control scrambled siRNA and VHL-GFP, Δ C157-GFP, or GFP-NES. Where indicated, cells were treated with 8 μ M ActD or 10 μ M LMB for 3 h after the 72-h incubation after transfection. Insets in B–E show Hoechst staining of DNA; scale bars, 10 μ m. Graphs in B–D represent relative nuclear:cytoplasmic ratios of fluorescence intensity. (F–I) Nuclear accumulation of TD-NEM-containing proteins in the absence of eEF1A is not due to an overall decrease in protein translation. (F) A549 cells were either mock-transfected or transfected with 100 nM eEF2 siRNA (10791) for 72 h and then subjected to Western blot analysis using anti-eEF2 antibody. Actin was used as a loading control. (G) A549 cells were untransfected, mock-transfected, or transfected with eEF1A or eEF2 siRNA for 72 h. Total cellular protein levels were measured using a standard protein quantification method. The decrease in protein levels in mock- or siRNA-transfected cells was calculated relative to protein levels in untransfected cells. (H) A549 cells were mock-transfected or transfected with eEF1A or eEF2 siRNA for 48 and 72 h. At the indicated times, cells were pulse-labeled for 30 min with [35 S]Met. Labeled whole cell lysates (WCL) were separated on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane, and translational activity was visualized by autoradiography (top panel). The two bottom panels show immunoblots from the same membrane, using eEF1A and eEF2 antibodies. (I) For determining the effect of eEF2 silencing, cells were transfected with only 100 nM eEF2 siRNA or cotransfected with VHL-GFP and 100 nM eEF2 siRNA for 72 h. Endogenous PABP1 was detected by immunofluorescence using a PABP1 antibody. For determining the effect of cycloheximide, cells were either untransfected or transiently transfected with VHL-GFP followed by treatment with cycloheximide for 2 h. Insets, Hoechst staining of DNA; scale bars, 10 μ m.



strate a correlation between inhibition of TD-NEM nuclear export and eEF1A binding and suggest the possibility that ActD and DRB may block nuclear export of VHL by altering its interaction with eEF1A.

Second, we tested the effect of silencing endogenous eEF1A using RNA interference. Silencing of eEF1A with two independent siRNAs markedly reduced the levels of eEF1A protein (Figure 4A) but did not result in measurable cell death as quantified by propidium iodide (PI) exclusion (data not shown). Supposing that eEF1A is required for transcription-dependent nuclear export, we reasoned that silencing of its protein levels should alter the steady state localization of TD-NEM-containing proteins in a manner reminiscent to the nuclear accumulation induced by ActD or DRB. Silencing eEF1A led to nuclear accumulation of endogenous PABP1 and transiently expressed VHL-GFP and Δ C157-GFP, which was similar to that induced by ActD treatment, when compared with untreated cells or those transfected with a scrambled siRNA (Figure 4, B–D). However, localization of the soluble cytoplasmic fusion protein, GFP-NES, which is altered by LMB, was not affected by eEF1A knockdown or ActD treatment (Figure 4E). Because eEF1A is highly abun-

dant it is difficult to achieve a complete knockdown of its protein levels, most likely resulting in residual eEF1A. We hypothesized that silencing eEF1A and treating with ActD, which partially inhibits interaction between eEF1A and VHL (Figure 3A), should result in an additive effect because the residual eEF1A would be blocked by ActD. Indeed, transfecting cells with eEF1A siRNA followed by ActD treatment resulted in a significant increase in the nuclear/cytoplasmic ratio of endogenous PABP1 and transiently expressed VHL-GFP and Δ C157-GFP (Figure 4, B–D). In fact, we observed a complete nuclear shift of Δ C157-GFP (Figure 4D), a protein that fails to interact with most VHL-binding proteins (Figure 1C). To eliminate the possibility that nuclear accumulation is due to a block in translation elongation, we silenced the elongation factor eEF2 (Figure 4F) using RNA interference to achieve a comparable decrease in protein synthesis and translational activity as that obtained with eEF1A silencing (Figure 4, G and H). Silencing eEF2 did not have an effect on the steady-state localization of either endogenous PABP1 or transiently expressed VHL-GFP (Figure 4I). Treatment with cycloheximide, an inhibitor of protein translation, also had no effect (Figure 4I), confirming that the change in steady-

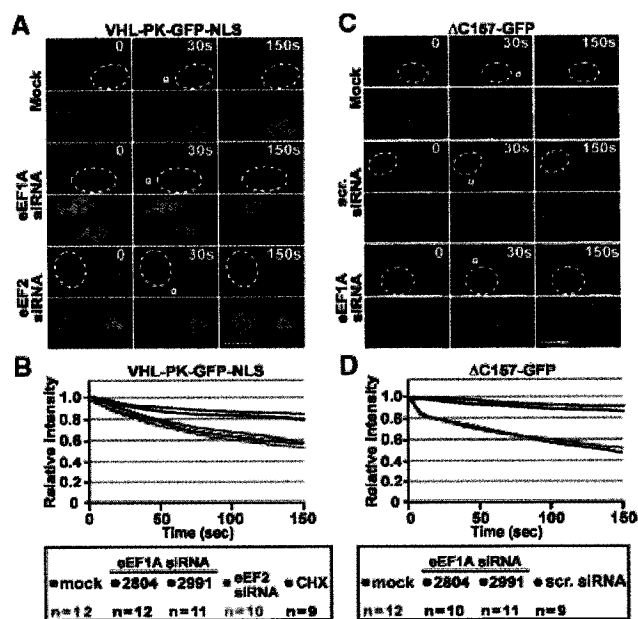


Figure 5. eEF1A is required for nuclear export of TD-NEM-containing proteins in living cells. (A–D) Silencing of eEF1A alters nuclear export of TD-NEM-containing proteins. MCF-7 cells were either mock-transfected or cotransfected with 100 nM eEF1A siRNA (2991 or 2804) or control siRNA and VHL-PK-GFP-NLS (A and B) or ΔC157-GFP (C and D) for 48 h before being submitted to the live cell FLIP nuclear export assay. In B cells were also treated with cycloheximide (CHX) for 1 h before cytoplasmic FLIP analysis. Cells were repetitively photobleached in a small cytoplasmic area (white box) within a cell of interest (dotted circle outlines the cell nucleus). The loss of nuclear fluorescence was monitored and plotted on a graph. The line graphs represent mean values obtained from the indicated number of cells used (n) per condition. Scale bars, 10 μ m.

state localization was not due to a requirement of ongoing protein synthesis.

siRNA-mediated silencing of eEF1A protein decreased the nuclear export rate of VHL-PK-GFP-NLS and ΔC157-GFP to levels similar to those observed upon treatment with ActD when compared with mock or control scrambled siRNA (Figure 5, A–D). However, silencing of eEF1A did not have an effect on the nuclear export rate of a classical nuclear export sequence (NES-PK-GFP-NLS; refer to Figure 9C). This indicates that silencing this translational factor does not alter the nuclear-cytoplasmic trafficking in general. Likewise, silencing eEF2 or treatment with cycloheximide confirmed that the reduced rate of export of VHL-PK-GFP-NLS was not due to the requirement of ongoing protein synthesis (Figure 5, A and B) but specifically from the decreased levels of endogenous eEF1A. To further uncouple the function of eEF1A in translation from its role in nuclear export and to provide additional evidence for a role in TD-NEM mediated nuclear export, we performed in vitro nuclear export assays. This assay serves to: 1) uncouple ongoing translation with nuclear export activity of eEF1A because there is negligible ongoing translation in the in vitro export assay and 2) eliminate the possibility for the loss of unknown factor(s) due to the partial arrest in translation in siRNA treatment for 48 and 72 h. Addition of cellular lysate depleted from eEF1A, using eEF1A specific antibodies (Figure 6A and see *Materials and Methods*), resulted in a significant decrease in nuclear export of VHL-PK-GFP-NLS in digitonin-permeabilized cells when compared with nondepleted lysate or lysate in-

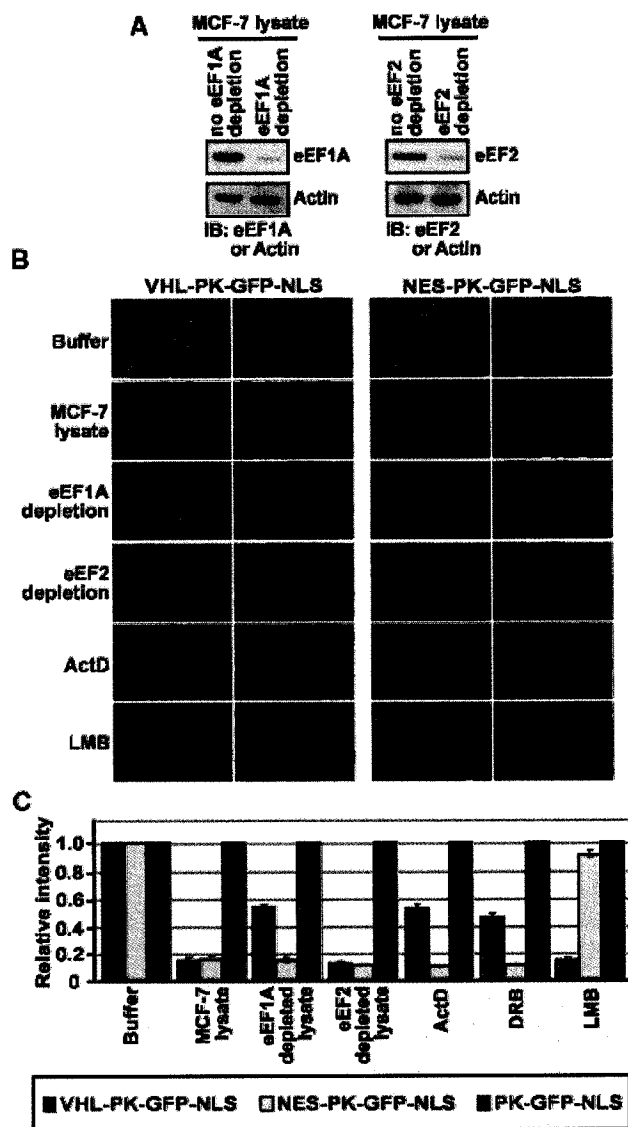
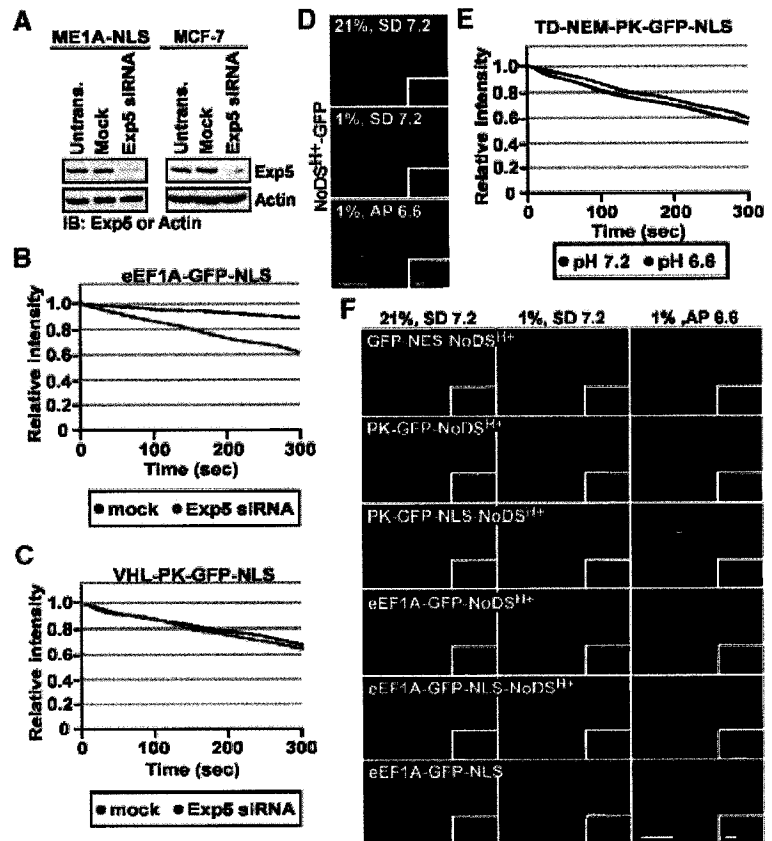


Figure 6. eEF1A is required for nuclear export of TD-NEM-containing proteins in vitro. (A) MCF-7 lysate containing 0.5 mg of protein (see Figure 9D) was depleted of eEF1A or eEF2 using eEF1A or eEF2 specific antibodies (see *Materials and Methods* for details). The depletion of eEF1A and eEF2 was assessed by Western blot analysis. Actin was used as a control. (B and C) MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin and incubated with transport buffer alone, MCF-7 lysate, or MCF-7 lysate that was depleted of eEF1A or eEF2. Where indicated cells were pretreated with 8 μ M ActD, 25 μ g/ml DRB, or 10 μ M LMB for 3 h before permeabilization with digitonin, and the nuclear export assay was performed using undepleted MCF-7 lysate containing the same concentrations of drugs as was used in the pretreatment. All conditions were performed in the presence of ATP-, GTP-, and ATP-regenerating system. Nuclear export was monitored by loss of nuclear fluorescence (B). Relative loss in nuclear fluorescence was calculated and plotted on a graph (C).

cubated with an irrelevant Flag antibody (Figure 6, B and C). Addition of ActD or DRB to living cells before digitonin treatment resulted in a similar decrease in nuclear export of VHL-PK-GFP-NLS (Figure 6, B and C). However, addition of cellular lysate depleted of eEF2 (Figure 6A) did not have an effect (Figure 6, B and C). In vitro nuclear export of

Figure 7. The function of eEF1A in nuclear export of TD-NEM-containing proteins is Exp5-independent and exerted from the cytoplasmic side of the nuclear envelope. (A–C) Nuclear export by TD-NEM is independent of Exp5. (A) ME1A-NLS (MCF-7 cells stably expressing eEF1A-GFP-NLS) and MCF-7 cells were transfected with 100 nM Exp5 siRNA for 48 h and then lysed and subjected to Western blot analysis using an anti-Exp5 antibody. Actin was used as a loading control. ME1A-NLS cells (B) or MCF-7 cells transiently expressing VHL-PK-GFP-NLS (C) were mock-transfected or transfected with 100 nM Exp5 siRNA and incubated for 48 h after which nuclear export was analyzed by the live cell FLIP nuclear export assay, as previously described. Loss of nuclear fluorescence was plotted on a graph (B and C). (D–F) eEF1A is not targeted to the nucleolus by the NoDS^{H+}. (D) MCF7 cells were cultured in standard media (SD) and transiently transfected to express the indicated GFP-tagged constructs. Cells were replenished with either fresh standard media (SD, pH 7.2) or acidification permissive media (AP, initial pH 7.2) that allows maximal extracellular acidification to pH 6.6 (see *Materials and Methods*). Cells either remained in normoxia (21% O₂) or transferred to hypoxia (1% O₂) for 18 h. Extracellular pH at the endpoint is indicated on each panel. (E) MCF-7 cells transiently expressing TD-NEM fused to PK-GFP-NLS were treated the same as in D. Cytoplasmic FLIP was performed to assess nuclear export activity. Loss of nuclear fluorescence was monitored and plotted on a graph. (F) MCF-7 cells transiently expressing the indicated constructs were incubated in the same conditions as in D. The localization of GFP-tagged fusion proteins was assessed by fluorescent microscopy. Insets in D and F show Hoechst staining of DNA; scale bars, 10 μ m.



NES-PK-GFP-NLS, which is sensitive to LMB (Figure 6, B and C), was not affected by either eEF1A or eEF2 depletion or by ActD and DRB treatment (Figure 6, B and C). These observations verify the results obtained with siRNA-mediated silencing of eEF1A and demonstrate that indeed eEF1A is required for nuclear export of TD-NEM. These results demonstrate that eEF1A binds to and is involved in nuclear export of TD-NEM-containing proteins and are consistent with a previous report showing that eEF1A is required for export of molecules in a yeast genetic system (Grosshans *et al.*, 2000a).

eEF1A Mediates Nuclear Export from the Cytoplasmic Side of the Nuclear Envelope

We examined the role of Exportin-5 (Exp5) in TD-NEM-mediated nuclear export since this exportin plays a role in nuclear export of eEF1A. siRNA-mediated silencing of endogenous Exp5 (Figure 7A) essentially abolished the ability of an eEF1A-GFP-NLS fusion protein to engage in nuclear export (Figure 7B) as previously shown by other groups (Bohnsack *et al.*, 2002; Calado *et al.*, 2002), though, in our cellular system, the steady-state localization of this fusion protein was mainly nuclear (see Figure 7F). In contrast, silencing of Exp5 did not disturb the nuclear export rate of VHL-PK-GFP-NLS (Figure 7C). These data suggest that the export pathway utilized by TD-NEM is independent of Exp5 and, consistent with data obtained with ActD and DRB, argues the possibility that tRNA may play a more minor role in this process.

This data raised the possibility that eEF1A may promote nuclear export of TD-NEM-containing proteins without having to enter the nucleoplasm. This would be consistent

with a model proposed by Grosshans *et al.* (2000) and McGuire *et al.* (2007) that eEF1A can stimulate nuclear export of tRNA from the cytoplasmic side of the nuclear envelope and that eEF1A ongoing nuclear import rate, if any, is very slow (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). To test this, we designed an assay based on the nucleolar detention properties of the previously identified NoDS^{H+} (nucleolar detention signal mediated by H⁺), which targets proteins for static detention by the nucleolar architecture of acidotic cells (Mekhail *et al.*, 2005, 2006, 2007). We reasoned that, upon NoDS^{H+} activation, an eEF1A-NoDS^{H+} fusion protein would be captured and detained by the nucleolus only if eEF1A is able to enter the nucleoplasm. NoDS^{H+}-GFP displays a diffuse nucleocytoplasmic localization pattern similar to GFP alone in neutral pH but accumulated in the nucleolus in acidotic cells, as previously described (Figure 7D and Mekhail *et al.*, 2005, 2006). Similarly, NES-NoDS^{H+}-GFP, which can diffuse into the nucleus but has a steady-state cytoplasmic localization due the presence of a strong and classical NES, is targeted to the nucleolus during acidosis (Figure 7F). PK-GFP-NoDS^{H+} failed to accumulate in the nucleolus of acidotic cells since it cannot import into the nucleoplasm, in contrast to the same fusion protein with an NLS (Figure 7F). eEF1A-GFP-NoDS^{H+} did not accumulate in the nucleolus suggesting that eEF1A does not import into the nucleoplasm (Figure 7F). Addition of a NLS caused the complete nucleolar sequestration of eEF1A-NoDS^{H+} in acidosis indicating that this protein can be captured by the nucleolus once imported into the nucleus (Figure 7F). NoDS^{H+}-containing proteins that display a similar cytoplasmic distribution at steady state to eEF1A, but can import into the nucleoplasm, are captured by the nucleolus in acidic pH

(Figure 7F and see Mekhail *et al.*, 2005). The rate of nuclear export of TD-NEM-containing proteins is similar regardless of the extracellular pH (Figure 7E) providing evidence that eEF1A can function in nuclear export of TD-NEM-containing proteins without having to enter the nucleus.

eEF1A Specifically Interacts with the TD-NEM Nuclear Export Sequence

As expected, we found that both residues 114–138 of VHL and residues 296–317 of PABP1 alone, which encode TD-NEM and mediate transcription-dependent nuclear export activity, were sufficient to bind to eEF1A (Figure 8, A and B). Single amino acid substitutions of key residues in the D_xG_x₂D_xL motif of VHL markedly reduced binding to eEF1A and nuclear export activity (Figure 8, A, C, and D). These data further demonstrate a clear correlation between nuclear export activity of TD-NEM and its ability to interact with eEF1A.

eEF1A Is Required for Nuclear Export of TD-NEM

To verify the involvement of eEF1A during transcription-dependent nuclear export our last criterion was to test the requirement for eEF1A in the nuclear export of the minimal TD-NEM sequence. We have previously shown that TD-NEM of both VHL and PABP1 mediate nuclear export of a PK-GFP-NLS reporter protein in a manner that is sensitive to ActD, but insensitive to LMB, an inhibitor of the classical NES/CRM1 nuclear export pathway (Khacho *et al.*, 2008). In contrast, nuclear export of the classical NES is inhibited by LMB but is unaffected by ActD (Khacho *et al.*, 2008). This demonstrates that nuclear export through TD-NEM operates independently of the classical CRM1/NES-mediated nuclear export pathway. siRNA-mediated silencing of eEF1A results in a decreased nuclear export rate of TD-NEM from both VHL and PABP1 to levels similar to those observed upon treatment with ActD (Figure 9, A and B). However, silencing of eEF1A did not have an effect on nuclear export of NES/CRM1 nuclear export pathway operates independently of eEF1A. These results also support the involvement of eEF1A in TD-NEM-mediated nuclear export because the decreased export of TD-NEM upon silencing of eEF1A is not due to a decrease in protein synthesis or a general effect on nuclear-cytoplasmic trafficking.

Next we tested the requirement of eEF1A to mediate nuclear export of TD-NEM in an *in vitro* nuclear export assay. Addition of decreasing amounts of cellular lysate to digitonin-permeabilized cells expressing TD-NEM-PK-GFP-NLS demonstrated the presence of a limiting factor in the lysate, which is required for mediating TD-NEM nuclear export (Figure 9D). Addition of cellular lysate depleted from eEF1A, using eEF1A specific antibodies (Figure 9E and see *Materials and Methods*), resulted in a significant decrease in nuclear export of TD-NEM of VHL and PABP1 in digitonin-permeabilized cells when compared with nondepleted lysate or lysates incubated with an irrelevant Flag antibody (Figure 9F and G). Addition of ActD to living cells before digitonin treatment resulted in a similar decrease in nuclear export (Figure 9G). These observations verify the results obtained with siRNA-mediated silencing of eEF1A and demonstrate that indeed eEF1A is required for nuclear export of TD-NEM.

DISCUSSION

VHL and PABP1 share the common characteristic of engaging in a nuclear export pathway that is dependent on ongoing

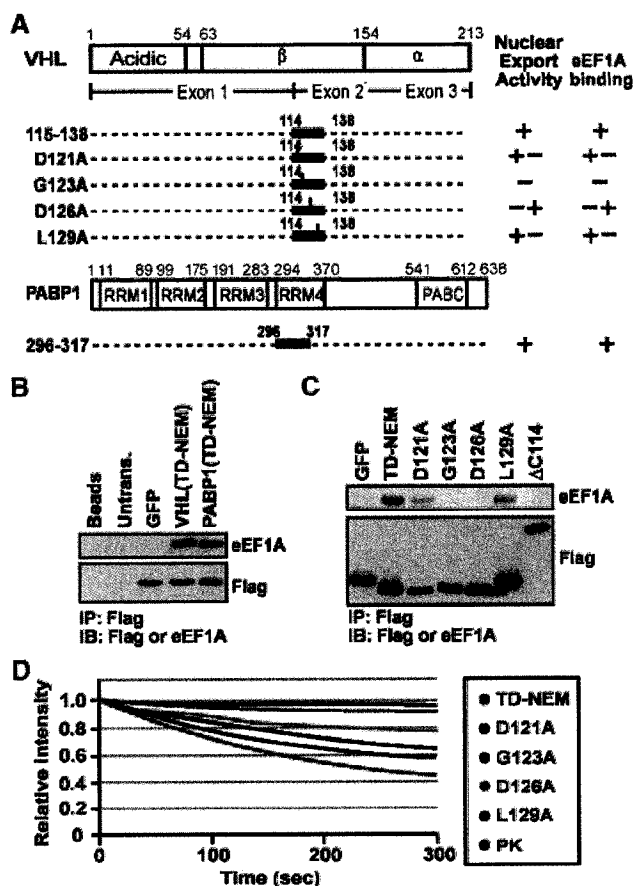
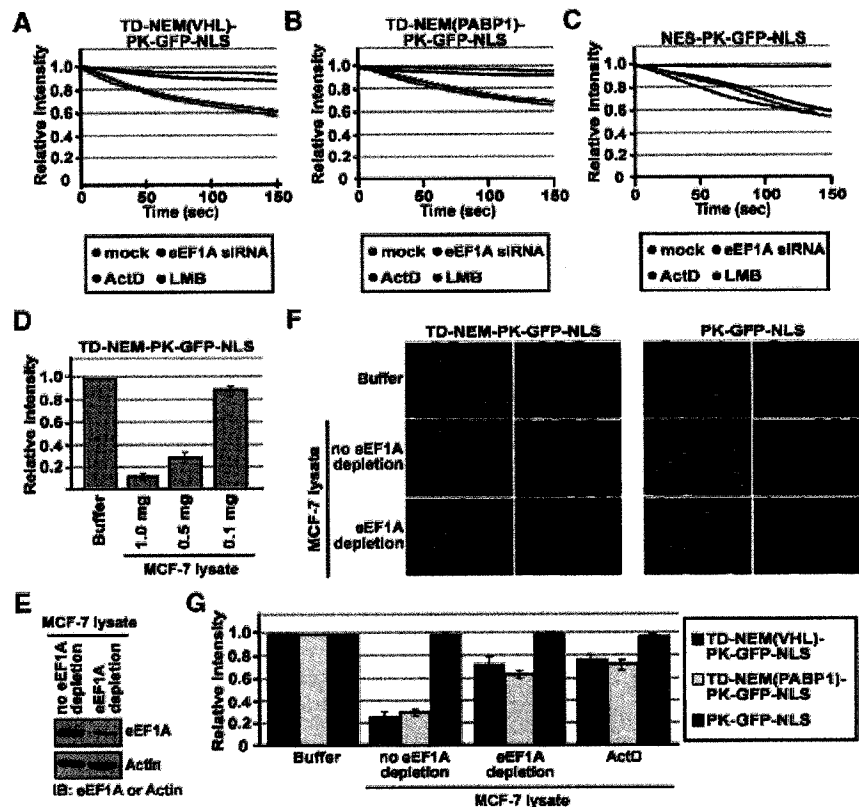


Figure 8. TD-NEM is sufficient for eEF1A interaction. (A) Schematic diagram depicts the TD-NEM of VHL encoded within residues 115–138 and alanine substitution of the key residues of TD-NEM. Schematic diagram also depicting the TD-NEM of PABP1 encoded between residues 296 and 317. (+), (+ –), (– +), and (–) indicate, in a decreasing order, the ability of the fusion proteins to interact with eEF1A or export from the nucleus. (B) The transcription-dependent nuclear export sequence, TD-NEM, of VHL and PABP1 is sufficient to interact with endogenous eEF1A. MCF-7 cells transiently expressing GFP alone or the TD-NEM sequences of VHL or PABP1 tagged with Flag and GFP were submitted to immunoprecipitation with anti-Flag beads and immunoblotted with anti-Flag or anti-eEF1A antibodies. TD-NEM refers to residues 114–138 of VHL and residues 298–317 of PABP1. (C) Conserved residues in TD-NEM are required for eEF1A interaction. TD-NEM refers to residues 114–138 of VHL. Flag and GFP-tagged D121A, G123A, D126A, and L129A indicate the amino acid that was substituted to alanine within residues 114–138 of VHL. Immunoprecipitation and Western blot was as described above. (D) Conserved residues in TD-NEM are required for efficient nuclear export. MCF-7 cells transiently expressing TD-NEM, D121A, G123A, D126A, or L129A fused to PK-GFP-NLS or expressing PK-GFP-NLS alone were subjected to the live cell FLIP nuclear export assay. The loss of nuclear fluorescence during cytoplasmic FLIP was monitored and graphed.

RNA PolII activity, but independent of the classical CRM1/NES nuclear export pathway. We have previously shown that transcription-dependent nuclear export of VHL and PABP1 is mediated by a novel nuclear export motif, TD-NEM. Here we report that TD-NEM-mediated nuclear export involves an essential member of the translational machinery, the translation elongation factor eEF1A. eEF1A is a novel VHL and PABP1 interacting protein that binds specifically to the TD-NEM sequence. siRNA-induced si-

Figure 9. eEF1A is required for TD-NEM-mediated nuclear export. (A–C) TD-NEM nuclear export requires eEF1A and is independent of the CRM1/NES nuclear export pathway. MCF-7 cells were transiently co-transfected with the indicated constructs and 100 nM eEF1A siRNA for 48 h before being assessed by the live cell FLIP nuclear export assay. Where indicated, cells were transfected with the indicated constructs then treated with either 2 μ M ActD or 10 μ M LMB for 1 h before cytoplasmic FLIP. The loss of nuclear fluorescence was monitored and graphed. (D–G) eEF1A is required for nuclear export of TD-NEM in an in vitro export assay. (D) Protein levels in an MCF-7 cellular lysate were quantified using standard protein quantification methods. Buffer alone or cellular lysate containing different protein amounts in the presence of the ATP-, GTP-, and ATP-regenerating system were added to digitonin-permeabilized cells transiently expressing TD-NEM-PK-GFP-NLS. Nuclear export was monitored by loss of nuclear fluorescence. Relative loss in nuclear fluorescence was calculated and plotted on a graph. (E) MCF-7 lysate containing 0.5 mg of protein was depleted of eEF1A using eEF1A specific antibody (see *Materials and Methods* for details). The depletion of eEF1A was assessed by Western blot analysis. Actin was used as a control. (F and G) MCF-7 cells transiently expressing the indicated constructs were permeabilized with digitonin and incubated with transport buffer alone, MCF-7 lysate, or MCF-7 lysate that was depleted of eEF1A. Where indicated cells were pretreated with 8 μ M ActD for 3 h before permeabilization with digitonin and the nuclear export assay was performed using undepleted MCF-7 lysate containing 8 μ M ActD. All conditions were performed in the presence of ATP-, GTP-, and ATP-regenerating system. Nuclear export was monitored by loss of nuclear fluorescence (F). Relative loss in nuclear fluorescence was calculated and plotted on a graph (G).



lencing or antibody-mediated depletion of eEF1A decreases TD-NEM nuclear export kinetics. Inhibition of RNA PolII-dependent transcription impairs the TD-NEM/eEF1A interaction thereby decreasing TD-NEM-mediated nuclear export. Inhibition of eEF1A did not effect NES-mediated nuclear export of proteins, suggesting that eEF1A and CRM1 operate independently. Because eEF1A exhibits a predominantly cytoplasmic localization, we propose that its functional role is orchestrated from the cytoplasmic side of the nuclear envelope. The results presented here highlight the essential role of cytoplasmic export factors in the coordination of events that lead to export from the nuclear to the cytoplasmic compartment. Meanwhile, these results also provide a link between the translational apparatus and the subcellular trafficking machinery demonstrating that these two central pathways in basic metabolism can cooperate to sustain the highly dynamic nature of cellular processes.

Regulated and signal-mediated transport of proteins between the nucleus and the cytoplasm is critical for many cellular processes. Several studies have reported the need for ongoing RNA PolII activity to actively shuttle proteins between the nuclear and cytoplasmic compartments. Most studies have focused on the role of ongoing transcription in nuclear import of proteins such as hnRNPs (Pinol-Roma and Dreyfuss, 1991, 1992, 1993; Siomi *et al.*, 1997). However, there are also proteins that necessitate RNA PolII-mediated transcription to efficiently export from the nucleus, including VHL and PABP1 (Afonina *et al.*, 1998; Lee *et al.*, 1999; Groulx and Lee, 2002; Griffis *et al.*, 2004; Zhang *et al.*, 2005; Khacho *et al.*, 2008). In an attempt to understand the mechanism of

transcription-dependent nuclear export we set out to identify components involved in this nuclear export pathway. In doing so, we have identified a member of the translational machinery, eEF1A, as a factor involved in the transcription-dependent nuclear export pathway. The correlation between binding and nuclear export activity argues that the interaction between TD-NEM and eEF1A is biochemically relevant. Removal of TD-NEM from full-length VHL totally abolished binding to eEF1A. TD-NEM alone from both VHL and PABP1, which consists of only a few residues, bound to eEF1A. However, mutations of key residues within the DxG₂Dx₂L consensus sequence of TD-NEM, particularly G123A, measurably hindered the ability of TD-NEM to bind to eEF1A and mediate export of reporter proteins. Yet, it is the data obtained with the functional assays that substantiates a role for eEF1A in TD-NEM-dependent nuclear export. Silencing of endogenous eEF1A alters the steady-state localization of full-length endogenous PABP1 and transiently expressed VHL, and the nuclear export competent VHL truncation mutant Δ C157. The reduction of nuclear export rate in a FLIP nuclear export assay of cells treated with siRNA against eEF1A argues that eEF1A has a direct role in the nuclear export activity of TD-NEM in living cells though a potential role of cytoplasmic retention cannot be formally excluded. However, depletion of endogenous eEF1A from a cellular lysate resulted in a decrease in nuclear export activity of TD-NEM-containing proteins in an in vitro nuclear export assay arguing that eEF1A is directly involved in nuclear export. Treatment with ActD or DRB partially abrogated binding of eEF1A with TD-NEM, providing a possible

explanation for their ability to block TD-NEM activity and nuclear export of VHL and PABP1. Although TD-NEM-mediated nuclear export does not appear to occur via mRNA export, we cannot exclude the possibility that other RNA PolII-dependent RNA species may be involved. Put together, these data, in addition to the binding results, support a role for eEF1A in TD-NEM-dependent nuclear export.

Several lines of evidence suggest that eEF1A itself is a key element in the coordination of TD-NEM nuclear export, rather than its contribution to protein translation as a member of the translational machinery. First, acute inhibition of protein translation by cycloheximide did not have an effect on TD-NEM-mediated nuclear export. Second, chronic inhibition of translation was tested by silencing of another translation elongation factor, eEF2, such that a comparable decrease in steady-state protein levels and translational activity, as in eEF1A silencing, was achieved. Knockdown of eEF2 did not have an effect on the localization or the nuclear export activity of TD-NEM-containing proteins compared with silencing of eEF1A. In addition, these experiments suggest that blockage of protein synthesis during the period of the experiments did not deplete a protein whose synthesis would have been required for nuclear export of TD-NEM-containing proteins. Finally, the role of eEF1A in TD-NEM-mediated nuclear export was addressed using *in vitro* nuclear export assays. This assay uncouples ongoing translation with nuclear export activity of eEF1A and it eliminates the possibility for the loss of unknown factor(s) required for nuclear export. Together these results aid to uncouple the role of eEF1A in TD-NEM-mediated nuclear export and protein synthesis.

Work conducted by several groups, including the present study, have failed to detect endogenous or exogenous eEF1A in the nuclear compartment. In fact, Calado *et al.* (2002) proposed the Exp5 pathway as a way to exclude eEF1A from nuclei if it were ever to enter, such as after cell division. In this study we demonstrate that TD-NEM-mediated nuclear export is independent of the Exp5 pathway. Also, the nucleolar retention experiments shown here, in addition to data from other groups, suggest that eEF1A may exert its function in TD-NEM-dependent nuclear export from the cytoplasmic side of the nuclear envelope. Because the nucleolar retention experiments were performed using exogenous eEF1A, we cannot formally exclude the possibility that a small population of endogenous eEF1A is capable of entering the nuclear compartment. In fact, this is an intriguing possibility that would easily explain the role of eEF1A in nuclear export of RNA species and TD-NEM proteins. It is difficult to assess whether this is the case. Consistent with a possible cytoplasmic role for eEF1A, is the work by Grosshans *et al.* (2000) that reported a role for cytoplasmic eEF1A in nuclear export of tRNAs in yeast, where nuclear accumulation of mature tRNAs was observed in strains with reduced amounts or mutated eEF1A. Because eEF1A was undetectable in the nuclear compartment, Grosshans *et al.* postulated that eEF1A may function at the cytoplasmic face of the nuclear pore to facilitate the release of charged tRNAs from the aminoacyl-tRNA synthase. Interestingly, another cytoplasmic protein, Cexp1, has been recently identified as a component of the tRNA export pathway. In fact, given that Cexp1 associates with the NPC by interacting with Nup116p, it was proposed that Cexp1 collects aminoacyl-tRNAs from nuclear export receptors at the cytoplasmic side of the NPC and transfer them to eEF1A using a channeling mechanism (McGuire and Mangroo, 2007). Now we have evidence to support a role for eEF1A in nuclear export of TD-NEM-containing proteins. Because a common theme for cytoplasmic nuclear export factors is their involvement

in the final steps of transport, it is possible that eEF1A functions in receiving proteins as they pass through the NPC. In this sense, eEF1A may utilize its GDP/GTP binding properties to release proteins in the cytoplasm, similar to the manner by which RanGTP hydrolysis to RanGDP results in the release of exportin-cargo complexes.

Previously, cytoplasmic eEF1A has been implicated in the nuclear export pathway of tRNA in yeast. We propose that eEF1A is a cytoplasmic component of the TD-NEM-dependent nuclear export pathway in mammalian cells. The fact that eEF1A may function only from the cytoplasmic side of the nuclear envelope implies the existence of a yet unidentified nuclear exporter that would interact with TD-NEM-containing cargo in the nucleus to facilitate its passage through the NPC. However we cannot rule out the possibility that the TD-NEM/eEF1A system operates independently of known classical nuclear export pathways. Future work in unraveling other components of this export pathway will further uncover the mechanism of transcription-dependent nuclear export of proteins and clarify the precise role of eEF1A during this process.

