Role of eEF1A in the Nuclear Export of the VHL Tumour Suppressor Protein

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ABSTRACT

The ability of proteins to engage in nuclear-cytoplasmic shuttling is required for their proper function. The nuclear export of the von Hippel Lindau (VHL) tumour suppressor protein is necessary for the proteasomal degradation of the hypoxia inducible factor alpha (HIFα). Studies have identified that the nuclear export of VHL and other proteins encoding a Transcription-Dependent Nuclear Export Motif (TD-NEM) is independent of the classical CRM1 nuclear export pathway but requires ongoing transcription. Furthermore, the eukaryotic elongation factor 1 alpha (eEF1A) was identified as a mandatory component of the TD-NEM-mediated nuclear export machinery. In this study, we have uncovered the ability of eEF1A to mediate the nuclear export of proteins by accessing the nuclear compartment in its inactive, GDP-bound form. Although previously thought of as a strictly cytoplasmic protein, work conducted in this thesis has shown that eEF1A is a nuclear-cytoplasmic shuttling protein and this ability is required for the effective export of proteins encoding a TD-NEM.

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List of Abbreviations

aa-tRNA	Aminoacylated tRNAs
Act D	Actinomycin D
APC	Adenomatous Polypsis Coli
BARD1	Breast Cancer Associated Protein
BRCA1	Ovarian Cancer Susceptibility Protein 1
CNS	Central Nervous System
CRM1	Chromosome Region Maintenance
Cul2	Cullin 2
DRB	5,6-Dichlorobenzimidazole
eEF1	Eukaryotic Translation Factor 1
eEF1A	Eukaryotic Elongation Factor 1 Alpha
eEF1B	Eukaryotic Elongation Factor 1 Beta
eIF5A	Eukaryotic Initiation Alpha
Exp-t	Exportin-t
Exp-5	Exportin 5
F	Flag tag
FG	Phenylalanine-Glycine
FLIP	Fluorescence Loss in Photobleaching
GDP	Guanosine Diphosphate
GFP	Green Fluorescence Protein
GLUT1	Glucose Transporter 1
GMP-PNP	5'-Guanylyl Imidodiphosphate
GTP	Guanosine Triphosphate
HIF	Hypoxia Inducible Factor
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HREs	Hypoxia Response Elements
LMB	Leptomycin B
MCF-7	Human Breast Adenocarcinoma
mRNA	Messenger RNA

NLS	Nuclear Localization Signal
NPC	Nuclear Pore Complex
PABP1	Poly(A)-Binding Protein 1
PBS	Phosphate Buffered Saline
PHDs	Prolyl Hydroxylase Domain-Containing Enzymes
pk	Pyruvate Kinase
PK1	Protein Kinase Inhibitor 1
PVDF	Polyvinylidene Fluoride
RanBP1	Ran Binding Protein 1
RanGAP	GTPase Activating Protein
RanGEF	Guanine Nucleotide Exchange Factor
RCC	Renal Cell Carcinomas
RNA Pol II	RNA Polymerase II
rRNA	Ribosomal RNA
SCF	Skp1-Cdc53-F-box Multiprotein Complex
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TD-NEM	Transcription-Dependent Nuclear Export Motif
TGFα	Transforming Growth Factor-α
tRNA	Transfer RNA
VGEF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau
WCL	Whole Cell Lysate
Xpo-t	Exportin-t

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1.0 INTRODUCTION

1.1 NUCLEOPLASMIC SHUTTLING OF PROTEINS

1.1.1 Compartmentalization of Eukaryotic Cells.

A distinguishing characteristic of eukaryotic cells is the observable division of the nucleus and cytoplasm. The nucleus is surrounded by a double-membrane nuclear envelope, punctuated by proteinaceous structures referred to as nuclear pore complexes (NPCs) (Anderson, 1953; Feldherr, 1962; Izaurralde et al., 1999; Rout and Aitchison, 2001; Wente, 2000). The nucleus houses and protects the cell's genomic DNA, whereas the cytoplasm is the site of protein synthesis and production. This cellular compartmentalization requires efficient nuclear-cytoplasmic transport pathways to ensure effective and rapid communication between the nucleus and cytoplasm. The functions of proteins and different RNA species are not limited to their origin of biogenesis, thereby requiring regulated mechanisms of bidirectional trafficking. Nuclear proteins synthesized in the cytoplasm are required to be imported into the nucleus and different RNA species, such as ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA), which are transcribed in the nucleus, require nuclear export for their biological function (Gorlich, 1998).

1.1.2 Movement through the Nuclear Pore Complex.

NPCs are the gatekeepers of the nucleus, selectively facilitating the movement of molecules and proteins. Extensive studies using electron microscopy have revealed that the overall structure of NPCs are highly conserved among eukaryotes (Akey and Radermacher, 1993; Alber et al., 2007; Beck et al., 2004; Heese-Peck and Raikhel, 1998; Hetzer et al., 2005; Hinshaw et al., 1992; Kiseleva et al., 2004; Tran and Wente, 2006).

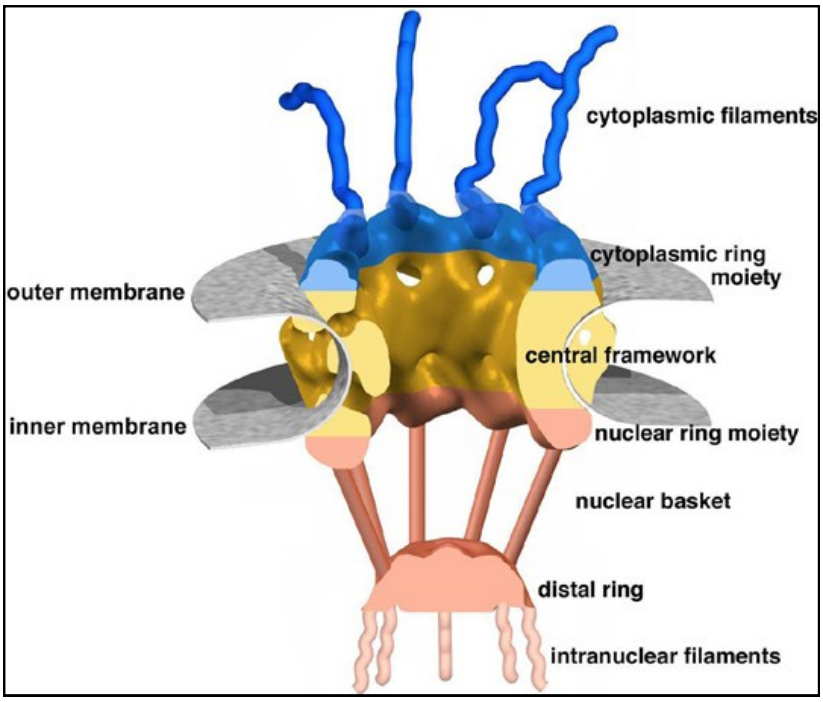
The tripartite architecture of the NPC includes a central framework consisting of eight spokes, cytoplasmic filaments and a nuclear basket (Figure 1A) (Alber et al., 2007; Fahrenkrog and Aebi, 2003; Heese-Peck and Raikhel, 1998). Approximately 30 distinct proteins, termed nucleoporins, make up the NPC and are distributed symmetrically or asymmetrically between the nuclear and cytoplasmic periphery (Alber et al., 2007; Cronshaw et al., 2002; Rout et al., 2000; Wente, 2000). Nucleoporins contain phenylalanine-glycine (FG) repeats which were observed to be highly unstructured (Bayliss et al., 2000; Denning et al., 2003; Denning et al., 2002). Ions and small proteins (< 40 kDa) cross the NPC through diffusion, whereas larger proteins and molecules require active transport to translocate across the NPC (Figure 1B) (Keminer and Peters, 1999; Paine, 1975; Paine et al., 1975; Peters, 1986).

Although the precise mechanism of protein translocation remains to be fully understood, multiple models have been proposed to explain the movement of proteins through the NPC. The selective-phase model proposes that weak hydrophobic interactions between the meshwork of FG-nucleoporins in the central channel can be disrupted by transport receptors, thereby allowing the passage of proteins (Ribbeck and Gorlich, 2002). Further studies expanded on the selective-phase model by explaining that FG-nucleoporins form sieve-like structures with hydrogel-like properties (Frey and Gorlich, 2007; Weis, 2007). Furthermore, the two-gate model suggests that nucleoporins situated in the central channel of the NPC forms a cohesive meshwork of filaments, in contrast to nucleoporins found in the nuclear basket which behave as non-cohesive filaments (Patel et al., 2007). It is therefore proposed that gate one is the physical meshwork barrier created by

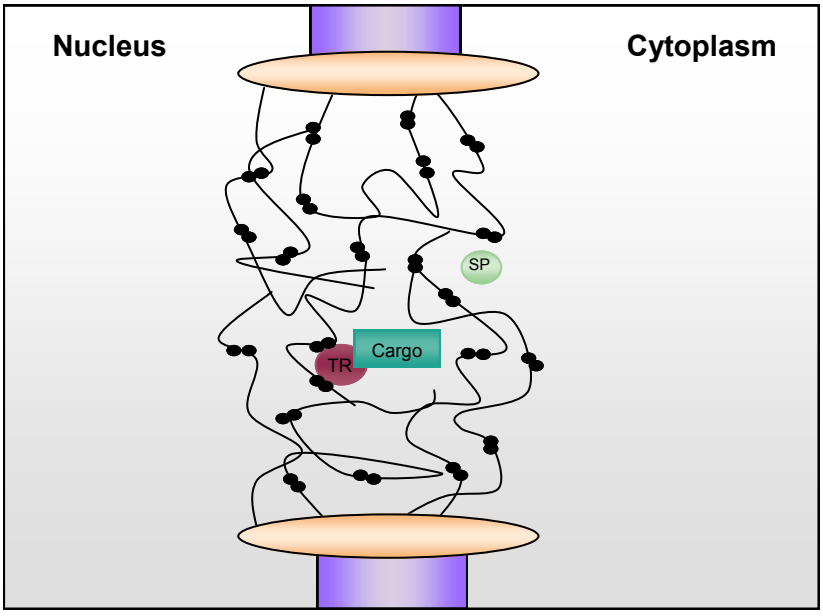
Figure 1: The Nuclear Pore Complex.

A) A cross-section model of the nuclear pore complex depicting the architecture of its different components. **B)** Ions and small proteins (SP) can freely diffuse through the nuclear pore complex to enter the nucleus. Larger proteins require transport receptors (TR) to facilitate the translocation across the nuclear pore complex.

A


cytoplasmic filaments
cytoplasmic ring moiety
outer membrane
central framework
inner membrane
nuclear ring moiety
nuclear basket
distal ring
intranuclear filaments

B


Nucleus
Cytoplasm
SP
Cargo
TR

nucleoporins in the central channel and gate two is a virtual or entropic exclusion gate of nucleoporins that comprise the nuclear basket (Patel et al., 2007).

1.1.3 Nuclear-Cytoplasmic Shuttling of Proteins Required for Biological Functions.

A significant leap in the understanding of intracellular transport of proteins was realized with the "Signal Hypothesis" of Gunter Blobel, stating that proteins have "zip codes" which effectively target them to different cellular compartments (Davis et al., 2007; Hagmen, 1999; Heemels, 1999). Consequently, it is necessary for proteins to reach their proper cellular destination in order to perform their biological function. For example, transcription factors need to be recruited in the nuclear compartment to promote gene expression. However, entry into the nucleus is tightly regulated and requires a trigger signal for nuclear import, thereby requiring selective and specific transport pathways (Gorlich and Kutay, 1999; Hood and Silver, 1999). The manifestation of various diseases has been observed when nuclear-cytoplasmic shuttling processes have been disrupted or altered (Fabbro and Henderson, 2003). Mistargeting of tumour suppressor proteins can result in severe consequences, such as potentially activating the initiation and progression of cancer (Fabbro and Henderson, 2003). The tumour suppressor p53 is involved in maintaining the integrity of the genome by controlling cell cycle progression and cell survival (Woods and Vousden, 2001). However, the tumour suppressor function of p53 can be inhibited by mutational events which impedes its nuclear import (Davis et al., 2007; Fabbro and Henderson, 2003). Conversely, the dimerization of BARD1 (the breast cancer associated protein) with BRCA1 (the ovarian cancer susceptibility protein 1) blocks the nuclear export of BARD1, subsequently resulting in BARD1's inability to perform its proapoptitic function in the cytoplasm (Fabbro et al., 2002; Rodriguez et al.,

2004). A mutated form of the adenomatous polypsis coli (APC) tumour suppressor protein results in its inability to export into the cytoplasm, where it plays critical roles in reducing the transcriptional ability of β-catenin (Davis et al., 2007; Rosin-Arbesfeld et al., 2000). The functional inactivation of APC leads to tumour progression due to an increase in the transcriptional activity of β-catenin (Rosin-Arbesfeld et al., 2003). Hence, a protein's ability to reach its proper cellular compartment is crucial for maintaining properly functioning cells.