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Subcellular dynamics of the VHL tumor suppressor: on the move for HIF degradation

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The von Hippel-Lindau (VHL) tumor suppressor protein, the recognition component of an E3 ubiquitin ligase complex, recruits the α -subunit of the hypoxia-inducible factor (HIF α) for oxygen-dependent degradation. The ability of VHL to mediate efficient degradation of HIF α is also dependent on its oxygen/pH-regulated subcellular trafficking. Under aerobic conditions, VHL engages in nuclear-cytoplasmic trafficking that requires ongoing transcription and is mediated by a novel nuclear export motif, the transcription-dependent nuclear export motif (TD-NEM). Disease-causing mutations targeting TD-NEM restrain VHL from mediating efficient oxygen-dependent degradation of HIF α by altering its subcellular dynamics. In addition, decreasing the extracellular pH, during anaerobic metabolism, stabilizes HIF α by triggering the relocalization and static detention of VHL to nucleoli. Together, these recent findings support the critical role of subcellular trafficking and dynamic properties for the function of VHL in promoting HIF regulation and tumor suppression.

Inheritance of mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene predisposes individuals to the development of VHL disease [1]. This disorder affects one in 36,000 individuals and has over 90% penetrance by the age of 65 years, with no ethnic, racial, cultural or sexual bias [1]. VHL disease is characterized by the presence of hypervascular tumors in multiple organs, including the CNS (cerebellum, brainstem and spinal cord), retina, pancreas, adrenal gland, endolymphatic sac of the inner ear, epididymis (male), broad ligament (female) and kidneys [1,2]. Although the majority of tumors associated with VHL disease are benign, kidney cancer is malignant and is the principle cause of morbidity and mortality for VHL patients [2]. Conforming to Knudson's two-hit model of tumor suppressors, predisposition to VHL disease results from a germ-line mutation of the *VHL* tumor suppressor gene (FIGURE 1A) [3]. Tumors arise in VHL families when the remaining wild-type *VHL* allele is mutated or inactivated in a susceptible cell. In keeping with the Knudson two-hit model, biallelic inactivation of VHL is commonly observed in sporadic renal cell carcinomas (RCC; kidney cancer) [4].

The VHL tumor suppressor protein has been reported to be involved in multiple cellular processes, including extracellular fibronectin matrix assembly, microtubule stability and cilia

formation [5–7]. However, the most characterized and noteworthy role for VHL is reserved for its function in the ubiquitylation system. VHL is a component of the ubiquitylation system as it acts as the particle recognition motif of a Cullin-2-containing E3 ubiquitin ligase complex [1,8–12], referred to as VBC-Cul2 (FIGURE 1B). It is widely accepted that the primary function of VHL as part of this E3 ubiquitin ligase complex is to promote oxygen-dependent ubiquitylation and proteasomal degradation of the α -subunit of the hypoxia-inducible transcription factor (HIF α) [13,14]. There exists different subunits of HIF α , including HIF1 α , HIF2 α and HIF3 α ; however, for sake of simplicity, these will not be discussed in detail and will be referred to as HIF α [15]. Under aerobic conditions, when there is normal oxygen tension (normoxia), HIF α is hydroxylated at key proline residues in the oxygen-dependent degradation domain by enzymes known as prolyl hydroxylases (FIGURE 1C) [16,17]. This post-translational modification allows VHL to recognize HIF α and target it for ubiquitylation and subsequent degradation by the 26S proteasome (FIGURE 1C) [18,19]. A shift to low oxygen tension (hypoxia) prevents hydroxylation of HIF α due to the requirement of molecular oxygen for the function of prolyl hydroxylases [19–21]. This suppresses the recognition and degradation of HIF α by the VBC-Cul2 complex and leads to HIF α stabilization (FIGURE 1C). Once

Keywords

5,6-dichlorobenzimidazole (DRB) ■ actinomycin D ■ E3 ubiquitin ligase ■ O₂- and pH-dependent HIF regulation ■ pH-dependent nucleolar detention ■ protein dynamics ■ TD-NEM ■ transcription-dependent subcellular trafficking ■ tumor suppressor ■ VHL

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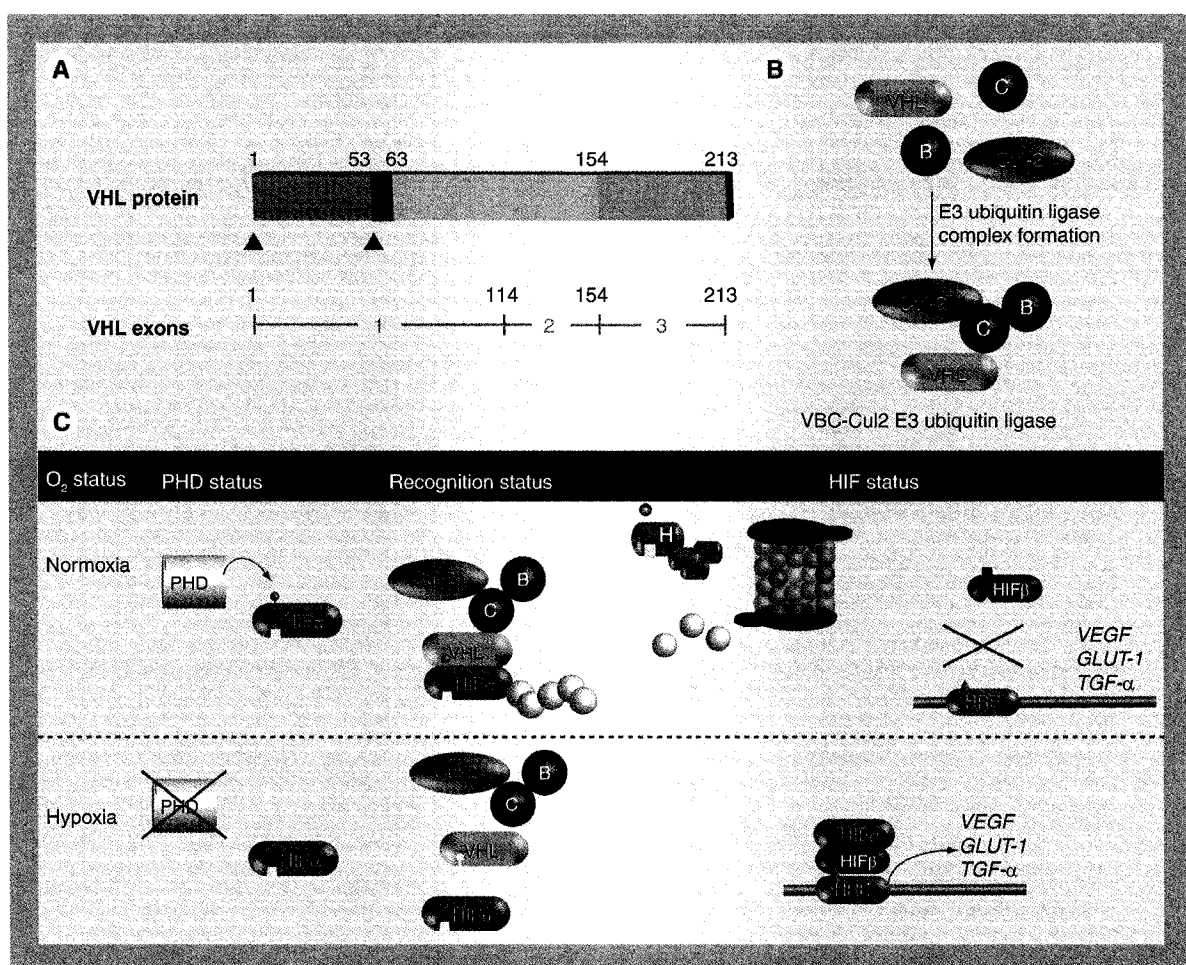


Figure 1. The VHL tumor suppressor protein is a component of an E3 ubiquitin ligase complex that targets HIF α for proteasomal degradation. (A) VHL is a 213 amino acid protein encoded by three exons. Red arrows indicate the two in-frame methionines. **(B)** Assembly of VHL into an E3 ubiquitin ligase complex, VBC-Cul2. **(C)** VBC-Cul2 mediates oxygen-dependent degradation of HIF α . C and B: Elongins B and C; Cul-2: Cullin-2; GLUT-1: Glucose transporter 1; HIF: Hypoxia-inducible factor; HRE: Hypoxia-response element; PHD: Prolyl hydroxylase; TGF- α : Transforming growth factor- α ; VHL: von Hippel-Lindau.

stabilized, the oxygen-regulated HIF α subunit heterodimerizes with its constitutively active partner HIF β to form an active transcription factor, together known as HIF (FIGURE 1C) [15].

HIF is a key element in the cellular response to hypoxia, a hallmark during the development of tumors, muscle stress and ischemic disorders [22,23]. The hypoxia-induced stabilization of HIF α and formation of the active HIF α /HIF β heterodimer results in the transcriptional induction of an array of genes, such as the vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) and transforming growth factor- α (TGF- α), among others, implicated in oxygen homeostasis [24,25]. Once activated, these genes modulate many aspects

of cell physiology, including angiogenesis, glycolysis and growth [22]. Although HIF is a key regulator of oxygen homeostasis, its dysregulation or untimely activation can be detrimental to the cell. In this sense, the essential role of VHL as the master regulator of HIF α is warranted, given that disruption of the VHL/HIF system can ultimately lead to the VHL cancer syndrome or sporadic RCC [24]. Loss of VHL function, through inactivating mutations, deletions or hypermethylation of the gene promoter, often prevent assembly with its substrate, HIF α , or core components of the E3 ubiquitin ligase, elongins B, C and Cullin-2, resulting in constitutive activation of HIF α [10,13,26]. This in turn induces a transcriptional program that alters

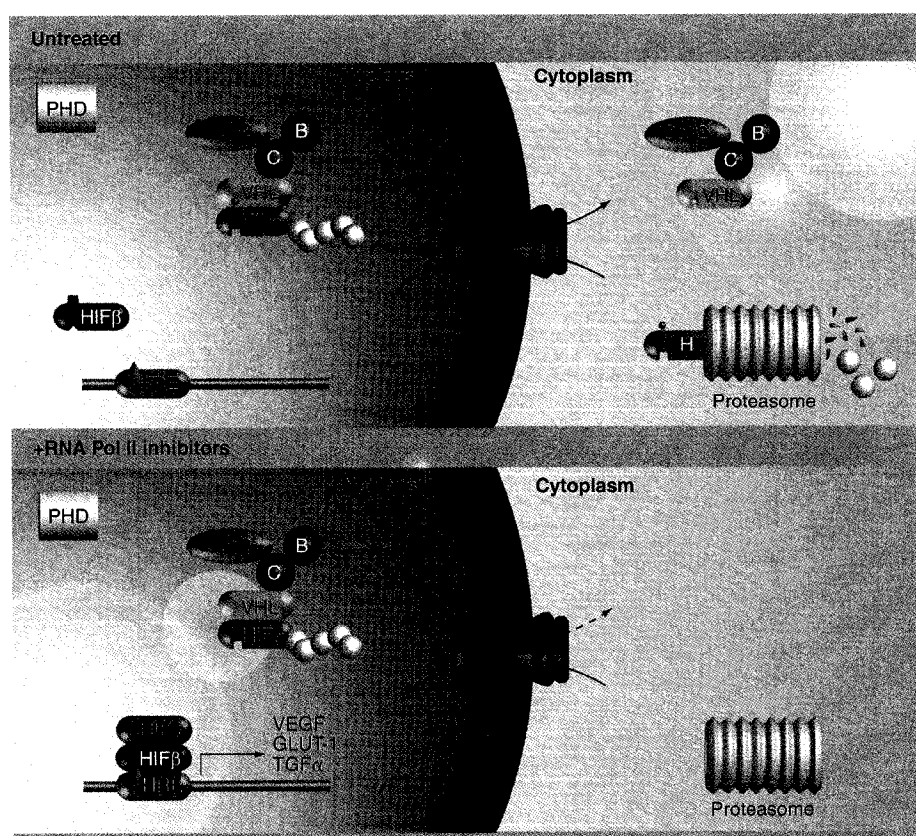


Figure 2. Transcription dependent subcellular trafficking of VHL is required for HIF α degradation. Nuclear–cytoplasmic trafficking of VHL is required for efficient degradation of HIF α . Inhibition of RNA Pol II-dependent transcription decreases the nuclear export activity of VHL and prevents efficient HIF α degradation.
C and B: Elongins B and C; Cul-2: Cullin-2; GLUT-1: Glucose transporter 1; HIF: Hypoxia-inducible factor; HRE: Hypoxia-response element; PHD: Prolyl hydroxylase; TGF- α : Transforming growth factor- α ; VHL: von Hippel–Lindau.

over 100 genes, ultimately leading to tumorigenesis. For this reason a great deal of effort has been invested into fully understanding the E3 ubiquitin ligase properties of VHL and how they contribute to coordinating the efficient and rapid degradation of HIF α in order to suppress tumorigenesis.

Subcellular trafficking of E3 ubiquitin ligases

In recent years it has become clear that most molecules are highly mobile and participate in networks that often require dynamic movement within subcellular compartments and highly efficient transport between different subcellular compartments. This newly found appreciation for protein dynamics has emerged particularly for E3 ubiquitin ligases. Due to the fact that E3 ubiquitin ligases serve as the substrate-recognition element, which dictate substrate

specificity, their function is important for progression of the ubiquitin-mediated degradation cascade. Considering the key role of E3 ubiquitin ligases in the regulation of many aspects of cellular functions and biological processes, it is not surprising that loss of function or deregulation of E3 ubiquitin ligases can result in the development of disease, including cancer [27]. Recently it has become evident that subcellular trafficking is also a prerequisite for the proper functioning of many E3 ubiquitin ligases [28]. It is now known that degradation of nuclear proteins by the ubiquitylation system often requires nuclear–cytoplasmic trafficking of E3 ubiquitin ligases. For example, efficient proteasomal degradation of the nuclear p53 tumor suppressor requires the continuous nuclear–cytoplasmic shuttling of the Mdm2 E3 ubiquitin ligase [29,30]. Disrupting the nuclear export of Mdm2, such as by cancer-causing point mutations, impairs

its ability to mediate degradation of p53 [31]. Another example is the ROC1SCF^{Ebw1a} E3 ubiquitin ligase, whose nuclear export is required for proteasomal degradation of the Smad3 transcription factor [32]. In this setting, the VHL-containing VBCul2 E3 ubiquitin ligase complex is not an exception in that the dynamic trafficking properties of VHL are required for the efficient and rapid degradation of nuclear HIF α [33,34].

Transcription-dependent subcellular trafficking of VHL

The VHL tumor suppressor protein exhibits a predominantly cytoplasmic localization for both endogenous VHL and exogenously expressed VHL-GFP in all tested tissues and cell lines [35–37]. The prominent cytoplasmic localization of VHL was shown to be altered by arrest of RNA Polymerase II (RNA PolII)-mediated transcription [36]. Inhibition of RNA PolII transcription results in a nuclear shift in VHL localization. These observations pointed to the idea that VHL was able to mobilize between the nuclear and cytoplasmic compartments. Soon after it

was determined, through cellular fusion experiments and *in vitro* export assays, that VHL is a nuclear–cytoplasmic shuttling protein (FIGURE 2) [36]. In fact, further studies identified this trafficking feature central to the tumor suppressor function of VHL [33,34].

Nuclear–cytoplasmic trafficking of proteins across the nuclear envelope results from a consecutive cycle of protein entry into the nucleus (nuclear import) and exit from the nucleus to the cytoplasmic compartment (nuclear export). The translocation of proteins across the nuclear envelope is generally mediated by soluble transport receptors that recognize specific transport signals, such as nuclear localization signals (NLSs) and nuclear export signals (NESs) [38]. With this in mind, the nuclear–cytoplasmic trafficking of VHL must be arranged by coordination of nuclear import and export pathways. However, identifying the nuclear export pathway was complicated by the fact that VHL does not contain a classical leucine-rich NES and is not sensitive to treatment with leptomycin, an inhibitor of the classical NES nuclear export pathway mediated by the

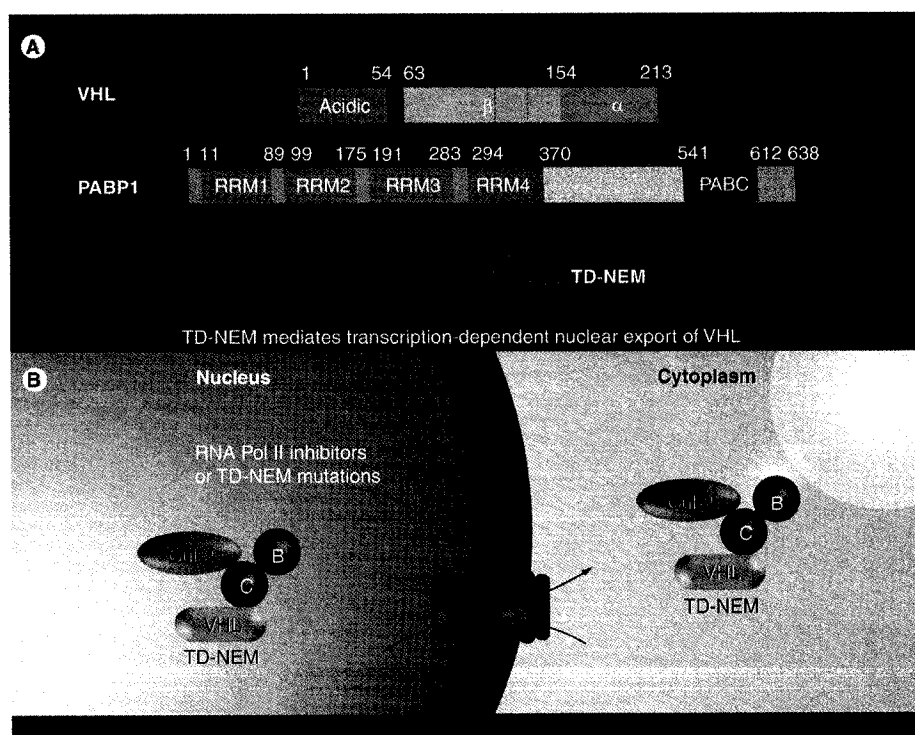


Figure 3. Transcription-dependent nuclear export motif mediates transcription-dependent nuclear export of VHL. (A) Mapping analysis and sequence comparison with PABP1 led to the identification of TD-NEM. **(B)** Inhibitors of RNA Pol II activity or mutations of key residues in TD-NEM block TD-NEM-mediated nuclear export of VHL. C and B: Elongins B and C; Cul-2: Cullin-2; TD-NEM: Transcription-dependent nuclear export motif; VHL: von Hippel–Lindau.

CRM1 transport receptor [33,36,39,40]. In fact, the nuclear accumulation ensued by inhibitors of RNA PolII-dependent transcription was a key observation in the identification of the subcellular trafficking properties of VHL. Treatment of cells with RNA PolII transcriptional inhibitors, such as actinomycin D (ActD) and 5,6-dichlorobenzimidazole (DRB), results in a clear nuclear shift in VHL localization in a reversible manner [33,34,36]. Cellular fusion and nuclear export assays have revealed that this nuclear accumulation was due, at least in part, to the inhibition of nuclear export [36,41]. This was also verified in live cells by photobleaching experiments, which are less abrasive than *in vitro* assays. In these experiments, VHL was fused to the large and amorphous pyruvate kinase (PK) to inhibit any diffusion across the nuclear membrane; GFP, to provide a fluorescence signal; and a strong NLS, to ensure that all starting material is nuclear [34]. Fluorescence loss in photobleaching (FLIP) on cells expressing VHL-PK-GFP-NLS confirmed that in the presence of ActD or DRB, there is a significant decrease in the nuclear export activity, as indicated by relative loss of nuclear fluorescence, compared with untreated cells or cells treated with leptomycin [34]. Together, these data supported the idea that firstly, VHL participates in dynamic nuclear–cytoplasmic trafficking, secondly, that nuclear export of VHL is independent of the classical CRM1/NES pathway, and thirdly, that VHL exports from the nucleus utilizing a mechanism that requires ongoing RNA PolII-dependent transcription.

Several nuclear substrates, such as p53, p27 and Smad3, are ubiquitinated in the nucleus but must undergo nuclear export for efficient ubiquitin-mediated degradation by the proteasome [32,42,43]. HIF α is a predominantly nuclear protein when it is stabilized during hypoxic conditions [33,44]. After HIF α is ubiquitinated in the nucleus by the VBCul2 complex, a detectable cytoplasmic signal becomes apparent before its degradation [33]. The observation that HIF α ubiquitination occurs in the nucleus, while its degradation is a cytoplasmic event, suggested that nuclear export is potentially required for the process. The importance of subcellular trafficking for efficient HIF α degradation was confirmed by the finding that treatment with inhibitors of RNA PolII activity, which interfere with the dynamic profile of VHL, caused a marked increase in the half-life of HIF α , without affecting its nuclear ubiquitination (FIGURE 2) [33].

Nuclear export of VHL by TD-NEM: a novel transport pathway

Although several studies have reported the dependency on ongoing RNA PolII activity for active nuclear–cytoplasmic shuttling, these have focused mostly on nuclear import of proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs) [45]. The idea that transcription-dependent nuclear export of proteins may be a *bona fide* nuclear export pathway was substantiated by the discovery that another protein, the poly(A)binding protein 1 (PABP1), also requires ongoing RNA PolII activity to export from the nucleus [46]. Similar to VHL, PABP1 is a predominantly cytoplasmic protein that shifts to a more nuclear localization upon arrest of RNA PolII-mediated transcription, using transcriptional inhibitors such as ActD and DRB [34,46]. The observation that nuclear export of VHL and PABP1 is transcription-dependent and independent of the classical NES/CRM1 pathway was the root for the identification of a novel nuclear export pathway.

Previous reports established that the sequence required for transcription-dependent nuclear export was embedded within the exon2-encoded β -domain of VHL [36,40]. Further mapping of the VHL protein and sequence alignment with PABP1 identified a conserved and discrete motif, encoded by the consensus sequence D_xG_x₂D_x₂L, which was shared by VHL and PABP1 (FIGURE 3A) [34]. This recently identified and novel nuclear export motif, named transcription-dependent nuclear export motif (TD-NEM), is required and sufficient to mediate nuclear export of VHL, as well as PABP1 and the cell-cycle regulator cyclin C [34]. Nuclear export through TD-NEM requires ongoing RNA PolII-mediated transcription (FIGURE 3B) and operates independently of the classical CRM1/NES-mediated nuclear export pathway [34]. Point mutations targeting the key conserved residues of TD-NEM abolish nuclear export and consequently disrupt the nuclear–cytoplasmic shuttling dynamics of VHL (FIGURE 3B) [34]. More recently, TD-NEM-mediated nuclear export has been shown to involve an essential member of the translational machinery, the translation elongation factor eEF1A [47]. eEF1A, an exclusively cytoplasmic protein, interacts specifically with TD-NEM of VHL and PABP1, and disrupting this interaction by point mutations of key TD-NEM residues prevents assembly and nuclear export [47]. siRNA-induced silencing or antibody-mediated depletion of eEF1A inhibited the TD-NEM nuclear export pathway both

in vivo and *in vitro* [47]. Interestingly, treatment with ActD or DRB partially abrogated binding of eEF1A with TD-NEM providing a possible explanation for the ability of these transcriptional inhibitors to block TD-NEM activity and nuclear export of VHL [47].

The essential role of nuclear–cytoplasmic trafficking of VHL for its tumor suppressor function has now been strengthened by the identification of its transcription-dependent nuclear export motif, TD-NEM. Naturally occurring point mutations within the key TD-NEM residues of VHL are associated with VHL disease. Within the consensus D_xG_xD_xL, substitution of the first aspartic acid residue to glycine (D121G) is a germ-line mutation associated with Type 2B VHL disease, characterized by high risk of RCC [48,49]. In addition, substitution of the second aspartic acid residue to tyrosine has been reported in individuals with polycythemia, [50]. These cancer-causing mutations alter the dynamic profile of VHL by decreasing the rate of export from the nucleus (FIGURE 3) [34]. Interestingly, expression of the D121G or D126Y mutants leads to an extended HIF α stability following reoxygenation of hypoxic cells, even though they maintain the ability to interact with HIF α [34]. In fact, the D121G mutant has been shown to maintain its ability to form an E3 ubiquitin ligase complex and to bind and polyubiquitylate HIF α *in vitro* [51], yet patients with this mutation develop classical tumors associated with VHL disease. This decreased HIF α degradation provides a strong correlation between the efficiency to mediate degradation of HIF α and nuclear export activity of VHL. It would be interesting to test if the VHL mutant G123A, which is not a cancer-causing mutation but has been previously shown to be nuclear export defective [34], would lead to *in vivo* tumorigenesis. It was previously thought that disease-causing mutations typically abrogate the tumor suppressor function of VHL by targeting the HIF α -binding region or the region required for formation of the VBCul2 E3 ubiquitin ligase complex. It is now clear that altering the dynamic profile of VHL, without affecting its E3 ubiquitin ligase function, *per se*, is detrimental to its ability to mediate efficient oxygen-dependent degradation of HIF α .

Subcellular dynamics of VHL & HIF degradation is regulated by extracellular H⁺

As mentioned earlier, VHL exhibits a predominantly cytoplasmic distribution under aerobic

conditions. However, a physiological decrease in the extracellular pH as a consequence of anaerobic respiration triggers a striking relocation of endogenous VHL, and VHL-GFP, to the nucleolus of cells (FIGURE 4). This phenomena was observed both in cultured cells and in the core of human tumors, where acidosis is commonly observed due to a hypoxic microenvironment [52–54]. Nucleolar relocation of VHL also occurs in normoxia following extracellular acidification by metabolically active cells, known as the Warburg effect, which is often observed in cancer cells (FIGURE 4) [52]. The extracellular pH required for relocation of VHL to nucleoli is cell-type-specific, but typically ranges within 6.60 to 5.80, which is well within the range observed in physiological and pathological settings such as development, ischemia and tumorigenesis [53,55,56].

Proteins often change their steady-state subcellular localization while retaining their dynamic profiles. However, using photobleaching techniques, it has been shown that the nucleolar architecture of acidotic cells captures and converts VHL to a static or immobile state [53]. This is in stark contrast to nucleolar resident proteins, such as B23 and fibrillarin, which exchange between subcellular compartments and retain their dynamic properties even in acidotic cells [53]. The detention of VHL in the nucleolus results in a consequent loss of its mobility across the nuclear envelope, and thus of nuclear–cytoplasmic trafficking. Interestingly, VHL remains sequestered in nucleoli, in a static state, even upon reoxygenation of hypoxic cells [52]. However, nucleolar VHL can revert back to its original subcellular localization and resume its dynamic nuclear–cytoplasmic trafficking simply by neutralization of the extracellular milieu [52]. Mapping analysis revealed that a new type of protein localization sequence named NoDS^{H⁺} (nucleolar detention signal regulated by H⁺) is able to detain VHL in the nucleolus [53,57]. The NoDS^{H⁺} is activated following an increase in extracellular hydrogen ion concentration and is inactivated after reversal to neutral pH conditions, causing rapid release of detained VHL into the nucleoplasm, where the protein resumes its dynamic profile [53,57]. Further analysis of the NoDS^{H⁺} revealed that this subnuclear targeting signal generally conforms to three rules: firstly, presence of at least one subnuclear targeting arginine domain RR(I/L)X₃r (STAD); secondly, two or more three-residue subnuclear targeting hydrophobic repeats L Φ (V/L) (STHD); and thirdly, preferential positioning of STADs in

low-disorder regions [57]. Interestingly, after a detailed search, several other proteins involved in basic cellular metabolism were found to harbor a NoDS^{H+} subnuclear targeting signal, including the heat-shock protein HSC70, the antiapoptotic cIAP2 and the catalytic subunit of DNA Polymerase- δ (DNA Pol- δ p125) [53,57]. These proteins have been shown to be targeted for nucleolar sequestration during anaerobic metabolism and acidification of the extracellular milieu in a manner reminiscent to VHL [57].

Extracellular acidosis has recently been identified as a mechanism, apart from hypoxia, by which HIF α can escape VHL-mediated ubiquitylation and proteasomal degradation. When a decrease in extracellular pH triggers nucleolar relocalization of VHL, there is a consequent loss of mobility of VHL within the nucleus and across the nuclear envelope (FIGURE 4) [53]. The pH-induced physical confinement of VHL to nucleoli allows HIF α to escape destruction in the presence of oxygen and activate its target genes (FIGURE 4) [52]. Thus, interfering with the dynamic profile of VHL inhibits its E3 ubiquitin ligase function, since it can no longer mediate HIF α ubiquitylation and degradation. The inactivation of VHL by nucleolar sequestration is insensitive to reoxygenation, allowing for prolonged HIF activation following transient hypoxic stress, and can only be reversed by the reinstatement of neutral extracellular pH [52,58]. Following neutralization of the extracellular pH, the nucleolus rapidly releases VHL from static detention, thereby restoring its mobility and ability to mediate HIF degradation [52,53,58]. A new model is now emerging in which pH-dependent regulation of VHL intersects with the previously identified oxygen-dependent control of HIF α . In fact, this was the first reported evidence that cells have evolved a mechanism to regulate molecular processes by reversibly switching participants, such as VHL, between a mobile and a static state.

Dynamic properties of VHL for other functions

Although much attention has been geared towards HIF regulation, VHL possesses several other HIF-independent as well as HIF-dependent functions that may contribute to tumor suppression. VHL associates with microtubules and has been shown to be involved in microtubule stabilization [6]. VHL protects microtubule depolarization *in vivo*, and thus maintains microtubule dynamics. The naturally occurring VHL point mutants Y98H and Y112H, which are associated

with type 2C VHL disease that predisposes individuals to development of adrenal gland tumors and cerebellar haemangioblastomas, disrupt the microtubule-stabilizing function of VHL [6]. VHL is also required for deposition of extracellular matrix (ECM) components, fibronectin and collagen IV. In addition, VHL-deficient cells exhibit increased activity of NF- κ B, a family of transcription factors that promotes tumor growth [59,60]. VHL was shown to be a kinase adaptor for the regulation of NF- κ B, which is required for suppression of tumorigenesis [61]. Loss of VHL results in cells with abnormal ECM deposition, which is the structural scaffolding surrounding cells, and may promote angiogenesis and invasion and metastasis of tumor cells [7]. Recently, VHL has also been implicated in the HIF-dependent regulation of E-cadherin, a protein involved in cell adhesion. Reduction of E-cadherin showed a marked increase in the invasiveness of RCC cells that were restored with functional VHL, implying a possible involvement for E-cadherin in promoting malignancy in RCC [62,63]. Together, these findings establish that the proper functioning of VHL is essential for many aspects of cell integrity. The involvement of VHL dynamic properties in these avenues has not yet been fully explored. However, considering the vital role of VHL dynamics for its proper functioning, it would not be surprising that subcellular trafficking and intracompartamental dynamics are also integrated in these pathways. For example, nucleolar sequestration of VHL function may trigger senescence, in a manner reminiscent to loss-of-function mutations [64]. It would be interesting, in the future, to test the impact of VHL dynamics on these aforementioned processes.

Conclusion

The advent of new technologies such as photo-bleaching in the field of cell biology has led to major conceptual changes in our understanding of protein dynamics and subcellular trafficking [65]. Previously, proteins were thought to be relatively immobile entities, localizing and functioning in distinct compartments. In recent years, it has become apparent that the function of many proteins is dependent on their dynamic profile. The VHL tumor suppressor serves as an excellent model to this emerging concept. It is now known that the transcription-dependent nuclear–cytoplasmic trafficking of VHL is essential for nuclear ubiquitylation and subsequent cytoplasmic degradation of HIF α . Mutations targeting subcellular trafficking or

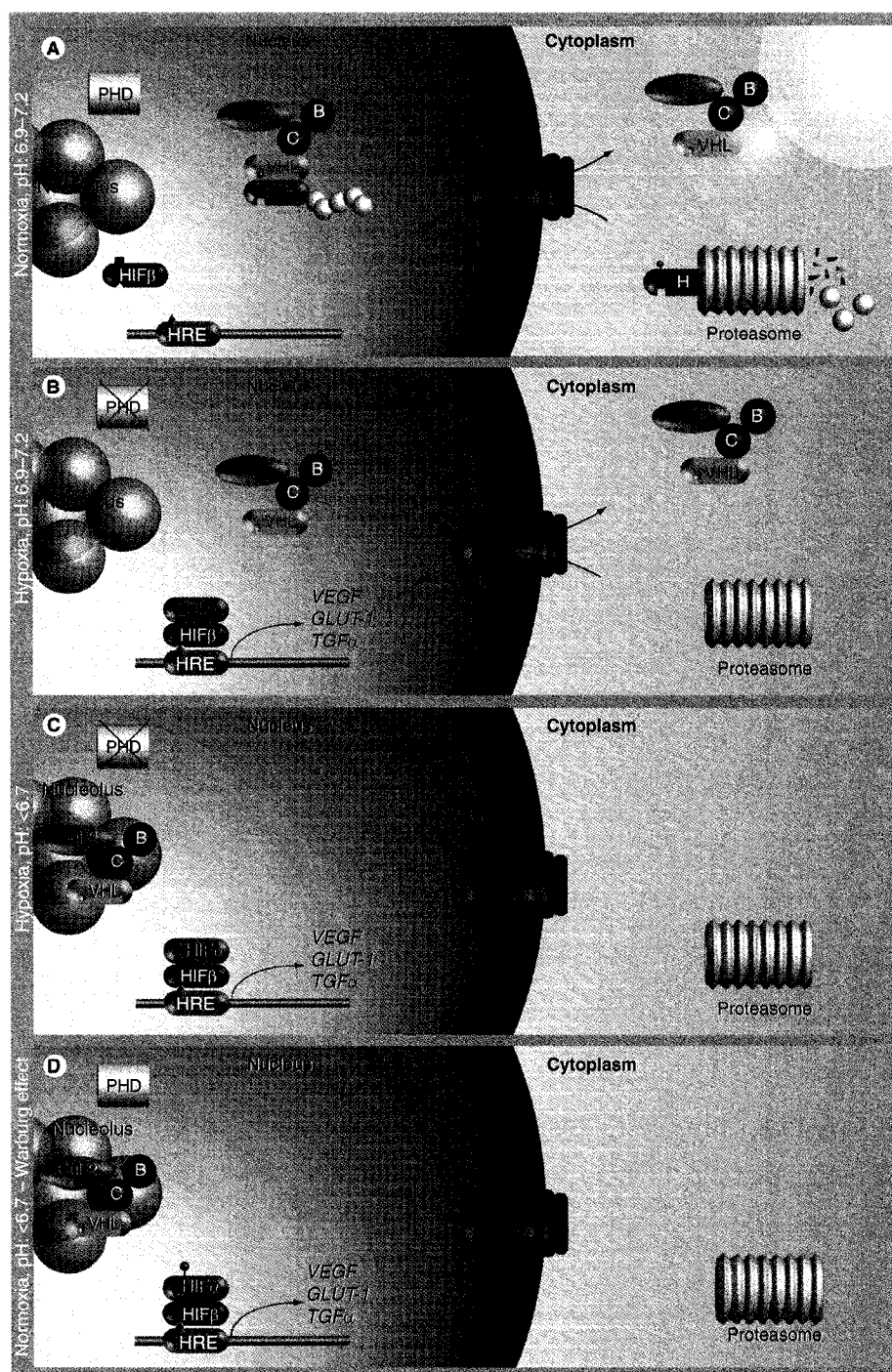


Figure 4. pH-dependent nucleolar detention of VHL prevents subcellular trafficking and HIFα degradation. (A) Under normal oxygen and pH levels, VHL shuttles continuously between the nucleus and cytoplasm to mediate efficient HIFα degradation. (B) During hypoxia, HIFα evades recognition by VHL and is stabilized. (C) Hypoxia-induced acidosis triggers the static detention of VHL in the nucleolus, preventing its subcellular trafficking and ability to degrade HIFα. (D) Acidosis can also be achieved in normoxic conditions by the Warburg effect. C and B: Elongins B and C; Cul-2: Cullin-2; GLUT-1: Glucose transporter 1; HIF: Hypoxia-inducible factor; HRE: Hypoxia-response element; PHD: Prolyl hydroxylase; TD-NEM: Transcription-dependent nuclear export motif; TGF-α: Transforming growth factor-α; VHL: von Hippel-Lindau.

the immobilization of VHL protein dynamics by pH-dependent nucleolar sequestration can abrogate function. Together, these recent findings provide evidence that the oxygen-/pH-regulated shuttling dynamics of VHL are instrumental for functional integrity, and are essential in the control of HIF activation, oxygen homeostasis and suppression of tumorigenesis.