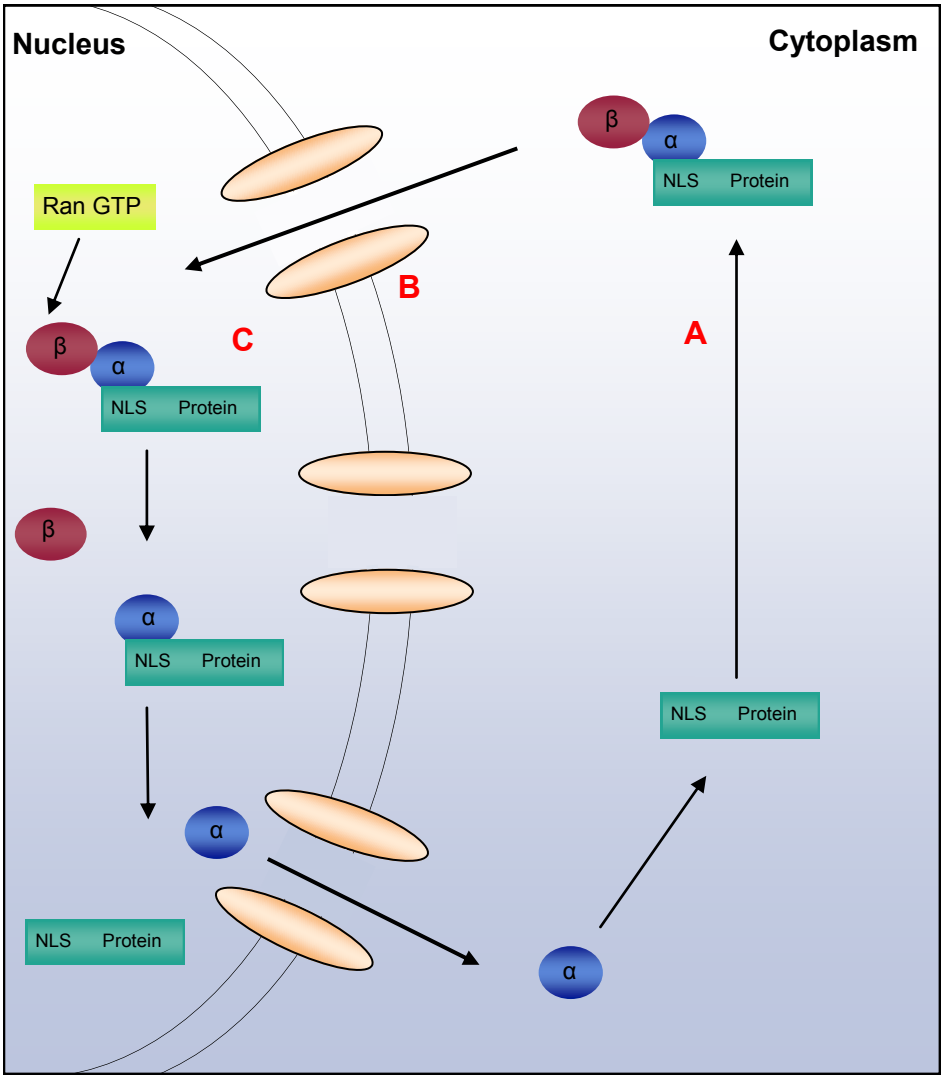
1.2 MECHANISMS OF NUCLEAR IMPORT

1.2.1 Nuclear Localization Signal (NLS)-Mediated Nuclear Import Pathway.

The discrimination of proteins intended for nuclear import is a fundamental step in maintaining specific and coherent active transport. A crucial requirement for active nuclear import of proteins is a distinct nuclear localization signal (NLS); first identified in SV40 large T-antigen (Kalderon et al., 1984a; Kalderon et al., 1984b; Lanford and Butel, 1984). The classical NLS is composed of one [monopartite : $(K/R)_{4-6}$] or two [bipartite: $(K/R)_2$ X_{10-12} $(K/R)_3$] stretches of basic amino acids, mainly lysines or arginines; where x represents a non-conserved residue and numbers represent the amount of repeats (Christophe et al., 2000; Dingwall and Laskey, 1991; Kalderon et al., 1984a; Robbins et al., 1991). The identification of discrete signals for nucleoplasmic transport is mainly recognized by members of the karyopherin β family, having functions in both nuclear import and export (Moroianu, 1998; van der Aa et al., 2006). Classical NLS sequences are recognized by importin α which in turn is recognized by importin β (Figure 2A) (Chi et al., 1995; Conti et al., 1998; Gorlich et al., 1994; Imamoto et al., 1995; Lange

Figure 2: Mechanism of the Classical NLS-Mediated Nuclear Import Pathway.

A) Proteins containing a nuclear localization signal (NLS) are recognized by importin α, which in turn is recognized by the β-karyopherin protein, importin β. **B)** Importin β mediates the translocation of the trimeric complex. **C.)** Once in the nucleus, the cargo protein is released upon Ran-GTP binding.


Nucleus
Cytoplasm
Ran GTP
β
α
NLS Protein
B
C
A
β
α
NLS Protein
β
α
NLS Protein
α
NLS Protein
α
NLS Protein

et al., 2007). Therefore, it is importin β which mediates the active translocation of NLS-containing proteins through the adapter protein, importin α (Gorlich et al., 1996; Gorlich et al., 1995a). Once the importin β /importin α /NLS-protein cargo complex is formed, the hydrophobic residues on the surface of the complex binds to phenylalanine-glycine (FG) repeats of nucleoporins, thereby triggering active translocation (Figure 2B) (Gorlich et al., 1995b; Moroianu et al., 1995; Radu et al., 1995; van der Aa et al., 2006). In the nucleus, the release of the cargo protein is accomplished by the binding of the small GTPase, Ran-GTP, to the N-terminus region of importin β (Figure 2C) (Lange et al., 2007; van der Aa et al., 2006). The liberation of the cargo protein from the complex allows the protein to function within the nuclear compartment.

1.2.2 Nuclear Import Pathways Independent of the Classical NLS.

Nuclear import of proteins containing a classical NLS has been thoroughly characterized and studied. Due to the abundance of proteins translocating through the NPC via the classical NLS, it is often thought to be the prototypical NLS and the most prevalent nuclear import pathway in the cell. However, no empirical studies have been conducted to determine the proportion of cargoes imported through this mechanism (Lange et al., 2007). There exist multiple other nuclear import pathways independent of the classical NLS pathway. For example, the β-catenin protein mentioned in section 1.1.3 lack a classical NLS and is able to bind directly to the nuclear envelope in the absence of karyopherins (Fagotto et al., 1998). Therefore, the nuclear import of β-catenin appears to be independent of the classical NLS-mediated pathway, although similarities may exists between the two pathways (Fagotto et al., 1998). Shorter NLS sequences, bearing no resemblance to the classical NLS, have also been identified; examples are the protein

Sam68 (P_2X_2R) and Cdc6 (S/T-P-X-K-R-L/I) (Ishidate et al., 1997; Takei et al., 1999).The heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein contains an import signal, termed M9, and is a stretch of 38 amino acids distinctly different from the classical NLS (Michael et al., 1995; Pollard et al., 1996). Nuclear import of M9-containing proteins are independent of importin β and α, however it is mediated by transportin (importin β2), a distant relative of importin β (Fridell et al., 1997; Michael et al., 1995; Nakielny et al., 1996; Pollard et al., 1996). Interestingly, the M9 sequence has been shown to perform functions in mediating both nuclear import and export (Michael et al., 1995; Pollard et al., 1996).

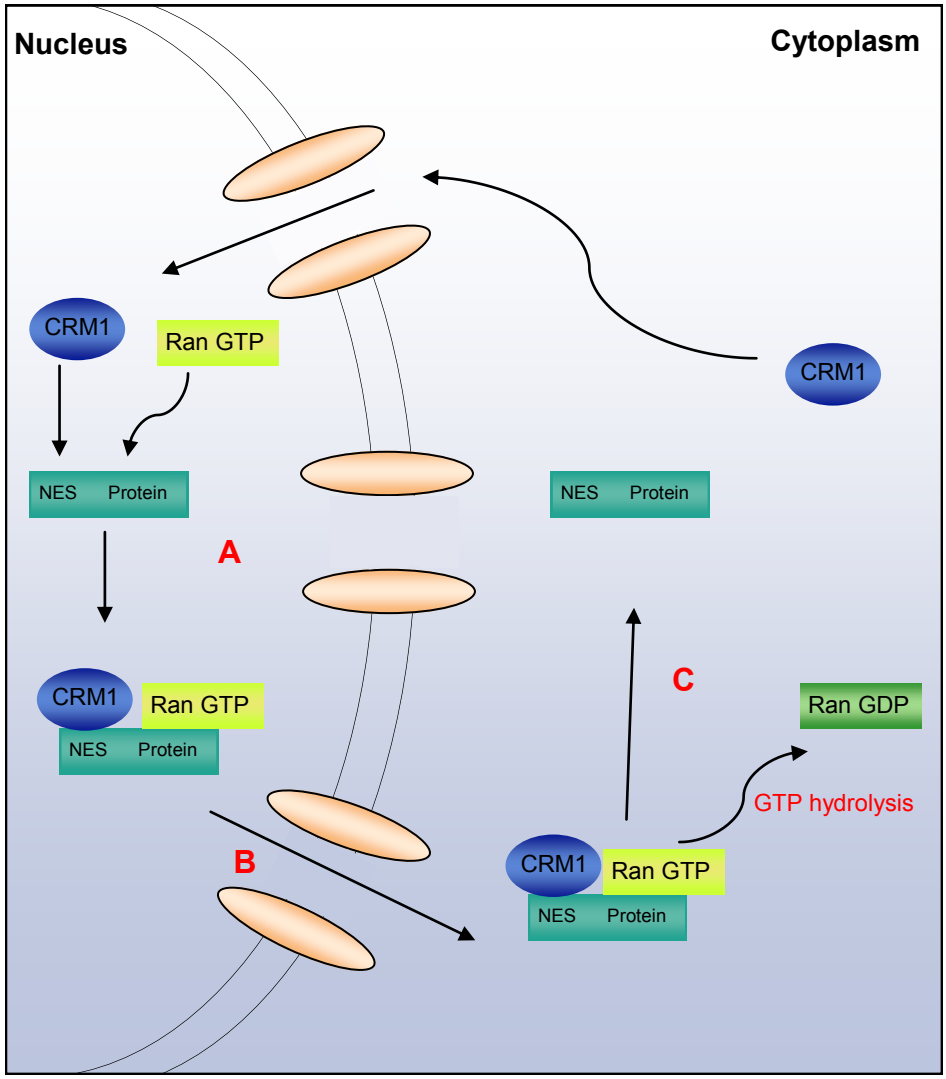
1.3 MECHANISMS OF NUCLEAR EXPORT

1.3.1 Nuclear Export Signal (NES)-Mediated Nuclear Export Pathway.

Steady-state localization of proteins are dependent on the interplay between the rate of nuclear import and export. Comparable to nuclear import, the export of proteins require a specific nuclear export signal (NES), which was first discovered in HIV-1 Rev protein and protein kinase inhibitor 1 (PK1) (Fischer et al., 1995; Wen et al., 1995). The classical NES consists of hydrophobic residues, consisting of three to four hydrophobic repeats of leucines or isoleucines, with a consensus sequence of: $LX_{(2,3)}(L/I/V/F/M)X_{(2,3)}.LX(L//I)$, where $X_{(2,3)}$ denotes any two or three amino acids (Bogerd et al., 1996; Kosugi et al., 2008). Nuclear export of proteins containing a NES is energy-dependent and is a saturable process; a strong indication that the process is receptor-mediated (Sweitzer et al., 2000). As mentioned in section 1.2.1, members of the karyopherin β family not only participate in nuclear import but also contribute in nuclear export. The discovery of the CRM1 exportin (Chromosome Region Maintenance or exportin 1) as a component of the

NES-export machinery was through the observation that treatment with leptomycin B (LMB) prevents nuclear export of the HIV-1 Rev protein (Fornerod et al., 1997a; Wolff et al., 1997). Further studies revealed that LMB binds directly to CRM1 and covalently modifies a cysteine residue, resulting in its inability to interact with the NES (Fornerod et al., 1997a; Kudo et al., 1999; Nishi et al., 1994). Reminiscent of nuclear import, the GTPase Ran is a critical component of nuclear export. The binding of CRM1 with proteins containing a NES is stabilized by the binding of Ran-GTP (Figure 3A) (Askjaer et al., 1998; Fornerod et al., 1997a). CRM1's affinity for nucleoporins aids in the docking of the trimeric complex (cargo protein/CRM1/RanGTP) to the NPC, however the precise mechanism of translocation across the NPC remains to be fully addressed and investigated (Figure 3B) (Neville et al., 1997; Stutz et al., 1996). The dissociation and the release of the cargo protein is accomplished by the hydrolysis of Ran-GTP, through the binding of RanGAP (GTPase activating protein) and its activator, RanBP1 (Ran binding protein 1) (Figure 3C) (Fornerod et al., 1997b; Gorlich and Mattaj, 1996; Kehlenbach et al., 1999; Koepp and Silver, 1996; Nigg, 1997). CRM1 without cargo is recycled back into the nuclear compartment without binding to other soluble factors (Zhang et. al., 2003). Ran-GTP therefore plays a crucial role not only in nuclear import, as mentioned in section 1.2.1, but is also a fundamental component of nuclear export. The directionality of nucleoplasmic transport is maintained by the asymmetric distribution of Ran; Ran likely bound to GTP in the nucleus due to the nuclear localization of the guanine nucleotide exchange factor, RanGEF (RCC1) and likely in the GDP-state in the cytoplasm due to the cytoplasmic localization of RanGAP (Gorlich and Mattaj, 1996; Koepp and Silver, 1996; Nakielny and Dreyfuss, 1999; Nigg, 1997).

Figure 3: Mechanism of the Classical NES-Mediated Nuclear Export Pathway. A) CRM1 recognizes proteins containing a nuclear export signal (NES) and the binding of Ran-GTP stabilizes the complex. **B)** The trimeric complex (protein/CRM1/Ran-GTP) is translocated across the nuclear pore complex via the ability of CRM1 to bind to nucleoporins. **C)** The hydrolysis of Ran-GTP releases the cargo protein in the cytoplasm.


Nucleus
Cytoplasm
CRM1
Ran GTP
NES Protein
A
CRM1
Ran GTP
NES Protein
B
CRM1
Ran GTP
NES Protein
C
Ran GDP
GTP hydrolysis
CRM1
NES Protein

1.3.2 Nuclear Export Pathways Independent of the Classical NES.

The discovery of the classical NES has resulted in profound advancements in understanding nuclear export. Nuclear export mediated by the classical NES/CRM1 is an extensively studied mechanism and is thought to be the major export pathway utilized by proteins. However, NES/CRM1-independent nuclear export processes for proteins and various RNA species have emerged. For example, the protein pUL69 of the β-herpesvirus human cytomegalovirus participates in nuclear export independent of CRM1 and is LMB insensitive (Lischka et al., 2001). Furthermore, pUL69 contains a novel and transferable signal which bears no similarity to the classical NES (Lischka et al., 2001). Although certain protein export machineries do not involve CRM1, the requirement for the karyopherin β family of transporters has not been diminished and remains a crucial component in mediating nuclear export. Examples include: Exportin-6 which mediates nuclear export of actin (Stuven et al., 2003), the eukaryotic initiation alpha (eIF5A) is exported through Exportin-4 (Lipowsky et al., 2000) and Exportin-7 transports p50RhoGAP and 14-3-3σ from the nucleus into the cytoplasm (Mingot et al., 2004). However, there are proteins that are unrelated to karyopherins which are capable of mediating nuclear export of molecules in a manner that is independent of the GTPase Ran cycle. The general mRNA export pathway requires a heterodimer transport machinery consisting of TAP and its cofactor, Nxt (p15) (Clouse et al., 2001; Gruter et al., 1998; Wiegand et al., 2002). Despite being unrelated to karyopherins, the heterodimer complex is able to interact with FG-nucleoporins, a critical step in translocation (Wiegand et al., 2002) (Fribourg et al., 2001; Levesque et al., 2001; Wiegand et al., 2002). Furthermore, certain molecules may utilize multiple nuclear export pathways, an example being tRNA.

A member of the karyopherin β family, Exportin-t (Xpo-t) in vertebrates (Arts et al., 1998; Kutay et al., 1998) and Los1p in yeast (Hellmuth et al., 1998; Sarkar and Hopper, 1998), was identified to interact with tRNA and effectively mediate its nuclear export in conjunction with Ran-GTP (Grosshans et al., 2000a). Aside from Xpo-t, recent studies have shown that Exp-5 is capable of mediating the nuclear export of tRNA and the eukaryotic elongation factor 1 alpha (eEF1A) (Bohnsack et al., 2002; Calado et al., 2002). Due to the numerous nuclear export pathways within the cell, it is understandable that certain nuclear export machineries are not as well characterized and its exact mechanism remains to be fully discovered; examples being the transcription-dependent nuclear export pathway utilized by the von Hippel Lindau (VHL) tumour suppressor protein and the Poly(A)-Binding Protein 1 (PABP1) (Afonina et al., 1998; Groulx and Lee, 2002; Lee et al., 1999).

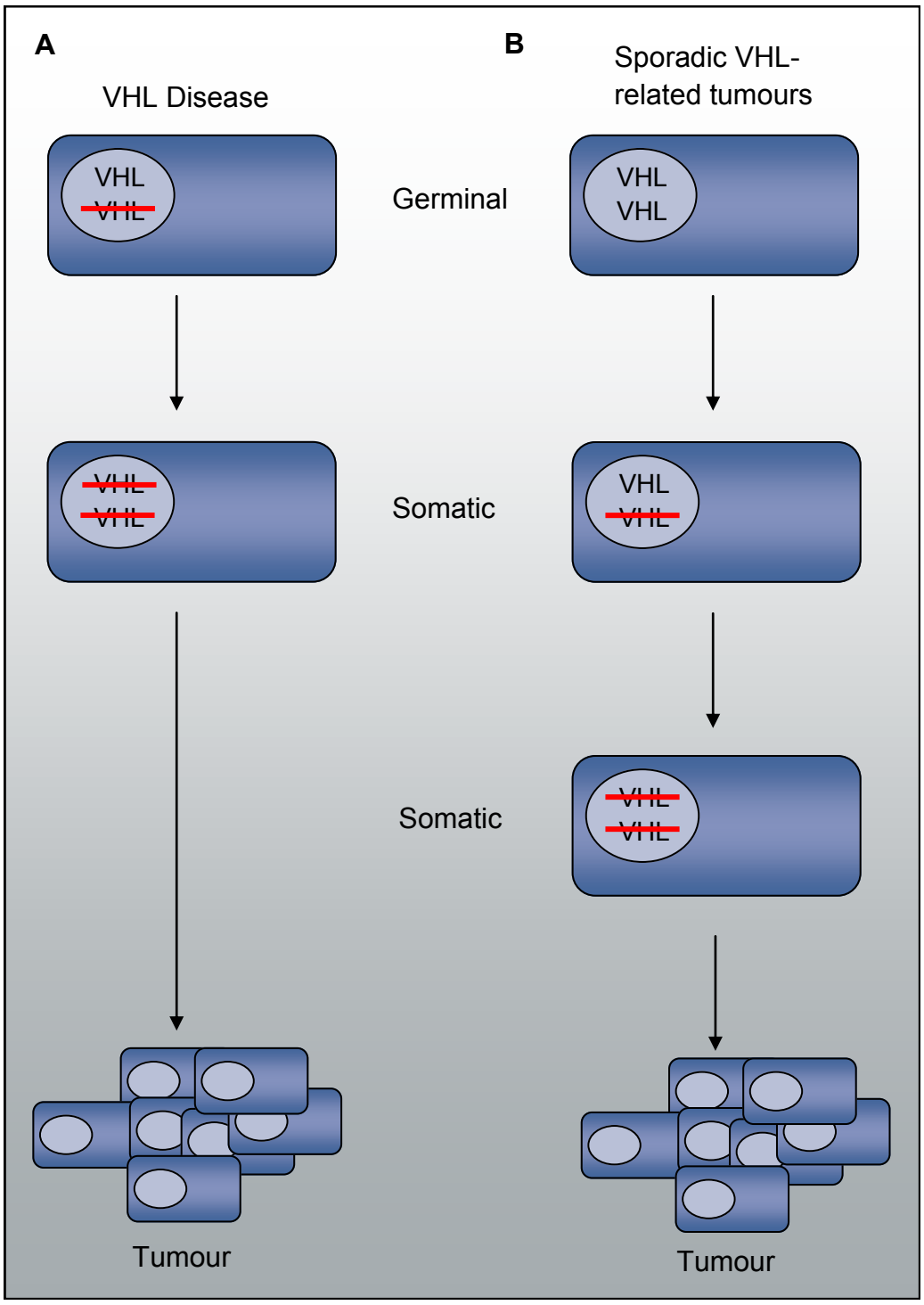
1.4 THE VON HIPPEL LINDAU TUMOUR SUPPRESSOR PROTEIN

1.4.1 VHL Disease.

The von Hippel Lindau (VHL) disease was first illustrated by Treacher Collins, a British surgeon (Collins, 1894) and was further described by Eugene von Hippel, a German ophthalmologist and a Swedish pathologist, Arvid Lindau (von Hippel, 1904; Lindau, 1927). VHL is a dominantly inherited cancer syndrome and is characterized by the presence of central nervous system (CNS) and retinal hemangioblastomas, renal clear cell carcinomas, and pheochromocytomas (Kim and Kaelin, 2004; Maher and Kaelin, 1997; Ohh and Kaelin, 2003; Richard et al., 1998). Individuals with VHL disease possess one wild-type VHL allele and one inactivated VHL allele; the subsequent loss or inactivation of the remaining wild-type VHL allele results in tumour or cyst formation (Figure 4A)

Figure 4: The Successive Loss of the VHL Tumour Suppressor Gene Leads to Disease.

A) VHL disease is the resultant of a germline mutation and a second hit to the remaining wild-type VHL allele, thereby resulting in tumour formation. **B)** Biallelic inactivation of VHL results in sporadic VHL tumours.


A
VHL Disease
B
Sporadic VHL-related tumours
VHL
VHL
Germinal
VHL
VHL
VHL
VHL
Somatic
VHL
VHL
VHL
VHL
Somatic
VHL
VHL
Tumour
Tumour

(Kim and Kaelin, 2004; Latif et al., 1993). Families afflicted with VHL disease are classified into the absence (Type 1) or presence (Type 2) of pheochromocytomas, a neuroendocrine tumour arising from the adrenal medulla (Brauch et al., 1995; Chen et al., 1995; Kim and Kaelin, 2004). Type 2 is further categorized into low risk (Type 2A) or high risk (Type 2B), depending on the individual's likelihood of developing kidney cancer (Kim and Kaelin, 2004). While tumours associated with VHL disease are often benign, kidney cancer is malignant and is the main cause of morbidity and mortality among VHL patients (Ohh and Kaelin, 2003). Furthermore, large scale studies have shown that there are prominent correlations between certain mutations and their clinical manifestations. For example, mutations associated with Type 1 VHL disease are usually the cause of nonsense mutations, deletions and microinsertions and Type 2 VHL disease is the resultant of missense mutations (Chen et al., 1995; Neumann and Bender, 1998; Zbar et al., 1996). Biallelic VHL inactivation, in accordance with the Knudson's Two-Hit model, occurs in sporadic renal cell carcinomas (RCC, kidney cancer) (Figure 4B) (Kim and Kaelin, 2004). The "first" and "second" hit occurs somatically and studies have shown that in approximately 50% of sporadic RCC VHL contains mutations and is hypermethylated in approximately 10-20% (Kim and Kaelin, 2004).

1.4.2 VHL is a Component of an E3 Ubiquitin Ligase Complex.

Initially, it was challenging to predict the biological function of VHL because based on early observations, the nucleotide or amino acid sequence of VHL was not homologous to any other known protein. However, through biochemical analysis, it was observed that VHL forms a multiprotein complex with elongin B, elongin C, Cullin 2 (Cul2) and Rbx1; termed collectively as the VBC-Cul2 complex (Figure 5) (Duan et al., 1995a; Duan et al.,

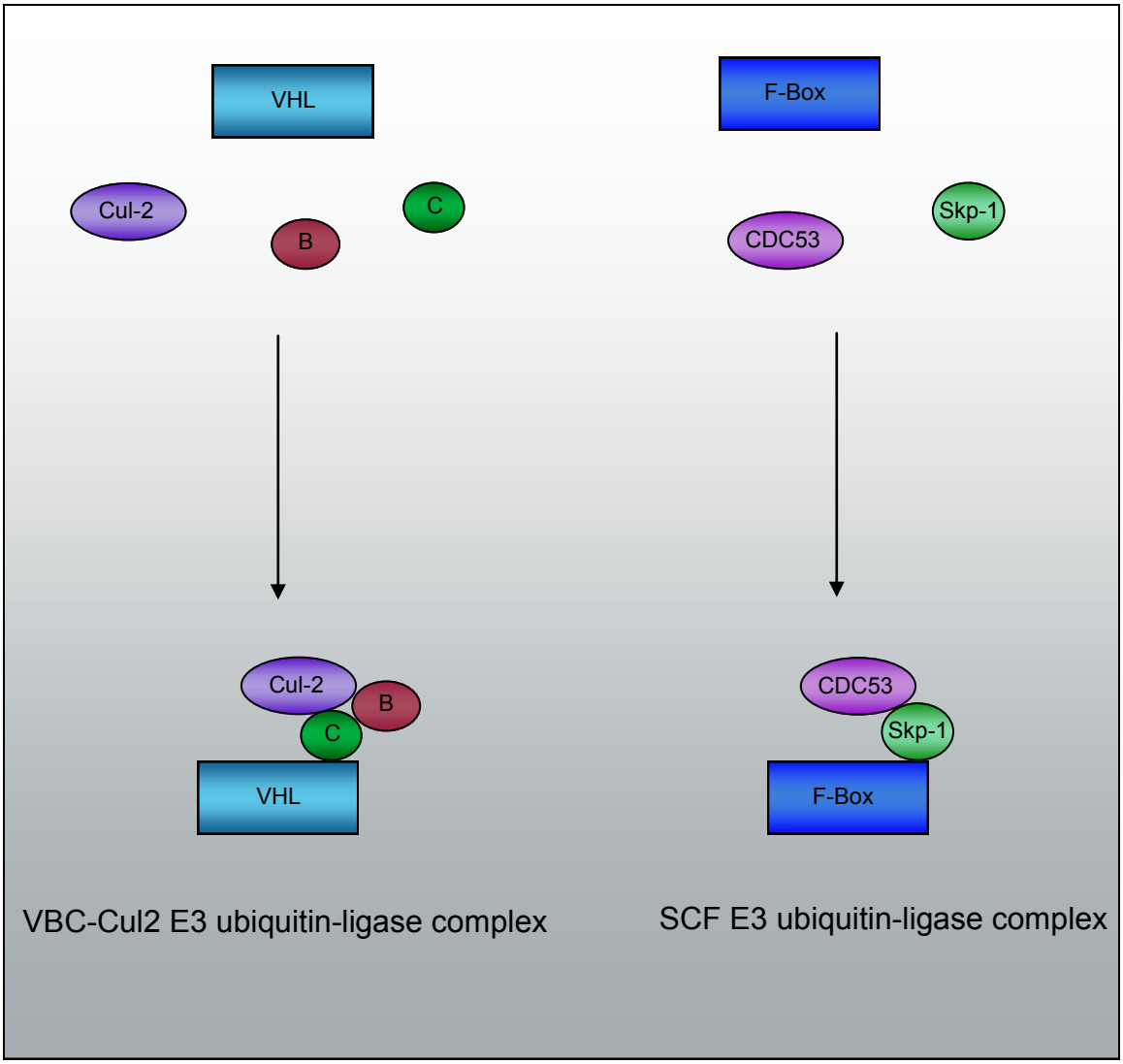
1995b; Kamura et al., 1999; Kamura et al., 2000; Kibel et al., 1995; Lonergan et al., 1998; Pause et al., 1997; Pause et al., 1999). Further experiments revealed that elongin C binds to the alpha domain of VHL, particularly within residues 157-171; which in turn serves as a docking site for Cul2 and the complex is stabilized through the interaction with elongin B (Duan et al., 1995a; Duan et al., 1995b; Lonergan et al., 1998; Ohh et al., 1999; Pause et al., 1999). Furthermore, when the primary sequences of elongin C and Cul2 were compared to other protein sequences, it was observed that elongin C and Cul2 resembled the yeast proteins Skp1 and Cdc53, respectively (Bai et al., 1996). Skp1 and Cdc53 interacts with an F-box protein and forms a multiprotein (SCF: Skp1-Cdc53-F-Box) E3 ubiquitin-ligase complex (Figure 5) (Deshaies, 1999; Zheng et al., 2002). Upon further analysis, the crystal structure of the VBC-Cul2 complex was observed to strongly resemble that of the SCF complex; thereby postulating the model that the VBC-Cul2 complex is an E3 ubiquitin-ligase complex (Stebbins et al., 1999). To further test the hypothesis that VBC-Cul2 is an E3 ubiquitin-ligase complex, VHL was immunoprecipitated in the presence of an E2 ubiquitin-conjugating enzyme. The result demonstrated that VHL exhibits ubiquitin-ligase activity, furthermore solidifying the premise that the VBC-Cul2 complex is an E3 ubiquitin-ligase complex (Iwai et al., 1999; Lisztwan et al., 1999).