Future perspective

The TD-NEM pathway utilized by VHL, among others, represents a novel pathway for protein export from the nuclear compartment. Studies aimed at dissecting this nuclear export pathway and elucidating the mechanism by which RNA PolII inhibitors abolish nuclear export activity should aid in further understanding the subcellular trafficking properties of VHL. Investigations are also due in deciphering the mechanism of NoDS^{H+}-mediated nucleolar detention, and will help in understanding the physiological regulation of VHL subcellular dynamics. It is thought that cellular adaptation to hypoxia and acidosis plays a central role in the evolution of malignancies. A common outcome of the inherent property of cancer cells to produce lactate, either as a consequence of hypoxia or the Warburg effect, is the acidification of their extracellular milieu *in vivo*. NoDS^{H+}-mediated

targeting of VHL to nucleoli has been observed, not only in cultured cells, but also in the acidic core of tumors [52,54]. The presence of nucleolar VHL in tumors not associated with VHL disease suggests that this may be as a general program to enable cell survival within the harsh conditions of the tumor microenvironment while maintaining the ability to promote angiogenesis, which is required for tumor growth. Alterations in pH or mutations disrupting pH-responsiveness of VHL may have various implications, particularly since HIF is strongly integrated in disease as well as normal physiology. It would be interesting to test if variants of VHL that fail to be captured by the nucleolus would maintain HIF regulation during *in vivo* acidosis. Characterization of the exact mechanism of VHL nucleolar targeting may, in the future, uncover novel cancer therapeutics.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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Executive summary

Subcellular trafficking of E3 ubiquitin ligases

- Nuclear export of E3 ubiquitin ligases is often required for ubiquitin-mediated degradation of nuclear substrates.

Transcription-dependent subcellular trafficking of VHL

- The von Hippel-Lindau (VHL) tumor suppressor protein exhibits a predominantly cytoplasmic localization, but accumulates in the nucleus in the presence of inhibitors of RNA PolII-dependent transcription.
- VHL engages in nuclear-cytoplasmic shuttling dynamics that require ongoing RNA PolII activity.
- Transcription-dependent trafficking of VHL is essential for efficient oxygen-dependent degradation of the α -subunit of the hypoxia-inducible transcription factor (HIF α).

Nuclear export of VHL by TD-NEM: a novel transport pathway

- Transcription-dependent trafficking of VHL is mediated by a novel nuclear export motif, the transcription-dependent nuclear export motif (TD-NEM), encoded by the consensus sequence DxGx₂Dx₂L.
- Naturally-occurring and cancer-causing mutations target key conserved residues in TD-NEM and alter the subcellular trafficking dynamics of VHL.
- Disease-causing mutations of key TD-NEM residues restrain the ability of VHL to efficiently mediate oxygen-dependent degradation of HIF α by altering its nuclear export dynamics without affecting the VHL/HIF interaction.

Subcellular dynamic of VHL & HIF degradation is regulated by extracellular H⁺

- A decrease in extracellular pH as a consequence of anaerobic metabolism results in the relocation and static detention of VHL in the nucleolus.
- pH-dependent targeting of VHL to nucleoli is mediated by the NoDS^{H+}.
- During anaerobic metabolism, the physiological increase in extracellular H⁺ prevents the subcellular trafficking of VHL and leads to the stabilization of HIF α regardless of oxygen tension.

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APPENDIX B- Coauthor Publications and Published Highlights

Perspectives

Oxygen Sensing by H^+

Implications for HIF and Hypoxic Cell Memory

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ABSTRACT

Hypoxia and acidosis are common features of several physiological and pathological situations, including cancer and stroke. The HIF (hypoxia-inducible factor) transcription factor plays a seminal role in orchestrating cellular responses to alterations in oxygen availability. HIF is degraded in normal oxygen tension by the VHL (von Hippel-Lindau) tumor suppressor protein but stabilized by hypoxia to activate an array of genes implicated in oxygen homeostasis including vascular endothelial growth factor. Cells respond to a comparatively mild decline in oxygen tension by converting to an anaerobic state of respiration and secreting lactic acid. We recently reported that a decrease in environmental pH triggers sequestration of VHL into the nucleolus neutralizing its ability to degrade HIF. This implies that cells have evolved a parallel mechanism of HIF activation that responds to changes in oxygen levels by sensing extracellular $[H^+]$. Here we discuss the implications of this new VHL regulatory mechanism on oxygen homeostasis and hypoxic cell memory.

Ischaemia occurs in cardiac arrest, stroke, muscle stress, cancer, and normal development.¹⁻⁴ Within these physiological and pathological settings, the HIF (hypoxia-inducible factor) transcription factor activates genes implicated in cellular response to low oxygen availability. Among others, HIF induces genes that modulate angiogenesis, glycolysis, growth and pH regulation.⁵⁻⁷ In the presence of oxygen, the α subunit of HIF (HIF α) is post-translationally modified at key proline residues by enzymes known as prolyl hydroxylases (PHDs) (Fig. 1A).⁸⁻¹² This allows the VHL tumor suppressor, a component of an E3 ubiquitin ligase (Fig. 1A), to recognize HIF and target it for degradation (refs. 8-24 and reviewed in ref. 25). PHDs require molecular oxygen and hypoxia prevents hydroxylation of HIF.^{8-12,26,27} This has led to the notion that PHDs act as "oxygen sensors" gauging oxygen tension and determining whether cells should engage in HIF activation.

Cells adapt to a decrease in oxygen tension by engaging in metabolic fermentation following an increase in the glycolytic rate. This results in acidification of the extracellular milieu due to excess production of lactic acid. Acidosis has been reported to be more than an innocent bystander in this process. In fact, low pH values exert protective effects under numerous physiological and pathological settings.²⁸⁻³² This led us to investigate the VHL-HIF system in hypoxic cells that are allowed to undertake the natural route to acidosis.³³ We found that hypoxia-induced acidosis triggered relocalization of VHL from a diffuse nuclear-cytoplasmic pattern to nucleoli (Fig. 1B).³³ VHL remained confined to nucleoli upon reoxygenation but reverted to its original nuclear-cytoplasmic distribution when neutral pH conditions were reinstated. Nucleolar localization of VHL also occurred in normoxia following extracellular acidification by metabolically active cells (Warburg Effect).^{34,35} Nucleolar sequestration of VHL stabilized HIF and activated its targets. Therefore, we reasoned that an increase in the levels of hydrogen ions might trigger nucleolar sequestration of VHL in hypoxia or normoxia to elicit a mechanistically reversible loss of VHL function followed by HIF activation.

We propose a model in which pH-dependent regulation of VHL intersects with the previously identified oxygen-dependent control of HIF. Acidosis is observed in ischaemic tissues and is a function of the hypoxic stress as defined by the severity and the duration of the decrease in oxygen concentration as well as the microenvironmental buffering capacity. Once achieved, acidosis triggers nucleolar confinement of VHL abolishing its ability to degrade HIF. Nucleolar sequestration-dependent inactivation of VHL is refractory to oxygen levels but not to the reinstatement of neutral extracellular pH. We thus speculate that prolonged HIF stabilization by acidosis-dependent nucleolar sequestration of VHL serves as a mechanism of hypoxic cell memory (Fig. 2) that enables cells to discount

the effect of reoxygenation. Since acidosis is known to protect cells against several different toxic effects including anoxic cell killing and glucose starvation,²⁸⁻³² it is reasonable to suspect that cells would benefit from a hypoxic cell memory. In accordance with this concept, the levels of HIF and the products of its target genes remain elevated following ischaemia-reperfusion, and this is associated with several protective effects.^{36,37}

A closer examination of our model (Fig. 2) identifies a central role for pH-dependent regulation of VHL function in oxygen sensing. Cells can engage in anaerobic fermentation and secrete lactic acid once oxygen concentration decreases by 50–70% of normal levels.³⁸ This argues that cells can sense mild hypoxia and produce sufficient H⁺ to trigger HIF activation before PHD inactivation (Fig. 2).^{11,12,38} According to this model, a “respiratory enzyme” would act as the oxygen sensor and regulate HIF activation by directing cells towards the citric acid cycle or fermentation as originally postulated by Otto Heinrich Warburg.^{34,35}

Disruption of the extracellular matrix is thought to facilitate tumor vascularization. In addition to causing unregulated HIF activation, loss of VHL function also disrupts fibronectin deposition.³⁹⁻⁴² Therefore, inactivation of VHL by nucleolar sequestration may serve the dual role of preventing extracellular matrix assembly and promoting HIF activation in hypoxic-acidotic cells thereby crafting a highly favourable environment to stimulate angiogenesis. This is supported by mathematical models predicting a role for H⁺ in tumor development, including disruption of the extracellular matrix.^{43,44} Nucleolar sequestration may also abolish uncharacterized VHL functions or even yet allow VHL to assume new duties within the nucleolus. It will be of outmost importance to test whether key VHL functions, in addition to its regulatory role in HIF activation, are affected by the localization of this tumor suppressor to the nucleolus.

Inactivating mutations within the *VHL* gene are associated with von Hippel-Lindau disease—a cancer syndrome predisposing individuals for the development of retinal angioma, central nervous system hemangioblastoma, pheochromocytoma and renal clear cell carcinoma (RCC).²⁵ Loss of HIF regulation by VHL is believed to play a role in cancers retaining wild-type VHL through inactivation of PHDs in hypoxic tumors.⁵ Since acidosis is commonly observed within the core of tumors, it is conceivable that inactivation of VHL may occur by pH-dependent nucleolar sequestration. This would imply that VHL-expressing tumors are not under any selective pressure to acquire inactivating mutations within this gene since its product can be efficiently inactivated by acidosis.

Figure 2. Oxygen sensing by H⁺ and hypoxic cell memory. A moderate decrease in oxygen concentration is accommodated by a shift to metabolic fermentation leading to acidification of the extracellular environment through excess lactic acid production. Extracellular acidosis leads to redistribution of VHL to nucleoli and stabilization of HIF α prior to inactivation of PHDs. Further decrease in oxygen prevents hydroxylation of HIF α . Reoxygenation of acidotic cells reactivates PHDs without affecting nucleolar VHL. This prolongs HIF stability and hypoxic cell memory until neutral conditions are restored.

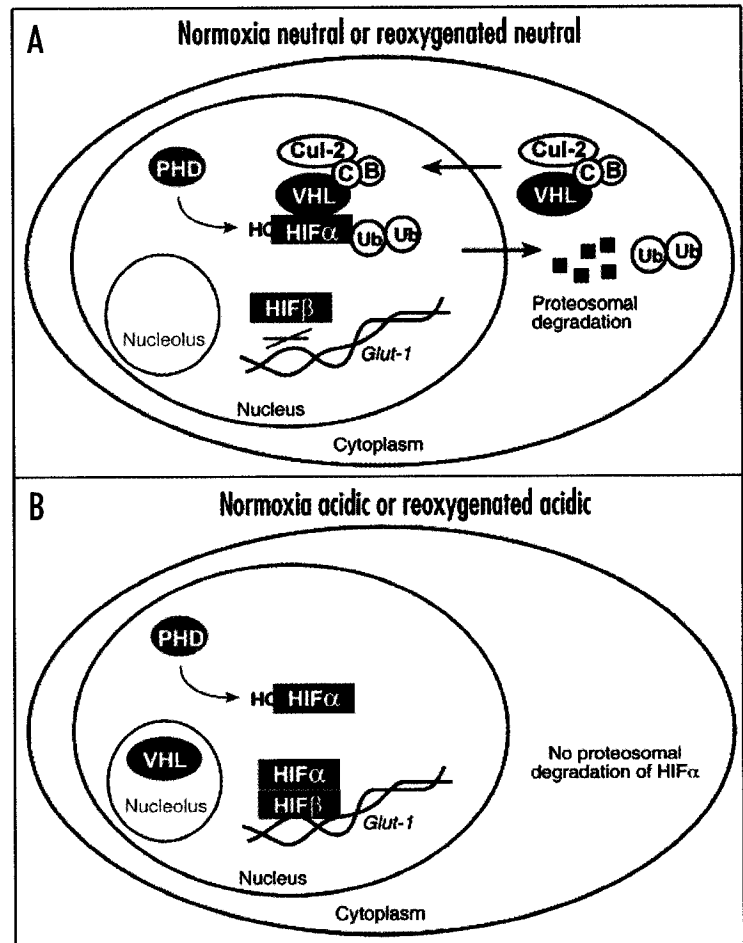
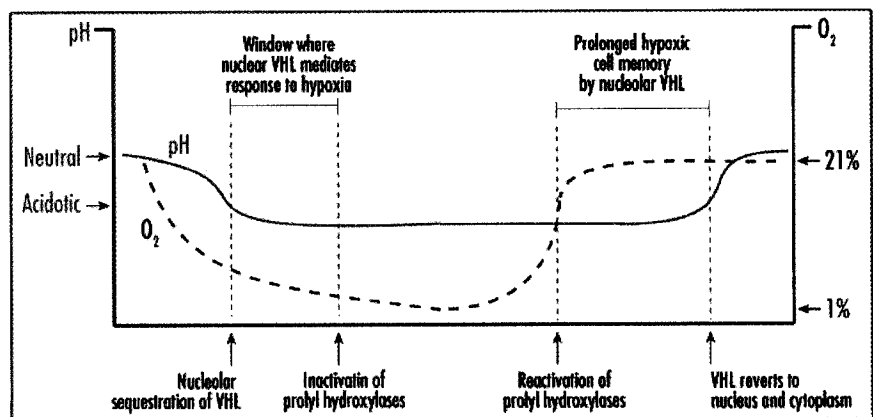


Figure 1. HIF Regulation by VHL. (A) In the presence of oxygen, prolyl hydroxylase (PHD) post-translationally modifies HIF α to increase its affinity towards VHL. The latter acts as a recognition motif of an E3 ubiquitin ligase complex, which includes cullin-2, elongins B and C. VHL targets hydroxylated HIF α for cullin-2-mediated ubiquitination in the nucleus and subsequent cytoplasmic degradation.^{14,49} Hypoxia inhibits PHD allowing HIF α to heterodimerize with the constitutively expressed HIF β and activates HIF target genes, including glucose transporter-1 (*Glut-1*). (B) Hypoxia-induced acidosis results in a pH-dependent relocalization of VHL to nucleoli and stabilization of HIF. Nucleolar sequestration of VHL prevents it from targeting HIF for degradation in reoxygenated acidotic cells even in the presence of reactivated PHD. VHL remains in the nucleolus until neutral pH conditions are restored. Nucleolar sequestration of VHL following reoxygenation of hypoxic-acidotic cells prolongs HIF stability and hypoxic cell memory. Although formation of the E3 ligase complex is not required for nucleolar localization of VHL, the subcellular distribution of cullin-2, elongins B and C is still unknown under acidosis.



In a 1999 *Nature* "News and Views", Jeffrey Bachant and Stephen Elledge hypothesized that the nucleolus can serve as an organelle by which cellular processes can be regulated through physical confinement of proteins.⁴⁵ Our data are consistent with this hypothesis and suggest a common pH-dependent regulatory mechanism of VHL function. This regulatory process is similar to that observed in another system in which the MDM2 E3 ubiquitin ligase is confined to nucleoli to prevent the degradation of p53 in response to physiological cues.⁴⁶⁻⁴⁸ Therefore, the nucleolus is implicated in both the activation and inactivation of tumor suppressor proteins, depending on the stress signal.^{33,46-48}

In conclusion, our recent study identifies nucleolar sequestration as a mechanism of inactivation of HIF destruction by VHL. Due to the abundance of evidence supporting the role of HIF in disease and normal physiology, alterations in pH, or mutations affecting the pH-responsiveness of VHL, may therefore have various important implications. The challenge will be to identify the role of nucleolar VHL within such physiological settings in vivo. Furthermore, it is possible that nucleolar sequestration of VHL might occur in response to signals other than acidosis, implying a more complex system of oxygen sensing than previously appreciated. Characterization of the exact mechanism of nucleolar localization might assist in the identification of such signals and could uncover novel therapeutic targets.

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Regulation of ubiquitin ligase dynamics by the nucleolus

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Cellular pathways relay information through dynamic protein interactions. We have assessed the kinetic properties of the murine double minute protein (MDM2) and von Hippel-Lindau (VHL) ubiquitin ligases in living cells under physiological conditions that alter the stability of their respective p53 and hypoxia-inducible factor substrates. Photobleaching experiments reveal that MDM2 and VHL are highly mobile proteins in settings where their substrates are efficiently degraded. The nucleolar architecture converts MDM2 and VHL to a static state in response to regulatory cues that are associ-

ated with substrate stability. After signal termination, the nucleolus is able to rapidly release these proteins from static detention, thereby restoring their high mobility profiles. A protein surface region of VHL's β -sheet domain was identified as a discrete [H⁺]-responsive nucleolar detention signal that targets the VHL/Cullin-2 ubiquitin ligase complex to nucleoli in response to physiological fluctuations in environmental pH. Data shown here provide the first evidence that cells have evolved a mechanism to regulate molecular networks by reversibly switching proteins between a mobile and static state.

Introduction

Conjugation of ubiquitin (ubiquitylation) to proteins destines them for very different fates in the cell (Weissman, 2001; Muratani and Tansey, 2003; Ciechanover, 2005). Although targeting proteins for degradation via the 26S proteasome is the best-studied role of ubiquitylation, this modification is integral to several biochemical pathways including receptor internalization (Terrell et al., 1998), chromatin maintenance (Muratani and Tansey, 2003) and DNA repair (Russell et al., 1999; Gillette et al., 2001). The ubiquitin system is sustained by the interaction of multiple dynamic molecular networks that begin with the loading of ubiquitin onto an ubiquitin-activating enzyme (E1). The ubiquitin moiety is then transferred to a ubiquitin-conjugating enzyme (E2), and finally, a ubiquitin protein ligase (E3) catalyzes the transfer of ubiquitin from E2 to the lysine residue of a specific substrate, thereby altering its cellular fate.

There are many more E3s in the cell than there are E1s and E2s combined, and it is thought that E3s determine the

specificity of substrate recognition within the ubiquitin system. The function of a ubiquitin ligase can be regulated by controlling the ligase or its substrate at various levels such as post-translational modifications, interactions with regulatory factors, or subcellular localization (Petroski and Deshaies, 2005).

The complexity of E3 regulatory mechanisms is well demonstrated by the mechanisms controlling the degradation of the p53 tumor suppressor protein (Michael and Oren, 2003). The murine double minute protein MDM2 ubiquitin ligase targets p53 for ubiquitylation in the nucleus followed by nuclear export and degradation by cytoplasmic 26S proteasome (Momand et al., 1992; Oliner et al., 1993; Freedman and Levine, 1998; Roth et al., 1998). Various signals can alter the function of MDM2 within this setting. DNA damage rapidly activates the ataxia telangiectasia mutated protein, which phosphorylates MDM2 to prevent the ubiquitylation of p53 (Appella and Anderson, 2001). Replicative senescence induces the tumor suppressor ARF to bind MDM2 and inactivate it by both immediately reducing its ability to recognize p53 in the nucleoplasm (Llanos et al., 2001) and translocating MDM2 to the nucleolus (Tao and Levine, 1999; Weber et al., 1999), a major nuclear compartment (Carmo-Fonseca et al., 2000). Similarly, perturbations to ribosomal biogenesis induce the ribosomal protein L11 to bind MDM2 and inhibit its function by relocating it to the nucleolus (Lohrum et al., 2003).

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Abbreviations used in this paper: ActD, actinomycin D; AP, acidification-permissive; B23, rRNA processing-factor nucleophosmin; FIB, rRNA processing factor fibrillarin; FLIP, fluorescence loss in photobleaching; HIF, hypoxia-inducible factor; iFRAP, inverse FRAP; MDM2, murine double minute protein; NES, nuclear export sequence; NoDS^{H+}, [H⁺]-responsive nucleolar detention signal; NoLS, nucleolar localization sequence; NoRS, nucleolar retention sequence; PEG, polyethylene glycol; RS, ribosomal stress; SD media, standard media; VHL, von Hippel-Lindau.

The online version of this article contains supplemental material.

Functional regulation of E3s by the nucleolus has also been observed in the von Hippel-Lindau (VHL) tumor suppressor/hypoxia-inducible factor (HIF) system (for review see Kaelin, 2002; Mekhail et al., 2004a). HIF activates an array of genes that mediate cellular response to low oxygen availability (Semenza, 2000). In the presence of oxygen, the α subunit of HIF (HIF α) is post-translationally modified by enzymes known as prolyl hydroxylases (PHDs). This allows the VHL tumor suppressor, the particle recognition motif of an elongin C/Cullin-2 ubiquitin ligase, to recognize HIF α and target it for nuclear ubiquitylation. VHL-mediated shuttling of HIF α to the cytoplasm then results in its destruction by the 26S proteasome (Lee et al., 1999; Groulx and Lee, 2002) in a manner reminiscent of the MDM2/p53 system. Several physiological cues can modulate the function of VHL within this setting. PHDs require molecular oxygen and hypoxia prevents hydroxylation of HIF, allowing it to evade recognition by VHL and degradation. In addition, we previously reported that a decrease in environmental pH triggers the relocation of VHL to the nucleolus, neutralizing its ability to degrade nuclear HIF even in the presence of oxygen (Mekhail et al., 2004a,b).

The nucleolus has traditionally been viewed as a factory for the production of ribosomes (Lam et al., 2005). More recently, this nuclear compartment has been linked to numerous cellular activities including cell cycle control (Shou et al., 1999, 2001; Visintin et al., 1999; Azzam et al., 2004), DNA damage repair (van den Boom et al., 2004), and tRNA processing (Paushkin et al., 2004). Although the nucleolus has a distinct set of "resident" proteins, it is now clear that these proteins are in continuous flux between the nucleolus and other cellular compartments (Dundr et al., 2000, 2002; Phair and Misteli, 2000; Chen and Huang, 2001; Misteli, 2001; Carmo-Fonseca, 2002; Andersen et al., 2005; Tsai and McKay, 2005). This dynamic nature is facilitated by a fundamental characteristic of nuclear compartments; that is the lack of a delineating membrane. For example, thousands of molecules of the rRNA processing factor fibrillarin (FIB), which displays steady-state nucleolar localization, exit the nucleolus each second (Phair and Misteli, 2000). The highly dynamic properties of proteins in the nucleus follow a stochastic model of high molecular mobility to ensure efficient functional interactions (Misteli, 2001). An advantage of such probabilistic movement is the ability to achieve rapid responses to signaling cues. For example, a slight increase in the quantity of a modified protein results in a relatively high probability of encountering its target. As mentioned above, resident nucleolar proteins are dynamic molecules that can functionally engage in subcellular trafficking between the nucleolus and other cellular compartments. Therefore, it remains unclear how the highly dynamic nucleolus inactivates the function of E3 enzymes, as these macromolecules would be predicted to retain their dynamic nature and maintain functional molecular interactions.

Here, we report the unexpected observation that the nucleolar architecture is able to reversibly capture and alter the dynamic properties of ubiquitin ligases. We show that VHL and MDM2 are highly mobile proteins that can be statically detained by the nucleolus to prevent functionally required molec-

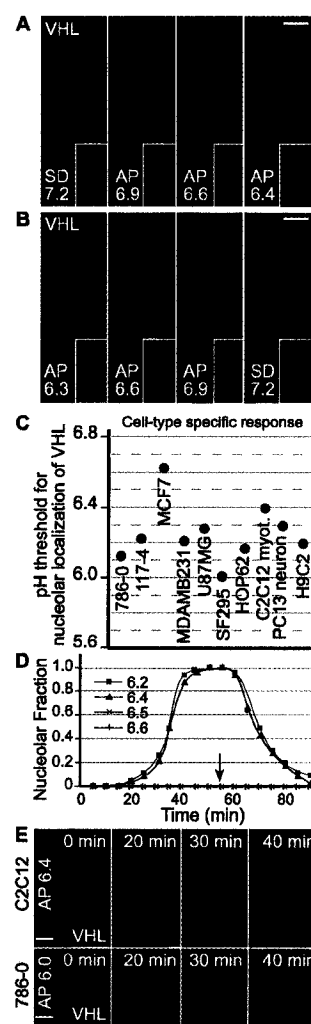


Figure 1. pH-dependent kinetics of VHL subcellular trafficking. (A) C2C12-differentiated myotubes were cultured in standard (SD) media and infected to express adenovirus-introduced VHL-GFP. Cells were replenished with either fresh SD media (pH 7.2) or different AP media (initial pH 7.2) that allow maximal extracellular acidification to pH 6.9, 6.6, 6.4, 6.2, or 6.0 (see Materials and methods). Cells were transferred to hypoxia (1% O₂) for 18 h. VHL redistributed to nucleoli only in conditions of pH 6.4 or lower (images of pH 6.2 and 6.0 are not depicted). Extracellular pH is indicated on each panel. Insets show Hoechst staining of DNA. Bars (A, B, and E), 10 μ m. (B) After localization of VHL-GFP to the nucleolus, myotubes were replenished with AP media previously maximally acidified to indicated pH levels, or with fresh SD media. VHL reverted to nucleocytoplasmic distribution only when the pH of the media was higher than 6.4. (C) Different cell types were treated as in A and B to identify the pH thresholds required for nucleolar targeting of VHL ($n = 40$ for each tested pH value). (D) Kinetics of VHL nucleolar localization in acidosis and release after neutralization in differentiated myotubes. Cells were exposed to different AP media as in A to induce VHL nucleolar redistribution, and all media were neutralized to pH 7.2 by addition of NaOH (arrow). Time zero indicates time at which the pH of AP media reached 6.4. For the 6.5 and 6.7 sets, time zero is matched to that of the pH 6.4 set. (E) Cells were incubated in AP media (initial pH 7.2) that blocks acidification beyond pH 6.0. After reaching the acidification level matching the pH threshold required to trigger nucleolar targeting of any given cell-type (time zero), the localization of VHL-GFP was monitored over time. Upon reaching the pH threshold, VHL shifts from mainly cytoplasmic to mainly nucleoplasmic before triggering nucleolar localization.

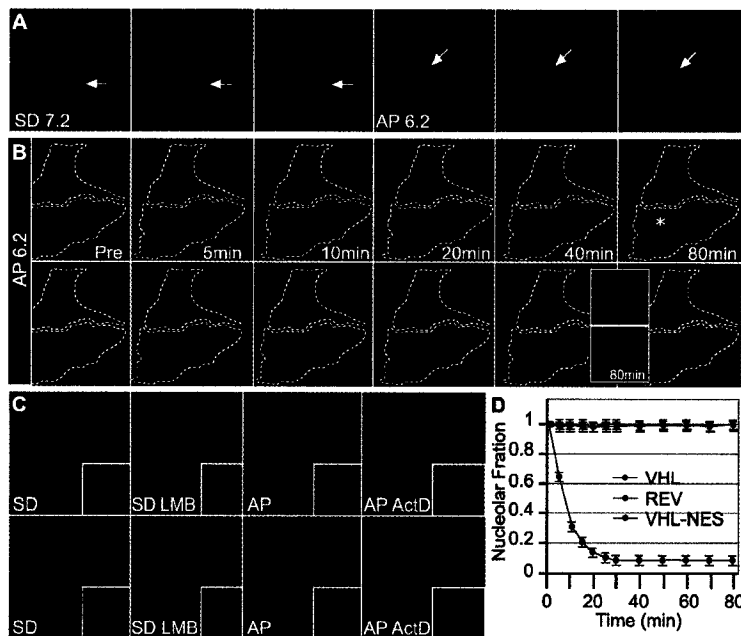


Figure 2. Kinetics of nucleolar VHL and REV. (A and B) MCF7 cells cotransfected to express VHL-GFP and REV-BFP were incubated in hypoxia in SD or AP conditions. After localization of VHL-GFP to the nucleolus, cells were either fixed and immunostained for nucleolar B23 (A) or treated with 10 μ g/ml ActD and monitored over time (B). Arrows in A indicate the same position in the cell and dashed cellular outlines are shown in B. Insets in B show a time-matched set that was not treated with ActD. (C and D) VHL-GFP-NES fusion protein is not released from nucleoli after ActD treatment. MCF7 cells were transfected to express VHL-GFP, VHL-GFP-NES, or GFP-NES alone and transferred to hypoxia in SD or AP media. Shown are images 50 min after AP media reached the pH 6.5 threshold. Where indicated, leptomycin B (LMB) was added when AP media reached pH 6.65 (usually 30–40 min) before pH 6.5 time point. Insets in C show Hoechst staining of DNA. (D) Ratiometric fluorescence measurements of hypoxic-acidotic cells initially displaying nucleolar REV-BFP, VHL-GFP, and VHL-GFP-NES after ActD treatment.

ular interactions in response to physiological cues. Based on these data, we suggest that cells have evolved a mechanism to regulate the function of proteins by reversibly switching them between mobile and static states.

Results

[H⁺]-regulated kinetics of VHL subcellular trafficking

Ischemic tissues or hypoxic cells normally acidify their extracellular milieu as a physiological consequence of anaerobic glycolysis. This is best exemplified by muscle fatigue, in which myotubes produce lactic acid after exposure to hypoxia. Study of the ubiquitin ligase component VHL within this setting revealed its functional regulation by changes in environmental H⁺ concentrations (Mekhail et al., 2004a). VHL engages in nuclear/cytoplasmic trafficking in neutral conditions but accumulates in the nucleolus upon a decrease in extracellular pH, a process that results in stabilization of its substrate HIF. Differentiated myotubes can be incubated in standard (SD) media, which prevents fluctuations in pH, or in acidification-permissive (AP) media, which is prepared to enable hypoxic cells to acidify their extracellular milieu to varying degrees (see Materials and methods) (Mekhail et al., 2004a). VHL-GFP is observed in its typical diffuse nuclear cytoplasmic distribution under neutral pH conditions, independent of oxygen tension (Fig. 1 A, left; Mekhail et al., 2004a). A rapid redistribution of VHL-GFP to nucleoli was observed only when hypoxic myotubes were incubated in AP media that allow the myotubes to acidify their environment to pH 6.40 or lower (Fig. 1, A and D; see Fig. S1, A and B, for cell viability controls, available at <http://www.jcb.org/cgi/content/full/jcb.200506030/DC1>), indicating the existence of a pH threshold required for triggering nucleolar localization of VHL. The threshold displayed cell type-

specific differences within the 6.60–5.80 pH range (Fig. 1 C; Fig. S2). VHL efficiently reverted to a diffuse nuclear-cytoplasmic localization under hypoxic conditions only when cells were replenished with media at values higher than the pH threshold required to trigger nucleolar localization (Fig. 1, B–D). Closer examination of this system reveals that the relocation of VHL to the nucleolus is a two-step process, where the protein displays an initial shift from mainly cytoplasmic to mainly nucleoplasmic localization before initiating any detectable targeting to the nucleolus, in all tested cell lines (Fig. 1 E; Fig. S1, C and D). These data suggest that the subcellular trafficking dynamics of VHL are regulated by a multilayered cellular mechanism that gauges environmental hydrogen ion concentrations.

Different kinetics of nucleolar VHL and resident nucleolar proteins after transcriptional inhibition

Due to the role of the nucleolus in ribosomal biogenesis, perturbations to transcription, such as by treatment with low levels of actinomycin D (ActD), alter the trafficking properties of steady-state nucleolar proteins between the nucleolus and the nucleoplasm (Chen and Huang, 2001; Andersen et al., 2005). For example, the human immunodeficiency virus (HIV) mRNA exporter REV is a dynamic nucleolar protein that redistributes to the nucleoplasm and cytoplasm after transcriptional inhibition under both neutral (unpublished data; Stauber et al., 1995; Daelemans et al., 2005) and acidic (Fig. 2 A; Fig. 2 B, top) conditions. In contrast, the nucleolar localization of VHL in acidosis persisted in the absence of transcription (Fig. 2 B, bottom). Similar results were obtained in experiments using VHL-BFP and REV-GFP (unpublished data). The ability of REV to rapidly alter its steady-state distribution under these conditions is greatly enhanced by its strong nuclear export sequence (NES). We therefore tested whether fusion of this NES

to VHL would enable it to release from nucleoli of acidotic cells after transcriptional inhibition. VHL-GFP-NES fusion localized almost exclusively to the cytoplasm under neutral conditions but was restricted to nucleoli at steady-state under acidosis (Fig. 2 C). ActD treatment failed to release the VHL-GFP-NES fusion protein from nucleoli (Fig. 2, C and D), suggesting that the subcellular trafficking dynamics of VHL in the nucleolus significantly differ from the dynamics of resident nucleolar proteins.

Physiological regulation of VHL ubiquitin ligase dynamics by the nucleolus

We therefore used FRAP to assess how the nucleolus affects the dynamic properties of GFP-tagged VHL in living cells (Lippincott-Schwartz et al., 2003). Specific cellular regions expressing fusion proteins were bleached with the use of a laser pulse that irreversibly quenches the GFP signal, and the recovery of signal in the bleached area was recorded by time-lapse confocal microscopy. The kinetics and extent of recovery of fluorescence in a cellular region after bleaching are reflective of the dynamics of the studied fluorescent chimeras.

Cells cultured under standard neutral conditions displayed an essentially complete recovery of VHL-GFP fluorescence within seconds of bleaching nucleoplasmic (Fig. 3, A and G) or cytoplasmic (see Fig. 7 D) regions. We first assessed the capacity of the nucleolus to sustain dynamic shuttling under acidosis by monitoring resident nucleolar proteins, such as the rRNA-processing factors fibrillarin (FIB) and nucleophosmin (NPM or B23), as well as the RNA polymerase I preinitiation factor upstream binding factor 1 (UBF1). Acidosis did not alter the steady-state distribution of any of the studied resident nucleolar proteins (Fig. 3, D and E) compared with neutral conditions. In addition, these proteins displayed a rapid pH-independent recovery of fluorescence after bleaching of a single nucleolus within cells with multiple nucleoli (Fig. 3, C and F), indicating dynamic protein shuttling between nucleoli of acidotic cells. In contrast, nucleolar VHL failed to display recovery of fluorescence under the same culture and bleaching parameters (Fig. 3, B and G), suggesting that acidosis alters the mobility profile of VHL. Similar to previous reports, reduction of the temperature from 37 to 22°C did not have any significant effect on the kinetics or extent of recovery of any of the tested proteins in the nucleus or cytoplasm (unpublished data; see Phair and Misteli, 2000). These data suggest that the redistribution of VHL to the nucleolus in response to increases in extracellular hydrogen ion concentrations may alter its general dynamic characteristics in the cell.

The dynamics of VHL were next analyzed by fluorescence loss in photobleaching (FLIP) experiments (Lippincott-Schwartz et al., 2003). In FLIP, a living cell is repeatedly hit with a laser beam in the same region. Loss of fluorescence in an area outside the bleached spot is reflective of protein mobility between that area and the bleached spot. A rapid loss of VHL-GFP fluorescence was observed in essentially the whole nucleus after repetitive bleaching of a small nucleoplasmic region in cells incubated under neutral conditions (Fig. 4 A). Studies presented further in this report (Fig. S3, available at

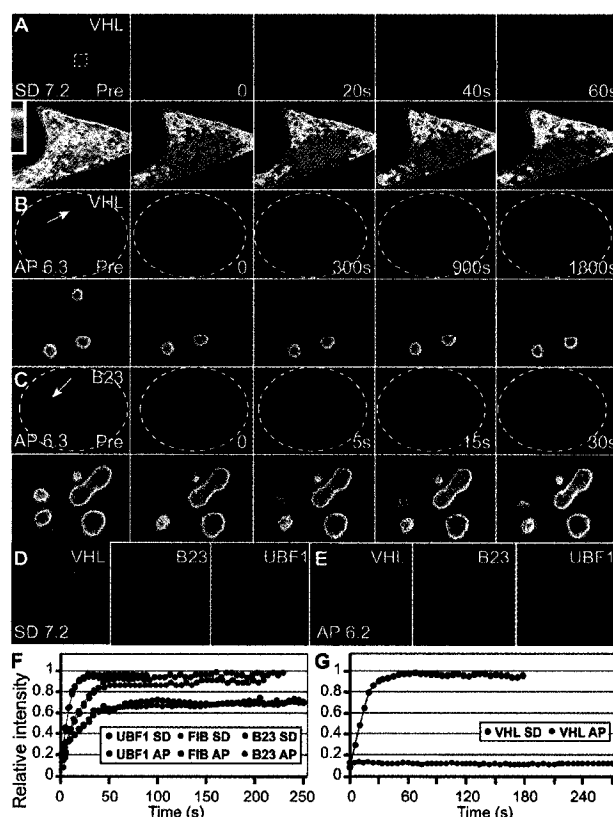


Figure 3. FRAP analysis reveals that VHL does not shuttle between nucleoli in acidosis. MCF7 cells transiently transfected to express low levels of VHL-GFP, B23-GFP, FIB-GFP, or UBF1-GFP were incubated in hypoxia under SD or AP conditions. (A–C) Cells were imaged before and after bleaching of indicated nucleoplasmic regions (dashed square) or specific nucleoli (arrows) within nuclei (dashed circles). Post-bleach time is indicated in seconds. Areas of interest are shown as pseudocolored panels to better illustrate minimal changes in fluorescence. (D and E) Steady-state localization of GFP-tagged proteins after incubation in SD or AP media under hypoxia. (F and G) Quantitation of recovery kinetics. Fluorescence intensity in the bleached region was measured and expressed as relative recovery (see Materials and methods). For quantitation, at least 10 cells were analyzed for each result.

<http://www.jcb.org/cgi/content/full/jcb.200506030/DC1>) will study the nuclear-cytoplasmic trafficking properties of VHL. These observations indicate that VHL participates in dynamic molecular networks.