1.4.3 The Hypoxia Inducible Factor Alpha (HIFα) is a Substrate of the VBC-Cul2 Complex.

The maintenance of oxygen homeostasis is one of the fundamental requirements of living organisms. The delicate balance of oxygen supply and demand are strictly regulated in order to ensure proper development and physiology (Giaccia et al., 2004). In response to

Figure 5: The VBC-Cul 2 Complex Resembles the Yeast SCF E3 Ubiquitin Ligase Complex.

VHL interacts with elongin B, elongin C and Cullin-2 to form the VBC-Cul2 complex. Interestingly, the VBC-Cul2 complex resembles the yeast SCF E3-ubiquitin ligase complex.


VHL
Cul-2
B
C
F-Box
CDC53
Skp-1
Cul-2
B
C
VHL
CDC53
Skp-1
F-Box
VBC-Cul2 E3 ubiquitin-ligase complex
SCF E3 ubiquitin-ligase complex

low oxygen levels or hypoxia, the transcriptional upregulation of a large group of genes required to re-establish equilibrium is accomplished by the hypoxia inducible factors (HIFs) (Axelson et al., 2005; Semenza, 1999; Semenza, 2003). HIF is a heterodimeric complex and was discovered to be composed of two basic helix-loop-helix proteins of the PAS family (Per/Arnt/SIM family): a constitutively expressed HIFβ and one of the three HIFα subunits: HIF1α, HIF2α or HIF3α (Semenza, 2000; Wang et al., 1995). Together, these proteins act as a DNA-binding complex and can therefore bind to the hypoxia response elements (HREs) of target genes; activating the transcription of more than 60 genes involved in the regulation of oxygen homeostasis (Maxwell et al., 2001; Semenza, 2003; Semenza and Wang, 1992; Wang et al., 1995). Examples of genes which are transcriptionally activated are the vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) and the transforming growth factor-α (TGFα), as well as other genes which encode proteins involved in angiogenesis, glucose metabolism, pH regulation, cell survival and cell proliferation (Carmeliet et al., 1998; Gerber et al., 1997; Iyer et al., 1998; Semenza, 2000). Studies have shown that in VHL-defective tumour cells, there is an overproduction of hypoxia-inducible mRNAs (Gnarra et al., 1996; Iliopoulos et al., 1996; Siemeister et al., 1996; Stratmann et al., 1997). However, when functional VHL was reintroduced, the hypoxia-inducible mRNA levels returned to normalcy (Iliopoulos et al., 1996). Furthermore, it was observed that HIFα is polyubiquitylated in the presence of VHL and accumulates in VHL-defective cells; thereby resulting in the upregulation of downstream target genes, such as VEGF (Maxwell et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000). Overall, this led to the

identification that HIFα is a substrate of the VBC-Cul2 E3 ubiquitin ligase complex.

1.5 THE PROTEOSOMAL DEGRADATION OF HIFα

1.5.1 VHL targets HIFα for Proteasomal Degradation.

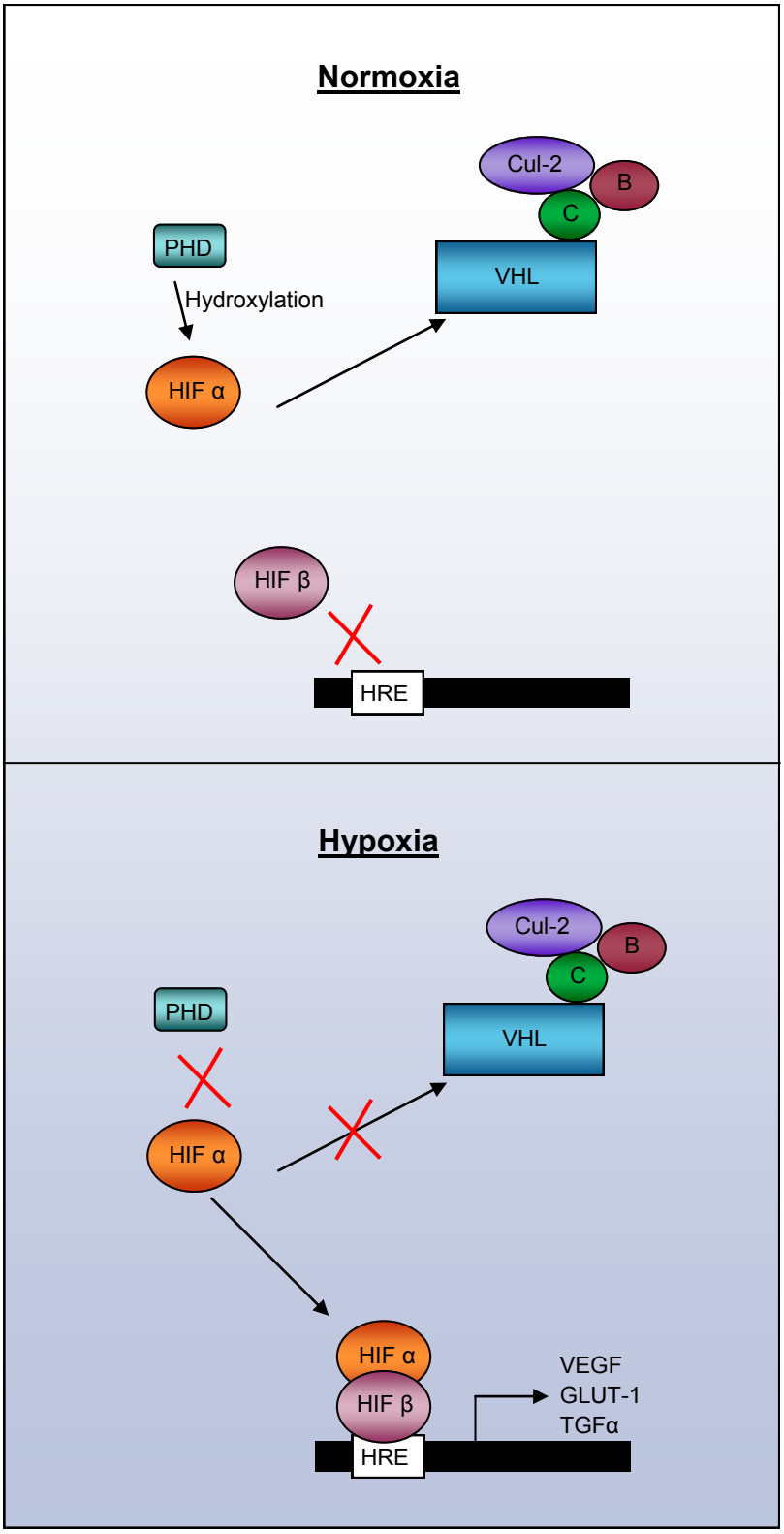
Structural and binding experiments revealed that HIFα is a substrate of the VBC-Cul2 complex, specifically binding to the β-domain of VHL (Ivan et al., 2001; Jaakkola et al., 2001; Ohh et al., 2000; Yu et al., 2001). Remarkably, this interaction only occurs in the presence of oxygen and not during low oxygen tension or hypoxia (Figure 6) (Jaakkola et al., 2001; Yu et al., 2001). Further research soon revealed that two specific prolyl residues of HIFα are hydroxylated by prolyl hydroxylase domain-containing enzymes 1, 2 and 3 (PHDs) in the presence of oxygen (Figure 6) (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). PHDs, which consist of a tetramer containing two hydroxylase units and two protein disulfide isomerase subunits, require oxygen, ferrous iron and 2-oxoglutarate to perform their enzymatic function (Masson et al., 2001). Therefore, HIFα can only be recognized by the VBC-Cul2 E3 ubiquitin ligase complex if it is hydroxylated at key proline residues under aerobic conditions, effectively targeting HIFα for proteasomal degradation; whereas the consequence of hypoxia is its stabilization due to its inability to interact with VHL (Figure 6) (Ivan et al., 2001; Jaakkola et al., 2001). Once stabilized, HIFα can interact with the constitutively expressed HIFβ and form the active HIF transcription factor, thereby resulting in the upregulation of downstream target genes (Figure 6).

1.5.2 Nuclear Export of VHL is required for the Proteasomal Degradation of HIFα.

The 26S proteasome is a multisubunit, ATP-driven complex which effectively degrades ubiquitinated proteins (Coux et al., 1996; Kloetzel, 2001). The process of ubiquitination

Figure 6: The VHL Tumour Suppressor Protein Targets HIFα for Oxygen Dependent Proteasomal Degradation.

The interaction between VHL and HIFα is oxygen-dependent. The hydroxylation of HIFα at key proline residues enables VHL to recognize and bind HIFα, effectively targeting HIFα for proteasomal degradation. However, under hypoxic conditions, hydroxylation does not occur and therefore results in the stabilization of HIFα. Once stabilized, HIFα can interact with the constitutively expressed HIFβ and form the active HIF transcription factor, resulting in the upregulation of downstream target genes.


Normoxia
Cul-2
B
C
PHD
VHL
Hydroxylation
HIF α
HIF β
HRE
Hypoxia
Cul-2
B
C
PHD
VHL
HIF α
HIF α
HIF β
HRE
VEGF
GLUT-1
TGFα

marks protein with ubiquitin and destines proteins for degradation via the 26S proteosome (Hershko and Ciechanover, 1998; Weissman, 2001). The 26S proteasome is composed of four stacked rings (28 subunits) forming a cylindrical chamber; as well as two outer rings - which act as "gates" for the entrance and release of substrates (Bedford et al., 2010 ; Groll et al., 1997). Proteins tagged with ubiquitin are recognized by the outer regulatory cap and are subsequently unfolded, resulting in the passage of proteins through the cylindrical chamber, where proteolysis occurs (Chen and Hochstrasser, 1996; Deveraux et al., 1994; Fenteany et al., 1995; Glickman et al., 1998). However, a prerequisite of proteasomal degradation of nuclear proteins is the ability of the E3 ubiquitin ligase complex to participate in nuclear-cytoplasmic trafficking (Scheffner, 1999). For example, the Mdm2 E3 ubiquitin ligase complex continuously shuttles in and out of the nucleus, consequently resulting in the efficient degradation of the p53 tumour suppressor protein (Freedman and Levine, 1998; Momand et al., 1992; Oliner et al., 1993; Roth et al., 1998). Failure of Mdm2 to export out of the nucleus results in its inability to mediate proteasomal degradation of p53 (Lindstrom et al., 2007). Similarly, the VBC-Cul2 complex requires nuclear-cytoplasmic trafficking to degrade HIFα because although VHL has a predominantly cytoplasmic localization, the ubiquitylation of HIFα occurs in the nucleus (Figure 7) (Groulx and Lee, 2002; Lee et al., 1996). Studies have shown that the ubiquitylation of HIFα is mediated by VHL because ubiquitylation of HIFα does not occur in VHL-negative cells (Groulx and Lee, 2002). Furthermore, it was observed that after HIFα is ubiquitylated in the nucleus through its interaction with the VBC-Cul2 complex, a cytoplasmic signal of HIFα was noticed before its degradation (Groulx and Lee, 2002). These sets of observations suggest that the

proteasomal degradation of HIFα is dependent on the capability of VHL to engage in nuclear export (Figure 7).

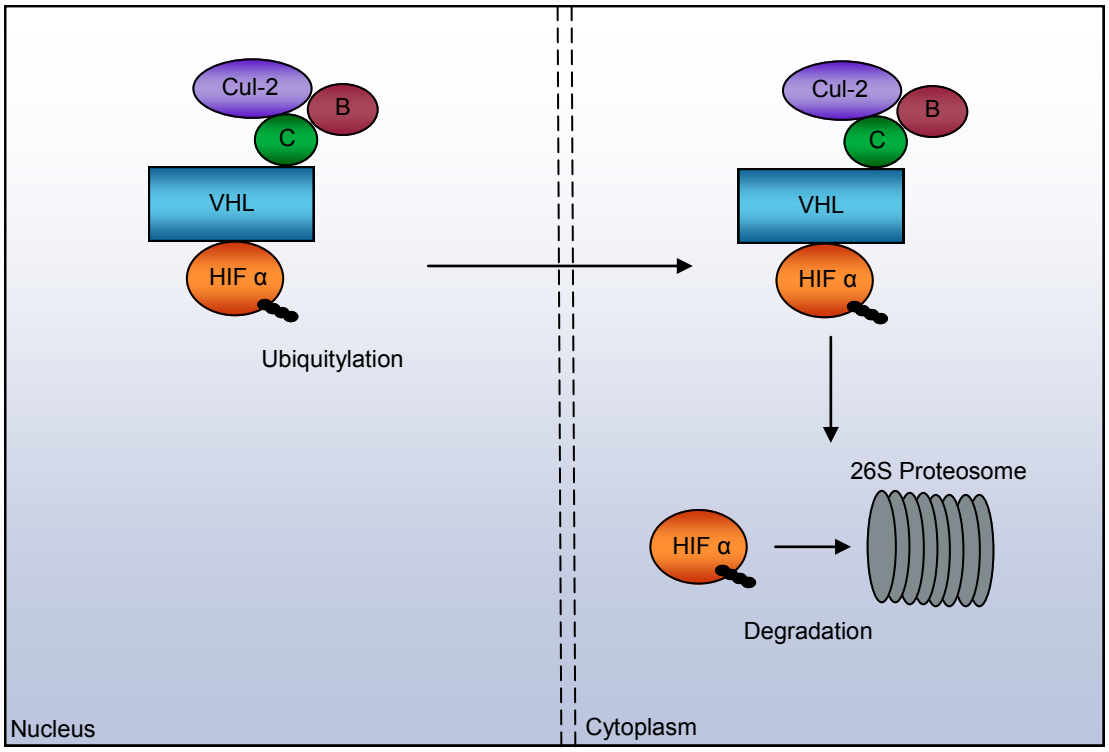
1.6 NUCLEAR-CYTOPLASMIC TRAFFICKING OF VHL

1.6.1 Transcription-Dependent Trafficking of VHL.

The VHL tumour suppressor protein can be found in the mitochondria, endoplasmic reticulum, nucleus and in the cytoplasm (Lee et al., 1996; Schoenfeld et al., 2001; Shiao et al., 2000). Studies with VHL fused with the green fluorescence protein (VHL-GFP) exhibited properties of endogenous VHL, both in localization, its ability to form the VBC-Cul2 complex and in mediating E3 ubiquitin ligase activity (Corless et al., 1997; Lee et al., 1996; Lee et al., 1999; Los et al., 1996; Ye et al., 1998). Furthermore, it appears that VHL's ability to mediate the degradation of HIFα is dependent on its nuclear export. As previously mentioned, VHL has a predominantly cytoplasmic localization. However, the localization of VHL was significantly altered when RNA Polymerase II (RNA Pol II) transcriptional activity was inhibited; a key observation in the subcellular trafficking properties of VHL (Lee et al., 1999). Cells treated with RNA Pol II transcriptional inhibitors, such as Actinomycin D (Act D), 5,6-dichlorobenzimidazole (DRB) or α-amanatin, resulted in a nuclear accumulation of VHL-GFP, both in transiently and stably expressing cells, as well as in different cell lines (Groulx and Lee, 2002; Lee et al., 1999). Furthermore, the removal of the reversible RNA Pol II transcriptional inhibitor, DRB, restored the predominantly cytoplasmic localization of VHL (Lee et al., 1999). Moreover, experiments using cellular fusion and nuclear export assays clearly demonstrated that VHL is a nuclear-cytoplasmic shuttling protein and its

Figure 7: Nuclear-Cytoplasmic Shuttling of the VHL Tumour Suppressor Protein is Required for the Proteasomal Degradation of HIFα.

VHL mediates the ubiquitylation of HIFα in the nucleus. To effectively degrade HIFα via the 26S proteasome, nuclear-cytoplasmic shuttling of the VBC-Cul2 complex is required.


Cul-2
B
C
VHL
HIF α
Ubiquitylation
Cul-2
B
C
VHL
HIF α
26S Proteosome
HIF α
Degradation
Nucleus
Cytoplasm

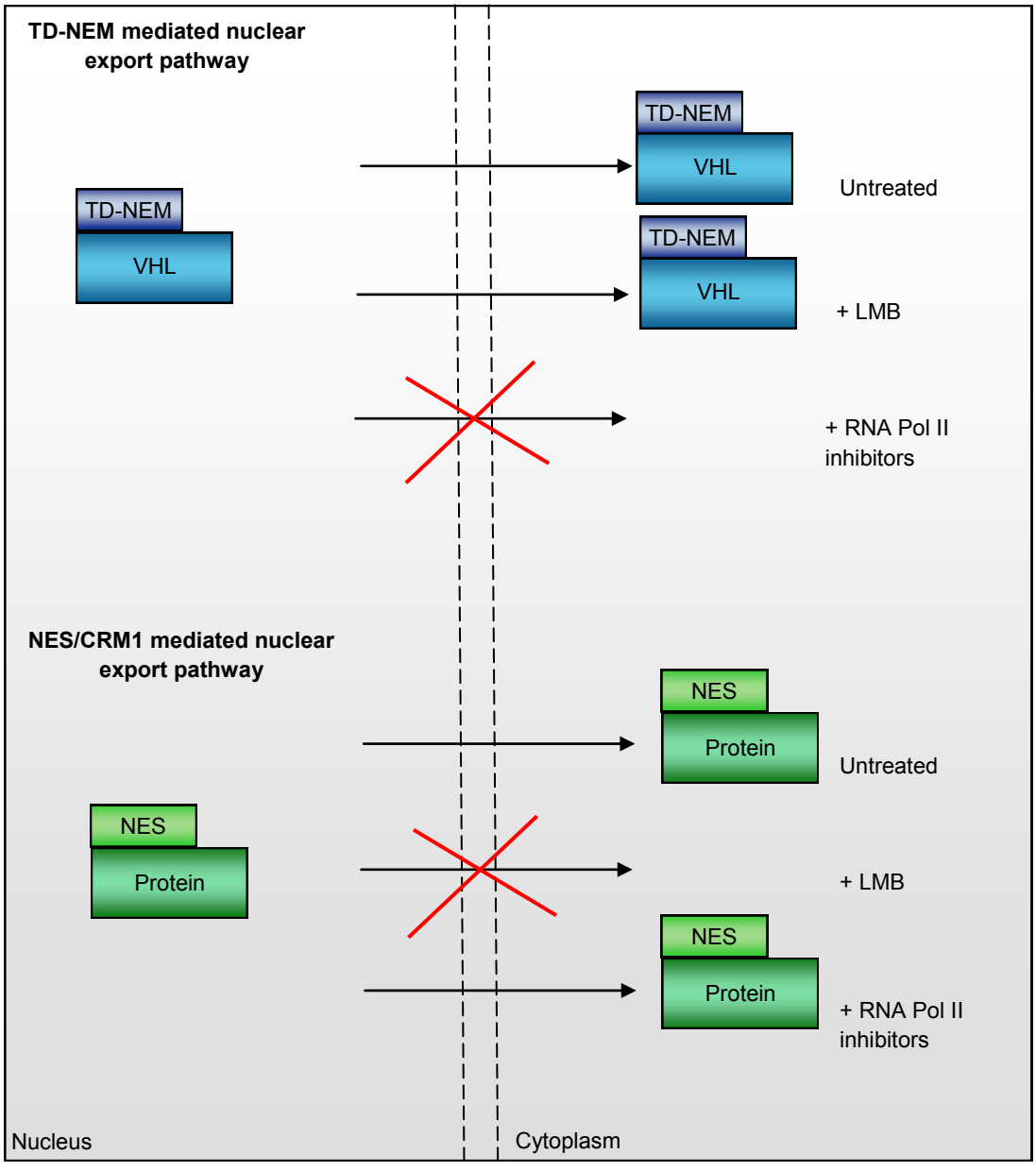
ability to export is dependent on ongoing RNA Pol II transcription (Groulx and Lee, 2002; Lee et al., 1999).

1.6.2 The Transcription-Dependent Nuclear Export Motif (TD-NEM) of VHL Mediates its Nuclear Export.

The ability of VHL to engage in nuclear-cytoplasmic shuttling has been clearly demonstrated through the alteration of its steady-state localization; a significant threefold increase in the nuclear compartment upon the inhibition of RNA Pol II transcription (Lee et al., 1999). The crystal structure of VHL revealed that this protein contains two distinct domains: a β domain (residues 64-154) and an α domain (residues 157-189) (Stebbins et al., 1999). Studies demonstrated that residues involved in mediating the transcription-dependent nuclear export capabilities of VHL is within the exon 2-encoded β domain (Bonicalzi et al., 2001). Within the residues of exon 2, the classical NES sequence was not detected and the steady state distribution of VHL was not affected when treated with LMB, an inhibitor of NES/CRM1 nuclear export pathway (Figure 8) (Bonicalzi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999; Yoshida and Horinouchi, 1999). This set of observations prompted the investigation of a novel export mechanism that is independent of the NES/CRM1 classical nuclear export pathway and one that requires ongoing RNA Pol II transcription. Mutational analysis revealed that the nuclear export of VHL is mediated by a novel and discrete nuclear export motif, $DXGX_2DX_2L$, termed Transcription-Dependent Nuclear Export Motif (TD-NEM) (Figure 8) (Khacho et al., 2008b). The TD-NEM of VHL was identified to be within residues 114-138 and this discrete motif was also present in the Poly(A)-Binding Protein 1 (PABP1), which was also observed to require ongoing RNA Pol II activity to export out of the nucleus

Figure 8: The Transcription-Dependent Nuclear Export Motif (TD-NEM) Mediates the Transcription-Dependent Nuclear Export of VHL.

VHL contains a novel export motif, termed the TD-NEM, which effectively mediates its nuclear export. Nuclear export of VHL is insensitive to LMB treatment but is sensitive to treatment of RNA Pol II inhibitors, in contrast to proteins containing a NES. Proteins participating in nuclear export via the classical NES are sensitive to LMB treatment but are insensitive to treatment of RNA Pol II inhibitors.


TD-NEM mediated nuclear export pathway
TD-NEM
VHL
TD-NEM
VHL
Untreated
TD-NEM
VHL
+ LMB
+ RNA Pol II inhibitors
NES/CRM1 mediated nuclear export pathway
NES
Protein
NES
Protein
Untreated
+ LMB
NES
Protein
+ RNA Pol II inhibitors
Nucleus
Cytoplasm

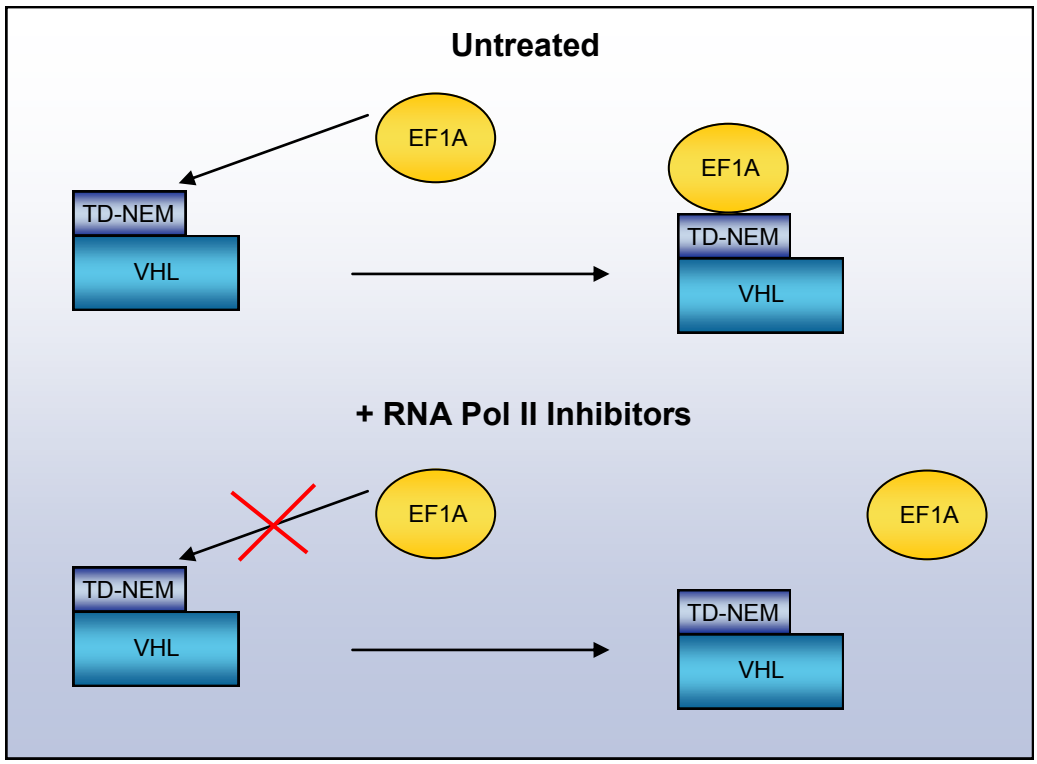
(Afonina et al., 1998; Khacho et al., 2008b). In comparison to the Rev NES, photobleaching experiments revealed that the rate of TD-NEM mediated nuclear export was 70%-80% of that observed for the Rev NES; signifying a strong capability of mediating nuclear export since the Rev NES is the strongest LMB-sensitive NES identified thus far (Henderson and Eleftheriou, 2000; Khacho et al., 2008b). Furthermore, TD-NEM alone from VHL fused to other proteins, such as pyruvate-kinase tagged with GFP (pk-GFP), was able to engage in transcription-dependent nuclear export, as observed in living cells and *in vitro* assays (Khacho et al., 2008b). Taken together, these results identify the TD-NEM as a novel export motif utilized by proteins in engaging nuclear egression in a manner that requires ongoing RNA Pol II-transcription.

1.6.3 eEF1A is a Mandatory Component of VHL's Nuclear Export Machinery.

Multi-protein complexes are required to mediate proficient nuclear export of a wide variety of proteins and RNA species. As previously mentioned in section 1.3.1, nuclear export of proteins containing a NES are recognized by CRM1 in conjunction with Ran-GTP, thereby effectively ensuring nuclear export of proteins (Askjaer et al., 1998; Fornerod et al., 1997a). However, nuclear export of VHL and PABP1 has been demonstrated to be independent of the classical CRM1/NES nuclear export pathway (Bonicalzi et al., 2001; Groulx and Lee, 2002; Lee et al., 1999; Yoshida and Horinouchi, 1999). These proteins utilize a novel export motif, termed the TD-NEM, and require ongoing RNA Pol II mediated transcription (Khacho et al., 2008b). To further understand the RNA Pol II transcription-dependent nuclear export machinery utilized by VHL, PABP1 and other proteins containing a TD-NEM, novel interacting proteins were investigated utilizing immunoprecipitation, silver staining and mass spectrometry

Figure 9: eEF1A Interacts Specifically with the TD-NEM of VHL.

eEF1A is a component of the transcription-dependent nuclear export pathway of VHL and was shown to specifically interact with the TD-NEM. Inhibition of RNA-Pol II-mediated transcription prevented the interaction between VHL and eEF1A.


Untreated
EF1A
TD-NEM
VHL
EF1A
TD-NEM
VHL
+ RNA Pol II Inhibitors
EF1A
TD-NEM
VHL
EF1A
TD-NEM
VHL

(Khacho et al., 2008a). Utilizing said techniques, eEF1A was identified as a novel interacting partner of VHL and PABP1 (Khacho et al., 2008a). Interestingly, eEF1A was determined to bind specifically to the TD-NEM and removal of this discrete motif prevented eEF1A binding and inhibited nuclear export (Figure 9) (Khacho et al., 2008a). In addition, treatment with inhibitors of RNA Pol II transcription, such as Act D or DRB, inhibited the interaction between VHL and eEF1A (Figure 9) (Khacho et al., 2008a). To further uncouple the exact function or role of eEF1A within this nuclear export pathway, eEF1A was silenced and the localization of VHL was monitored (Khacho et al., 2008a). It was observed that silencing eEF1A resulted in a nuclear accumulation of VHL; reminiscent of the nuclear accumulation observed of cells treated with Act D or DRB (Figure 10) (Khacho et al., 2008a). Fluorescence loss in photobleaching (FLIP) experiments revealed that the nuclear accumulation detected was due to a decrease in nuclear export (Khacho et al., 2008a). eEF1A is therefore a novel interacting protein of VHL and PABP1 and is a mandatory component of the transcription-dependent nuclear export pathway machinery.