Next, cells were transfected to express low levels of VHL-GFP to allow for a complete redistribution of the protein chimera to the nucleolus after acidification in hypoxia (Fig. 4 B, first panel; see Mekhail et al., 2004a). Under these conditions, VHL-GFP fluorescence in the nucleolus was unaffected by repetitive bleaching of a nucleoplasmic (Fig. 4, B and F) or cytoplasmic (unpublished data) region. In stark contrast, resident nucleolar proteins rapidly dissociate from the nucleolus in hypoxic-acidotic cells (Fig. 4, C and F). We next examined whether interaction with the nucleolar architecture is required for acidosis-mediated modification of VHL dynamics. Cells were thus transfected to express higher levels of VHL-GFP, saturating nucleolar binding sites and preventing the full redistribution of the fluorescent protein chimera to the nucleolus

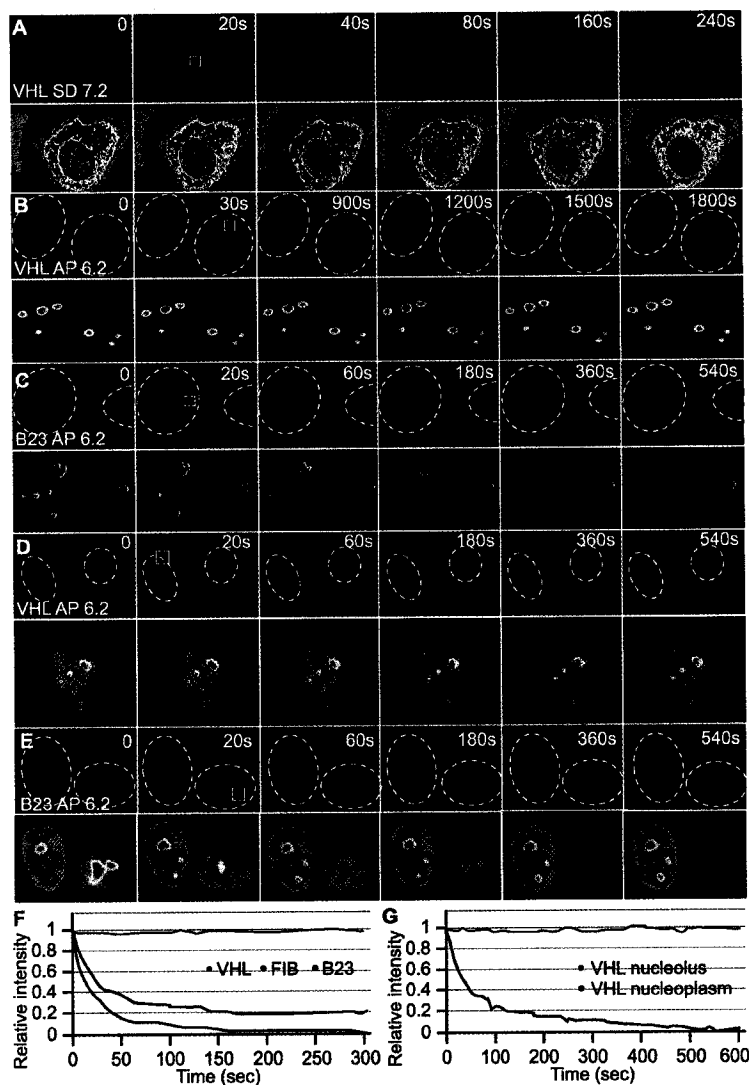


Figure 4. FLIP analysis reveals that nucleolar VHL does not traffic between the nucleolus and nucleoplasm in acidosis. MCF7 cells transiently transfected to express low (A–C, and F) or high (D, E, and G) levels of VHL-GFP or B23-GFP were incubated in hypoxia under SD or AP conditions. At time points matching the relocation of VHL-GFP (low levels set) to nucleoli, all cells were submitted to a FLIP analysis where nucleoplasmic regions (white squares) within specific nuclei (dashed circles) were repeatedly bleached. Cells were imaged between pulses. (F and G) Corresponding kinetics of loss of fluorescence.

after acidification in hypoxia (Fig. 4 D, first panel). This establishes two different protein pools in the cell—nucleoplasmic and nucleolar. Although repetitive bleaching of a nuclear region in these cells resulted in complete loss of nucleoplasmic fluorescence, nucleolar VHL-GFP signal remained constant over the course of the experiment (Fig. 4, D, E, and G). No significant bleaching was observed in neighboring nuclei in all of the experiments. Similar to nuclear VHL-GFP under neutral conditions, the nucleoplasmic pool of VHL-GFP in acidosis was able to engage in nuclear export as revealed by inverse FRAP (iFRAP) analysis of living cells (Lippincott-Schwartz et al., 2003), thereby indicating that this nucleoplasmic pool retains functional interactions with the nucleopore architecture in acidosis (Fig. S3). These data indicate that the interaction of VHL with the nucleolar architecture is required for acidosis-mediated modification of VHL protein dynamics.

We next studied VHL dynamics using polykaryon fusion assays, which provide an alternative approach to photobleaching in assessing changes in subcellular trafficking of proteins (Walther et al., 2003). Cells expressing VHL-GFP were fused in

a standard polyethylene glycol (PEG) fusion assay. VHL remains nuclear-cytoplasmic in polykaryonic cells (Fig. 5 A, a and b; Lee et al., 1999). Transfer to hypoxia resulted in acidification of the media and VHL-GFP displayed its typical two-step localization process to the nucleolus (Fig. 5 A, c–g). It is important to note that nucleolar VHL signal was equally distributed between the nuclei of a polykaryonic cell (Fig. 5 A), indicating that VHL-GFP displays no preference for the nucleoli of one nucleus over another. Next, we cocultured VHL-GFP-expressing and nonexpressing cells under standard conditions, then transferred them to hypoxia in AP media. After the redistribution of VHL-GFP to nucleoli, cells were rapidly fused and replenished with their own acidified AP media. This process yielded a significant number of polykaryonic cells where the fluorescence observed in the cell is only associated with nucleoli of only one or two nuclei, whereas other nuclei displayed no fluorescence (Fig. 5 B). VHL-GFP failed to exhibit any change in localization up to 3 h after fusion. In contrast, under the same conditions B23-GFP (Fig. 5 C) and REV-GFP (unpublished data) redistributed from the nucleoli of a single cell to the nucleocytoplasm and nucleoli of the acceptor

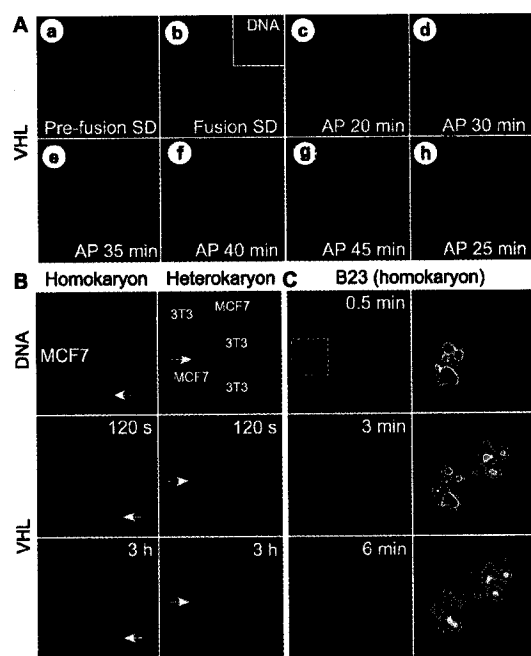


Figure 5. Long-term detention of VHL within the nucleolar space revealed by the inability of VHL to release from nucleoli in a polykaryon fusion assay. (A) MCF7 cells transiently expressing VHL-GFP were fused in a standard PEG fusion assay and incubated in SD media for 30 min (b). Inset shows Hoechst staining of DNA. Cells were replenished with AP media and transferred to hypoxia. VHL-GFP localization was monitored after reaching the pH 6.5 threshold (c–g). Nuclei within a polykaryonic cell were always synchronized in the rates of nucleolar appearance of VHL-GFP. This is not necessarily the case for monokaryonic cells in close proximity under AP conditions (h). (B) Unaltered MCF7 cells were cocultured under standard conditions with either MCF7 (homokaryon assay) or NIH 3T3 (heterokaryon assay) cells transfected to transiently express VHL-GFP. Cells were then transferred to hypoxia in AP media. After nucleolar localization of VHL-GFP, cells were fused and monitored by time-lapse microscopy. Hoechst staining of DNA was used to identify donor and acceptor cells. Arrows indicate the same position in the cell. (C) Unaltered MCF7 cells were cocultured under standard conditions with MCF7 cells transfected to transiently express B23-GFP. Cells were cultured in AP media, fused and monitored as in B. Pseudocolored zooms of area indicated by dashed square are shown.

(nontransfected) cells of polykaryons. In addition to bleaching experiments, results from the fusion assays reveal a role for the nucleolus in regulating the subcellular dynamic profile of the VHL tumor suppressor.

Static detention of MDM2 and VHL ubiquitin ligase by the nucleolus

MDM2 displays a diffuse nuclear localization under standard culture conditions. FRAP and FLIP experiments revealed that MDM2 is a highly dynamic protein within this setting (Fig. 6 A; Fig. S4 A, available at <http://www.jcb.org/cgi/content/full/jcb.200506030/DC1>). MDM2 localizes to the nucleolus in response to perturbations in ribosomal biogenesis after treatment with low levels of ActD that inhibit RNA polymerase I (Fig. 6 B; Fig. S4 B; Lohrum et al., 2003). MDM2 is unable to target p53 for degradation under these conditions. We therefore assessed the dynamics of MDM2 after relocation to the nucleolus. When nucleolar, the dynamic profile of MDM2 significantly changed

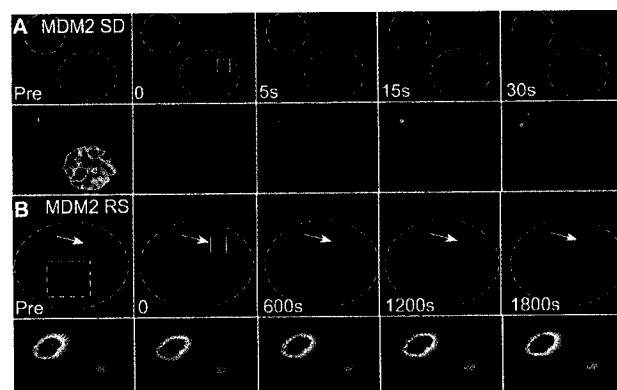


Figure 6. FRAP analysis reveals that the redistribution of MDM2 from nucleoplasm to nucleoli in response to perturbations in ribosomal biogenesis alters general MDM2 dynamic state. MCF7 cells transfected to express low levels of MDM2-GFP were cultured either under standard conditions (A), or ribosomal stress (RS) (B) induced by ActD treatment (see Lohrum et al., 2003 and Materials and methods). Cells were submitted to FRAP analysis as described for VHL in Fig. 3 by bleaching the indicated nucleoplasmic regions (white squares) or specific nucleoli (arrows) within specific nuclei (dashed circles). Pseudocolored zoom of area indicated by dashed rectangle is shown in B.

as GFP fluorescence did not exhibit any recovery/redistribution in FRAP experiments after bleaching of the nucleolus (Fig. 6 B) or loss in FLIP experiments after repetitive bleaching of a nucleoplasmic (Fig. S4 B) or cytoplasmic (unpublished data) region. Similar to VHL (Fig. 4, D and G), the interaction of MDM2 with the nucleolar architecture is required for modification of its trafficking dynamics as evidenced by the quick recovery of MDM2 in the nucleoplasm of transcriptionally inhibited cells expressing high levels of the protein in FRAP (Fig. 7 A). We next evaluated the dynamics of VHL and MDM2 ligases within the nucleolar space. VHL and MDM2 did not exhibit any fluorescence recovery after bleaching of an area within the nucleolus (Fig. 7, A and C). In contrast, B23 remained localized to nucleoli at steady-state within our experimental settings (SD, AP, and RS), retaining its highly mobile properties, though prolonged incubation with ActD resulted in B23 accumulating in the nucleoplasm (Fig. 7 B; unpublished data). Upon photobleaching, a border is created between the bleached area and the gradient of concentration established by the remaining fluorescent molecules (Fig. 7 C). For moving proteins, this border changes its shape as well as its position within the field of vision over time. Statically detained cellular components do not exhibit significant changes in these variables. We therefore compared the characteristics of borders of concentration gradients established by bleaching fluorescently labeled proteins when localized to different regions of the cell. Although analysis of concentration gradient borders in the nucleoplasm and cytoplasm revealed a highly dynamic profile of protein mobility, borders established within the nucleolar space neither changed in shape nor moved within the field of vision for up to 2 h after bleaching (Fig. 7, C–F; unpublished data). A similar static protein profile was observed for MDM2 in the nucleolus (unpublished data). These findings suggest that VHL and MDM2 are targeted for static detention in the nucleolus in response to physiological cues.

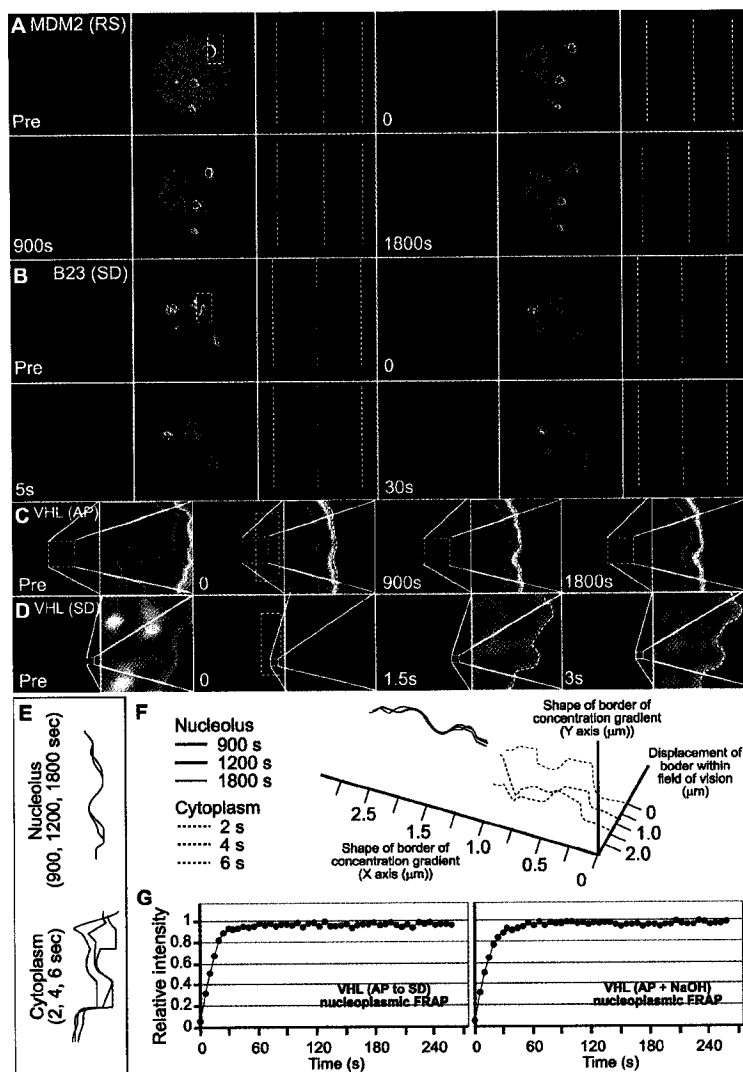


Figure 7. Reversible static detention of VHL and MDM2 by the nucleolar architecture. MCF7 cells were transfected to express GFP-tagged MDM2 (A), B23 (B), or VHL (C–G) and incubated under SD, AP, or RS conditions as indicated. A region representing about half of the nucleolar space (white squares) of a single nucleolus was bleached and cells were monitored by time-lapse fluorescence microscopy. Note the absence of any changes in fluorescence for nucleolar VHL and MDM2. Zoom of a single nucleolus is shown in C. Changes in the shape of the border (dashed white outline set by bleaching) of the protein concentration gradient from different time points as in C and D are overlayed in E. (F) Borders with changing (cytoplasm) or unaltered (nucleolus) shapes (plotted on X and Y axes) were monitored for their displacement away from their original position within the field of vision. Only borders set within the nucleolar space did not exhibit any movement. (G) After hypoxia-induced acidification of AP media and confinement of VHL-GFP to the nucleolus, NaOH was added to neutralize AP media (to pH 7.2) or cells were replenished with fresh SD media (pH 7.2) to induce the reversion of VHL-GFP to the nucleocytoplasm (also see Fig. 1). FRAP analyses in which small nucleoplasmic regions were bleached reveal that VHL-GFP resumes its high mobility profile after a return to neutral pH conditions.

Detention of ubiquitin ligases by the nucleolar architecture is a reversible process

Data presented so far suggest that the nucleolus captures the highly mobile VHL for static detention upon the establishment of cell type-specific extracellular pH threshold. We asked whether this process is reversible and if VHL can be released from nucleoli to recover its highly mobile state. VHL rapidly reverts to a diffuse nuclear cytoplasmic localization only after the reinstatement of neutral pH conditions under hypoxia or normoxia conditions (Fig. 1; Mekhail et al., 2004a). After reversion, fluorescence rapidly recovered after bleaching small nucleoplasmic regions (Fig. 7 G). These data indicate that the regulated inactivation of ligases after targeting to the nucleolus relies on their transient conversion to static participants of particular molecular networks.

Identification of a novel, discrete, pH-responsive nucleolar detention signal
Targeting of proteins to nucleoli is achieved by nucleolar localization sequence (NoLS) or nucleolar retention sequence

(NoRS). These sequences are relatively large and differ considerably from nuclear import or export sequences, which are comprised of only a few amino acid residues. Therefore, we decided to identify the domain of the VBC/Cul-2 complex that mediates [H⁺]-regulated nucleolar sequestration of VHL. VHL is a component of the multi-protein ubiquitin ligase complex that targets the transcription factor HIF for proteasomal destruction. The complex is composed of at least VHL, elongin B, elongin C, Cullin-2, and Rbx1 (VBC/Cul-2) (Fig. 8 A; Kaelin, 2002). The $\Delta C157$ deletion mutant of VHL, which is defective in E3 ligase complex formation (Fig. 8 A), retains the ability to target a GFP reporter to nucleoli in acidosis (Fig. 8 B, first four panels) (Pause et al., 1997; Cockman et al., 2000; Bonicalzi et al., 2001; Mekhail et al., 2004a). In contrast, Cullin-2 failed to target a GFP reporter to nucleoli of VHL-deficient cells under the same conditions (Fig. 8 B). Although these data suggest that complex formation is not required for nucleolar targeting of VHL, immunoprecipitation analysis of acidotic cells suggested that VHL can still assemble within the VBC/Cul-2 complex under acidic conditions (Fig. 8 C). Furthermore, when

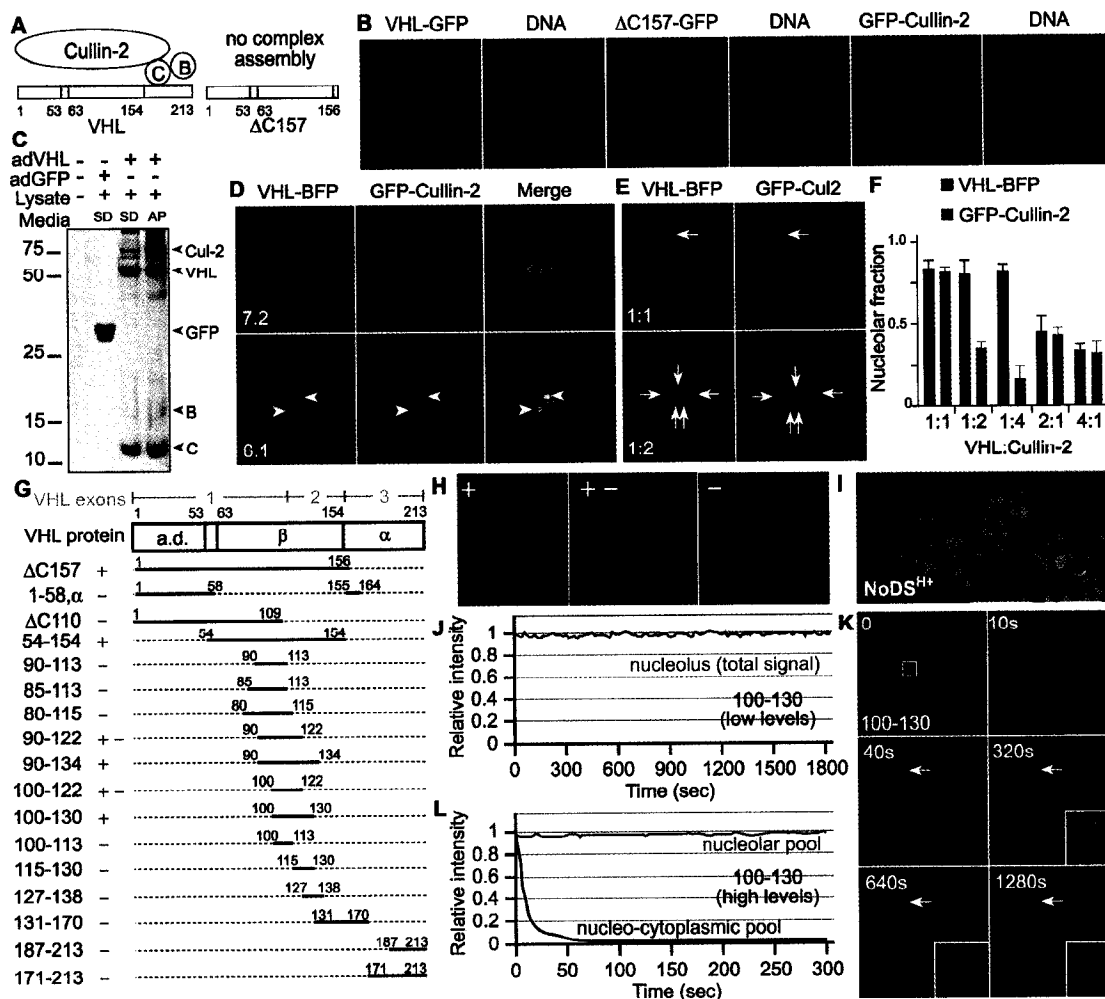


Figure 8. pH-responsive nucleolar detention signal (NoDS^{H+}) allows VHL to target the VBC/Cul-2 ubiquitin ligase complex for static detention in the nucleolus. (A) Schematic representation of the VBC/Cul-2 complex. $\Delta C157$ is a mutant of VHL that fails to assemble the complex. Positions of different amino acids of VHL are indicated. (B) Complex formation is not required for nucleolar localization. VHL-deficient 786-O cells were infected to express GFP-tagged VHL, $\Delta C157$, or Cullin-2 and transferred to hypoxia in AP media. Acidosis triggered the nucleolar relocation of VHL-GFP and $\Delta C157$ -GFP, but not of GFP-Cullin-2. (C–F) VHL targets components of its ubiquitin ligase complex to the nucleolus. (C) VHL-deficient 786-O cells were left uninfected or infected to express flag-tagged VHL-GFP or GFP alone and incubated in hypoxia in SD or AP media. When ~60% of the cells in AP conditions displayed ~30% nucleolar accumulation of VHL-GFP, cells were lysed and submitted to anti-flag immunoprecipitation and silver staining. Acidosis did not cause any sudden disruption of the VBC/Cul-2 complex. (D) VHL-deficient 786-O cells cotransfected to transiently express VHL-BFP and GFP-Cullin-2 (1:1 ratio) were transferred to hypoxia in SD or AP conditions. Cullin-2 colocalized with VHL to the nucleolus in acidosis (arrowheads). (E and F) VHL-deficient 786-O cells were cotransfected to transiently express VHL-BFP and GFP-Cullin-2 to varying ratios (indicated on panels and graph). VHL is the limiting factor in the nucleolar colocalization (arrows) of VHL and Cullin-2. (G) VHL mapping analysis. MCF7 cells transfected to transiently express indicated GFP-tagged proteins were incubated in AP media for 20 h in hypoxia. Schematic of VHL exons and derived amino acid domains are shown. Nucleolar localization was scored according to representative images in (H). Amino acid residues 100–130 of the VHL protein were identified as the minimal domain to recapitulate the nucleolar localization potential of the wild-type VHL tumor suppressor protein in acidosis. "a.d." denotes acidic domain. (I) VHL(100–130) represents a surface pocket on the VHL protein as revealed by molecular modeling (PyMOL). Each amino acid within that sequence is represented in a different color for better visualization. MCF7 cells were transfected to transiently express VHL(100–130)-GFP at low (J) or high (K and L) levels and transferred to hypoxia under AP conditions. Upon reaching a plateau for nucleolar targeting, cells were submitted to FLIP analysis in which a nucleoplasmic region (white square in K) was repeatedly bleached. Insets in K show a pseudocolored zoom of a nucleolus (arrow).

cotransfected with at least an equimolar amount of VHL, Cullin-2 colocalized with VHL in the nucleoli of acidotic cells (Fig. 8, D–F). Cullin-2 is also immobile in the nucleolus of acidic cells, suggesting that VHL responds to changes in extracellular pH and dictates the dynamic status of the assembled VBC/Cul-2 E3 ligase complex (unpublished data). Together, these data identify a novel role for the VHL tumor suppressor in regulating the subcellular trafficking dynamics of the VBC/

Cul-2 ubiquitin ligase complex by targeting it to the nucleolus in response to an increase in environmental H⁺ concentrations.

Mapping analyses of MDM2 and its associated proteins have previously identified small amino peptide sequences that can target a GFP reporter protein to the nucleolus in response to various physiological signals (Weber et al., 2000; Lohrum et al., 2003). Deletion mutant analysis of VHL was therefore conducted to identify minimal nucleolar detention sequences.

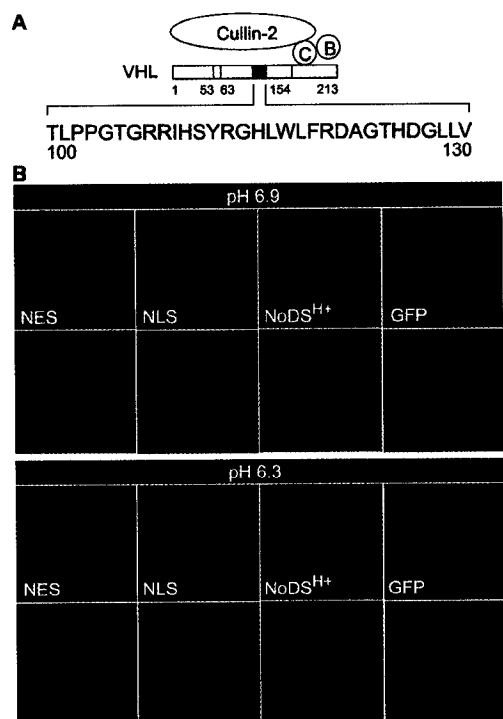


Figure 9. Characteristics of the NoDS^{H+} sequence. (A) Shown is a schematic representation of the VBC/Cul-2 complex. The NoDS^{H+} of VHL is highlighted in red and its constituent amino acids (residues 100–130 of VHL, inclusively) are indicated. (B) MCF7 cells were transiently transfected to express GFP-tagged NES, NLS, NoDS^{H+} or GFP alone. Cells were incubated in hypoxia under AP conditions with maximal acidifications of pH 6.9 or 6.3.

Although several regions within the β -domain of VHL displayed relatively weak nucleolar localization activity in response to acidosis, a domain encoding residues 100–130 recapitulated the nucleolar targeting capability of wild-type VHL (Fig. 8, G–I). VHL(100–130) efficiently mediated the nucleolar detention of a GFP reporter in acidosis as revealed by FLIP experiments (Fig. 8 J). Neutralization of the media released VHL(100–130) into the nucleoplasm where it resumed its dynamic mobility profile (unpublished data). In addition, similar to the wild-type protein (Fig. 4, D and G), increasing the expression level of VHL(100–130) created a static nucleolar and a dynamic nucleocytoplasmic pool (Fig. 8, K and L). Unlike VHL(100–130), previously identified NLS and NES sequences fail to respond to increases in extracellular hydrogen ion concentrations (Fig. 9), highlighting the functional specificity of the herein identified domain. These findings identify a novel and discrete protein domain as a new type of protein localization sequence that we now refer to as [H⁺]-responsive nucleolar detention signal (NoDS^{H+}).

Discussion

We provide evidence that the nucleolar architecture serves as a scaffold to convert highly mobile ubiquitin ligases to static participants of their molecular networks in response to physiological cues. This has various implications for our understanding of

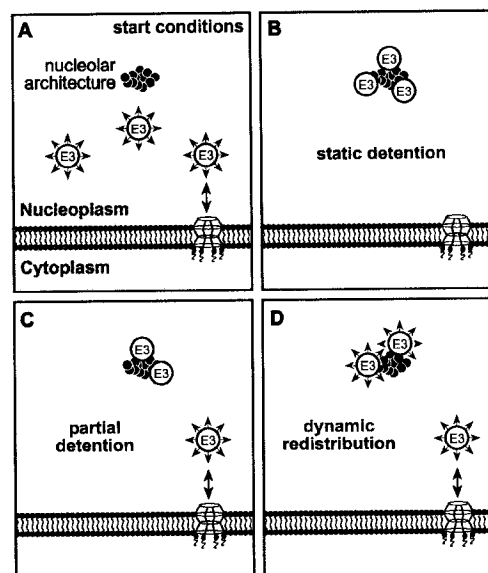


Figure 10. Regulation of ubiquitylation networks by the nucleolus. (A) Ubiquitin ligases follow a stochastic model of molecular mobility to ensure functional interactions such as those with the functional nuclear pore architecture at the nuclear envelope. (B) Complete static detention of ligases within the nucleolar space abolishes these required molecular interactions. (C) Detention of a fraction of the protein population results in a static nucleolar pool while a second pool sustains dynamic functions in the nucleoplasm or cytoplasm. (D) Dynamic change in the steady-state distribution of a protein from mainly nucleocytoplasmic to mainly nucleolar allows the protein to assume dynamic functions in the nucleolus and other cellular compartments.

the role of nuclear compartments in regulating the output of dynamic molecular networks. Unlike certain core histones, which ensure chromatin stability by adopting a constitutive profile of relative immobility (Abney et al., 1997; Kimura and Cook, 2001), most proteins, including heterochromatin protein-1 (Cheutin et al., 2003; Festenstein et al., 2003; Maison and Al-mouzni, 2004), follow a stochastic model of high molecular mobility to ensure efficient functional interactions. We propose a model by which dynamic molecular networks, such as the ubiquitylation system, are built on complex interactions between mobile and relatively static participants. According to this model, modulation of these interactions through regulation of the dynamic state of the participants alters the output of the network. It is known that the interaction of the VBC/Cul-2 and MDM2 ubiquitin ligases with the functional nuclear pore architecture is required for nuclear export and subsequent degradation of their substrates (Fig. 10 A) (Momand et al., 1992; Oliner et al., 1993; Freedman and Levine, 1998; Roth et al., 1998; Lee et al., 1999; Groulx and Lee, 2002). Although constituents of the nuclear pore can move between subcellular compartments, functional pore architecture is confined to the nuclear envelope and persists for long periods of time within well-defined spatial regions (Rabut et al., 2004). Therefore, eliminating the physical interaction between an immobile and a mobile participant only requires the immobilization of the dynamic participant at a different spatial coordinate. In the herein described system, key interactions are abolished after static de-

tention of the ubiquitin ligases within the nucleolar space, a phenomenon that alters network output (i.e., degradation of substrates) (Fig. 10 B) as previously shown by work from our and other groups (Tao and Levine, 1999; Weber et al., 1999; Lohrum et al., 2003; Mekhail et al., 2004a). These data suggest that static nucleolar detention selectively abolishes ubiquitin ligase functions requiring interactions with immobile constituents of the ubiquitylation networks. Whether VHL or MDM2 retain other functions when sequestered in nucleoli, or assume new roles, remains unknown.

The redistribution of dynamic nucleo-cytoplasmic proteins to the nucleolus can be classified in three main categories. First, complete nucleolar detention results in the conversion of a mobile protein to a static participant of its molecular network (Fig. 10 B). Second, detention of a fraction of the protein population results in a static nucleolar pool while a second pool sustains dynamic functions in the nucleoplasm or cytoplasm (Fig. 10 C). Third, dynamic change in the steady-state distribution of a protein from mainly nucleo-cytoplasmic to mainly nucleolar allows the protein to assume dynamic functions in the nucleolus and other cellular compartments (Fig. 10 D). It is possible that a single protein can be targeted to the nucleolus through different mechanisms (Fig. 10, B–D) to custom-tailor specific dynamic profiles in response to different signals. Alternatively, ubiquitin ligases can be regulated through nonnucleolar mechanisms, such as inactivating post-translational modifications, to control ubiquitylation without altering general dynamic properties of the ligase in the cell.

Specific aminopeptide sequences, such as NLS, NES, and NoLS, target proteins to various cellular regions. Mapping analysis of the VHL tumor suppressor protein identified a new type of protein localization sequence, NoDS^{H+}, which is activated after a decrease in extracellular pH to target proteins for static detention in the nucleolus. NoDS^{H+} is inactivated after a return to neutral pH conditions, causing rapid release of detained proteins into the nucleoplasm. The NoDS^{H+} is one of the first discrete domains that have been identified to target proteins to the nucleolus and differs considerably from other NoLS and NoRS signals in its size and mode of regulation. The NoDS^{H+} is characterized by the presence of several arginine residues (Fig. 9 A) that are known to be involved in targeting proteins to the nucleolus. It is possible that these residues are involved in pH-regulated targeting of VHL to nucleoli, whereas other residues play a role in static detention. Further investigation will be required to decipher the mechanisms by which extracellular hydrogen ions activate the NoDS^{H+} of VHL. It will also be important to screen proteins for similar sequences, as they could play vital roles in altering general protein dynamics and metabolism in response to changes in extracellular hydrogen ion concentration. Consistent with the hypothesis that nucleolar sequestration may be a general phenomenon is the recent report that the nucleolus can capture and release several proteins in response to different cellular cues (Andersen et al., 2005).

In conclusion, our findings highlight the role of the nucleolus in regulating protein dynamics, localization, and function. We propose a model by which, via reversible interactions with the nucleolar architecture, ubiquitin ligases alternate between

dynamic and static states to alter the output of their complex molecular networks. There is ample evidence that proteins are highly mobile molecules that function through stochastic interactions with binding partners. This paper provides evidence that cells have evolved a mechanism to regulate molecular networks by switching proteins between mobile and immobile states and highlight the role of the nucleolus in sequestering molecules.

Materials and methods

Cells and materials

C2C12 and PC12 cells from the American Type Culture Collection (Manassas, VA) were differentiated by lowering the serum concentration from 5 to 0.5% or by addition of NGF (50 ng ml⁻¹), respectively, before infection with adenoviruses. 786-0 (VHL-defective), U87MG, HOP62, MCF7, MDA-MB-231, SF295, and H9C2 cells were also obtained from the American Type Culture Collection. VHL-negative 117 cells were a gift from James Gnarra (Louisiana State University, Baton Rouge, LA). 786-0 (Lee et al., 1999), 117 (Mekhail et al., 2004a), or MCF7 cells stably expressing VHL-GFP were generated as described previously (Lee et al., 1999). Where indicated, fluorescein diacetate (5 μ M) and propidium iodide (2 μ M) (Sigma-Aldrich) were added to cells 20 min from endpoint.

Cell culture

Normoxic cells were incubated at 37°C under a 5% CO₂ environment. Hypoxia was achieved by incubation in a hypoxic chamber at 37°C under a 1% O₂, 5% CO₂, and N₂-balanced atmosphere. Acidosis (VHL) (Mekhail et al., 2004a) and ribosomal perturbation (MDM2) (Lohrum et al., 2003) experiments were conducted as previously described. For SD or AP conditions, buffer-free medium (DME; Invitrogen) was freshly prepared and supplemented with 5% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. NaHCO₃ (44 mM) was added and the pH was adjusted to 7.2 (SD) or 5.4–7.2 (AP) with HCl. Air was bubbled into both media at 22°C, which stabilizes the pH at 7.2. AP media slowly reverted to its original pH (5.4–7.2) under hypoxia, whereas the SD medium remained at pH 7.2. MDM2 ribosomal stress (RS) conditions were induced in SD media by addition of 15 nM ActD (Calbiochem) for the last 2 h of a 6-h treatment with 20 μ M MG132 (Calbiochem) (Lohrum et al., 2003). Transfected or adenovirus-infected cells were grown for 24 h under standard conditions before any treatment.

Plasmids and adenoviruses

VHL and its variants and mutants were cloned between an NH₂-terminal Flag-tag and a COOH-terminal GFP-tag and into pcDNA3.1, as previously described (Bonicalzi et al., 2001; Groulx and Lee, 2002). Adenoviruses were produced using the Cre-lox recombination system. Cullin-2 construct is previously described (Groulx et al., 2000). We sincerely thank Tom Misteli (National Cancer Institute, Bethesda, MD) for providing UBF1-GFP and FIB-GFP constructs; Mark Olson (University of Mississippi Medical Center, Jackson, MS) for B23-GFP construct; Gang Pei (Shanghai Institute of Biological Sciences, Shanghai, China) for MDM2-GFP; and Uri Alon (Weizmann Institute of Science, Rehovot, Israel) and Galit Lahav (Harvard Medical School, Boston, MA) for MDM2-YFP. Transient transfections were conducted with Effectene transfection reagent (QIAGEN).

Immunoprecipitation

Cells lysis and immunoprecipitations were conducted as previously described (Groulx and Lee, 2002). In brief, M2 beads were added to total cell lysates containing 1 mg of protein. After 1 h tumbling at 4°C, beads were washed several times, eluted with flag peptides. Eluates were boiled before undergoing Western blotting. Gels were silver stained according to manufacturer's protocol (Bio-Rad Laboratories).

Immunoblotting

Samples were separated on denaturing polyacrylamide gels containing SDS and transferred to methanol-activated polyvinylidene difluoride membrane (NEN Life Science Products). Membranes were blocked in skimmed milk before incubation with antibodies to VHL (BD Biosciences) (Kibel et al., 1995; Corless et al., 1997; Mekhail et al., 2004a) or HIF-2 α (Novus) (Mekhail et al., 2004a). After washing with a 0.2% Tween-PBS solution, membranes were blotted with a secondary antibody conjugated to HRP (Jackson ImmunoResearch Laboratories) and detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

Immunofluorescence microscopy

Cells were seeded onto coverslips and fixed with prechilled (to -20°C) methanol for 10 min followed by acetone for 1 min. An anti-B23 mAb (Sigma-Aldrich) was used. Cells were incubated for 1 h with a primary antibody solution containing 10% FBS and 1% Triton X-100 (vol/vol). Cells were washed several times in PBS before 1 h incubation with a secondary Texas red-labeled antibody (Jackson ImmunoResearch Laboratories). Images of fixed cells were captured with a microscope (Axioskop 2 MOT PLUS; Carl Zeiss Microimaging, Inc.) using a digital charged-coupled device camera (Qimaging). Compartmental fluorescence was measured as described previously (Lee et al., 1999; Mekhail et al., 2004a).

Photobleaching and microscopy

Cells cultured on 40-mm-diameter glass coverslips were visualized on a confocal microscope (MRC 1024; Bio-Rad Laboratories) in an FCS2 environmental chamber (Biopetechs) maintained at 37°C or, where indicated, directly into 35-mm dishes with coverslip bottoms. A $60\times$ plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to five rapid pulses of a 488-nm argon laser at 100% power and image acquisition was conducted at 1% of full laser power. For FRAP experiments, images were collected at 1- or 5-s intervals. Recovery of the fluorescent signal within a bleached region was calculated as described by Phair and Misteli (2000) following $I_{\text{rel}} = (I_{\text{ij}}/I_{\text{ij}0}) * (T_{\text{ij}0}/T_{\text{ij}})$, where T_{ij} is the total cellular intensity at time t , $T_{\text{ij}0}$ is total cellular intensity before bleach, $I_{\text{ij}0}$ is the intensity in the bleached area before bleach, and I_{ij} is the intensity in the previously bleached area at time t . For iFRAP nuclear export experiments the whole cytoplasm was bleached and cells were monitored in 30-s intervals. Relative loss of total fluorescence in the unbleached nucleus was calculated as $I_{\text{rel}} = (I_{\text{ij}}/I_{\text{ij}0}) * (N_{\text{ij}0}/N_{\text{ij}})$, where I_{ij} is the average intensity of the unbleached nucleus at time point t , $I_{\text{ij}0}$ is the average prebleach intensity of the nucleus of interest, and $N_{\text{ij}0}$ and N_{ij} are the average total cellular fluorescence intensities of a neighboring cell in the same field of vision at prebleach or at time point t , respectively. For FLIP experiments, cells were repeatedly bleached and imaged at 5-s intervals and fluorescence loss in unbleached areas was calculated similar to iFRAP calculations to account for any losses in fluorescence by normalizing the fluorescence in the cell of interest to that of a neighboring cell. Where indicated, cycloheximide ($20 \mu\text{g ml}^{-1}$) was added 1 h from endpoint. For all bleaching experiments, at least 10 datasets were analyzed for each result. Average pixel intensities were normalized for background fluorescence. Images of living cells from experiments that do not implicate bleaching were captured with a microscope (Axiovert S100TV; Carl Zeiss Microimaging, Inc.) equipped with a $40\times/1.2$ C-Apochromat water immersion objective using a digital charged-coupled device camera (Empix). Pseudocoloring for bleaching and fusion experiments was achieved by applying the gradient map function of Photoshop (Adobe) to a montage of picture frames prepared with Image J (National Institutes of Health, Bethesda, MD) software. Other software packages used to capture images, analyze the data, and generate graphs include Northern Eclipse (Empix), Excel (Microsoft), and FreeHand (Macromedia).

Polykaryon assay

For VHL-GFP relocation to the nucleolus in homokaryon fusion assays, cells were transfected according to manufacturer's protocol to express fluorescently labeled proteins and incubated under standard conditions for 24 h (Lee et al., 1999). Usually between 40 and 60% of cells presented strong fluorescence. Cells were washed twice with warm PBS and fused for 2 min by addition of a warm 50% solution of PEG in PBS (Sigma-Aldrich). PEG was removed thoroughly by four washes with warm PBS and cells were incubated for 30 min under standard conditions. Cells were then replenished with AP media (see Cell culture) and transferred to hypoxia. After acidification, cells were monitored for the distribution of VHL-GFP in polykaryonic cells. For VHL-GFP and B23-GFP dynamic trafficking assays (Fig. 5, B and C), cells were transfected to express GFP-tagged proteins or left unaltered. The cells were then mixed at a 1:10 ratio, plated in 35-mm-diameter culture dish with a girded coverslip as its base, and exposed to hypoxia in AP media. After acidification and redistribution of VHL-GFP to nucleoli, cells were fused by PEG treatment as described above. This process yielded a significant number of polykaryonic cells where the fluorescence observed in the cell is only associated with nucleoli of only one or two nuclei, whereas other nuclei within the same polykaryonic cell displayed no fluorescence. Hypoxic cells were then rapidly washed twice with nonbuffered acidic media (pH 6.0–6.5), replenished with their original acidified AP media, and cells were monitored by fluorescence microscopy.

Online supplemental material

Fig. S1 shows characteristics of cells and VHL subcellular trafficking in hypoxia-acidosis. Fig. S2 shows that both forms of VHL relocate to the nucleolus in response to the same pH threshold in cells stably expressing the GFP-tagged proteins. Fig. S3 shows a comparison of nuclear export of VHL under neutral and acidic conditions using iFRAP. Fig. S4 shows how FLIP analysis indicates that the redistribution of MDM2 from nucleoplasm to nucleoli alters general MDM2 dynamic state. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200506030/DC1>.

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Report

Restriction of rRNA Synthesis by VHL Maintains Energy Equilibrium Under Hypoxia

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ABSTRACT

Biological evolution abides by an unbending rule obligating organisms to maintain energy equilibrium. Hypoxia reduces cellular energy supply and is thus thought to be deleterious. We report that cells have evolved pH-sensitive mechanisms to maintain energy equilibrium by lowering energy demand. We found that fermentation-induced acidosis allows hypoxic cells to maintain energy equilibrium and viability under hypoxia by restricting ribosomal biogenesis, the most energy-demanding cellular process. Acidosis triggers nucleolar condensation, decreases precursor rRNA synthesis, reduces the dynamic profile of the RNA polymerase I preinitiation factor UBF1 and its interaction with the promoter of rRNA genes (rDNA). These changes require the pH-dependent interaction of the statically detained von Hippel-Lindau tumor suppressor protein (VHL) with rDNA. This phenomenon is promoted by, but does not require, activation of the hypoxia-inducible factor (HIF), a transcription factor implicated in extracellular acidification, energy production and oxygen homeostasis. Abrogating this program by silencing VHL expression, competing rDNA-VHL interaction or preventing environmental acidification triggers energy starvation and cell death under hypoxia. Our data suggest that oxygen-starved cells maintain energy equilibrium by gauging the environmental concentration of H⁺ to statically detain VHL to nucleolar rDNA and restrict ribosome production. These findings also provide an explanation for the protective effect of acidosis in ischemic settings such as development, stroke and cancer.

INTRODUCTION

Introduction of molecular oxygen into an anaerobic biosphere over 2.2 billion years ago triggered a drastic reorganization and expansion of complex molecular networks.¹ Under normal oxygen tension (normoxia), contemporary eukaryotic cells use this diatom to maximize the amount of energy extracted from glucose catabolism. Glycolytic conversion of each glucose into two pyruvate molecules generates only two ATPs. However, each pyruvate is then transferred to mitochondria to be converted into acetyl coenzyme-A (AcCoA) via pyruvate dehydrogenase (PDH). Further mitochondrial catabolism of AcCoA through the citric acid cycle (CAC) and the passage of high-energy electrons along a transfer chain (electron transfer chain, ETC) where molecular oxygen is the last acceptor generate a large amount of ATP and replenish glycolysis with nicotinamide adenine dinucleotides (NAD⁺), a scarce cellular coenzyme that cannot be used stoichiometrically.

Cells facing low oxygen tension (hypoxia) shift to, or rely more on, anaerobic metabolism, which is defined here as the sum of processes generating ATP without utilizing oxygen, in an effort to maintain energy equilibrium and viability.²⁻⁴ Mitochondrial respiration is repressed both passively by the scarcity of oxygen as an electron acceptor and actively by the hypoxia-inducible factor (HIF), a heterodimeric transcription factor involved in the maintenance of oxygen homeostasis.^{5,6} In the presence of oxygen, enzymes known as prolyl hydroxylases (PHDs) hydroxylate key proline residues within the α subunit of HIF (HIF α) (reviewed in ref. 7). These post-translational modifications allow the von Hippel-Lindau tumor suppressor (VHL) to mediate the ubiquitination of HIF α in the nucleus. VHL-mediated nuclear export then targets HIF α for degradation via the 26S proteasome in the cytoplasm.^{8,9} PHDs require molecular oxygen and hypoxia prevents hydroxylation of HIF α , allowing it to evade recognition by VHL. Stabilized HIF α dimerizes with the constitutively expressed β subunit of HIF (HIF β). HIF can then activate its target genes, which include glucose transporter-1 (Glut-1), vascular endothelium growth factor (VEGF), and carbonic anhydrase IX (CAIX).

Following a shift to hypoxia, initial production of reactive oxygen species (ROS) by an ETC destabilized by decreasing oxygen levels contributes to the induction of HIF,¹⁰⁻¹⁵ which in turn activates the PDH inhibitor pyruvate dehydrogenase kinase-1 (PDK-1).¹⁶⁻¹⁸ The latter actively represses mitochondrial respiration to prevent toxic accumulations of ROS and ensures that no pyruvate is wasted in the form of AcCoA. Instead, hypoxic cells maximize anaerobic energy production by both triggering the Pasteur effect, a HIF-dependent increase in glycolytic rate,^{5,19} and converting pyruvate to lactate through fermentation, which efficiently replaces mitochondria with regard to the task of replenishing glycolysis with NAD⁺.² This causes extracellular acidosis as lactate molecules (La⁻) are coupled to hydrogen ions and released in the environment in the form of lactic acid (La-H⁺). Although the production of lactic acid is often viewed as a terminal event of anaerobic metabolism, it has long been recognized that acidosis protects cells facing limited oxygen tension in various physiological and pathological settings, including muscle exercise, tumor development, and ischemic disorders.²⁰⁻²⁶ We previously found that hypoxia-induced acidosis triggers nucleolar sequestration of VHL. This allows HIF to evade destruction and activate its target genes, including those involved in different aspects of energy supply, suggesting the existence of a hypoxic cell memory.²⁷⁻²⁹

While hypoxic cells increase glycolytic ATP production, this process does not compensate for the loss of mitochondrial respiration resulting in energy starvation and cell death under prolonged hypoxia.³⁰ We reasoned that either hypoxia drives energy shortage or that anaerobic metabolism encompasses an additional program that guards energy balance and viability. In light of the defensive effects of acidosis in ischemic settings, we hypothesized that cells have evolved a mechanism utilizing fermentation protons to maintain energy equilibrium by increasing the production or decreasing the consumption of energy.

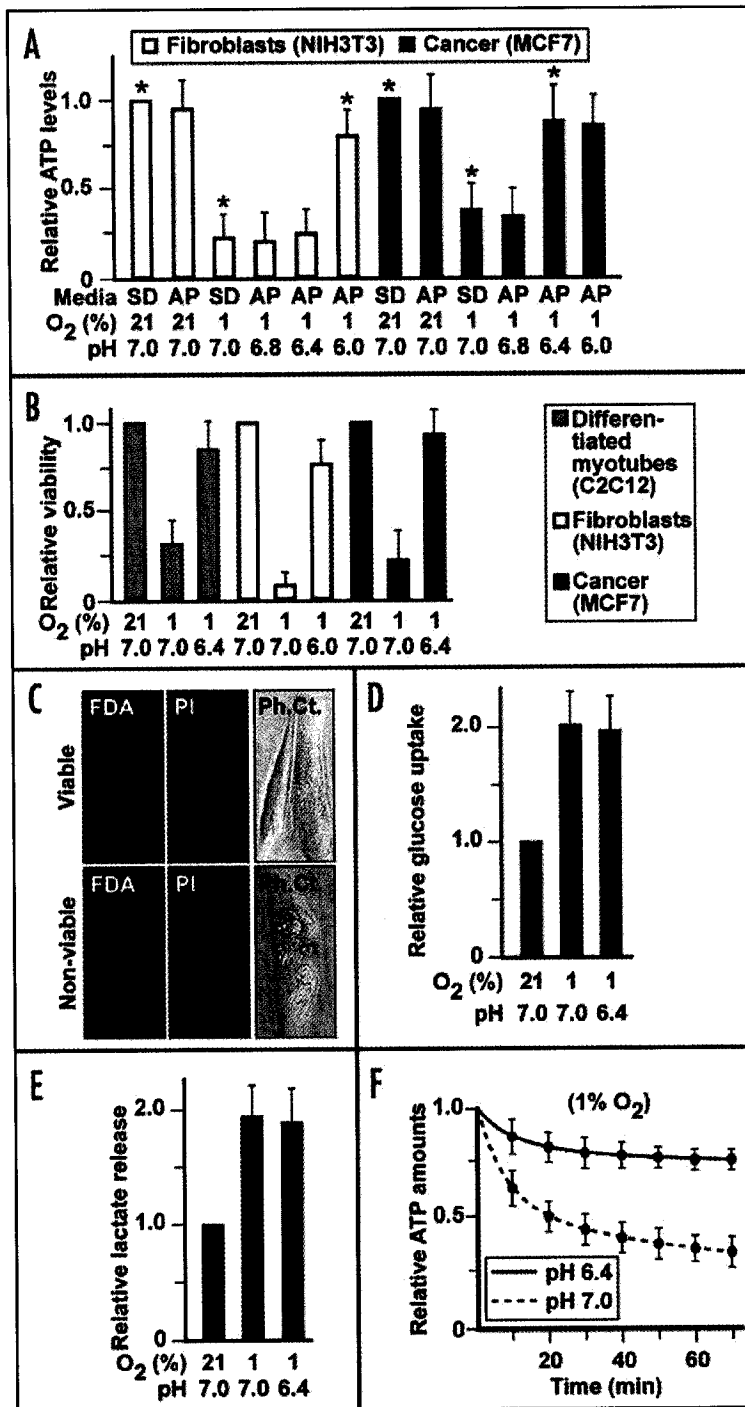
Here, we report that cells regulate energy demand by sensing the environmental concentration of hydrogen ions. H⁺ produced under hypoxia promotes interactions between VHL and rRNA genes (rDNA) to reduce rRNA synthesis. This silencing program restricts ribosomal biogenesis, the most energy-demanding cellular process,³¹⁻³³ to preserve energy equilibrium and viability. Our data suggest that cells maintain constant ATP levels regardless of oxygen tension by controlling and gauging the environmental concentration of hydrogen ions. These findings also provide an explanation for the protective effect of acidosis in ischemic settings such as development, stroke and cancer.

RESULTS

H⁺ limits energy expenditure in hypoxia. To study how cellular energy levels are altered under anaerobic metabolism (see introduction for definition), we incubated cells from various tissue origins in standard (SD) media, that prevents fluctuations in pH, or in acidification-permissive (AP) media, which is prepared to enable cells to naturally acidify their extracellular milieu to varying degrees under hypoxia (see experimental procedures).^{27,29} Similar to previous reports, hypoxia induced significant reductions in total ATP levels when cells were incubated under standard conditions (Fig. 1A).³⁰ Unexpectedly, ATP concentrations were preserved when hypoxic cells acidified their environment to cell-type-specific pH thresholds (Fig. 1A). The establishment of acidosis was associated with increased cellular viability as revealed by fluorescein-diacetate (FDA) staining and propidium iodide (PI) exclusion in costaining experiments

(Fig. 1B and C). We next set out to determine if H⁺-dependent maintenance of energy levels is the consequence of increased production or decreased consumption of ATP. Increases in glucose uptake and lactate release, which are triggered by hypoxia, were insensitive to fluctuations in pH (Figs. 1D and E), suggesting that acidosis does not increase ATP levels by modulating glycolysis, the Pasteur effect, or possible residual mitochondrial respiration. To study the effect of pH on energy consumption, hypoxic cells were treated with 6-deoxyglucose (6-DOG) to inhibit glycolytic energy production and ATP levels were monitored. Energy expenditure was lower in acidotic cells as demonstrated by a slower rate of ATP depletion (Fig. 1F). In addition, ATP levels under hypoxia-neutral conditions declined before any apparent cell death (Fig. 1A and B, and data not shown), suggesting that mechanisms triggering the reduction in energy levels are upstream of the processes affecting viability. Taken together, this suggests that cells rely on fermentation protons and the ensuing acidosis to preserve cellular energy levels and viability by restricting energy demand to levels matching limited supply.

pH-dependent nucleolar condensation and restriction of rDNA transcription. While examining several cellular energy-parameters, we noticed that nucleoli of cells engaged in anaerobic acidosis exhibit changes that are characteristic of the transcriptional silencing of rDNA. Light microscopy revealed that nucleoli undergo a decrease in size (Fig. 2A). This was confirmed by assessment of the localization of the green fluorescent protein (GFP)-tagged B23 protein (Fig. 2A and B). While short exposure (3 h) of cells to RNA polymerase-I (RNAPI) inhibitors such as treatment with low concentrations of actinomycin-D (Act-D) only decrease nucleolar size, prolonged exposure (8 h) to these drugs results in the toxic fragmentation of nucleoli (Fig. 2A, and asterisks and inset in 2B).³⁴ Unlike such toxic pharmacological treatments, prolonged incubation under physiological acidosis (up to 30 h) did not induce any nucleolar fragmentation (Figs. 2, A and B), suggesting that acidosis might inhibit RNAPI and restrict ribosomal biogenesis in a fashion that does not disrupt nucleolar integrity. Next, immunohistochemistry analysis was performed on sections from glioblastoma tumors grown in the flanks of nude mice. Staining for endogenous B23 protein revealed that nucleoli of cells positioned closer to the core of tumors, which is thought to be more acidic,³⁵⁻³⁷ exhibit a noticeably more condensed phenotype compared to nucleoli at the periphery (Fig. 2C). Fibrillar centers constitute the nucleolar subcompartment where rDNA resides. Silencing of RNAPI is usually accompanied by the coalescence of fibrillar centers (FCs) into a few relatively well-defined foci, a phenomenon associated with the segregation of nucleolar subcompartments.³⁴ Coalescence of FCs under acidosis was revealed by assessment of the localization of the GFP-tagged FC protein RNAPI preinitiation factor upstream binding factor 1 (UBF1) (Fig. 2D and E). DNA-associated proteins, like UBF1, exhibit increased residence time on DNA under transcriptional silencing conditions.³⁸ The dynamic properties of GFP-tagged UBF1 were thus analyzed by fluorescence loss in photobleaching (FLIP) experiments.³⁹ In FLIP, a living cell is repeatedly hit with a laser beam in the same region. Loss of fluorescence in an area outside the bleached spot is reflective of protein mobility between that area and the bleached spot. A rapid loss of UBF1-GFP fluorescence was observed in essentially the whole nucleus following repetitive bleaching of a small nucleoplasmic region in cells incubated under neutral conditions (Fig. 2F and G), highlighting the dynamic character of the protein chimera. UBF1-GFP displayed an H⁺-dependent increase in its residence time on rDNA with 34% of



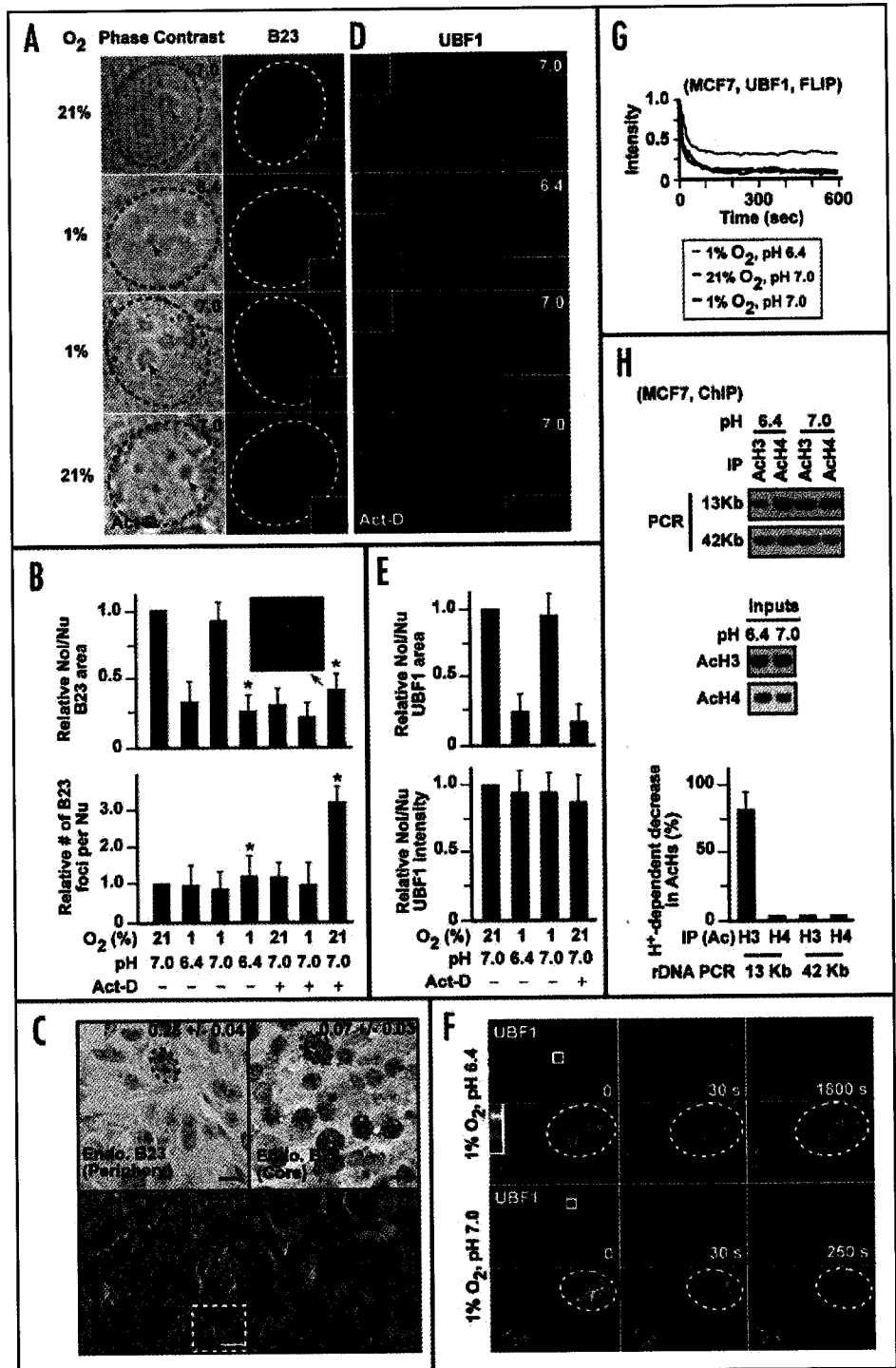
the protein pool still detectable after a 30 min FLIP experiment within coalesced FCs (Fig. 2F and G). As previously reported, UBF1-GFP was highly mobile at earlier time points (data not shown and see Ref. 29). None of these changes occurred when cells were prevented from acidifying their extracellular milieu under anaerobic conditions (Fig. 2A–G). Thus, we reasoned that by temporarily restricting ribosomal anabolism, the most energy-consuming cellular process (50–80% of total consumption),^{31–33} fermentative protons would match anaerobic energy demands with limited supply. Consistent with this notion, chromatin crosslinking and immunoprecipitation (ChIP) revealed a decrease of about 75% in the level of acetylated

Figure 1. Acidosis reduces cellular energy demand. (A) Acidosis preserves cellular energy levels under hypoxia. Fibroblasts or breast carcinoma cells were cultured either in standard media (SD, initial pH 7.0) or different acidification permissive media (AP, initial pH 7.0) that allow maximal hypoxia-induced extracellular acidification to pH 6.8, 6.4, or 6.0. Cells were then cultured under normoxia (21% O₂) or hypoxia (1% O₂) for 22 h, endpoint pH was measured, and cellular ATP levels were measured and normalized to cell number. Single asterisk denotes conditions of interest which will be tested hereon after. N is 9 for three independent experiments performed in triplicates, values are means, and bars represent standard error. Shown normoxic AP media had maximal acidification potential of pH 6.0 but media remained at pH 7.0 at endpoint. (B and C) H⁺ preserves viability. Breast carcinoma cells, fibroblasts, or differentiated myotubes were cultured as in (A). FDA and PI were added 3.5 h later for 30 min. Viability scores are ratios of the number of FDA-positive cells relative to the total number of FDA-positive and PI-positive cells (B). Scores were obtained from three independent experiments (B) and representative myotubes are shown in (C). Bars represent standard error. (D and E) H⁺ production under hypoxia does not affect glycolysis or the Pasteur effect. MCF7 cells were cultured as in (A) then glucose uptake and lactate release were measured and normalized to cell number. (F) H⁺ reduces energy demand under hypoxia. MCF7 cells were cultured as in (A). Following a 20 min 6-deoxyglucose treatment, ATP levels were measured and normalized to cell number and to the difference of initial ATP concentrations before addition of drug.

histone H3 (AcH3) associated with the transcriptional termination site of rDNA (Fig. 2H, and see Fig. 4E for schematic of an rDNA gene unit), a region often hyperacetylated by RNAPI-activating signals such as Myc expression.^{40,41} The 5'-external transcribed spacer (5'-ETS) region of precursor rRNA (pre-rRNA) is rapidly processed following rDNA transcription and measurement of its levels by reverse transcriptase PCR (RT-PCR) directly reflects the rate of RNAPI activity (Fig. 4E).⁴⁰ RT-PCR analysis employing primers specific to 5'-ETS revealed an H⁺-dependent decrease in pre-rRNA synthesis (Fig. 3A), which occurred before reductions in the levels of rRNA products were observed (Fig. 3B). A regulatory role for H⁺ in rDNA transcription was supported by an acidosis-dependent reduction in the level of BrUTP incorporation into nascent rRNA in BrUTP pulse-chase experiments (Fig. 3C, notice that asterisk-marked green panels have triple the exposure time relative to other panels for the purpose of presentation) and by an H⁺-dependent decrease in the levels of UBF1 preinitiation factors bound to the rDNA promoter in ChIP experiments (Fig. 3D and E). rRNA processing was essentially unaltered by oxygen or pH levels since direct ethidium bromide staining revealed equimolar reductions of all three rRNA products (Fig. 3A) and since the vectorial path of BrUTP-labeled rRNA emanating from the nucleolus was preserved (Fig. 3C). No difference was observed in the ability of RNA polymerases II and III to synthesize ARPP-P0/Actin and 5S rRNA, respectively (Fig. 3A and B). Thus, acidosis restricts rDNA transcription under hypoxia.

Acidosis-dependent nucleolar condensation and restriction of rDNA transcription requires nucleolar VHL. Next, we set out to identify biochemical characteristics of the pH-dependent process modulating nucleolar condensation. Morphological changes to the nucleolus and reductions in pre-rRNA synthesis were observed only after reaching cell-type-specific hydrogen ion concentrations correlating with pH thresholds required to target the VHL tumor suppressor for static nucleolar detention (Figs. 2, 3 and 4A).^{27,29}

Figure 2. Fermentation protons trigger nucleolar condensation. (A and B) Acidosis-dependent nucleolar condensation under hypoxia. MCF7 cells expressing B23-GFP were cultured under indicated conditions for 22 h and nucleoli were assessed by phase contrast imaging or GFP fluorescence. Representative pictures are shown in (A) and measurements of the ratio of nucleolar GFP area (all nucleoli within a nucleus) relative to nuclear area (outlined by dashed circles revealed by Hoechst staining of DNA in insets) (Nol/Nu area) are shown in (B) (N is 30 cells analyzed from three independent experiments and bars represent standard error). Extracellular pH is indicated on each panel. Act-D was added 3 h from endpoint, where indicated, and single asterisks (B) denote longer treatments (30 h acidosis, 8 h Act-D). (C) Nucleolar condensation observed in the acidic core of glioblastoma tumors grown in the flanks of nude mice. Sections (8 μ m) were either submitted to H&E staining or to immunohistochemistry analysis to detect endogenous B23 protein. Ratios of nucleolar B23 to nuclear area were 0.25 and 0.07 near the tumor periphery and the core, respectively. Scale bars represent 5 μ m (black) or 20 μ m (yellow). (D–G) pH-dependent alteration of both steady-state and dynamic profiles of rDNA-interacting proteins under hypoxia. UBF1-GFP expressed in MCF7 cells cultured as in (A) was assessed for changes in steady-state distribution (D and E) (N is 30 cells analyzed from three independent experiments and bars represent standard error) or in dynamic profile by FLIP analysis (F and G). Insets in (D) show Hoechst staining of DNA or zoom of specific nucleoli (dashed squares). For FLIP, nucleoplasmic regions (white squares) were repeatedly bleached while cells are imaged between pulses, and pseudocolored images with nuclear outlines (dashed circles) highlighting changes in GFP fluorescence (F) and corresponding kinetics of loss of fluorescence (G) are shown. (H) ChIP analysis performed on MCF7 cells cultured for 22 h under indicated conditions reveals an H⁺-dependent reduction in the levels of acetylated histone H3 bound to rDNA near the rDNA transcriptional unit termination site 13 Kb from the start site. Representative gels and graph of quantitation of the changes in histone acetylation are shown. Results are normalized to cell number and inputs, and minimal IgG-associated background was subtracted in the quantitation process where results are means from three independent ChIP experiments and bars represent standard error.



Immunohistochemistry analysis performed on sections of glioblastoma tumors revealed that the localization of endogenous VHL shifts from mainly cytoplasmic in cells near the tumor surface to mainly nucleolar deeper in the core of tumors, which is thought to be more acidic (Fig. 4B).^{35–37} Confocal microscopy of GFP-tagged VHL revealed that the tumor suppressor first appears in the nucleolus within well-defined foci (Fig. 4C, left panel) similar to condensed rDNA regions (Fig. 2D).³⁴ We used fluorescence recovery after photobleaching (FRAP) to assess the kinetic properties of VHL-GFP within these early foci and determine the potential involvement of this tumor suppressor in H⁺-dependent rDNA silencing.^{38,39,42}

Specific cellular regions expressing fusion proteins were bleached with the use of a laser pulse that irreversibly quenches the GFP signal, and the recovery of signal in the bleached area was recorded by time-lapse confocal microscopy. The kinetics and extent of recovery of fluorescence in a cellular region following bleaching are reflective of the dynamics of the studied fluorescent chimeras. While nucleoplasmic VHL was highly mobile in hypoxic cells under neutral conditions, the protein was statically detained within these early subnucleolar foci (Fig. 4C and D), suggesting a role for VHL in the acidosis-dependent restriction of rDNA transcription.³⁸

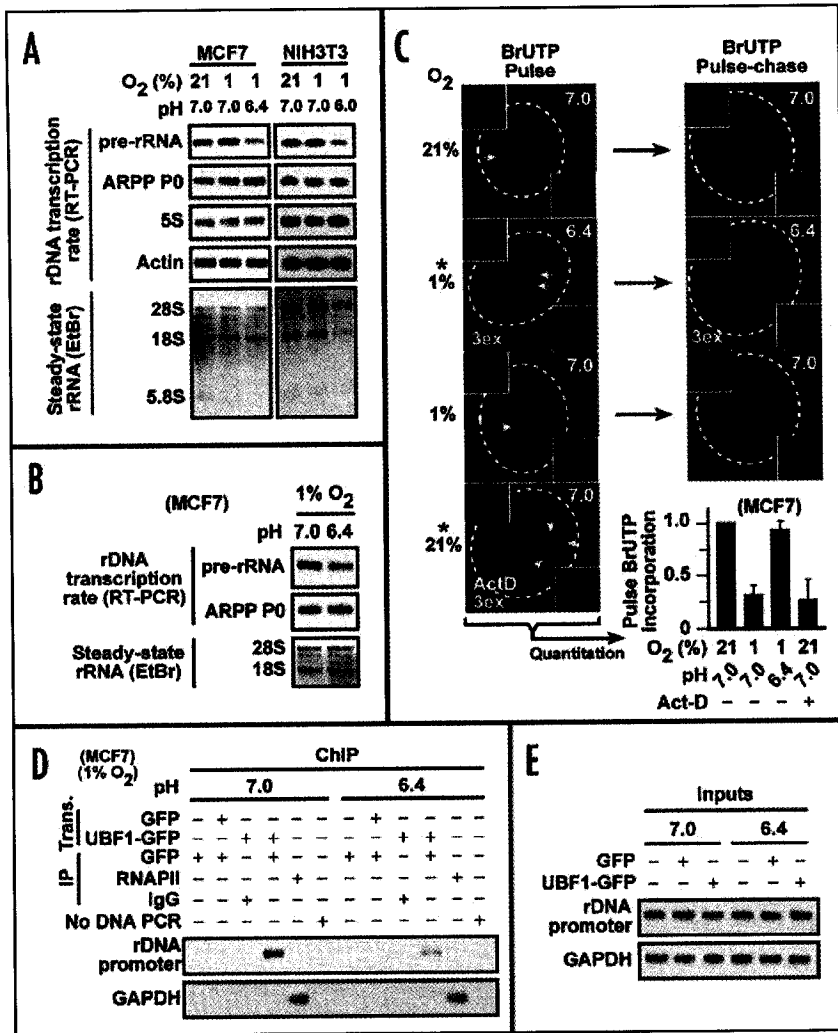


Figure 3. Fermentation protons restrict rDNA transcription. (A) H⁺ reduces the levels of pre-rRNA under hypoxia. RT-PCR analysis was performed on mRNA normalized by cell number from cells cultured for 22 h under indicated conditions. Steady-state levels of 28S, 18S and 5.8S rRNAs were revealed by direct ethidium bromide (EtBr) staining. Results are representative of three independent experiments. (B) pH-dependent reductions in pre-rRNA levels are observed prior to any detectable changes in the steady state levels of 28S and 18S rRNA as revealed by RT-PCR and EtBr staining of samples from MCF7 cells incubated for 18 h in hypoxia. (C) H⁺ lowers BrUTP incorporation in nascent rRNA but does not affect the vectorial path characteristic of rRNA processing emanating from the nucleolus. Nascent rRNA was pulsed with BrUTP and visualized by immunofluorescence before or after a 10 min chase. Asterisk-marked green panels were captured at triple the exposure time (3ex) relative to other conditions to better illustrate BrUTP incorporation patterns but all quantitation analysis were based on images obtained at a 1ex exposure time (N is 30 cells and bars represent standard error). Insets are zooms of arrow-marked regions. (D and E) ChIP analysis reveals that acidosis lowers the levels of UBF1 protein bound to the rDNA promoter. MCF7 cells were transfected to express UBF1-GFP, GFP alone or left unaltered. Cells were incubated under indicated conditions for 22 h. Extracts from equal number of cells were submitted to immunoprecipitations using indicated specific antibodies followed by PCR using primers specific to the rDNA promoter. Binding of RNAPII to the GAPDH gene served as positive control and input controls are shown (E).

ChIP analysis revealed that nucleolar VHL was physically linked, directly or indirectly, with the intergenic spacer of rDNA around 27 Kb from the transcriptional start site (Fig. 4E and F).⁴³ Silencing of endogenous VHL expression in MCF7 cells by use of small-interfering RNA (siVHL), which was equally functional under neutral and acidic conditions (Fig. 4G), abolished H⁺-dependent nucleolar condensation (Fig. 4H). VHL-deficient cells⁷ failed to condense nucleoli, a phenomenon that was rescued by the reintroduction of hemagglutinin-tagged VHL (HA-VHL) by stable transfection (Fig. 4I). Neutralization of the extracellular milieu by addition of NaOH or replenishing the cells with fresh neutral SD media abolished nucleolar condensation only after essentially all of the VHL protein pool was released from nucleoli (Fig. 4J and K), suggesting that condensation does not require complete sequestration of the VHL protein pool.