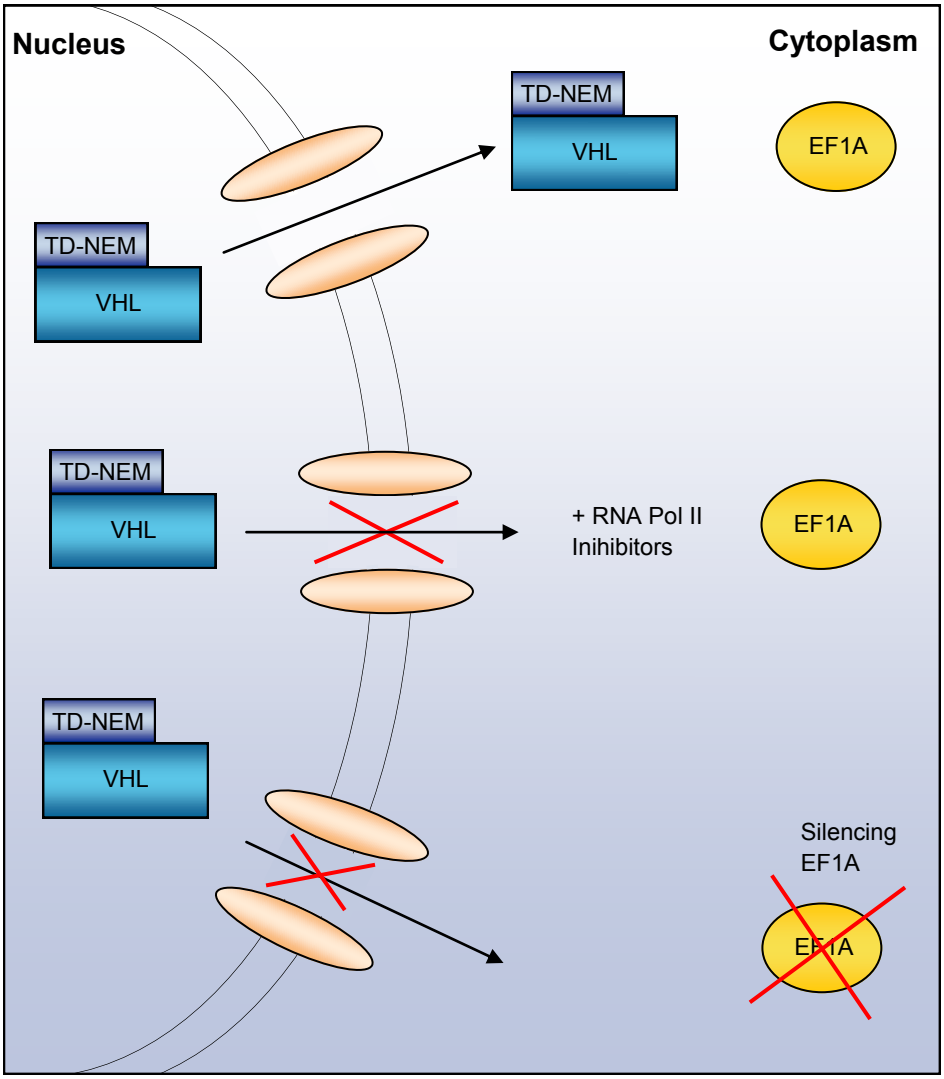
1.7 EUKARYOTIC ELONGATION FACTOR 1 ALPHA

1.7.1 The Canonical Role of eEF1A.

Although an essential component of the transcription-dependent nuclear export machinery, eEF1A's canonical role is in protein translation. The eukaryotic translation factor 1 (eEF1) is composed of a guanine nucleotide-binding protein (G-protein), eEF1A, and a guanine nucleotide exchange factor (GEF), eEF1B (Merrick and Nyborg, 2000). G-proteins are molecular switches; enabling a guanosine triphosphate (GTP) or guanosine diphosphate (GDP) -bound state (Bourne et al., 1991). Furthermore, eEF1A is a member

Figure 10: eEF1A is a Mandatory Component of the TD-NEM Mediated Nuclear Export Pathway of VHL.

Silencing eEF1A inhibited the nuclear export of VHL, reminiscent of the nuclear accumulation and inhibition of nuclear export observed when treated with RNA Pol II-inhibitors, such as DRB or Act D.


Nucleus
Cytoplasm
TD-NEM
VHL
EF1A
TD-NEM
VHL
TD-NEM
VHL
+ RNA Pol II
Inihibitors
EF1A
TD-NEM
VHL
Silencing
EF1A
EF1A

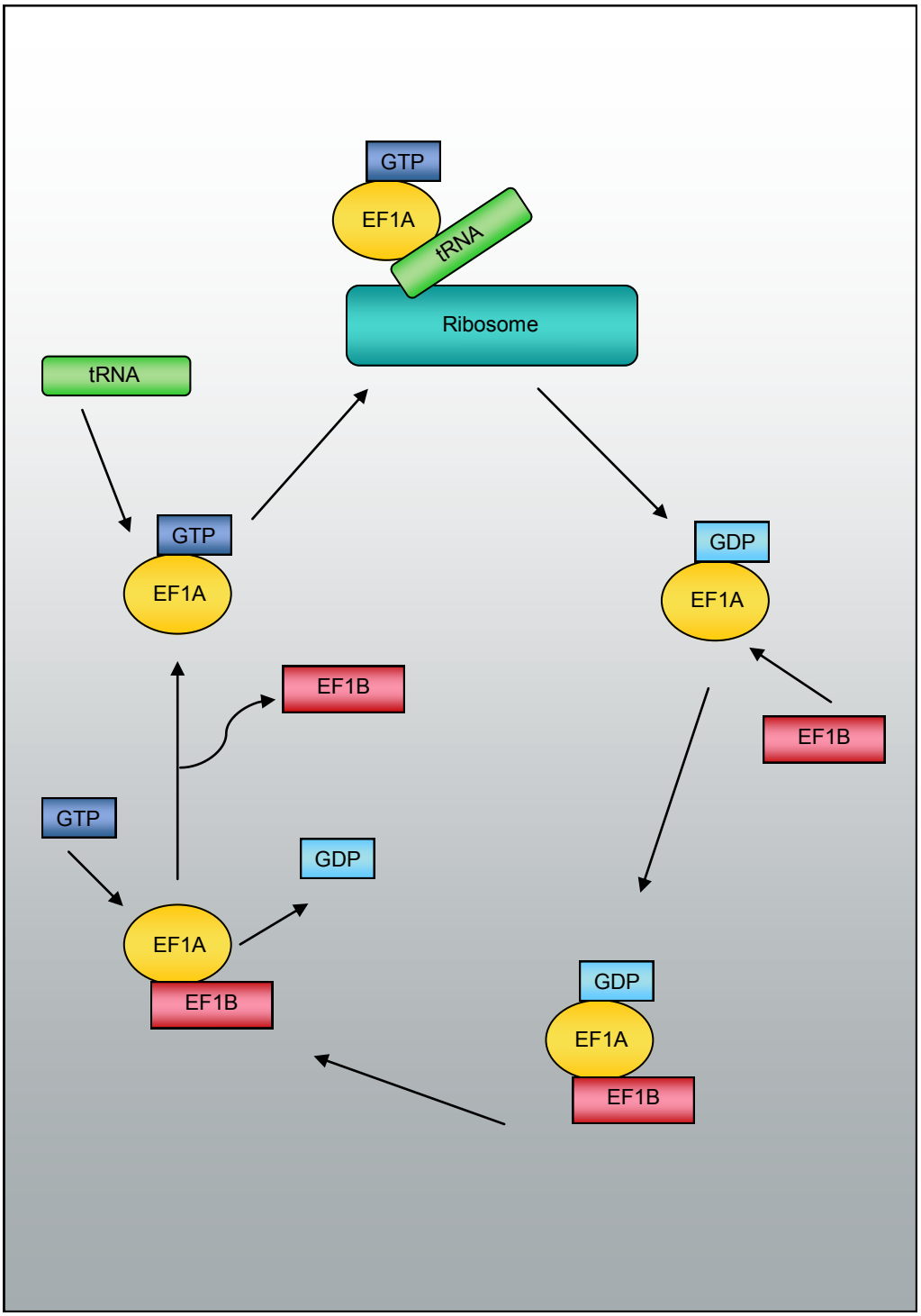
of the translation factor family of GTPases and is capable of GTP hydrolysis upon activation (Merrick and Nyborg, 2000). eEF1A (termed EF-Tu in prokaryotes) is in its active form when bound to GTP. In its active form, eEF1A-GTP recruits aminoacylated tRNAs (aa-tRNA) to form a ternary complex and to deliver the aa-tRNA to the A-site of the elongating ribosome (Figure 11) (Andersen et al., 2003; Carvalho et al., 1984). The ribosome triggers GTP hydrolysis if the mRNA codon exposed in the A-site of the ribosome matches the anti-codon of the aa-tRNA, thereby resulting in the release of eEF1A-GDP (Figure 11) (Andersen et al., 2003). The intrinsic rate of GDP release is extremely slow; thereby requiring the activity of eEF1B (also known as EF-Ts in prokaryotes) to facilitate the exchange from eEF1A-GDP to eEF1A-GTP (Janssen and Moller, 1988; Pittman et al., 2006; Saha and Chakraburtty, 1986; Slobin and Moller, 1978). The C-terminus, which possess the nucleotide exchange activity, of the alpha subunit of eEF1B (eEF1Bα) binds to eEF1A-GDP, effectively resulting in the release of GDP (Figure 11) (Perez et al., 1998; van Damme et al., 1990) (Gromadski et al., 2007). GTP then binds to the eEF1A : eEF1B complex, which causes the dissociation of eEF1B, subsequently resulting in a GTP-bound state of eEF1A (Figure 11) (Gromadski et al., 2007). The active GTP-bound state of eEF1A is once again capable of recruiting aa-tRNA to perform its canonical role in translation.

1.7.2 Alternative Roles of eEF1A.

As one of the most abundant proteins in the cell, it is not surprising that eEF1A is involved in multiple cellular processes aside from its canonical role in protein translation. Aside from its function in the transcription-dependent nuclear export machinery mentioned in section 1.6.3, eEF1A has been identified to participate in numerous other

Figure 11: The GTP/GDP Cycle of eEF1A.

eEF1A is a G-protein and be bound to either GTP or GDP. eEF1A in its active form (eEF1A-GTP) recruits aa-tRNA and delivers it to the elongating ribosome. Upon codon-anticodon match, eEF1A-GTP is hydrolyzed and eEF1A-GDP is released from the ribosome. EF1Bα binds to eEF1A-GDP to facilitate the release of GDP. The binding of GTP to the eEF1A-EF1B complex causes the dissociation of EF1B, thereby resulting in eEF1A-GTP.


GTP
EF1A
tRNA
Ribosome
tRNA
GTP
EF1A
GDP
EF1A
EF1B
EF1B
GTP
GDP
EF1A
EF1B
GDP
EF1A
EF1B

cellular processes. eEF1A isolated from the slime mold, *Dictyostelium discoideum*, was observed to bind and bundle actin filaments *in vitro* (Demma et al., 1990; Yang et al., 1990). Furthermore, mutations in domains II and III resulted in functional impairment and disorganization of the actin cytoskeleton *in vivo*; clearly demonstrating the role of eEF1A in actin bundling and organization (Gross and Kinzy, 2005; Gross and Kinzy, 2007). eEF1A may also play an important role in microtubule dynamics, although the exact function remains to be fully understood. eEF1A isolated from carrot cells has been shown to stabilize and bundle microtubules (Durso and Cyr, 1994; Moore et al., 1998); whereas eEF1A purified from *Xenopus laevis* egg extracts acted as a microtubule severing factor (Shiina et al., 1994). It has also been reported that eEF1A plays a role in apoptosis or programmed cell death in metazoans. In experiments of serum withdrawal, higher levels of eEF1A expression resulted in a faster rate of cell death in cultured mouse fibroblast (Duttaroy et al., 1998). However, in mammalian cells, the exact function of eEF1A in apoptosis remains to be further investigated due to the presence of the two isoforms of eEF1A; differing in tissue and developmental-specific expression patterns, as well as varying in protein interaction partners (Kahns et al., 1998; Lee et al., 1992). As mentioned in section 1.6.3, eEF1A is required in mediating the transcription-dependent nuclear export of VHL, PABP1 and other proteins containing a TD-NEM (Khacho et al., 2008a; Khacho et al., 2008b). In budding yeast, mutations or reduced expression of eEF1A resulted in a defect in the nuclear export of aa-tRNA; thereby identifying an alternative aa-tRNA nuclear export pathway independent of Los1p (Grosshans et al., 2000a; Grosshans et al., 2000b). The exact mechanism and function of eEF1A in mediating nuclear export remains to be fully understood. Since eEF1A is an abundant

cytoplasmic protein, studies have hypothesized that the function of eEF1A is on the cytoplasmic side of the nuclear envelope (Bohnsack et al., 2002; Calado et al., 2002).

1.7.3 eEF1A can access the Nuclear Compartment in Yeast Cells.