Pre-rRNA levels were insensitive to pH in VHL-deficient cells but stable expression of HA-VHL in these cells restored H⁺-dependent reductions in the levels of the precursor (Fig. 5A). Silencing endogenous VHL expression in MCF7 cells abolished acidosis-dependent restriction of ribosomal biogenesis (Fig. 5B). ΔC157 is a deletion mutant of VHL that localizes to the nucleolus and can compete with the wild-type tumor suppressor protein for nucleolar localization.²⁷ ΔC157 failed to induce nucleolar condensation when expressed in VHL-deficient cells incubated under hypoxia-acidosis, indicating that this mutant can be used as a dominant negative molecule

(dnVHL) (data not shown). Expression of dnVHL into MCF7 cells abolished the constriction imposed by H⁺ on ribosomal anabolism (Fig. 5C). These findings suggest that acidosis-mediated restriction of nucleolar rDNA requires the H⁺-dependent relocation of VHL to the nucleolus.

H⁺/VHL-dependent nucleolar rDNA remodeling is promoted by, but does not require, HIF activity. Mechanistically, hypoxia and acidosis promote HIF stabilization by inhibiting PHDs and triggering nucleolar sequestration of VHL, respectively.^{7,27,29} Stabilized HIF activates several genes implicated in the cellular oxygen homeostatic response under hypoxia, including promotion of the Pasteur effect through activation of genes encoding glycolytic enzymes or cofactors.^{5,19} Introduction of dominant negative HIF (dnHIF) into MCF7 cells reduced the rate at which they acidified their extracellular milieu, as expected (Fig. 6A).^{44,45} However, nucleolar condensation was still observed in these cells upon reaching pH 6.4 (Fig. 6A and B). We next tested the direct involvement of HIF activation in the observed pH-dependent control of rDNA under low oxygen tension by incubating MCF7 cells expressing dnHIF (see ref. 44 for full characterization of this molecule) in SD media under hypoxia for 16 h (pH stable at 7.0), which stabilized both HIF-1α and HIF-2α but abrogated their ability to activate Glut-1 (Fig. 6C, lanes 1–6). Cells were then replenished for 4 h with AP media conditioned to acidosis by direct addition of HCl (pH stable at 6.4) (Fig. 6C) or by another batch of cells (data not shown). Although HIF activity remained abrogated after acidification of dnHIF-expressing cells as revealed by Glut-1 mRNA levels (Fig. 6C, lanes 7–9),^{27,29} nucleolar condensation still occurred under hypoxia-acidosis (Fig. 6D). Slight variations in the levels of reactive oxygen species, which reflect mitochondrial status,

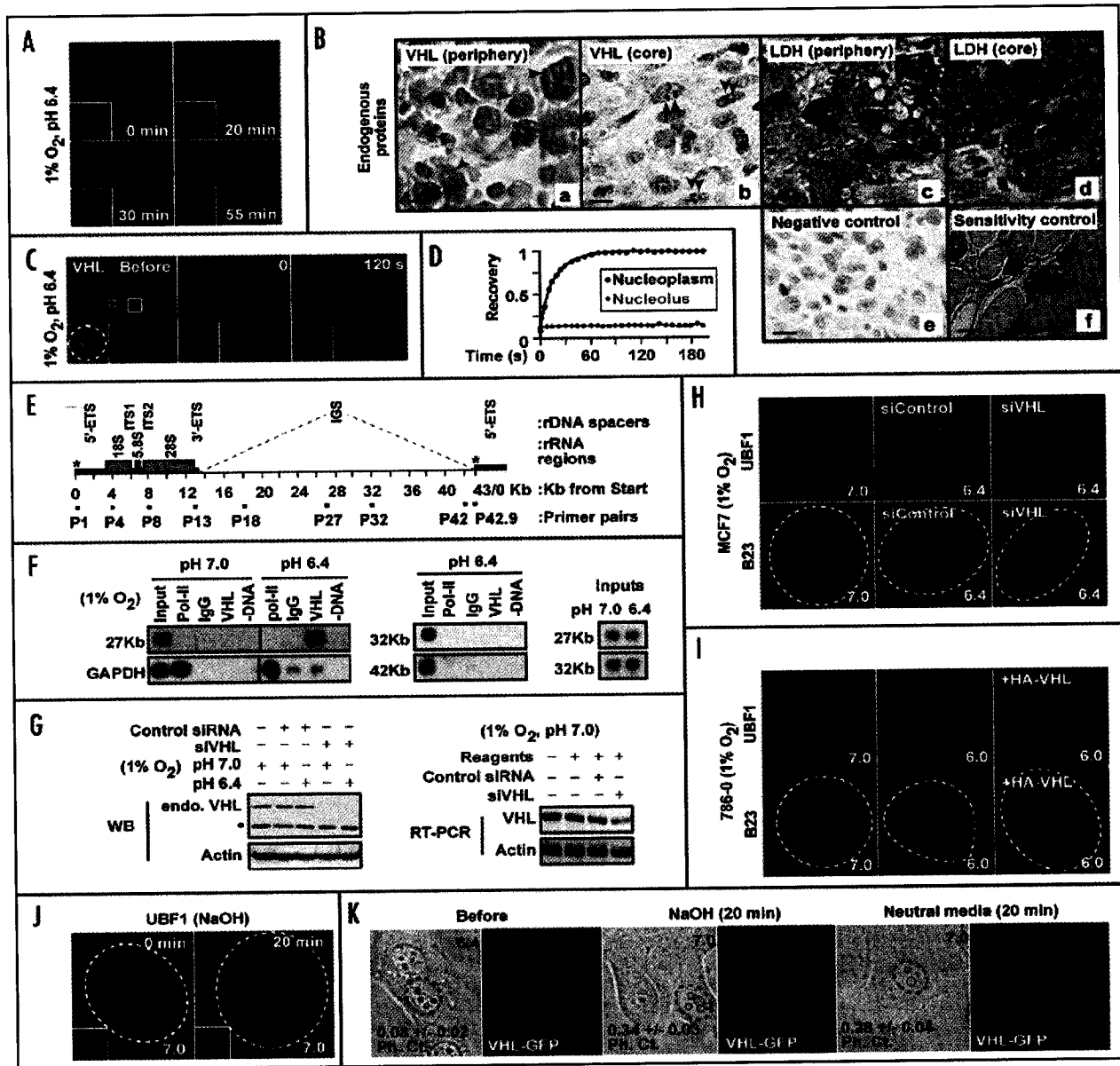


Figure 4. Nucleolar condensation implicates VHL-rDNA interactions. (A) pH-dependent targeting of VHL to the nucleolus. MCF7 cells stably expressing GFP-tagged VHL were incubated under hypoxia for 17 h (t_0) and VHL was monitored for changes in steady state distribution. Insets show Hoechst staining of DNA. (B) Endogenous VHL localizes to nucleoli in the acidic core of tumors. Glioblastoma (U87MG) sections (8 μ m) (a-e) or sections of surrounding muscle tissue (f) were submitted to immunohistochemistry analysis using antibodies recognizing endogenous VHL (a and b) or LDH (c, d and f). Note that while VHL localization changes from mainly cytoplasmic near the tumor periphery to an exclusively nucleolar distribution near the core of the tumor, LDH is cytoplasmic independent of distance from tumor surface. Scale bars represent 5 μ m. (C and D) FRAP analysis reveals H^+ -dependent static detention of VHL in subnucleolar foci reminiscent of condensed fibrillar centers. Nucleoplasmic fluorescence was first reduced by five laser pulses (yellow square) before performing FRAP. Inset is a zoom of area marked by white square showing a specific nucleolus (dashed circle). Cells were imaged before and after bleaching arrow-marked focus (C) and kinetics of fluorescence recovery was revealed by time-lapse microscopy and plotted against the kinetics of VHL in the nucleoplasm of another cell incubated under the same conditions (D). (E) Human rRNA gene unit. Primer pairs (■) for ChIP and their positions in Kb relative to the transcription start site (*) are shown. E/I-TS, external/internal transcribed spacer; IGS, intergenic spacer. (F) ChIP analysis reveals VHL/rDNA interactions. MCF7 cells expressing adenovirus-introduced VHL-GFP were incubated under hypoxia for 22 h then ChIP was performed. VHL interacts with the IGS of rDNA around the 27 Kb region. Controls are the pH-independent interaction of RNAPII with the GAPDH gene, the absence of any detectable interaction of VHL with other regions of rDNA (only two of these regions are shown as others yielded similar results), and inputs. (G) Characterization of siVHL. Western blotting (WB) and RT-PCR analysis reveal that transiently expressed siVHL silences endogenous VHL expression in hypoxic MCF7 cells independent of pH. Results from cells expressing a random siRNA control (siControl) are also shown. (H to K) VHL is required for H^+ -dependent nucleolar condensation after a 22 h hypoxic incubation but reintroduction of HA-tagged VHL into VHL-deficient 786-0 cells by stable expression sensitizes these cells to the effect of H^+ on rDNA (I). MCF7 cells were transfected to transiently express UBF1-GFP (J) or infected to express VHL-GFP (K) then transferred to hypoxia to induce acidosis and nucleolar condensation. Acidotic media was then neutralized by addition of sodium hydroxide (NaOH) or cells were replenished with fresh SD media for 20 min (J and K). Ratios of nucleolar to nuclear area with SEM are shown for each panel in (K). Certain nuclei (dashed circles), nucleoli (red circles) and subnucleolar foci (arrows) are outlined to highlight nucleolar condensation states. Extracellular pH is indicated on each panel.

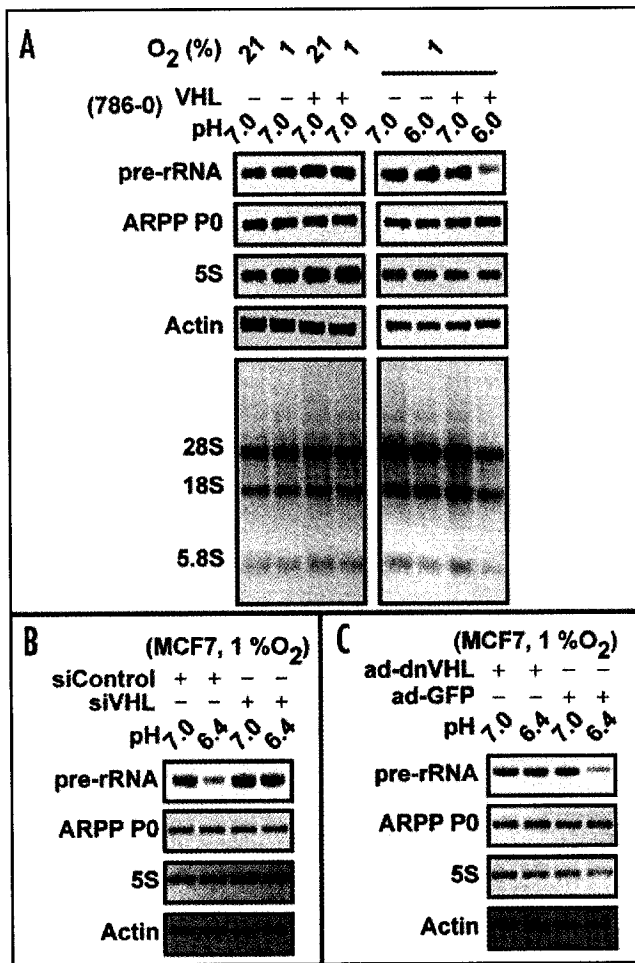


Figure 5. Nucleolar VHL is required for acidosis-dependent restriction of rDNA transcription. (A) VHL-deficient 786-0 cells do not exhibit H⁺-dependent rDNA silencing characteristics after a 22 h incubation under indicated conditions. Reintroduction of HA-tagged VHL into VHL-deficient 786-0 cells by stable expression sensitizes these cells to the effect of H⁺ on rDNA. (B and C) Silencing VHL expression with transiently transfected siVHL (B) or competing nucleolar-VHL interactions with adenovirus-introduced dnVHL (C) desensitizes MCF7 cells to the effects of acidosis on nucleolar rDNA.

can have significant effects on HIF activity under hypoxia.¹⁰⁻¹⁵ Since HIF activity was not affected by pH under hypoxia (Fig. 6C, lanes 7 and 8, compared to lanes 4 and 5), it is reasonable to suspect that ROS production is similar under both neutral and acidic conditions within our experimental settings. This suggests that the observed effects of H⁺ on nucleoli are not due to gross pH-dependent differences in ROS production or mitochondrial status. Taken together, this reveals that H⁺/VHL-dependent nucleolar rDNA remodeling under hypoxia is promoted by, but does not require, HIF activity.

Intersection of pH and oxygen in the H⁺/VHL-dependent control of nucleolar rDNA. We found that cells submitted to hypoxia-induced acidosis triggered nucleolar condensation and reductions in the levels of pre-rRNA, both of which persisted following reoxygenation until neutral pH conditions were reinstated (Fig. 6E). Treatment of cells with AP media that had previously been conditioned to acidosis by another batch of cells in hypoxia (Fig. 6F, lanes 2 and 5, compared to lanes 1 and 4) or by direct addition of HCl (Fig. 6F, lanes 3 and 6, compared to lanes 1 and 4) (pH stable

at 6.4) triggered nucleolar condensation and reduced pre-rRNA levels both in the presence or absence of oxygen. SD media that was similarly conditioned by cells under hypoxia failed to induce any change in nucleolar morphology or rDNA transcription (Fig. 6F and data not shown). This reveals that H⁺ does not require lactate molecules or hypoxia to trigger the observed phenotype.

Acidosis-dependent restriction of ribosomal biogenesis in the presence of oxygen also required VHL as siRNA-mediated silencing of endogenous VHL expression in MCF7 cells abolished the phenomenon (Fig. 6G). Substitution of key proline residues within HIF-1 α or HIF-2 α to alanine generates HIF α variants (vHIF-1 α and vHIF-2 α) that evade proteasomal degradation and activate HIF target genes such as glucose transporter-1 (Glut-1) in the presence of oxygen (Fig. 6H).^{46,47} Introduction of vHIF-1 α or vHIF-2 α (Fig. 6H) or dnHIF (data not shown) failed to affect pre-rRNA levels (Fig. 6H) or induce nucleolar condensation in normoxia (data not shown). Taken together, these findings suggest that pH overrides oxygen levels in the H⁺/VHL-dependent control of rDNA.

Abrogating H⁺-dependent rDNA restriction prevents acidosis from sustaining energy equilibrium and viability under hypoxia. We noticed that a small fraction of the MCF7 cell population (less than 2%), which failed to target VHL to the nucleolus under acidosis,²⁷ incorporated trypan blue under hypoxia-acidosis (Fig. 7A). Thus, we tested if abrogating rDNA silencing would prevent acidosis from preserving cellular energy equilibrium and viability. Silencing endogenous VHL expression in MCF7 cells prevented acidosis-dependent preservation of ATP levels and viability under hypoxia (Fig. 7B). VHL-deficient 786-0 cells failed to maintain ATP concentrations or exhibit protection by acidosis under hypoxia but were resensitized to both of these effects of acidosis following the re-establishment of HA-VHL expression (Fig. 7B and C). These experiments were performed at time points where endogenous VHL was confined to nucleoli (Fig. 7D), where the tumor suppressor is known to be statically detained (Fig. 4D and see ref. 29), eliminating the potential implication of nonnucleolar functions of VHL in the herein observed energy homeostatic program. As expected, cellular viability was unaffected by dnHIF-mediated blockade of HIF activity under all tested conditions (Fig. 7E). Although dnVHL introduced into VHL-deficient cells is statically detained by the nucleolar architecture,²⁹ this dominant negative molecule did not sensitize these cells to the protective effect of acidosis nor did it increase toxicity under hypoxia (Fig. 7F). In stark contrast, introduction of dnVHL at levels sufficient to compete with the wild-type protein pool into both 786-0 cells stably expressing reintroduced HA-VHL as well as MCF7 cells abolished the protective effect of acidosis under hypoxia (Fig. 7F and G).²⁷ These results suggest that oxygen-starved cells conserve their limited energy by gauging the environmental concentration of hydrogen ions and restricting ribosome production.

DISCUSSION

We report that cells regulate energy demand by sensing environmental H⁺ concentration. Cells maintain their total energy levels and remain viable regardless of oxygen tension only when allowed to undertake the natural route to acidosis, a process explained by H⁺-dependent reduction in cellular ATP expenditure. We demonstrate that acidosis restricts ribosomal biogenesis, the most energy-demanding cellular process, at the level of pre-rRNA synthesis. Determination of the cell-type-specific pH thresholds required for RNAPI transcriptional silencing lead us to investigate

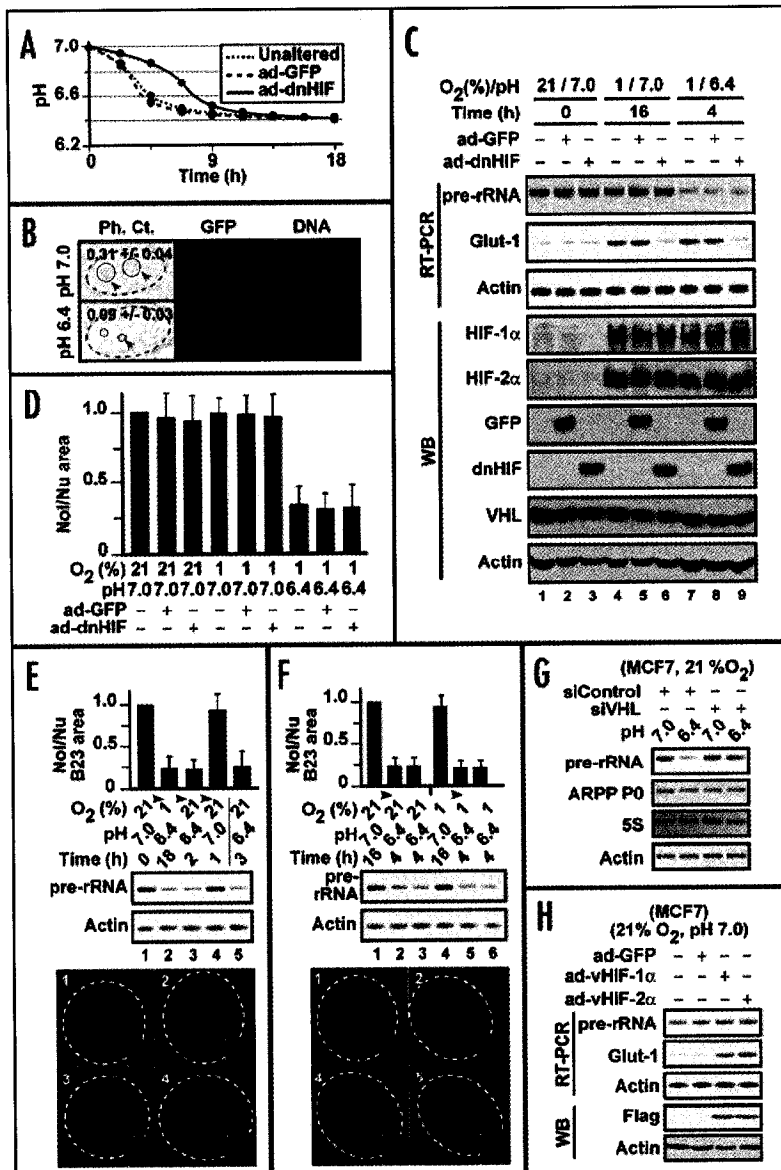


Figure 6. H⁺/VHL-dependent nucleolar rDNA remodeling is promoted by, but does not require, HIF activity. (A and B) HIF activity promotes extracellular acidification. Adenovirus-mediated introduction of GFP-tagged dnHIF, but not GFP alone, lowers the rate of acidification of the extracellular milieu by MCF7 cells under hypoxia (A) but does not prevent nucleolar condensation which is observed in representative MCF7 cells expressing GFP-tagged dnHIF (22 h hypoxia) (B). Arrow heads highlight differences in nucleolar size. (C and D) Acidosis-dependent modulation of rDNA under hypoxia does not require HIF activity. MCF7 cells that are unaltered or expressing adenovirus-introduced GFP-tagged dnHIF or GFP alone were incubated in SD media under hypoxia for 16 h (pH stable at 7.0) then replenished for 4 h with AP media that had been conditioned to acidosis (pH stable at 6.4) by direct addition of the exogenous acid HCl. Cells were submitted to RT-PCR and western blotting (WB) at different time points (C) and quantitation of nucleolar condensation was performed on phase contrast images of cells from a parallel set (N is 30 cells, values are means, and bars represent standard error) (D). (E) Nucleolar condensation and reductions in pre-rRNA levels triggered by hypoxia-induced acidosis persist after oxygenation until neutral pH is reinstated. MCF7 sets transfected to express B23-GFP (graph) or left unaltered (gels) were grown under standard conditions in normoxia (lane 1) then replenished with AP media and transferred to hypoxia for 22 h resulting in extracellular acidification (lane 2). Cells were then reoxygenated for 2 h (lane 3) before neutralization of the media with NaOH for another hour (lane 4). Control cells that were reoxygenated for 3 h without neutralization are also shown (lane 5). Cells were monitored for changes in B23 distribution (N is 30 cells, values are means, and bars represent standard error) and mRNA was isolated for RT-PCR analysis. Representative images corresponding to numbered lanes of gels are shown. (F) Acidosis can trigger nucleolar condensation and reductions in pre-rRNA levels in the presence of oxygen. MCF7 sets transfected to express B23-GFP (graph) or left unaltered (gels) were cultured in normoxia (lane 1) or hypoxia (lane 4) in SD media for 16 h then replenished with AP media that was previously conditioned to pH 6.4 by another batch of hypoxic MCF7 cells (lanes 2 and 5) or by direct addition of HCl (lanes 3 and 6). Cells were monitored for changes in B23 distribution (N is 30 cells, values are means, and bars represent standard error) and mRNA was isolated for RT-PCR analysis. Representative images corresponding to numbered lanes of gels are shown. Gels are representative of three independent experiments. (G) Silencing VHL expression with transiently transfected siVHL desensitizes MCF7 cells to the effects of acidosis on nucleolar rDNA in normoxia. (H) Expression of HIF variants that evade degradation in the presence of oxygen fail to induce nucleolar condensation in normoxia. MCF7 cells expressing adenovirus-introduced HIFα variants were submitted to RT-PCR and Western blot analyses.

the potential implication of the H⁺-dependent nucleolar targeting of VHL in this process. Nucleolar VHL interacts with the IGS of rDNA and was required for H⁺-dependent nucleolar condensation and reduction of rRNA synthesis. Abrogating this program by disrupting the expression or nucleolar accumulation of VHL abolishes the ability of acidosis to lower cellular energy demand, triggers energy starvation and decreases viability. H⁺/VHL-dependent nucleolar rDNA remodeling under hypoxia is promoted by, but does not require, HIF activity. These findings suggest that H⁺ plays a crucial role in basic metabolism and provide a potential explanation for the protective effect of acidosis under ischemic settings.

We would like to amend the model of basic cellular metabolism by adding a central role for H⁺ in modulating the demand side of the cellular energy equation. We propose a model where fermentation, in addition to sustaining ATP production by replenishing glycolysis with NAD⁺ under hypoxia, also generates H⁺ to restrict ribosomal biogenesis, thereby limiting energy expenditure (Fig. 8). We envision two different paths that could lead to the induction of this energy-saving process. In the first scenario, increased lactic

acid production at 25–75% of maximal cellular oxygen uptake (VO₂max),² levels likely not low enough to directly stabilize HIF,⁴⁸ would target VHL to the nucleolus resulting in rDNA silencing and HIF activation to both restrict ATP consumption and promote energy production by glycolysis, respectively (Fig. 8). Alternatively, sharper reductions in oxygen could first stabilize HIF, which then induces anaerobic glycolysis allowing fermentation to generate the hydrogen ions needed to lower cellular energy demands. Therefore, we propose that cells maintain constant ATP levels regardless of their relative engagement in aerobic or anaerobic metabolism, contrasting the traditional idea that hypoxia leads to energy starvation.

We share a view of the nucleolus where it acts as a hub that ensures cellular adaptation to alterations in environmental parameters.^{49,50} In addition to excluding VHL from certain molecular networks to promote the transcription of hypoxia-inducible genes,²⁹ static

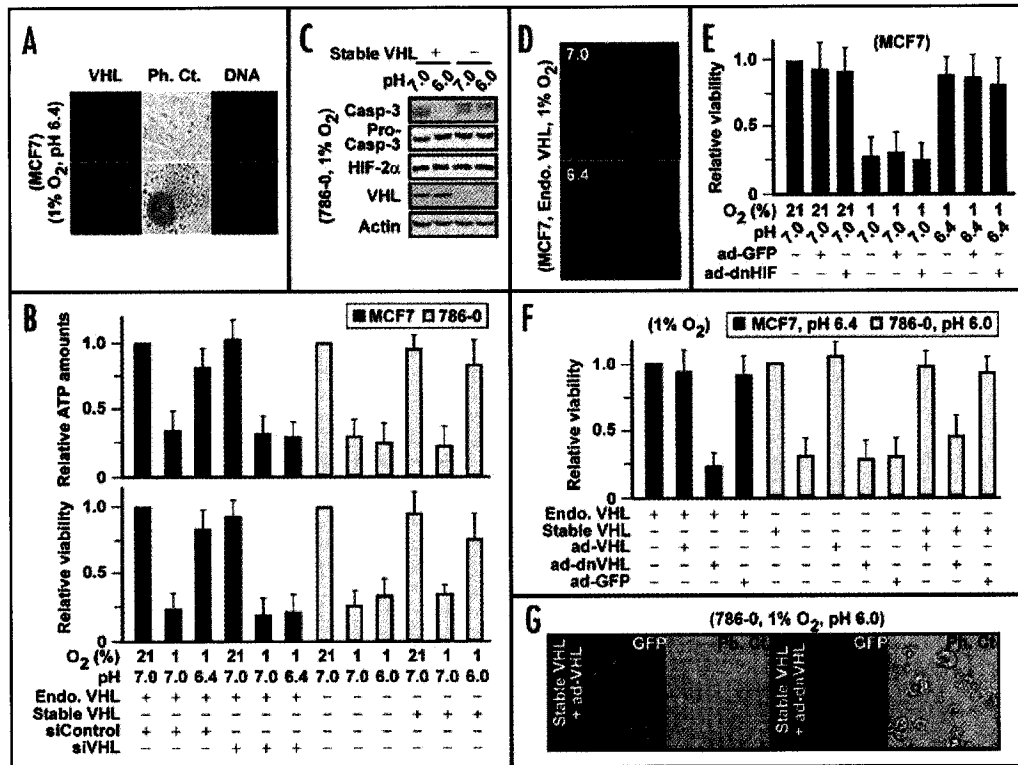


Figure 7. Abrogating H^+ -dependent rDNA restriction by silencing VHL expression or competing VHL-rDNA interactions prevents acidosis from sustaining energy equilibrium and viability under hypoxia. (A) Correlation between nucleolar localization of VHL and survival under hypoxia-acidosis. MCF7 cells incubated for 22 h under hypoxia in AP media were submitted to trypan blue staining. Shown in lower panels is a representative cell of the small number of cells (less than 2%) that did not exhibit nucleolar VHL localization and stained positive for trypan blue under acidosis. (B) Abrogation of H^+ -dependent restriction of ribosomal biogenesis by induced or naturally occurring loss of VHL expression abolishes acidosis-dependent maintenance of energy equilibrium and viability under hypoxia. Transfection of MCF7 cells with siVHL, but not siControl, desensitizes the cells to the protective effects of acidosis. VHL-deficient 786-0 cells were sensitized to acidosis by stable transfection-mediated reintroduction of HA-tagged VHL. Cells were analyzed for ATP levels at 22 h (9 wells from three independent experiments performed in triplicates were assessed, values are means and bars represent standard error) and for viability by the FDA/PI cotreatment method 4 h later (viability scores from three independent experiments are shown). (C) 786-0 cells that are either deficient for VHL expression or stably expressing reintroduced HA-tagged VHL were cultured for 26 h in hypoxia under indicated conditions and assessed for viability by caspase-3 immunoblotting where actin and other proteins were also detected as controls. (D) Immunofluorescence analysis with an antibody specific to endogenous VHL reveals its nucleolar distribution in MCF7 cells incubated for 22 h in hypoxia under AP conditions. (E) Acidosis-dependent preservation of viability does not require HIF activity. MCF7 cells that are unaltered or expressing adenovirus-introduced GFP-tagged dnHIF or GFP alone were incubated under indicated conditions for 26 h and submitted to an FDA/PI viability assay. (F) MCF7 cells or 786-0 cells (with or without stably expressed HA-VHL) were infected with an adenovirus expressing VHL-GFP, dnVHL-GFP, GFP alone, or left unaltered. Cells were then incubated for 26 h under indicated conditions and submitted to an FDA/PI viability assay. (G) Representative GFP fluorescence or phase contrast endpoint images of cells in (F).

nucleolar detention introduces VHL into a network that silences rDNA. Several molecules, including the replication factor PCNA as well as the transcription and repair factor TFIIH, respond to various signals and can alternate between dynamic and static states through interactions with different DNA domains.^{38,42,51,52} It is tempting to speculate that at least some of these molecules are implicated in molecular interactions reflective of “symbiotic relationships”, similar to that of VHL with rDNA, where chromatin is modulated by statically detained proteins, which in turn abandon dynamic functions in other molecular networks.

The history of nucleolar evolution indicates that the IGS of rDNA underwent a disproportionate expansion in size relative to rRNA transcriptional units.⁴³ IGS is known to play key roles in the regulation of rDNA.⁵³ For example, non-protein-coding IGS transcripts are required for the establishment and maintenance of specific heterochromatic configurations at the promoter of a subset of rDNA arrays.⁵⁴ Interestingly, we found that VHL interacts with IGS at regions containing repeats proposed to act as enhancers for RNAPI

transcription.⁵⁵ A possible role for nucleolar VHL in rDNA silencing could consist of inactivating such sequences by direct chemical modification, by preventing their interactions with regulatory molecules, or by recruiting silencing proteins. Whether evolutionary expansion in IGS size contributed to the evolution of the pH-dependent rDNA silencing program described here and whether ethanol fermentation activates similar mechanisms in different species or phyla is of considerable interest but remains to be determined.

Only a certain subpopulation of rRNA genes is active at any given time in the cell.⁴¹ pH levels could be modulating rDNA transcription through two different approaches that are not necessarily mutually exclusive. Cells can control the number of transcripts that are made from each gene. Alternatively, as there are hundreds of rRNA genes, another strategy would be to turn subsets of the rRNA gene pool either “on” or “off”. Since both mechanisms could be rapid and efficient, future work should aid in identifying the exact approach that is employed by acidosis. Unlike acidosis, several previously identified regulated rDNA silencing signals result in

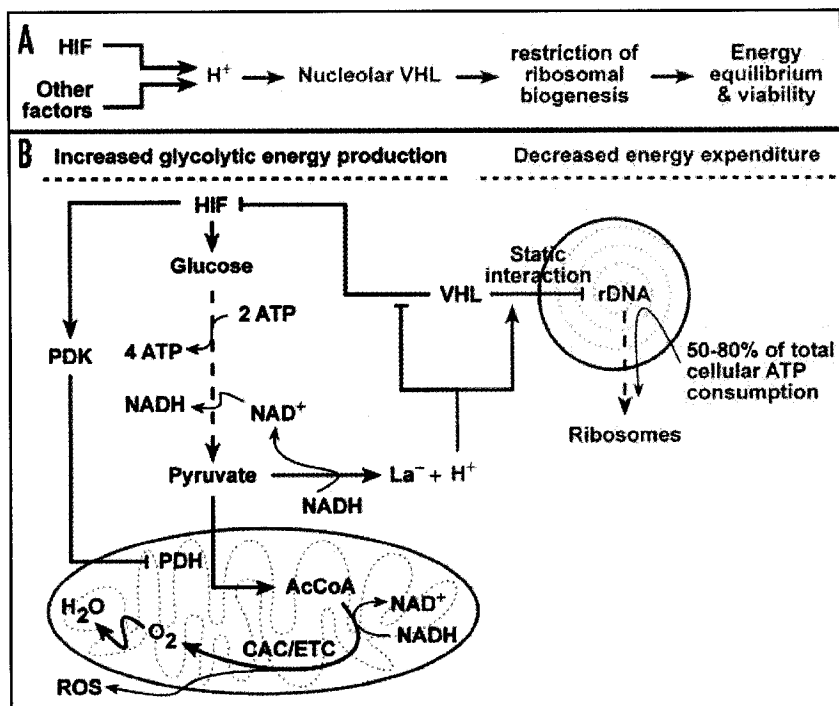


Figure 8. Model for the modulation of cellular energy equilibrium by H^+ . (A) Several factors, including HIF activation under hypoxia, promote H^+ production and acidosis. Cells sensing increases in the environmental hydrogen ion concentration target VHL to the nucleolus to participate in the restriction of the energy-demanding process of ribosomal biogenesis preserving energy equilibrium and viability under low energy supply. (B) The role of H^+ in anaerobic metabolism. Fermentation, in addition to sustaining ATP production by replenishing anaerobic glycolysis with NAD^+ , also generates H^+ to restrict ribosomal biogenesis and limit energy consumption. Two different paths could lead to the induction of this energy-saving process. First, increased lactic acid production at 25–75% of VO_2 max, levels not low enough to directly induce HIF, targets VHL to the nucleolus where it silences rDNA to restrict ATP consumption. Nucleolar sequestration of VHL also induces HIF to actively block mitochondrial respiration and promote energy production by anaerobic glycolysis. Alternatively, sharper reductions in oxygen first stabilize HIF, which then induces anaerobic glycolysis allowing fermentation to generate the hydrogen ions needed to lower cellular energy demands.

EXPERIMENTAL PROCEDURES

Cells and materials. C2C12 cells from ATCC (Manassas, VA) were differentiated by lowering the serum concentration from 5% to 0.5%. MCF7, 786-0 (VHL-defective), U87MG, and NIH3T3 cells were also obtained from ATCC (Manassas, VA). The generation of 786-0 cells stably expressing HA-VHL is previously described.⁶¹ Viability was assessed with a 20 min and 5 min pretreatment with fluorescein diacetate (FDA, 5 μ M) and propidium iodide (PI, 2 μ M) (Sigma, St. Louis, MO), respectively. FDA and PI are unaffected by pH as neutralization of an acidic set or acidification of a neutral set immediately prior to addition of the dyes yielded essentially the same results. 6-deoxyglucose (6-DOG, 6 mM), sodium azide (NaN_3 , 0.02%, v/v), and actinomycin-D (Act-D, 10 μ g·ml⁻¹, Calbiochem) was used where indicated. 6-DOG is insensitive to pH as glucose uptake and lactate release equally decreased following treatment of hypoxic cells with the drug under both neutral and acidic conditions.

Cell culture. Normoxic cells were incubated at 37°C under 5% CO_2 environment. Hypoxia was achieved by incubation in a hypoxic chamber at 37°C under a 1% O_2 , 5% CO_2 and N_2 -balanced atmosphere. Acidosis experiments were conducted as previously described.^{27,29} For standard (SD) or acidosis-permissive (AP) conditions, buffer-free medium (DMEM; Invitrogen, Carlsbad, CA) was freshly prepared and supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. Unless otherwise indicated, $NaHCO_3$ was added at a 44mM (or 18 mM for normoxia experiments) and the pH was adjusted to 7.0 (SD) or 6.0–7.0 (AP) with HCl. Air was bubbled into both media at 22°C, which stabilizes the pH at 7.0. AP media slowly reverted to its original pH (6.0–7.0) under hypoxia, whereas the SD medium remained at pH 7.0. Transfected or adenovirus-infected cells were grown for 24 h under standard conditions before any treatment.

Plasmids and adenoviruses. VHL, its variants and mutants were cloned between an N-terminal Flag-tag and a C-terminal GFP-tag and into pcDNA3.1, as previously described.^{8,27} Adenoviruses were produced using the Cre-lox recombination system. We sincerely thank Tom Misteli (NCI, NIH, Bethesda, MD) for providing the UBF1-GFP construct, and Mark Olson (University of Mississippi

nucleolar disintegration, a phenomenon that is believed to be caused by the complete loss of RNAPI activity and is associated with decreased viability.^{56,57} Acidosis might preserve nucleolar integrity by the fact that it only decreases, rather than completely abolish, rDNA transcription or by actively stabilizing the nucleolar architecture through another pathway. Identification of these processes should help identify precise and targeted approaches to test if restriction of rDNA under hypoxia-neutral conditions, without affecting the expression or nucleolar localization of VHL, would mimic acidosis and prevent energy starvation. We noticed that the occupancy of a fraction of VHL's nucleolar binding sites by dnVHL was sufficient to completely abrogate H^+ -dependent rDNA silencing. A possible explanation of these findings is that the stepwise spreading of VHL, and possibly other proteins,^{58,59} along the rDNA lattice restricts pre-rRNA synthesis but this remains to be tested. Such a model would be similar to that proposed for another system; here the stepwise spreading of the yeast Sir2 protein and its associated proteins along the DNA lattice is required for transcriptional silencing.⁶⁰

Sustainable biological systems balance the production and expenditure of energy. Introduction of oxygen into an anaerobic biosphere over 2.2 billion years ago triggered a drastic reorganization and expansion of complex molecular networks.¹ The advent of mitochondrial respiration and the steady rise in atmospheric oxygen facilitated the evolution of energy-demanding metabolic pathways otherwise incompatible with anaerobic energy production. Contemporary organisms that provisionally retreat to the less sophisticated energy generators under low oxygen tensions must therefore decrease cellular energy consumption to preserve equilibrium and avoid the deleterious effects of energy starvation. Our work reveals that cells rely on H^+ to accurately harmonize cellular energy demands with shifting supply and provides an explanation for the protective effect of acidosis in ischemic and hypoxic settings.

Medical Center, Jackson Mississippi) for providing the B23-GFP construct. Transient transfections were conducted with Effectene transfection reagent (Qiagen, Valencia, CA).

Reverse transcriptase PCR. Analysis was conducted using previously described primers and procedures.^{40,44,62-65} Briefly, cycling parameters for messengers were as follows. Pre-rRNA 5'-ETS: 95°C for 3 min, followed by 25 cycles of [95°C for 1 min, 65°C for 1 min, 72°C for 1 min] and then 72°C for 5 min; ARPP P0: 95°C for 2 min, followed by 23 cycles of [95°C for 1 min, 58°C for 30 s, 72°C for 1 min], and then 72°C for 3 min; 5S rRNA: 94°C for 3 min, followed by 23 cycles of [95°C for 30 s, 58°C for 30 s, 72°C for 1 min], and then 72°C for 5 min; VHL: 95°C for 3 min, followed by 25 cycles of [94°C for 1 min, 55°C for 1 min, 72°C for 1 min], and then 72°C for 5 min; β -actin: 94°C for 2 min, followed by 25 cycles of [94°C for 20 s, 60°C for 20 s, 72°C for 40 s], and then 72°C for 10 min.

BrUTP-labeling and detection of nascent rRNA. BrUTP experiments were performed as described.⁶⁶ RNAPII transcription was inhibited with a 2 h α -amanitin (10 μ g.mL⁻¹) pretreatment. Cells, BrUTP-pulsed only, or pulsed and chased for 10 min, were formaldehyde-fixed and methanol-permeabilized. Nascent rRNA was detected by immunofluorescence analysis using a monoclonal anti-BrdU antibody (Roche Diagnostics, Penzberg, Germany) and an anti-mouse fluorescein-labeled secondary antibody (Roche Diagnostics, Penzberg, Germany). As previously reported,⁶⁶ 15–20% of cells were labeled and images of cells were captured with an Axioskop 2 MOT PLUS microscope (Zeiss, Thornwood, NY) using a digital charged-coupled device camera (Qimaging, Burnaby, BC).

Chromatin crosslinking and immunoprecipitation. Experiments were performed using the EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate Biotechnologies, Lake Placid, NY). Crosslinked cells were lysed and DNA was sonicated to 100–500 bp use of a Sonifier-450 sonicator (Branson; output 3.0, duty cycle 30%). Antibodies detect Flag-M2 (1 μ g) (Sigma, St. Louis, MO), RNA pol-II (1 μ g), acetylated H3 (10 μ g), acetylated H4 (10 μ l) (Upstate Biotechnologies). PCR conditions were 94°C for 3 min, followed by 45 cycles of [94°C for 20 s, 59°C for 30 s, 72°C for 30 s], and then 72°C for 2 min.⁴⁰ Primers P1 (952 to 1030), P4 (3990 to 4092), P8 (8204 to 8300), P13 (12855 to 12970), P18 (18155 to 18280), P27 (27366 to 27477), P32 (32734 to 32859), P42 (41982 to 42075), and P42.9 (42943 to 43033), were (from Ref. 40) and nucleotide positions from start site are based on the Genbank rDNA sequence with accession number U13369.

Photobleaching and microscopy. As described,²⁹ cells were cultured on glass coverslips with a 40 mm diameter in an FCS2 environmental chamber (Biophtechs) maintained at 37°C or directly into 35 mm dishes with coverslip bottoms and visualized with a confocal microscope (MRC 1024; Bio-Rad Laboratories). A 60X plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to five rapid pulses of a 488 nm argon laser at 100% power and image acquisition was conducted at 1% of full laser power. For FRAP experiments, images were collected at 10s intervals. Recovery of the fluorescent signal within a bleached region was calculated as described²⁹ following $I_{rel} = (I_t/I_{(0)}) * (T_{(0)}/T_{(t)})$, where $T_{(t)}$ is the total cellular intensity at time t , $T_{(0)}$ is total cellular intensity before bleach, $I_{(0)}$ is the intensity in the bleached area before bleach, and $I_{(t)}$ is the intensity in the previously bleached area at time t . For FLIP experiments, cells were repeatedly bleached and imaged at 5s intervals and fluorescence loss in unbleached areas was calculated for any losses in

fluorescence by normalizing the fluorescence in the cell of interest to that of a neighboring cell according to $I_{rel} = (I_t/I_{(0)}) * (N_{(0)}/N_{(t)})$, where $I_{(t)}$ is the average intensity of the unbleached nucleus at time point t , $I_{(0)}$ is the average prebleach intensity of the nucleus of interest, and $N_{(0)}$ and $N_{(t)}$ are the average total cellular fluorescence intensities of a neighboring cell in the same field of vision at prebleach or at time point t , respectively. For all bleaching experiments, at least three datasets were analyzed for each result. No differences in protein dynamics were observed when cycloheximide (20 μ g.mL⁻¹) was added 1 h from endpoint. Average pixel intensities were normalized for background fluorescence. Images of living cells from experiments that do not implicate bleaching or of immunofluorescence experiments were captured with a microscope (Axiovert S100TV; Carl Zeiss MicroImaging, Inc.) equipped with a 40X C-Apochromat water immersion objective with a 1.2 NA using a digital charged-coupled device camera (Empix). Pseudocoloring and software packages used to capture images, analyze data, and generate graphs are previously described.²⁹

siRNA. Cells were transiently transfected with double-stranded 21-nucleotide-long small interfering RNA (siRNA) to VHL (siVHL) or with a scramble control (siControl) (Ambion, Austin, TX). See Figure 5G for characterization and controls.

ATP, glucose, and lactate measurements. For ATP, concentrations were measured with the CellTiter-Glo Luminescent Assay (Promega, WI) using a LUMIstar Galaxy luminometer (BMG Labtechnologies, Durham, North Carolina) according to manufacturer's protocol. 15000 Cells plated in 100 μ l of media per well of a 96-well plate (Dyex Technologies, Chantilly, VA) were used. Glucose uptake and lactate release were measured by analysis of media using the Glucose Hexokinase Assay (Sigma, Saint Louis, MO) and the Lactate Reagent and Lactate Standard Set (Trinity Biotech, Saint Louis, MO), respectively, according to manufacturer's protocol.

Western blot analysis. Samples were prepared and Western blots were performed as described.²⁷ Primary monoclonal antibodies recognize HA, Flag-M2 (Sigma, St. Louis, MO), VHL (BD Pharmingen, San Diego, CA), or Caspase-3 (Cell Signaling, Beverly, MA). A primary polyclonal antibody detecting β -actin (Sigma, St. Louis, MO) was also used. A secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was used and detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA).

Immunofluorescence. Cells were seeded onto coverslips and fixed with prechilled (to -20°C) methanol for 10 min followed by acetone for 1 min. An anti-VHL monoclonal antibody (BD Pharmingen, San Diego, CA) was used. Cells were incubated for 1h with a primary antibody solution containing 10% FBS and 1% Triton-X-100 (v/v). Cells were washed several times in PBS before 1 h incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch).

Nude mouse xenograft assays. Nude mouse xenograft assays were done as described elsewhere.⁶¹ In brief, 5 x 10⁶ viable U87MG glioblastoma cells (trypan blue dye exclusion method) were subcutaneously injected in the flanks of female nude mice (Charles River, Wilmington, MA). Tumors were excised two weeks post-injection according to the animal facility protocol (University of Ottawa).

Immunohistochemistry. Experiments were done blinded. Eight micrometer sections mounted on sialinized slides (DakoCytomation, Carpinteria, CA) were dewaxed in xylene and rehydrated using graded ethanol washes. For antigen retrieval, sections were immersed in preheated Dako target retrieval solution (DakoCytomation, Carpinteria, CA) and heat treated in a water bath. Primary antibodies

were anti-human monoclonal VHL (BD Pharmingen, San Diego, CA), anti-human monoclonal LDH (Sigma, St. Louis, MO) and anti-human monoclonal B23 (Sigma, St. Louis, MO), all used at 1/1000. Negative controls were performed for all experiments and included irrelevant primary immunoglobulins of the same isotype or species. Antigen/antibody complexes were revealed by the Envision system (DakoCytomation, Carpinteria, CA) according to the manufacturer's protocol. Sections were counterstained with hematoxylin for 10 s, dehydrated in graded ethanol washes, and mounted with coverslips.

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Identification of a Common Subnuclear Localization Signal[□]

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Proteins share peptidic sequences, such as a nuclear localization signal (NLS), which guide them to particular membrane-bound compartments. Similarities have also been observed within different classes of signals that target proteins to membrane-less subnuclear compartments. Common localization signals affect spatial and temporal subcellular organization and are thought to allow the coordinated response of different molecular networks to a given signaling cue. Here we identify a higher-order and predictive code, $\{[RR(I/L)X_3r]_{(n, n \geq 1)} + [L(\phi/N)(V/L)]_{(n, n \geq 1)}\}$, that establishes high-affinity interactions between a group of proteins and the nucleolus in response to a specific signal. This position-independent code is referred to as a nucleolar detention signal regulated by H^+ (NoDS^{H+}) and the class of proteins includes the cIAP2 apoptotic regulator, VHL ubiquitylation factor, HSC70 heat shock protein and RNF8 transcription regulator. By identifying a common subnuclear targeting consensus sequence, our work reveals rules governing the dynamics of subnuclear organization and ascribes new modes of regulation to several proteins with diverse steady-state distributions and dynamic properties.

INTRODUCTION

Biochemical processes occurring in the nucleus contribute to its compartmentalization, which facilitates the control of molecular networks (Chubb and Bickmore, 2003). Unlike the cytoplasm, compartmentalization in the nucleus does not rely on the concentration of molecules behind membranes to enhance biochemical reactions. Similar gene loci and regulatory proteins are concentrated within specific membrane-less nuclear substructures such as speckles, PML bodies, Cajal bodies, and nucleoli (Misteli, 2001, 2004; Chubb and Bickmore, 2003; Isogai and Tjian, 2003; Zimmer *et al.*, 2004).

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Abbreviations used: AP, acidification-permissive; B23, rRNA processing factor nucleophosmin; cIAP2, inhibitor of apoptosis 1; E3, ubiquitin ligase; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; HIF, hypoxia-inducible factor; HSC70, heat-shock cognate 71-kDa protein; NES, nuclear export signal; NLS, nuclear localization signal; NoDS^{H+}, nucleolar detention signal regulated by $[H^+]$; NoLS, nucleolar localization signal; NoRS, nucleolar retention signal; PBK1, ribosomal L1 domain-containing protein 1; RNF8, RING finger protein 8 ubiquitin ligase; SD medium, standard (medium); VHL, von Hippel-Lindau.

The temporal and spatial precision of intranuclear dynamics of nucleic acids and polypeptides is central to the proper control of the cell cycle, transcription, apoptosis, ubiquitylation, ribosomal biogenesis, and several other pathways (Shou *et al.*, 1999; Visintin *et al.*, 1999; Weber *et al.*, 1999; Dundr *et al.*, 2000, 2004; Barseguian *et al.*, 2002; Wong *et al.*, 2002; Isogai and Tjian, 2003; Leung *et al.*, 2004; Zaidi *et al.*, 2005). For example, silent gene loci at the nuclear periphery can move centripetally when activated to be repositioned away from repressive factors and closer to activators concentrated within particular subnuclear compartments (Kosak and Groudine, 2004; Misteli, 2004).

Several molecules rely on common peptidic sequences to localize to a given membrane-bound compartment. This includes nuclear localization/export signals (NLS or NES, respectively; Conti and Izaurralde, 2001; Kutay and Guttinger, 2005; Lee *et al.*, 2006) and cell membrane localization signals (Shikano *et al.*, 2005). Identification of such sequences has been instrumental in the functional characterization of a very large number of proteins. Some similarities have also been observed within each class of subnuclear targeting signal. This has been the case for the nucleolus, a major nuclear substructure that coordinates many cellular activities including ribosomal production (Lam *et al.*, 2005; Shaw and Doonan, 2005), cell cycle control (Shou *et al.*, 1999, 2001; Visintin *et al.*, 1999; Azzam *et al.*, 2004), DNA damage repair (van den Boom *et al.*, 2004), and tRNA processing (Pashkin *et al.*, 2004). However, signals mediating the localization of proteins to the nucleolus (nucleolar localization signal [NoLS]) can range from a few to over a hundred amino acids (Weber *et al.*, 2000; Catez *et al.*, 2002; Hiscox, 2002). Subnuclear localization signals also include nucleolar retention signals (NoRS; Tsai and McKay, 2005; Reed *et al.*, 2006), and nuclear matrix targeting signals (NMTS; Zeng *et al.*, 1997; Barseguian *et al.*, 2002; Chatterjee and Fisher, 2002; Zimmer

et al., 2004), as well as signals targeting proteins to splicing speckles (Caceres *et al.*, 1997). Shared localization signals are thought to allow the coordinated response of different molecular networks to a given signaling cue.

We had previously reported that the von Hippel-Lindau (VHL) tumor suppressor is targeted for static detention (i.e., change in steady-state distribution coupled to a loss of dynamic properties) by nucleoli in response to increases in the environmental concentration of hydrogen ions (Mekhail *et al.*, 2004a, 2005, 2006). The relocation of VHL to the nucleolus switches the tumor suppressor from hypoxia-inducible gene-silencing to rRNA gene (rDNA)-restrictive molecular networks. This results in an increase in the production and a decrease in the consumption of energy under low oxygen tension (hypoxia; Mekhail *et al.*, 2004a, 2005, 2006).

Initial mapping analysis revealed that a VHL fragment constituted of 30 amino acids is capable of targeting a green fluorescent protein (GFP) reporter protein for static detention in the nucleolus after an increase in extracellular hydrogen ion concentration (Mekhail *et al.*, 2005). We named this new type of protein localization sequence NoDS^{H+} (nucleolar detention signal regulated by H⁺). NoDS^{H+} is inactivated after a return to neutral pH conditions, causing rapid release of detained VHL proteins into the nucleoplasm where they resume their dynamic profile. It is primarily the extremely high affinity of NoDS^{H+} toward nucleoli that makes this subnuclear localization signal considerably different from NoLS and NoRS. However, some similarities do exist as all of these three types of nucleolar targeting sequences contain arginine residues.

Therefore, we reasoned that unraveling the details of the NoDS^{H+} of VHL could provide an explanation for this unusually high affinity for nucleoli. We envisioned that these details could also be used to predict the subnuclear coordinates, dynamic properties, and novel modes of regulation of proteins harboring similar signals. More importantly, identification of similar peptidic codes in other proteins would provide insight into the rules governing subnuclear organization, general protein dynamics, and the role of hydrogen ions in basic cellular metabolism. Consistent with the hypothesis that nucleolar sequestration may be a general phenomenon is the observation that the nucleolus can capture and release several proteins in response to different cellular cues (Andersen *et al.*, 2005).

Mutagenesis studies coupled to steady-state and protein dynamic analyses allowed us to identify and test common characteristics hidden within the highly complex and modular NoDS^{H+} subnuclear targeting sequence of VHL. In addition, we find that, unlike some previously reported subnuclear targeting sequences, NoDS^{H+} does not harbor NLS-like activity. These findings allowed us to identify many proteins that harbor a NoDS^{H+} subnuclear targeting signal. Overall, six of the six proteins studied were targeted to the nucleolus under acidosis regardless of the initial steady state distribution before signal activation. By identifying a common subnuclear targeting consensus sequence, our work reveals rules governing the dynamics of subnuclear organization and ascribes new modes of regulation to several proteins.

MATERIALS AND METHODS

Cells and Materials

MCF7 cells were obtained from ATCC (Manassas, VA). The generation of 786-0 cells stably expressing HA-VHL was previously described (Iliopoulos *et al.*, 1995).

Cell Culture

Normoxic cells were incubated at 37°C under 5% CO₂ environment. Hypoxia was achieved by incubation in a hypoxic chamber at 37°C under a 1% O₂, 5% CO₂, and N₂-balanced atmosphere. Acidosis experiments were conducted as previously described (Mekhail *et al.*, 2004a, 2005). For standard (SD) or acidosis-permissive (AP) conditions, buffer-free medium (DMEM; Invitrogen, Carlsbad, CA) was freshly prepared and supplemented with 5% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin-streptomycin. Unless otherwise indicated, NaHCO₃ was added at 44 mM (or 18 mM for normoxia experiments), and the pH was adjusted to 7.2 (SD) or 6.3 (AP) with HCl. Air was bubbled into both media at 22°C, which stabilized the pH at 7.2. The AP medium slowly reverted to its original pH (6.3) under hypoxia, whereas the SD medium remained at pH 7.2. Transfected or adenovirus-infected cells were grown for 24 h under standard conditions before any treatment.

Plasmids and Adenovirus Construction

VHL and VHL constructs were cloned between a Flag-tag and a C-terminal GFP and into pcDNA3.1, as previously described (Bonicalzi *et al.*, 2001; Groulx and Lee, 2002). RNF8, cIAP2, HSC70, and their predicted NoDS^{H+}, as well as PBK1 and HSP110, were cloned in the same manner as VHL. Cells were transiently transfected using Effectene Transfection Reagent (Qiagen, Mississauga, ONT, Canada).

Triton Solubility Assay

Cells were harvested in transport buffer containing 20 mM HEPES (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol (DTT), and a cocktail of protease inhibitors added shortly before use (leupeptin, 2 µg/ml; aprotinin, 2 µg/ml; and pepstatin A, 1 µg/ml). Cells were left on ice to equilibrate for 1 min, and Triton X-100 was added (1% vol/vol). Permeabilization was monitored by fluorescence microscopy with Hoechst stain 33258 (Sigma, St. Louis, MO), which only stained nuclei of permeabilized cells. Cells were then centrifuged, to separate triton-insoluble from soluble material, and lysed at a final concentration of 5% SDS in a manner to maintain equal final volume for both fractions.

Nucleolar Isolation by Sucrose Gradient

Nucleoli of MCF-7 cells were essentially isolated as previously described (Andersen *et al.*, 2002). Briefly, ~8 × 10⁷ cells were collected by trypsinization, incubated with a hypotonic solution (10 mM HEPES, 10 mM KCl, 1.2 mM MgCl₂, and 0.5 mM DTT in water) and homogenized using a Dounce tissue homogenizer until ~90% of cells were lysed, but nuclei remained intact. Lysates were centrifuged at 300 relative centrifugal force (rcf) for 7 min, and the pellet was resuspended in 0.25 M sucrose, 10 mM MgCl₂ solution (S1 solution) and layered over a 0.35 M sucrose, 0.5 mM MgCl₂ solution (S2 solution) before centrifuging at 2000 rcf for 7 min at 4°C. Pure nuclei obtained were resuspended in S2 solution and sonicated for 6–8 25-s bursts (with 15-s intervals) using a 60 Sonic dismembrator (Fisher Scientific, Pittsburgh, PA) set at power 6 (0.7 W). Sonicated material was layered over a 0.88 M sucrose, 0.5 mM MgCl₂ solution (S3 solution) and centrifuged 15 min at 3100 rcf at 4°C, and the nucleolar pellet was washed with S2 solution, centrifuged 7 min at 2000 rcf and resuspended in 0.5 ml of S2 solution for storage at –80°C. The purity of isolated nucleoli was assessed both by light microscopy and by Western blot using antibodies against either nucleolar (fibrillarin) or cytoplasmic (LDH) proteins.

Western Blot Analysis

Samples were prepared and Western blots were performed as described (Mekhail *et al.*, 2004a). Primary monoclonal antibodies recognize hemagglutinin (HA), Flag-M2, and lactate dehydrogenase (LDH; Sigma) and p125 and HSC70 (Abcam, Cambridge, MA). Primary polyclonal antibodies detecting RNF8, cIAP2 (Abcam) and fibrillarin (Santa Cruz Biotechnology, Santa Cruz, CA) were also used. A secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was used and detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer-Cetus, Boston, MA).

Immunofluorescence

Cells were seeded onto coverslips and fixed with prechilled (to –20°C) methanol for 10 min followed by acetone for 1 min. Anti-HSC70 (Santa Cruz Biotechnology), anti-cIAP2 and p125 (Abcam), and anti-B23 (Sigma) monoclonal antibodies were used. Cells were incubated for 1 h with a primary antibody solution containing 10% FBS and 1% Triton X-100 (vol/vol). Cells were washed several times in phosphate-buffered saline before 1-h incubation with a secondary Texas Red-labeled antibody (Jackson ImmunoResearch). Hoechst stain 33342 (Sigma) was added to visualize nuclei and coverslips were mounted using Fluoromount G (EMS, Hatfield, PA).

Photobleaching and Microscopy

As described by Mekhail *et al.* (2005), cells were cultured into 35-mm dishes with coverslip bottoms and visualized with a confocal microscope (MRC 1024; Bio-Rad Laboratories, Richmond, CA). A 60× plan Apo oil immersion lens with a 1.4 NA was used for bleaching and imaging. Indicated areas were exposed to five rapid pulses of a 488-nm argon laser at 100% power, and image acquisition was conducted at 1% of full laser power. For fluorescence recovery after photobleaching (FRAP) experiments, images were collected at 10-s intervals (or 1 s for highly mobile proteins). Recovery of the fluorescent signal within a bleached region was calculated as described by Mekhail *et al.* (2005). For fluorescence loss in photobleaching (FLIP) experiments, cells were repeatedly bleached and imaged at 5-s intervals, and fluorescence loss in unbleached areas was calculated to account for any losses in fluorescence by normalizing the fluorescence in the cell of interest to that of a neighboring cell according to $I_{rel} = (I_{(t)}/I_{(0)}) * (N_{(0)}/N_{(t)})$, where $I_{(t)}$ is the average intensity of the unbleached nucleus at time point t , $I_{(0)}$ is the average prebleach intensity of the nucleus of interest, and $N_{(0)}$ and $N_{(t)}$ are the average total cellular fluorescence intensities of a neighboring cell in the same field of vision at prebleach or at time point t , respectively. For all bleaching experiments, at least three datasets were analyzed for each result. Average pixel intensities were normalized for background fluorescence. Images of living cells from experiments that do not implicate bleaching or of immunofluorescence experiments were captured with a microscope (Zeiss Axiovert S100TV; Carl Zeiss MicroImaging, Thornwood, NY) equipped with a 40× C-Apochromat water immersion objective with a 1.2 NA using a digital charged-coupled device camera (Empix, Mississauga, ONT, Canada). Pseudocoloring and software packages used to capture images, analyze data, and generate graphs were previously described (Mekhail *et al.*, 2005).

Bioinformatic Analyses

Searches for candidate proteins with the subnuclear targeting consensus sequence (with up to 110 residues separating the arginine from the first three-residue hydrophobic repeat) were conducted on the UniProtKB/SwissProt database (Bairoch *et al.*, 2004) with the SynaREX function of My Genomics Resource Centre (MGRC) using motifs R-R-I/L-X₃-R-X₀₋₁₀₀-L-φ-V/L, L-φ-V/L-X₀₋₁₀₀-R-R-I/L-X₃-R, R-R-I/L-X₀₋₁₁₀-L-φ-V/L, L-φ-V/L-X₀₋₁₁₀-R-R-I/L, where φ symbolizes any hydrophobic residue. Retrieved entries were then filtered for low arginine domain disorder using the DisEMBL program (Linding *et al.*, 2003). Transmembrane proteins containing the consensus sequences were not eliminated from the list as acidosis could thus signal the nucleolar sequestration of these proteins.

RESULTS

Initial Dissection of a Subnuclear Targeting Signal Reveals Three Criteria

To study the activity of subnuclear targeting sequences within VHL, we incubated MCF7 cells in SD medium, which prevents fluctuations in pH, or in AP medium, which is prepared to enable cells to naturally acidify their extracellular milieu (see *Materials and Methods*; Mekhail *et al.*, 2004a, 2005, 2006). GFP-tagged VHL or a fragment constituted of its amino acids 100-130 completely relocate from a diffuse nucleocytoplasmic distribution to nucleoli only upon the establishment of acidosis (Figure 1A). We first set out to assess the requirement for residues 100-130 in the subnuclear targeting of the VHL tumor-suppressor protein. Deletion of residues 100-130 or of small sections within these residues did not abrogate targeting to nucleoli suggesting that other residues within VHL harbor similar targeting capacity (Figure 1B, lines 1–6 and 16). Stepwise mapping of VHL from the C-terminus revealed that a second nucleolar targeting domain may reside within amino-acids 152-186 (Figure 1B, lines 8–16). Closer examination of these VHL polypeptide sequences revealed three striking patterns (Figure 1B and Supplementary Figure S1). First, positive polypeptides contained at least one of two homologous arginine-rich segments constituted of residues 107-RRHSYR-113 or 176-RRLDIVR-182, with a putative consensus sequence RR(I/L)X₃R (Figure 1, B and C, and Supplementary Figure S1, A and B). Second, positive fragments contain at least one or two three-residue hydrophobic repeats located between the two arginine domains with each repeat starting with a leucine and ending with either a valine or leucine (LWL,

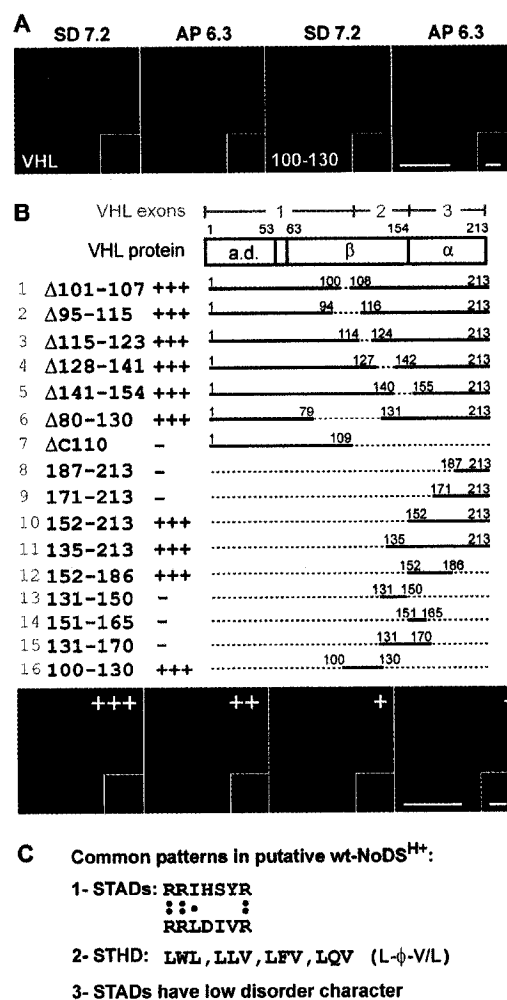
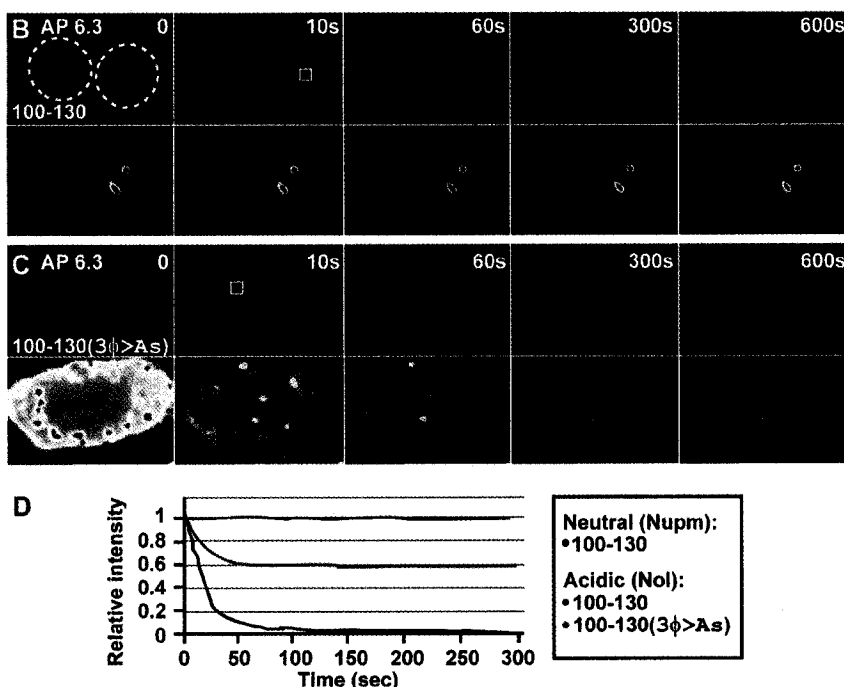


Figure 1. Initial mapping of VHL reveals high-order and multi-partite character of its pH-dependent nucleolar targeting signal. (A) Steady-state distribution of wild-type VHL and VHL(100-130) under neutral or acidic conditions. MCF7 cells were cultured in standard media (SD) and transiently transfected to express the indicated GFP-tagged polypeptides. Cells were replenished with either fresh SD medium (pH 7.2) or acidification permissive medium (AP, initial pH 7.2) that allows maximal extracellular acidification to pH 6.3 (see *Materials and Methods*). Cells were transferred to hypoxia (1% O₂) for 18 h. Extracellular pH at the end point is indicated on each panel. (B) VHL mapping analysis. MCF7 cells transfected to transiently express indicated GFP-tagged proteins were incubated in AP medium in hypoxia. Schematic of VHL exons and derived amino acid domains are shown. Nucleolar localization was scored according to representative images (bottom). For A and B, insets show Hoechst staining of DNA; scale bars, 10 μm. (C) Shared characteristics of VHL fragments displaying nucleolar detention under acidosis. Subnuclear targeting arginine domains (STADs) as well as subnuclear targeting hydrophobic domain (STHD) composed of three-residue hydrophobic repeats are shown.

LLV, LFV, LQV; Figure 1, B and C, and Supplementary Figure S1, A and B). Third, both arginine domains are located in the wild-type VHL protein within regions with low probability for structural disorder as calculated by the DisEMBL program (Figure 1C and Supplementary Figure S1C; Linding *et al.*, 2003). The arginine domains are located at regions of disorder probabilities of <0.03, 0.2, and 0.32 compared with threshold levels for disorder of 0.09, 0.5, and 0.43, following the predictors Hot-Loops, Remark-465, and

A	1	100-130	TLPPGTGRRHSYRGHLWLF RDAGTHDGLLV	+++	100%
	2	100-130 (3 ϕ >As)	TLPPGTGRRHSYRGHLWLF RDAGTHDGAAA	++	60%
	3	100-130 (3X>As)	TLPPGTGRRHSYRGHLWLF RDAGAAAGLLV	+++	100%
	4	100-122	TLPPGTGRRHSYRGHLWLF RDA	++	60%
	5	100-113	TLPPGTGRRHSYR	-	NA
	6	115-130	HLWLF RDAGTHDGLLV	-	NA
	7	106-122	GRRHSYRGHLWLF RDA	++	60%

Figure 2. Analysis of a minimal subnuclear targeting signal at the level of steady-state and dynamic phenotypes implicates arginine-rich segments and a stretch of three hydrophobic residues. (A) Mutational analysis of VHL's NoDS^{H+} confirms the importance of hydrophobic repeats and involvement of arginines in subnuclear targeting. MCF7 cells expressing indicated GFP-tagged proteins were incubated in AP medium in hypoxia. Steady-state nucleolar distribution (+/- rating) of VHL constructs and their affinity with the nucleolar architecture (represented by percentage of statically detained fractions as revealed by FLIP analysis) were scored. NA, not applicable. (B and C) MCF7 cells were transfected to express low levels of GFP-tagged VHL(100-130) without (B) or with a hydrophobic repeat substituted by alanines (C). Cells were then submitted to hypoxic treatment under AP conditions and FLIP analysis was performed. Bleached nuclear regions (squares) are shown and pseudocolored panels are included to better illustrate subtle changes in fluorescence intensity. See Supplementary Figure S2, A and B, for controls. (D) Quantitation of FLIP experiments shown in B and C and in Supplementary Figure S2A. Nupm, nucleoplasm; Nol, nucleolus.



Loops or Coil, respectively (Supplementary Figure S1C; Lindling *et al.*, 2003). Agreement between the different predictors supports a role for these arginine-rich sequences in high-affinity interactions. Sequences implicated in hit-and-run interactions such as NLSs are often characterized with high disorder (Lee *et al.*, 2006). No disorder pattern was observed for the three-residue hydrophobic repeats. Taken together, these observations suggest that this subnuclear targeting signal might conform to three main criteria (Figure 1C) to achieve high-affinity interactions with nucleoli: 1) At least one arginine domain (RR(L/L)X₃; hereinafter referred to as subnuclear targeting arginine domain [STAD]; data below will explain lower case r); 2) A number of three-residue hydrophobic repeats (referred to as subnuclear targeting hydrophobic domain [STHD]); and 3) Low disorder character of STADs might contribute to its subnuclear targeting activity.

Validation of NoDS^{H+} Subnuclear Targeting Criteria

To achieve higher sensitivity of detection of the effects of different mutations, we decided to test how they affect both the steady-state distribution as well as the dynamic profile under signal-activating conditions. The affinity of fluorescent protein chimeras for the nucleolus was determined by FLIP experiments (Lippincott-Schwartz *et al.*, 2003). In FLIP, a living cell is repeatedly hit with a laser beam in the same region. Loss of fluorescence in an area outside the bleached spot is reflective of protein mobility between that area and the bleached spot. Both VHL-GFP and 100-130-GFP, which are highly dynamic under neutral conditions, become statically detained by the nucleolar architecture under acidosis

(Figure 2, A, line 1, B, D, and Supplementary Figure S2A; Mekhail *et al.*, 2005). In stark contrast, the resident nucleolar protein B23 rapidly dissociates from the nucleolus in hypoxic-acidotic cells (Supplementary Figure S2B; Mekhail *et al.*, 2005). The 100-130 segment of VHL harbors two of the three-residue hydrophobic repeats of the wild-type protein (Figure 2A, line 1, and Supplementary Figure S1A, line 16). Substitution of the three hydrophobic residues 128-LLV-130 to alanines (Figure 2A, line 2) or deletion of a section spanning these residues (mutant 100-122; Figure 2A, line 4) prevented complete relocation to the nucleolus but nucleolar accumulation was still easily detectable under acidosis (++ steady-state score), suggesting a reduction in nucleolar binding affinity. Consistent with these observations, only 60% of the total protein pool of each of these fragments was still associated with nucleoli after 5- and 10-min FLIP experiments (Figure 2, A, lines 2 and 4, C, and D). In contrast, no change was observed when three nonhydrophobic residues were substituted to alanines (Figure 2A, line 3). Although fragments 100-113 and 115-130 failed to display any activity, 106-122 displayed a 10-min nucleolar affinity score similar to that of 100-122 (Figure 2A, lines 5-7, 3B, and Supplementary Figure S2C), providing a large range of retained activity for analysis by saturated mutagenesis.