Saccharomyces cerevisiae (yeast) cells exploit multiple tRNA nuclear export pathways to effectively export tRNA transcripts. In vertebrates, tRNA nuclear export is mediated by Exportin-t (Exp-t), a member of the β-importin family (Arts et al., 1998; Kutay et al., 1998). The yeast orthologue of Exp-t is Los1 in yeast cells (Hellmuth et al., 1998; Sarkar and Hopper, 1998). Remarkably, it was discovered that Los1 is an unessential gene because Los1 defective mutants resulted in relatively normal growth phenotype (Hurt et al., 1987). This observation led to the discovery of an alternative tRNA nuclear export pathway: Msn5 in yeast cells and Exp-5 in mammalian cells (Bohnsack et al., 2002; Calado et al., 2002; Takano et al., 2005). However, it appears that although Los1 and Msn5 have overlapping roles in the nuclear export of tRNA in yeast cells, their function appears to be distinct (Murthi et al., 2010). Knockdown of Exp-5 had little effect on the nuclear export of tRNA whereas the inhibition of Exp-t resulted in a considerable reduction in the export rate of tRNA (Arts et al., 1998; Lipowsky et al., 1999). A recent study utilizing epifluorescence and confocal imaging revealed that in yeast cells lacking msn5 (Δ msn5), Tef1 (yeast eEF1A), was observed to be in the nucleus and not completely excluded out (Murthi et al., 2010). Moreover, the distribution of eEF1A between the nucleus and cytoplasm in Δ msn5 cells were found to be evenly distributed, in contrast to the strictly cytoplasmic localization of eEF1A in normal cells (Murthi et al., 2010). This result thereby demonstrates that eEF1A has the capability of accessing the

nuclear compartment in yeast cells and this ability is at least in part dependent on msn5/Exp-5.

1.8 RATIONALE

The ability of VHL to engage in nuclear egression is crucial for the proteasomal degradation of HIFα (Groulx and Lee, 2002). However, VHL operates independently of the classical NES/CRM1 nuclear export pathway and is mediated by a discrete motif, the TD-NEM (Groulx and Lee, 2002; Khacho et al., 2008b; Lee et al., 1999). Proteins containing a TD-NEM, such as VHL and PABP1, require ongoing transcription and are sensitive to treatments with transcriptional inhibitors (Khacho et al., 2008a). Furthermore, eEF1A has recently been shown to be a novel interacting partner with VHL and a mandatory component of the transcription-dependent nuclear export machinery (Khacho et al., 2008b). eEF1A is an abundant cytoplasmic protein and its exact function in nuclear export remains to be further explored. Typically, proteins involved in nuclear import and export are found in the cellular compartment of origin and are capable of moving between the nucleus and cytoplasm. The ability of eEF1A to access the nucleus to participate in tRNA export has recently been demonstrated in budding yeast (Murthi et al., 2010). Therefore, although the steady-state localization of eEF1A is cytoplasmic, a population of eEF1A may have the capability of accessing the nuclear compartment and participating in the transcription-dependent nuclear export of proteins containing a TD-NEM. The goal of this study was to: 1- understand the involvement of a cytoplasmic protein in nuclear egression; and 2- comprehend the mechanism utilized by eEF1A to mediate nuclear export of proteins encoding a TD-NEM.

2.0 HYPOTHESIS

Mammalian EF1A shuttles between the nucleus and cytoplasm to mediate the nuclear export of proteins encoding a TD-NEM.

3.0 OBJECTIVES

3.1 To examine whether the nucleotide status of eEF1A has an effect on its ability to participate in nuclear import and export.

3.2 To identify domains of eEF1A required for nucleocytoplasmic shuttling.

3.3 To understand the requirement of ongoing transcription necessary for nuclear export of proteins.

4.0 MATERIALS AND METHODS

4.1 Cell Culture, Transfections and Drug Treatments.

MCF-7 (human breast adenocarcinoma) cells used in this study were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in a 37˚C and 5% CO_2 environment chamber with Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Fisher) and 1% penicillin-streptomycin (P-S). Cells were grown in a monolayer culture and passaged every 2-3 days. Transient transfections were accomplished using Effectine (Qiagen) transfection reagent as per manufacturer's instructions. The transfected cells were incubated for 24 hours before treatment with drugs or subjected to experimental manipulations. When indicated, cells were treated with a final concentration of 8 µM Act D (CalBioChem) for 6 hours prior to live cell fluorescence imaging. When described, 2 mM GMP-PNP (Sigma) or 2 mM GDP (Sigma) were incubated with the lysates before immunoprecipitation and immunoblotting.

4.2 Expression Vectors.

Human full length VHL, eEF1A, truncated and point mutants of eEF1A were cloned in between an NH_2-terminal Flag-tag and a COOH-terminal GFP tag into pcDNA 3.1 expression vector, as previously described in Bonicalzi et. al., 2001, Groulx and Lee, 2002 and Lee et. al., 1999. The restriction sites Apa1 and Xho1 were used to clone human full length, truncated and point mutants of eEF1A. The constructs F-GFP and F-GFP-NES were previously described in Groulx and Lee, 2002 and Lee et. al., 1999. cDNAs corresponding to VHL, eEF1A and eEF1A-^{252}DVY^{254}->AAA were inserted in between the Flag-tag and GFP tag of F-GFP-NLS or F-GFP-NES constructs using Apa1

and Xho1 restriction sites. The nuclear localization signal (NLS) was derived from the simian virus large T antigen SV40 as previously described in Groulx et. al., 2000 and Kalderon et. al., 1844. The nuclear export signal (NES) was derived from the human immunodeficiency virus REV as described in Khacho et. al., 2008b. The F-GFP-GFP-NLS and F-GFP-GFP-NES constructs consisted of two GFP cDNAs with either a NLS or a NES. For photobleaching experiments, the F-pk-GFP-NLS construct consisted of a pyruvate kinase (pk), which does not encode any localization determinant, GFP and a NLS.

4.3 Live Cell Fluorescence Imaging.

Experiments (apart from photobleaching) utilizing MCF-7 cells transiently expressing GFP were imaged using an Axiovert S100TV microscope (Carl Zeiss MicroImaging, Inc) equipped with a 40x/1.2 C-Apochromat water immersion objective. Using a digital charged-couple device camera (Empix) and a Northern Eclipse software package (Empix), images of the live cells were taken. Hoechst 33342 (Sigma) was used to stain the nuclei for imaging.

4.4 Live Cell Nuclear Import/Export Assay.

Cells were cultured onto 35-mm coverslip plates (Mat Tek) and subsequently transfected with fusion proteins. Proteins were either tagged with a nuclear export signal (NES) or a nuclear localization signal (NLS) to maintain a steady-state cytoplasmic and nuclear localization, respectively. Transfected cell were incubated in the cold room (4˚C) for 3 hours or at 37˚C. Hoechst 33342 (Sigma) was used to stain the nuclei. Cells were viewed and imaged as mentioned in section 4.3.

4.5 Fluorescence Loss in Photobleaching.

MCF-7 cells were cultured and transfected onto 35-mm coverslip plates (Mat Tek). Photobleaching was performed using a confocal microscope (L5M5 Pascal Laser Scanning Microscope, Carl Zeiss Canada), while cells were maintained in an environment chamber at 37˚C with 5% CO_2. Indicated areas in the cytoplasm (small white squares) represented the area that was bleached using a 488-nm argon laser at 20% of full laser power and image acquisition at 5% of full laser power. The size and distance from the nucleus of the cytoplasmic area bleached was kept constant. Bleaching and imaging was performed using a 63x plan Apo oil immersion lens with a 1.4 NA. To monitor nuclear export activity of proteins, a strong nuclear localization signal (NLS) was fused to the proteins to ensure similar levels of nuclear fluorescence. Fusion proteins also harbour GFP to monitor and measure fluorescence intensity. Fluorescence loss in the nucleus was measured using equations previously described in Mekhail et al., 2005 and Phair and Misteli, 2000. The equation: $I_{rel}=(I_{(t)}/I_{(0)})*(N_{(0)}/N_{(t)})$, where $I_{(t)}$ is the average intensity of the unbleached nucleus at time point t, $I_{(0)}$ is the average prebleached intensity of the nucleus, $N_{(0)}$ and $N_{(t)}$ are the average nuclear fluorescence intensity of a neighbouring cell in the same field of vision at a prebleach time or at time t, respectively. This calculation normalizes the fluorescence of the cell of interest to that of a neighbouring cell, thereby accounting for any loss in fluorescence due to picture acquisition. Pseudocolour images were presented to demonstrate subtle changes in nuclear fluorescence, red representing high fluorescence intensity and light blue representing low fluorescence intensity. The Northern Eclipse (Empix), Adobe

Photoshop and Microscope Excel were used to capture images, analyze data and generate graphs.

4.6 Subcellular Fractionation.

For experiments requiring fractionation, cells were incubated in buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 1mM sodium orthovanadate, 10 mg/ml digitonin with 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin. Permeabilization was performed for 10-15 minutes on ice with gentle shaking. The progress of the permeabilization was monitored using Hoechst 33258 (Sigma) (stains permeabilized cells only) and visualized using a fluorescence microscope. Permeabilization was stopped when the majority of the cells displayed stained nuclei. Permeabilized cells were centrifuged and the resultant supernatant was the digitonin-soluble or cytosolic enriched fraction. The pellet was washed in buffer and lysed with buffer containing 0.5% Igepal CA630, 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 1mM sodium orthovanadate with 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin for 2 hours. Lysates were subjected to another around of centrifugation and the resultant supernatant was the digitonin-insoluble or nuclear enriched fraction. Digitonin-soluble and insoluble fractions were used for immunoprecipitation and immunoblotting.

4.7 Immunoprecipitation and Immunoblotting.

Lysis buffer containing 0.5% Igepal CA630, 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 1mM sodium orthovanadate with 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin was used to lyse cells for immunoprecipitation. Digitonin-soluble and insoluble lysates were prepared as mentioned in section 4.6. Anti-Flag M2 beads

(Sigma) were added to cell lysates and incubated overnight at 4˚C while rotating. The following day, beads were washed in TBS (pH 7.4) containing Tris and NaCl several times before eluting with Flag peptide. Total cell lysates were obtained by washing cells several times in PBS, lysing cells in 4% SDS in PBS, shearing the DNA with 19-gauge needles and boiling for 5 minutes. Protein concentration was obtained using the bicinchoninic acid (BCA) method (Pierce). Samples were separated on denaturing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred onto PVDF (Polyvinylidene fluoride) membranes and blocked in a solution of PBS containing 0.2% Tween 20 and skimmed milk powder. The membranes were incubated in primary antibodies: Flag-M2 (Sigma), eEF1A (Millipore), eEF1Bα (Abcam), HA (Sigma), LDH (Sigma) and B23 (Sigma). Membranes were washed in 0.2% Tween-PBS and blotted with mouse secondary antibody conjugated to horseradish peroxidise (Jackson ImmunoResearch Laboratories). Proteins were detected using a western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

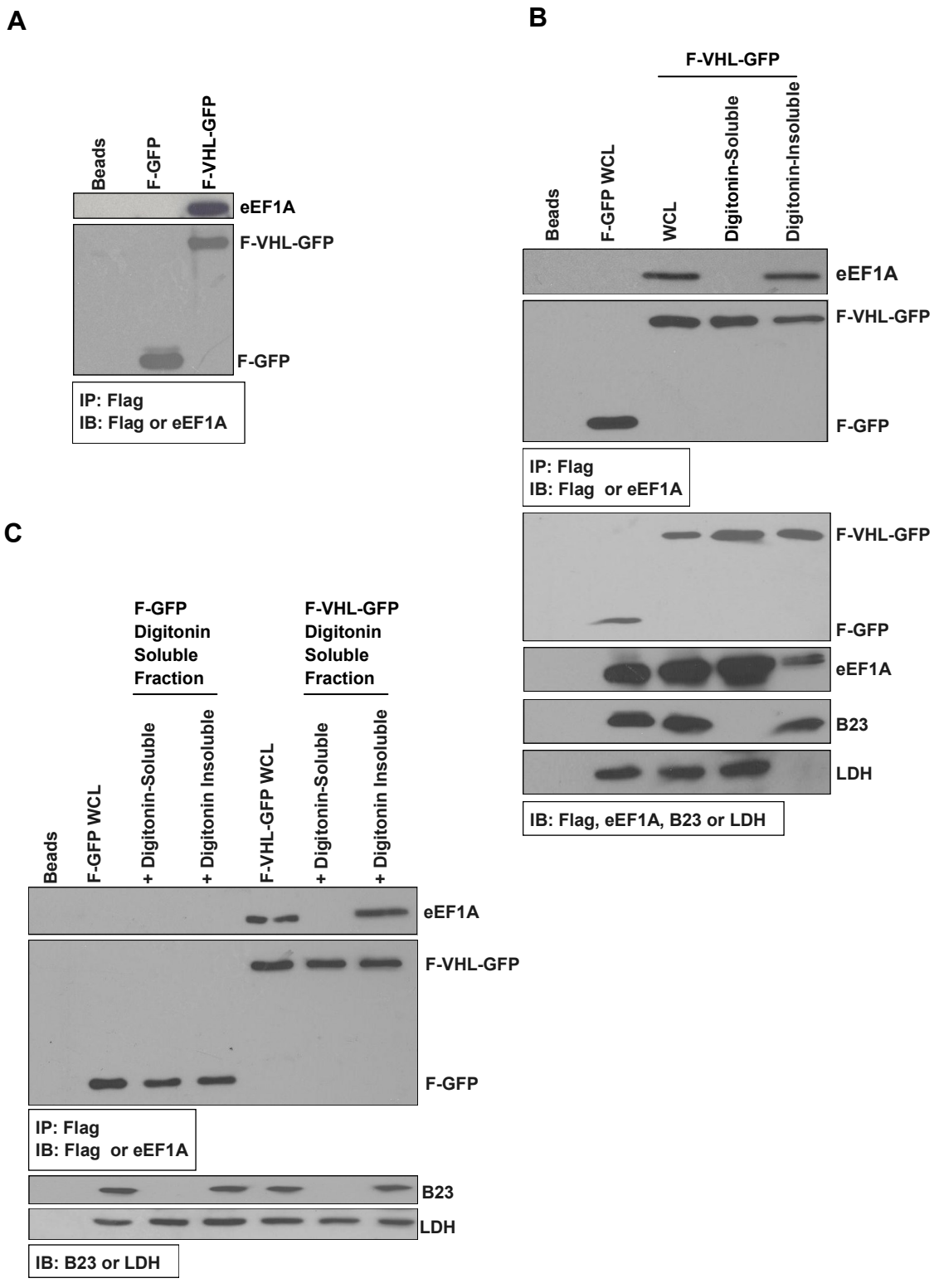
5.0 RESULTS

5.1 VHL Interacts with eEF1A in the Nuclear Fraction.

The nuclear export of VHL is independent of the classical CRM1/NES nuclear export pathway but requires ongoing RNA Pol II transcription (Groulx and Lee, 2002; Khacho et. al., 2008a; Lee at. al., 1999). Furthermore, a discrete motif, termed the TD-NEM, was identified to mediate the transcription-dependent nuclear export of VHL, PABP1 and other proteins encoding a TD-NEM (Khacho et. al., 2008a). Within this TD-NEM mediated nuclear export pathway, eEF1A was identified as a novel and mandatory interacting protein (Khacho et. al., 2008b). As previously observed and reported, eEF1A binds to VHL (Figure 12A), specifically to the TD-NEM (Khacho et. al., 2008b). To further investigate in which cellular compartment the interaction between VHL and eEF1A occurs, fractionation experiments utilizing digitonin were conducted. Fractionation experiments revealed that the binding between VHL and eEF1A occurred only in the digitonin-insoluble (nuclear enriched) fraction, a surprising observation due to the abundance of both proteins in the cytosol (Figure 12B). This observation led to the premise that VHL in the cytosolic fraction is the variant that is incompetent to bind to eEF1A. To understand the lack of interaction between VHL and eEF1A in the cytosolic fraction, despite the abundance of both proteins, the cytoplasmic enriched lysates (digitonin-soluble fraction) of VHL were incubated with additional digitonin soluble or digitonin insoluble lysates. Results show that the digitonin-soluble fraction of VHL has the capabilities of binding to eEF1A only when incubated with nuclear enriched lysates (Figure 12C). These results demonstrated that both nuclear and cytoplasmic VHL are

Figure 12: The Interaction between VHL and eEF1A.