Glycine and arginine residues have been randomly observed in NoLS signals (Weber *et al.*, 2000; Catez *et al.*, 2002; Hiscox, 2002). Fragment 106-122 harbors two glycine and four arginine residues (Figure 3A, line 1). Substitution of glycines (Figure 3A, lines 2 and 3) or R120 (Figure 3A, line 7) to alanine failed to affect localization or affinity scores. In contrast, single substitution of each of the other arginine

A	1	106-122	GRRHSYRGHLWLFDA	++	60%
	2	106-122-G106A	ARRHSYRGHLWLFDA	++	60%
	3	106-122-Gs>As	ARRHSYRGHLWLFDA	++	60%
	4	106-122-R107A	GARIHSYRGHLWLFDA	++	45%
	5	106-122-R108A	GRIHSYRGHLWLFDA	++	43%
	6	106-122-R113A	GRRHSYAGHLWLFDA	++	55%
	7	106-122-F119A-R120A	GRRHSYRGHLWLAADA	++	59%
	8	106-122-R108A-H110A	GRAIHSYRGHLWLFDA	++	43%
	9	106-122-GRR>As	AAAIHSYRGHLWLFDA	+	30%
	10	106-122-L116A	GRRHSYRGHAWLFDA	+	0%
	11	106-122-LWL>As	GRRHSYRGHAAAFDA	+	0%
	12	106-122-LWL>As-R107A	GARIHSYRGHAAAFDA	-	NA
	13	106-122-Rs>As	GAAIHSYAGHLWLFDA	-	NA
	14	106-122-Rs>Ds	GDDIHSYDGHLLWLFDA	-	NA
	15	106-122-Rs>Ks	GKKIHSYKGHLLWLFDA	-	NA
	16	106-122-Y112A	GRRHSARGHLWLFDA	++	58%

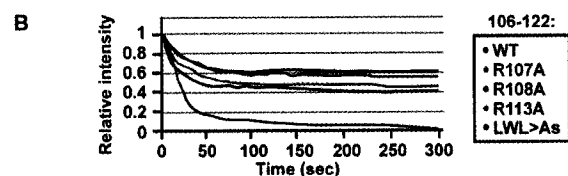


Figure 3. Saturated mutagenesis analysis of fragment 106-122 of VHL highlights three arginines and implicates multiple hydrophobic residues. (A) Effects of mutation of arginine, glycine, and hydrophobic residues within VHL 106-122 on nucleolar steady-state distribution and affinity scores. MCF7 cells expressing GFP-tagged VHL 106-122, or this segment with different mutations, were incubated in AP medium (pH 6.3). Steady-state nucleolar distribution (+/- rating) of VHL constructs and their affinity for the nucleolar architecture (represented by percentage of statically detained fractions as revealed by FLIP analysis) are shown. NA, not applicable. (B) Quantitation of FLIP experiments of the wild type or some mutant 106-122 segments shown in A. WT, wild-type sequence.

residues 107, 108, and 113 to alanine did not have a detectable effect on steady-state distribution but lowered affinity scores from 60 to 45, 43, and 55%, respectively (Figures 3, A, lines 1, and 4–6, B). Similar substitution of F119 or Y112 essentially had no effect on activity (Figure 3A, lines 7 and 16). Concomitant alteration of R107 and R108 rendered it difficult to distinguish nucleolar from nucleoplasmic signal and further lowered the affinity score to 30% (Figure 3A, line 9). Complete loss of nucleolar targeting was observed when all arginines were substituted to alanines, to the negatively charged glutamic acid, or to the positively charged lysines (Figure 3A, lines 13–15), suggesting that arginines cooperate in mediating nucleolar localization in a manner that is not recapitulated by charged amino acids. Histidine residues have been implicated in responses to changes in environmental parameters, including pH and oxygen (Ludwig et al., 2003; Murakami et al., 2004; Stewart et al., 2004; Ishikawa et al., 2005; Lee and Helmann, 2006; Ramsey et al., 2006). Substitution of H110, both alone (data not shown) or in conjunction with R108 (Figure 3A, line 8) did not alter activity. Considering the importance of LLV to this subnuclear targeting sequence (Figure 2, A, line 2, C, and D), we considered a role for another three-residue hydrophobic cluster that also starts with a leucine, 116-LWL-118. Substitution of L116 alone or of all three hydrophobic residues LWL to alanines (Figure 3A, lines 10 and 11) rendered it difficult to distinguish nucleolar from nucleoplasmic signal and completely abolished static detention capability (Figure 3, A, lines 10 and 11, B, and Supplementary Figure S2, C and D), suggesting that three-residue hydrophobic repeats play a key role in the observed phenomenon. Interestingly, concomitant mutation of LWL and only R107 seems to render the sequence completely insensitive to changes in pH levels as nucleoli remain black after the establishment of acidosis

A	1	162-186	CLQVVRSLVKPENYRRLDIVRSLEYE	++	60%
	2	162-186 (LQV>As)	CAAAVRSLVKPENYRRLDIVRSLEYE	+	0
B	1	100-113	TLPPGTGRRHSYR	-	NA
	2	HD1	LLVNQTELFV	-	NA
	3	100-113-HD1	TLPPGTGRRHSYRLLVNQTELFV	++	76%
	4	HD1-100-113	LLVNQTELFVTLPPGTGRRHSYR	++	76%
C	1	(107-113)	AARRHSYR	-	NA
	2	HD1	LLVNQTELFV	-	NA
	3	(107-113) HD1	AARRHSYRLLVNQTELFV	++	75%
	4	(107-113, R107A) HD1	AARRHSYRLLVNQTELFV	++	56%
	5	(107-113, R108A) HD1	AARRHSYRLLVNQTELFV	++	54%
	6	(107-113, I109A) HD1	AARRHSYRLLVNQTELFV	++	55%
	7	(107-113, R113A) HD1	AARRHSYRLLVNQTELFV	++	69%
D	1	RRR-HD1	AARRRRAALLVNQTELFV	-	NA
	2	RRA ₂ R-HD1	AARRRRAALLVNQTELFV	-	NA
	3	RRA ₄ R-HD1	AARRRRAALLVNQTELFV	-	NA
	4	RRIA ₃ R-HD1	AARRIAAALLVNQTELFV	++	58%
	5	RRIA ₂ R-HD1	AARRIAAALLVNQTELFV	++	66%
	6	(107-113) LLVA ₄ LFV	AARRHSYRLLVAAALFV	++	75%
	7	(107-113) LLVA ₂ LFV	AARRHSYRLLVAAALFV	++	74%
	8	(107-113) LLVLFV	AARRHSYRLLVLFV	++	75%
E	1	(176-182) HD1	ARRLDIVRLLVNQTELFV	++	74%
	2	(176-182)	ARRLDIVR	-	NA
	3	(176-182, L178A) HD1	ARRADIVRLLVNQTELFV	++	53%
	4	(176-182, I180A) HD1	ARRLDAVRLVNQTELFV	++	74%
	5	(176-182, L/I>As) HD1	ARRADAVRLVNQTELFV	++	54%

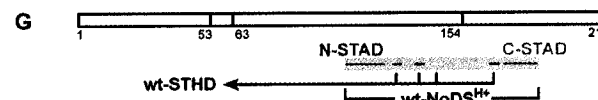
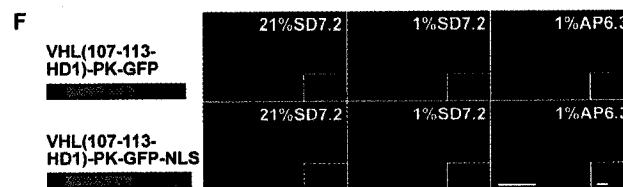


Figure 4. Wild-type NoDS^{H+} of VHL conforms to a set of rules and is composed of two subnuclear targeting arginine-rich domains and multiple hydrophobic repeats with precise clustering features. (A) Effects of amino acid replacement of a three-residue hydrophobic repeat within VHL 162-186 on the nucleolar distribution and affinity of this segment. (B) Analysis of the positioning of hydrophobic clusters relative to the STAD for nucleolar targeting and static detention. (C) Importance of arginine and isoleucine residues within the N-STAD. (D) Effects of disorder and spacing between arginine residues and hydrophobic repeats within the fusion construct 107-113-HD1 on nucleolar steady-state distribution and affinity. (E) Importance of arginine and leucine/isoleucine residues within the C-STAD. (F) VHL's NoDS^{H+} does not have an NLS activity. VHL 107-113-HD1 was fused to the diffusion-incompatible GFP-tagged pyruvate kinase, with or without the SV40 NLS sequence, and localization under neutral or acidic condition was monitored. Insets show Hoechst staining of DNA; scale bars, 10 μm. (G) Schematic of wt-NoDS^{H+} of the VHL tumor suppressor protein.

(Figure 3A, line 12), suggesting a cooperation between arginine residues within the N-terminal STAD (N-STAD) and hydrophobic residues in subnuclear targeting.

A fragment composed of VHL residues 162-186, which contains the three-residue hydrophobic cluster 163-LQV-165 and the C-terminal STAD (C-STAD), exhibited a double-plus nucleolar redistribution under acidosis and a 60% affinity score by FLIP (Figure 4A, line 1). Similar to the effect of mutating LWL in fragment 106-122 (Figure 3A, line 11), substitution of LQV to alanines in fragment 162-186 rendered it difficult to distinguish nucleolar from nucleoplasmic

signal and reduced the 10-min FLIP affinity score to nil (Figure 4A, line 2). It is important to note that this nonfunctional fragment still contains a section where the sequence VRSLVK is present (Figure 4A, line 2). This suggests that a two-residue leucine-containing hydrophobic cluster cannot compensate for the loss of LQV. Similar results were obtained for fragment 106-122(L116A), which still harbors a 117-WLF-119 hydrophobic cluster (Figure 3A, line 10).

The herein analyzed subnuclear targeting signal thus seems to be built with strict components that are organized within a flexible framework. The fragment constituted of residues 100-113, which contain the N-STAD, fails to exhibit any nucleolar targeting activity (Figure 2A, line 5, 4B, line 1). A relatively downstream fragment composed of residues 128-137 (hereon called hydrophobic domain 1 or HD1), which contains two hydrophobic clusters but no arginine residues, is also completely insensitive to changes in extracellular pH levels (Figure 4B, line 2). Thus, we predicted and found that fusion proteins 100-113-HD1 and HD1-100-113 exhibit significant activity, as reflected by a double-plus steady-state distribution pattern and a 76% nucleolar affinity score (Figure 4B, lines 3 and 4, and Supplementary Figure S2E). Similar results (75% affinity score) were obtained for the fusion protein 107-113-HD1 (Figure 4C, lines 1-3), further supporting data indicating that G106 is not important for subnuclear targeting (Figure 3A, lines 2 and 3). Single substitutions of arginine to alanine within 107-113-HD1 confirmed a more prominent role for R107 and R108 relative to R113 (Figure 4C, lines 4, 5, and 7, compared with 3A, lines 4-6). Interestingly, I109 seems to be as important as R108 in establishing high-affinity interactions with the nucleolus (Figure 4C, lines 5 and 6). However, removal of I109 along with 110-HSY-112 completely abolished nucleolar targeting capacity, irrespective of the spacing between the arginine residues (Figure 4D, lines 1-3). DisEMBL-mediated analyses revealed that while single or combined substitutions of the arginine or isoleucine residues to alanine or cysteine do not affect disorder levels of the 107-113-HD1 fragment or the wild-type VHL protein, combined substitutions of 110-HSY-112 to alanines introduce above threshold disorder levels within that region (Figure 4D, line 4, and Supplementary Figure S3, line 2; data not shown; Linding *et al.*, 2003). Consistent with this prediction, substitution of HSY within 107-113-HD1 to alanines decreased affinity to nucleoli from a detention score of 75% (Figure 2C, line 3) to 58% (Figure 4D, line 4). This suggests that residues embedded between the key arginines cooperate in the maintenance of low disorder, as expected for sequences involved in the establishment of high-affinity interactions (Lee *et al.*, 2006). Interestingly, as predicted by DisEMBL, artificial reduction of disorder below threshold levels via reduction of the spacing between I109 and R113 from three to two alanines increases affinity from 58 to 66% (Figure 4D, lines 4 and 5, and Supplementary Figure S3, lines 2 and 3). Alteration of spacing between the three-residue hydrophobic repeats increased or decreased their level of disorder but had no detectable effect on nucleolar affinity scores (Figure 4D, lines 6-8; data not shown), supporting the prediction that low disorder enhances the function of STADs but not the hydrophobic repeats (Supplementary Figure S1C). Mapping analysis of the C-STAD of VHL revealed that L178 behaves similarly to I109 of the N-STAD (Figure 4, E, lines 1-5, and C, line 6). Similar to previous reports, reduction of the temperature from 37 to 22°C did not have any significant effect on the kinetics or extent of recovery of any of the tested proteins in the nucleus or cytoplasm (data not shown and see Phair and Misteli, 2000). A chimeric protein containing the 107-113-HD1 seg-

ment of VHL fused to the nuclear diffusion incompatible GFP-tagged pyruvate kinase (PK-GFP) failed to exhibit any nuclear or nucleolar accumulation after a shift to acidosis (Figure 4F). Introduction of an NLS signal into the protein chimera resulted in nucleolar accumulation under acidosis, suggesting that NoDS^{H+} does not harbor NLS-like activity under either neutral or acidic conditions (Figure 4F). PK-GFP-NLS alone does not exhibit any nucleolar targeting activity under neutral or acidic conditions (data not shown). Interestingly, we noticed that both arginine domains are positioned on one side of the VHL molecule, whereas the three-residue leucine-containing hydrophobic repeats are clustered on the other face of the molecule (data not shown). Although several of our small constructs are efficiently targeted to nucleoli (Figures 1-4), we cannot completely rule out the possibility that this differential positioning of NoDS^{H+} components does not contribute in any way to the function of the sequence within the setting of the wild-type VHL protein. Taken together, these findings suggest that the analyzed subnuclear targeting signal (Figure 4G) generally conforms to three rules: 1) At least one STAD [RR(I/L)X₃r] (where r reflects a more accessory role for the last arginine); 2) Preferably, two or more three-residue hydrophobic repeats [L ϕ (V/L)]; and 3) STADs are preferably positioned within low disorder regions.

Rules Help Identify Additional Proteins Targeted to Nucleoli in Acidosis

We thus suspected that proteins that abide by these rules would be targeted for high-affinity interactions with the nucleolar architecture after signal activation. A search of human proteins in the SwissProt database (Bairoch *et al.*, 2004) using the SynaRex program of MGRC followed by filtering for low structure disorder of STADs with the DisEMBL program was performed (Supplementary Figure S4; data not shown; Linding *et al.*, 2003). This allowed us to generate a list of candidate proteins (Supplementary Table S1). We chose three of these—RING finger protein ubiquitin ligase/transcription regulator RNF8 (SwissProt entry O76064), inhibitor of apoptosis cIAP2 (Q13489), and DNA polymerase delta catalytic subunit p125 (P28340)—at random for analysis of the full-length proteins and their predicted subnuclear targeting sequences. As indicated in the SwissProt database, GFP-tagged wild-type RNF8 and cIAP2 displayed nucleoplasmic and nucleocytoplasmic distribution under standard growth conditions, respectively (Figure 5A). Both the GFP-tagged versions of RNF8 and cIAP2 as well as their predicted subnuclear targeting sequences alone exhibited a complete relocation to nucleoli upon acidosis as revealed by fluorescence microscopy as well as immunofluorescence colocalization studies with the resident nucleolar protein B23 (Figure 5, A, B, and G; data not shown). Similar relocation of endogenous cIAP2 and p125 was detected by immunofluorescence microscopy (Figure 5, C and D). Because of their inherent density, pure nucleoli can be isolated from cell culture using a combination of sonication and sucrose density centrifugation (see *Materials and Methods*; Andersen *et al.*, 2002). Endogenous RNF8, cIAP2, and p125 proteins behaved similar to VHL and were detected in nucleoli isolated from acidotic cells only (Figure 5E). In addition, detergent-based biochemical fractionation revealed that acidosis triggered a complete shift of endogenous cIAP2 and p125 from the triton-soluble to the triton-insoluble cellular fraction, which is enriched in resident nucleolar proteins such as fibrillarin (Figure 5F; Mekhail *et al.*, 2004a).

We next used FRAP to assess the kinetic properties of RNF8-GFP and cIAP2-GFP (Lippincott-Schwartz *et al.*, 2003).

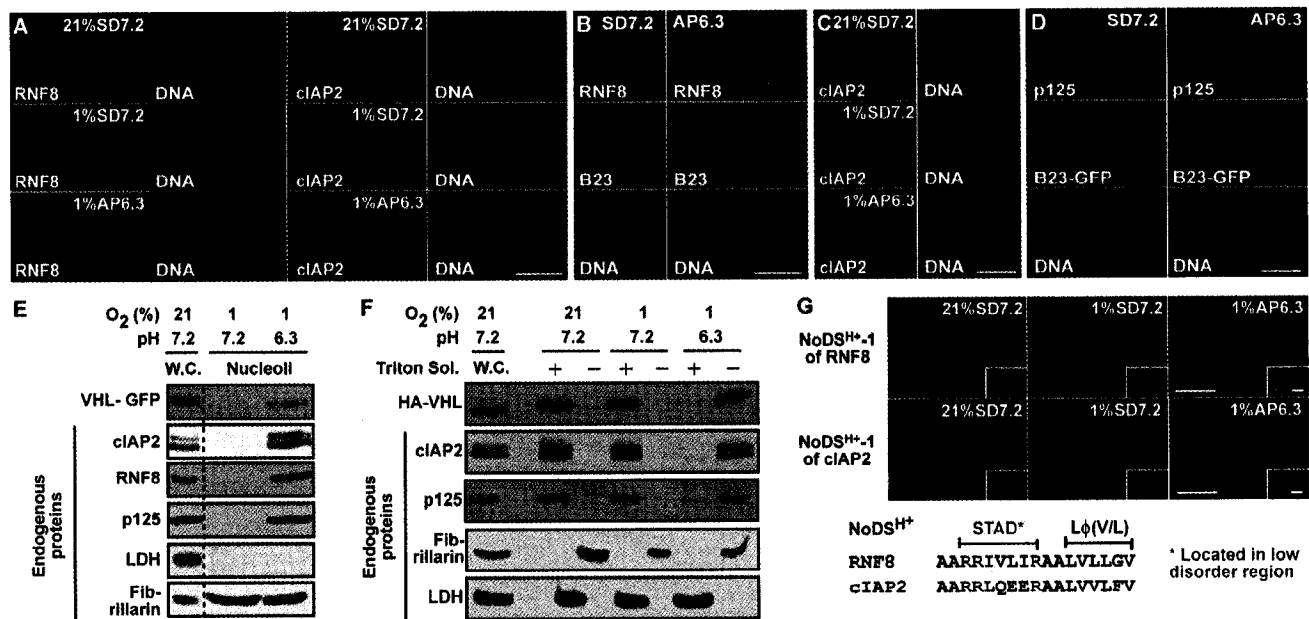


Figure 5. Confirmation of predicted H⁺-responsiveness of RNF8, cIAP2, and p125. (A) Steady-state distribution of GFP-tagged RNF8 and cIAP2 under SD or AP conditions in either normoxia (21% O₂) or hypoxia (1% O₂). (B) Acidosis-dependent colocalization of RNF8-GFP with endogenous B23 protein detected by immunofluorescence microscopy. (C) pH-dependent nucleolar localization of endogenous cIAP2 as revealed by immunofluorescence microscopy. (D) Endogenous p125 relocates to nucleoli in response to acidosis. MCF7 cells transiently transfected to express B23-GFP were cultured under indicated conditions and submitted to immunofluorescence analysis using an antibody specific to p125. (E) Western blot analysis of nucleoli isolated from MCF7 cells expressing adenovirus-introduced Flag-tagged VHL-GFP through a combination of sucrose-gradient centrifugation and sonication (see *Materials and Methods*) show that endogenous NoDS^{H+}-containing proteins accumulate in nucleoli under acidotic conditions. W.C., whole cell extract. (F) 786-0 cells stably expressing HA-VHL were incubated under SD or AP conditions and fractionated based on Triton X-100 solubility. Lysates were immunoblotted for HA, cIAP2, p125, nucleolar fibrillarlin, and cytoplasmic LDH. W.C., whole cell extract; Sol., solubility. (G) The NoDS^{H+} sequences of RNF8 and cIAP2 are sufficient to target a reporter GFP protein to nucleoli under acidosis. For each protein, a fusion of wild-type STAD and two three-residue hydrophobic repeats was GFP-tagged. MCF7 cells transiently transfected to express low levels of the fusion proteins were incubated under the indicated conditions and monitored by fluorescence microscopy. Insets show Hoechst staining of DNA. Scale bars, 10 μ m.

Specific cellular regions expressing fusion proteins were bleached with the use of a laser pulse that irreversibly quenches the GFP signal, and the recovery of signal in the bleached area was recorded by time-lapse confocal microscopy. The kinetics and extent of recovery of fluorescence in a cellular region after bleaching are reflective of the dynamics of the studied fluorescent chimeras. As previously reported, the resident nucleolar protein B23 retained a dynamic interaction with the nucleolar architecture under acidotic conditions as revealed by a rapid and full recovery of fluorescence after bleaching (Supplementary Figure S5C). Similar to VHL (Supplementary Figure S5, A and B; Mekhail et al., 2005), both RNF8 (Figure 6, A and B) and cIAP2 (Figure 6, C and D) abandoned a highly dynamic nucleoplasmic or nucleocytoplasmic profile, respectively, and became statically detained by nucleoli in response to acidosis.

We next evaluated the dynamics of RNF8 within the nucleolar space. RNF8 did not exhibit any fluorescence recovery after bleaching an area within the nucleolus (Supplementary Figure S5D). In contrast, B23 retains its highly mobile subnucleolar properties within our experimental settings (Supplementary Figure S2B and S5C; data not shown; Mekhail et al., 2005). We next examined whether interaction with the nucleolar architecture is required for acidosis-mediated modification of protein dynamics. We transfected cells to express higher levels of RNF8-GFP, saturating nucleolar-binding sites and preventing the full redistribution of the fluorescent protein chimera to the nucleolus after acidification in hypoxia (Figure S5E). This establishes two differ-

ent protein pools in the cell: nucleoplasmic and nucleolar. Although repetitive bleaching of a small nucleoplasmic region in a FLIP assay resulted in the complete loss of nucleoplasmic fluorescence, nucleolar RNF8-GFP signal remained constant over the course of the experiment (Supplementary Figure S5E). We next asked whether this process is reversible and if RNF8 can be released from nucleoli to recover its highly mobile state. After the reinstatement of neutral pH conditions, RNF8 rapidly reverted to a dynamic nucleoplasmic profile (Supplementary Figure S5F). Cullin-2 assemblies within the VHL ubiquitin ligase complex that targets HIF-1 α for degradation. Cullin-2 and HIF-1 α provide two examples that do not follow the subnuclear targeting rules and do not accumulate in nucleoli of VHL-deficient cells under acidosis (Supplementary Table S2; Mekhail et al., 2004a, 2005). Taken together, these results indicate that the subnuclear targeting rules are predictive in terms of both the targeting to a particular subnuclear compartment and the degree of affinity involved in these interactions.

Next, we decided to investigate the functional implications of nucleolar sequestration of RNF8. We have previously shown that static detention of the VHL tumor suppressor protein results in the stabilization of its main target, the α subunit of the hypoxia inducible factor (HIF- α ; Mekhail et al., 2004a). The RING finger protein RNF8 is known to interact with the retinoid X receptor α (RXR α) to enhance its transcription-stimulating activity, as demonstrated by the assessment of transcription of the RXR responsive element (RER)-containing cytosolic retinol binding

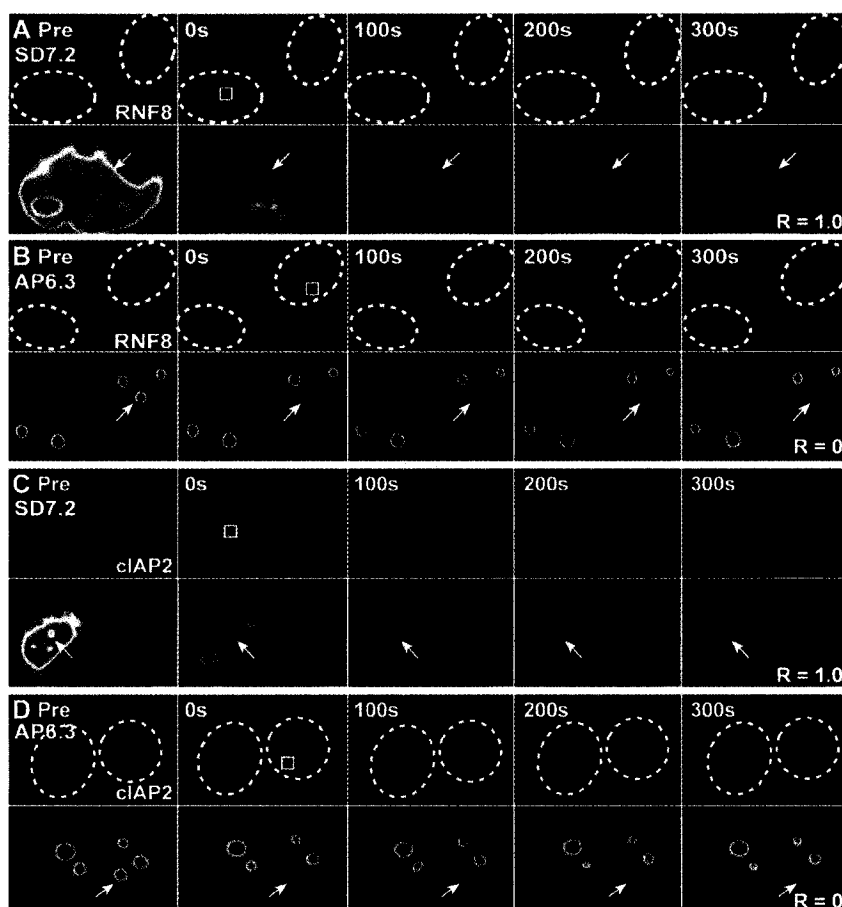


Figure 6. FRAP analysis confirms predicted changes to the dynamic character of RNF8 and cIAP2 upon signal activation. MCF7 cells, transiently transfected to express low levels of RNF8-GFP (A and B) or cIAP2-GFP (C and D), were incubated under indicated conditions and imaged before and after bleaching of the square-marked regions. Time after bleaching is indicated in seconds, and pseudocolored panels are included to better illustrate minimal changes in fluorescence. Fluorescence recovery value R is shown. See Supplementary Figure S5, A–C, for VHL-GFP and B23-GFP controls.

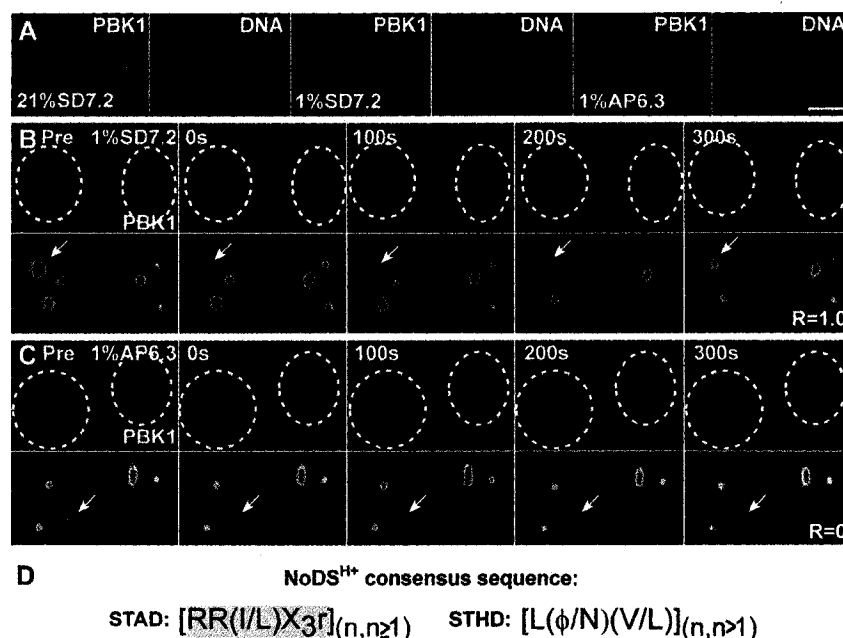
protein II gene (CRBP_{II} gene; Takano *et al.*, 2004). As expected, CRBP_{II} mRNA levels were greatly reduced only when cells were incubated under acidification-permissive conditions (Supplementary Figure S5G). We next transfected cells to express high levels of RNF8-GFP to create a pool of dynamic nuclear molecules (Supplementary Figure S5E). This rescued CRBP_{II} transcript levels in hypoxic-acidotic cells (Supplementary Figure S5G), as expected. Taken together, these findings reveal how the herein described rules and subnuclear targeting sequences can help uncover new modes of regulation of protein function.

Reverse Correlation of pH-responsive Proteins to the Rules

We next isolated nucleoli from cells incubated under either neutral or acidic conditions (Figure 7A). After the separation of purified nucleolar proteins by SDS-PAGE, a prominent ~70-kDa band that appeared only in nucleoli isolated from cells incubated under acidic conditions was sliced from the gel (Figure 7B). Analysis of the protein content of this band using MALDI-MS after trypsin digestion identified the HSC70 (P11142) heat-shock protein as its major constituent (Figure 7B). Unlike HSC70, other proteins detected by MALDI-MS in this band were also found in a parallel area of the gel cut from the neutral lane (data not shown). Nucleolar targeting of HSP70, an inducible homologue of HSC70, was previously reported to contribute to the recovery of nucleolar morphology after heat shock (Pelham, 1984). Endogenous HSC70, similar to VHL, was detected by immunofluorescence analysis performed on cells exposed to low pH conditions (Figure 7C) or by immunoblotting only in pure

nucleoli isolated from acidotic cells (Figure 7D). Examination of amino acid sequences revealed the presence of leucine-containing three-residue hydrophobic repeats (although here some of these repeats had the middle residue as N) and an arginine domain (composed of residues RRL) that was located within a low disorder region of the wild-type protein (Figure 7E and Supplementary Figure S6A). RRL still matches the consensus sequence $RR(I/L)X_3$, as the last arginine of the STAD sequence seems to play a more accessory role in subnuclear targeting (Figures 3 and 4). Further examination of the amino acid sequence of VHL revealed the presence of three more LXVs (two LPVs and one LNV). Therefore, we tested the response of GFP-tagged HSC70 and its predicted subnuclear targeting sequence (harboring RRL and the LNV and LLL hydrophobic repeats) to acidosis. We compared that response to that of another heat-shock protein, HSP110 (Q92598), which contains a double arginine that is not followed by a leucine or an isoleucine (Figure 7E) and also contains several three-residue hydrophobic repeats that do not match our consensus sequence (e.g., VVG, VVF, FQV, FVV). As predicted, acidosis triggered the relocation of HSC70 and its predicted domain alone, but not HSP110, to nucleoli (Figure 7, F and G), suggesting the possible existence of variants of the three residue hydrophobic repeats where the middle residue might be substituted for specific nonhydrophobic amino acids (e.g., LNV and LPV). This is supported by our mapping analysis of VHL since if we take the LPVs and LNV repeats of VHL into account, all triple-plus mutants would now have at least two hydrophobic repeats (Figures 1–4, and Supplementary Figure S1A). In

Figure 8. Validation of predicted alterations to the dynamic character of a NoDS^{H+}-containing resident nucleolar protein. (A) Steady-state distribution of GFP-tagged PBK1 transiently expressed in MCF7 cells incubated under indicated conditions. Hoechst staining of DNA is shown. Scale bar, 10 μ m. (B and C) FRAP analysis reveals that the nucleolar protein PBK1 is dynamic under neutral conditions but adopts a static detention profile in response to acidosis. MCF7 cells transiently transfected to express GFP-tagged PBK1 were incubated under indicated conditions and imaged before and after photobleaching of the arrow-marked nucleoli. Time after bleaching is indicated in seconds, and pseudocolored panels are included to better illustrate minimal changes in fluorescence. Fluorescence recovery value R is shown. (D) NoDS^{H+} consensus sequence R, critical arginines; r, less critical arginine; X, any amino acid; n, number of sequence elements; shading, preferably low intrinsic disorder.



nal and support a model where subnuclear targeting is a relative term, the extent of which is determined by the strength of interactions with nuclear substructures. In addition, this reveals that proteins can share regulated subnuclear targeting sequences irrespective of their steady-state distribution in the absence of signal activation.

DISCUSSION

Our analysis of the VHL tumor suppressor has revealed a set of rules governing its subnuclear targeting in response to an increase in extracellular hydrogen ion concentration. In principle, these rules are sequence-based and require the presence of at least one arginine domain (named STAD) and hydrophobic repeats (named STHD) as presented in the consensus sequence $\{[RR(I/L)X_3r]_{(n,n>1)} + [L(\phi/N)(V/L)]_{(n,n>1)}\}$. In combination, these criteria provide substantial restrictions that constitute a robust filter in sequence space. This allowed us to uncover the regulated subnuclear targeting of a number of proteins and their subnuclear targeting domains. In addition, we reverse correlated the rules to a protein blindly isolated from nucleoli of acidotic cells. Interestingly, an in silico-identified protein, which displays a steady-state nucleolar distribution under standard conditions, increases its affinity of interaction with the nucleolar architecture after signal activation. This highlights the sharing of regulated subnuclear targeting sequences between proteins irrespective of their steady-state distribution in the absence of signal activation. It is important to mention that proteins that do not follow some or all of these rules might still be targeted to nucleoli in response to the same environmental cues. For example, although we have tested the effect of changing specific amino acids to alanines in an effort to change the composition or disorder level of the sequences reported here, it is still possible that some of the key residues identified here can be replaced by other specific ones in different proteins. Also, although we have tested the effect of disorder on nucleolar targeting using different mutations, the absolute requirement for low disorder within the wild-type protein setting awaits further characterization. Nonetheless, 1)

we have shown that six randomly selected proteins of six that abide by these rules are responsive; 2) we have yet to stumble on a protein that harbors NoDS^{H+} but fails to undergo nucleolar sequestration in acidotic cells; 3) a protein that is enriched in nucleoli of acidotic cells follows the rules; and 4) five of five randomly selected proteins that do not encode a NoDS^{H+} did not localize to the nucleolus under acidosis. We expect that future work by us and others will help refine and possibly expand the consensus sequence presented here (Figure 8D). By identifying a common subnuclear targeting consensus sequence, our work reveals rules governing the dynamics of subnuclear organization and ascribes new modes of regulation to several proteins.

Proteins identified by the NoDS^{H+} rules include the anti-apoptotic cIAP2, transcriptional regulator RNF8, the heat shock protein HSC70, and ribosomal L1 domain-containing protein PBK1. The functional relevance of nucleolar detention of the VHL and MDM2 ubiquitin ligases is already known because this regulates their HIF/rDNA- and p53-regulatory functions, respectively (Tao and Levine, 1999; Weber *et al.*, 1999; Lohrum *et al.*, 2003; Mekhail *et al.*, 2004a, 2005, 2006). In addition, our findings suggest that H⁺-dependent nucleolar detention of RNF8 prevents it from acting as an enhancer of the transcriptional-stimulating activity of RXR α (Figure 5 and Supplementary Figure 5S; Takano *et al.*, 2004). Thus, we propose that reversible nucleolar detention is a general mechanism of regulation of protein function. For example, inactivation of the ATP-dependent chaperone HSC70 by nucleolar detention could allow the cell to rely more on the energy-independent HSP110, which does not localize to nucleoli under acidosis (Figure 7), when facing limited energy supply under hypoxia (Bukau and Horwich, 1998; Easton *et al.*, 2000). We had previously reported that acidosis and perturbations to ribosomal biogenesis target VHL and MDM2 to the nucleolus, respectively (Mekhail *et al.*, 2005). Here, we uncover that the same signal—acidosis—can specifically target several different proteins for static nucleolar detention. In addition to our previous work, findings presented here therefore suggest the existence of a potential complex pattern of regulation of molecular net-

works. pH-dependent static nucleolar detention of several proteins such as VHL and RNF8 eliminates key interactions they perform within certain molecular networks (Figure 5 and Supplementary Figure S5; see Mekhail *et al.*, 2004a,b, 2005, 2006). This could also be the case for some of the proteins that exhibit a steady-state localization to the endoplasmic reticulum under standard growth conditions but harbor an NoDS^{H+} (Supplementary Table S1). Identification of putative NoDS^{H+} sequences within these proteins supports the existence of broader cellular programs that remodel various molecular networks in response to environmental cues. We also know that pH-dependent nucleolar targeting of VHL, for example, introduces it within a molecular network that restricts rRNA biogenesis (Mekhail *et al.*, 2006). It is also possible that the other herein identified pH-responsive proteins also participate with VHL in the restriction of rRNA biogenesis. Nucleolar targeting of HSP70, an inducible homologue of HSC70, was previously reported to contribute to the recovery of nucleolar morphology after heat shock (Pelham, 1984). Therefore, further characterization of the sequences involved in the nucleolar targeting of these proteins in response to different environmental conditions could uncover general stress response sequence elements. Taken together, these findings support the proposal that the nucleolar proteome is dynamic and constantly changes its entity in response to environmental conditions (Andersen *et al.*, 2002, 2005).

We find that the high-affinity character of nucleolus-NoDS^{H+} interactions provided us with a large window where saturated mutagenesis was capable of revealing the relative contribution of different amino acids before complete loss of activity was observed. In addition, the regulated nature of the system provided us with an additional layer for the assessment of specificity. Different scenarios can be envisioned for the nature of biochemical interactions mediating static nucleolar detention. We previously reported that VHL interacts with the intergenic spacer of rRNA genes (rDNA) under acidosis (Mekhail *et al.*, 2006). Our data here cannot uncouple nucleolar localization and detention activities suggesting that STADs and STHDs cooperate in mediating the herein analyzed high-affinity interactions. Thus, one possibility is that different combinations of hydrophobic repeats confer features required for arginine domains to physically associate with their nucleolar binding sites. Another possibility is that the presence of arginine domains allows the hydrophobic repeats to act as anticodon-like (three residues per single repeat) structures that physically associate with specific regions of rDNA, depending on the nature of distribution of hydrophobic repeat recognition sites on the chromatin lattice. Although several of our smaller VHL fragments are efficiently targeted to nucleoli, we cannot completely rule out the possibility that the differential positioning of NoDS^{H+} components does not contribute in any way to the function or the regulation of the sequence within the setting of the wild-type VHL protein. Arginine/lysine-rich cryptic NoLS sequences have been identified in several proteins including HDM2, Coilin, and Survivin (Hebert and Matera, 2000; Lohrum *et al.*, 2000; Weber *et al.*, 2000; Catez *et al.*, 2002; Hiscox, 2002; Song and Wu, 2005). Therefore, it is possible that acidosis induces a conformational change in the wild-type VHL protein that changes the positioning of the STHD relative to the STADs to reveal the functional "cryptic" NoDS^{H+} sequence. Identification of potential posttranslational modifications within VHL could help uncover such a mechanism.

In conclusion, dissection of the NoDS^{H+} of VHL allowed us to identify a common subnuclear targeting consensus

sequence. Our work thus provides insight into the rules governing subnuclear organization/dynamics and ascribes new modes of regulation to several proteins. We thus propose that proteins with diverse steady-state distribution share the higher order code NoDS^{H+}, which determines their subnuclear coordinates under specific environmental cues once they enter the nucleus.

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