(A) eEF1A binds to VHL. Lysates of MCF-7 cells transiently expressing F-GFP or F-VHL-GFP were immunoprecipitated with anti-Flag beads and immunoblotted with either Flag or eEF1A antibodies. **(B)** Digitonin-insoluble fraction of VHL binds to eEF1A. Whole cell or digitonin-fractionated lysates of MCF-7 cells transiently expressing F-GFP or F-VHL-GFP were immunoprecipitated with anti-Flag beads and immunoblotted with either Flag or eEF1A antibodies. Lysates were immunoblotted with B23 (nuclear marker) and LDH (cytoplasmic marker) antibodies to control for the fractionation. **(C)** Digitonin-soluble fraction of VHL binds to eEF1A upon the addition of digitonin-insoluble lysates. The digitonin-soluble lysates of MCF-7 cells transiently expressing F-GFP or F-VHL-GFP were incubated with additional digitonin-soluble or digitonin-insoluble lysates, immunoprecipitated with anti-Flag beads and immunoblotted with Flag or eEF1A antibodies. Lysates were immunoblotted with B23 and LDH antibodies to verify fractionation.


A
Beads
F-GFP
F-VHL-GFP
eEF1A
F-VHL-GFP
F-GFP
IP: Flag
IB: Flag or eEF1A
B
F-VHL-GFP
Beads
F-GFP WCL
WCL
Digitonin-Soluble
Digitonin-Insoluble
eEF1A
F-VHL-GFP
F-GFP
IP: Flag
IB: Flag or eEF1A
F-VHL-GFP
F-GFP
eEF1A
B23
LDH
IB: Flag, eEF1A, B23 or LDH
C
F-GFP
Digitonin
Soluble
Fraction
F-VHL-GFP
Digitonin
Soluble
Fraction
Beads
F-GFP WCL
+ Digitonin-Soluble
+ Digitonin Insoluble
F-VHL-GFP WCL
+ Digitonin-Soluble
+ Digitonin Insoluble
eEF1A
F-VHL-GFP
F-GFP
IP: Flag
IB: Flag or eEF1A
B23
LDH
IB: B23 or LDH

competent to bind eEF1A; however, it appears that the nuclear enriched fraction harbours a central component required for the VHL-eEF1A interaction to occur.

5.2 The GTP/GDP Status of eEF1A Dictates its Ability to Bind to VHL.

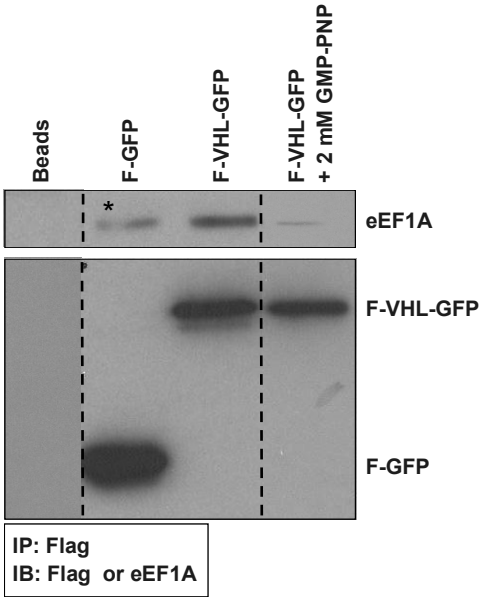
Fractionation experiments revealed that VHL in the nuclear and cytoplasmic compartments are capable of binding to eEF1A (Figure 12B and 12C). This observation suggested that the modifier between the VHL-eEF1A interaction may reside within the eEF1A protein. Since eEF1A is a member of the translation factor family of GTPases and can either be bound to GTP or GDP (Bourne et al., 1991), we speculated that the GTP/GDP exchange may play a part in eEF1A's ability to interact with VHL. Lysates transiently transfected with F-VHL-GFP were incubated in the absence or presence of 5'-Guanylyl imidodiphosphate **(**GMP-PNP), a non-hydrolysable analog of GTP, before being subjected to immunoprecipitation and immunoblotting. Results show that the addition of GMP-PNP resulted in a decrease of eEF1A binding to VHL (Figure 13). These observations prompted the further investigation of the GTP/GDP cycle of eEF1A and its potential involvement in eEF1A's ability to bind to VHL.

5.3 Point Mutant of eEF1A Incapable of Interacting with EF1Bα.

The majority of eEF1A is thought to be in the active, GTP-bound state due to 1.) the 30-fold higher concentration of GTP compared to GDP in cells and 2.) eEF1Bα (the sub-unit of eEF1B which possess the guanine nucleotide-exchange activity) can accelerate the rate of GDP dissociation by 700-fold (Andersen et al., 2001; Gromadski et al., 2007; Pittman et al., 2006). Despite the abundance of eEF1A in the GTP-bound state, our result shows that the interaction between eEF1A and VHL is hampered in the presence of GMP-PNP (Figure 13). Therefore, it appears that the nucleotide status of eEF1A may

Figure 13: The Interaction between VHL and eEF1A is Dependent on eEF1A's Nucleotide Status.

The interaction between VHL and eEF1A decreased in the presence of GMP-PNP, a non-hydrolysable analog of GTP. Lysates of MCF-7 cells transiently expressing F-GFP or F-VHL-GFP were either left untreated or incubated with 2 mM GMP-PNP before being subjected to immunoprecipitation with anti-Flag beads and immunoblotted with either Flag or eEF1A antibodies. Asterisk indicates non-specific band.


Beads
F-GFP
F-VHL-GFP
F-VHL-GFP
+ 2 mM GMP-PNP
*
eEF1A
F-VHL-GFP
F-GFP
IP: Flag
IB: Flag or eEF1A

play a role in regulating the interaction between VHL and eEF1A. We hypothesized that eEF1A in the translationally inactive-GDP bound form may be the variant that is competent to interact with VHL. We therefore sought to create a mutant form of eEF1A that would remain bound to GDP for a greater period of time in comparison to wild-type eEF1A. *In vitro* experiments have shown that because the intrinsic rate of the GTP/GDP exchange is slow in comparison to the measured rate of polypeptide elongation *in vivo*, a guanine-nucleotide exchange factor (which was later identified as the EF1B complex) is required to sustain protein synthesis (Janssen and Moller, 1988; Palmiter, 1975; Pittman et al., 2006; Saha and Chakraburtty, 1986; Slobin and Moller, 1978). Hence, to create a mutant form of eEF1A that would theoretically remain bound to GDP for a greater period of time in comparison to wild-type eEF1A, this mutant form of eEF1A should be incapable of binding to eEF1Bα in order to suppress the regenerative activity of eEF1Bα. Yeast and human eEF1A share an approximately 81% sequence identity and according to work conducted in yeast, amino acids 252-254 (within domain II) of eEF1A is an eEF1Bα binding site (Figure 14A) (Soares et al., 2009). To confirm whether this is indeed an eEF1Bα binding site for the human eEF1A protein, a mutant form of eEF1A was created which substituted the amino acids positioned at 252-254 (DVY) with alanines (AAA). This mutant form of eEF1A, termed eEF1A-^{252}DVY^{254}->AAA, was subjected to immunoprecipitation and immunoblotting. Results demonstrated that unlike wild-type eEF1A, eEF1A-^{252}DVY^{254}->AAA was unable to bind to eEF1Bα (Figure 14 B), confirming previously published data that amino acids 252-254 are indeed eEF1Bα binding sites (Soares et al., 2009). We therefore hypothesized that since eEF1A-^{252}DVY^{254}->AAA is incapable of binding to eEF1Bα (Figure 14B), the ability of eEF1A

Figure 14: The Inability of eEF1A-^{252}DVY^{254}->AAA to Bind to eEF1Bα.

(A) The amino acid sequence of human eEF1A. GTP/GDP, eEF1Bα and tRNA predicted binding sites are marked. Amino acids 252-254 are a key eEF1Bα binding site**.** **(B)** eEF1A-^{252}DVY^{254}->AAA does not bind to eEF1Bα. MCF-7 cells transiently expressing F-eEF1A-GFP, F-eEF1A-^{252}DVY^{254}->AAA-GFP or GFP alone were immunoblotted with anti-Flag beads and immunoblotted with either Flag or eEF1Bα antibodies. Asterisks indicate non-specific bands.

A

Eukaryotic Elongation Factor 1 Alpha (eEF1A):

GTP/GDP
1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL

EF1B
61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV

GTP/GDP
121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKK

181 IGYNPDTVAFVPISGWNGDNMLEPSANMPWFKGWKVTRKDNASGTTLLEALDCILPPTR

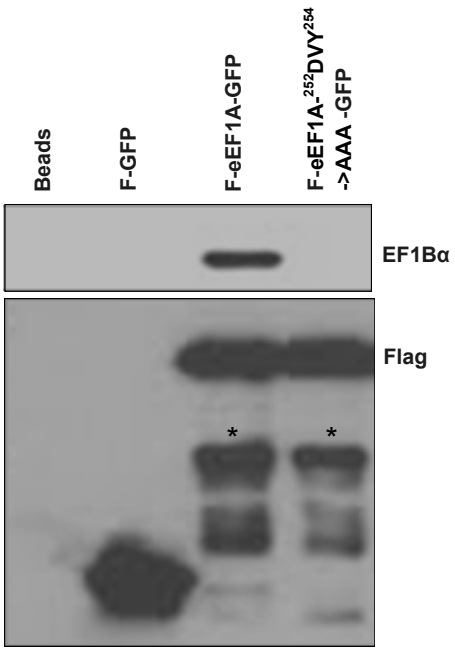
EF1B EF1B
241 PTDKPLRLPLQ**DVY**KIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS

tRNA EF1B/tRNA
301 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPV

361 LDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPP

EF1B
421 LGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK

B



IP: Flag
IB: Flag or EF1Bα

to convert from the GDP-bound state to the GTP-bound configuration is hampered, consequently resulting in a considerable pool of eEF1A in the GDP-bound state.

5.4 Cytosolic eEF1A-^{252}DVY^{254}->AAA Bound to GTP does not Interact with VHL.

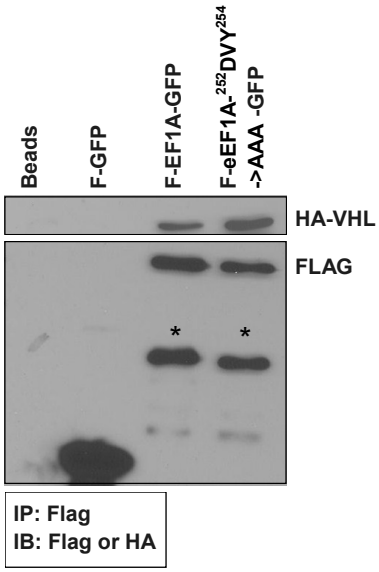
A distinguishing characteristic of the eEF1A-^{252}DVY^{254}->AAA mutant is its inability to bind to eEF1Bα (Figure 14B). The consequence is a mutant form of eEF1A which is predicted to spend a greater period of time bound to GDP at steady-state in comparison to wild-type eEF1A. Immunoprecipitation and immunoblotting of whole cell lysates (WCL) transiently transfected with F-eEF1A-GFP or F-eEF1A-^{252}DVY^{254}->AAA-GFP with HA-VHL depicted that F-eEF1A-^{252}DVY^{254}->AAA-GFP bound more strongly to VHL in comparison to F-eEF1A-GFP (Figure 15A). It is apparent that the inability of eEF1A-^{252}DVY^{254}->AAA to interact with eEF1Bα has altered its properties, consequently resulting in a mutant form of eEF1A with an enhanced affinity for VHL. To further study the enhanced binding capabilities of eEF1A-^{252}DVY^{254}->AAA with VHL, fractionation experiments were performed in the absence or presence of GMP-PNP, a non-hydrolysable analog of GTP. MCF-7 cells transiently co-expressing F-eEF1A-GFP or F-eEF1A-^{252}DVY^{254}->AAA-GFP with HA-VHL were digitonin fractionated and subjected to immunoprecipitation and immunoblotting. As expected, the cytosolic-enriched fraction (digitonin soluble fraction) of wild-type eEF1A was unable to bind to VHL, presumably because the majority of eEF1A is in the active, GTP bound state (Figure 15B). Conversely, the interaction between eEF1A-^{252}DVY^{254}->AAA and VHL occurred in the cytosolic-enriched or digitonin soluble fraction; this is in contrast to wild-type eEF1A where the binding to VHL occurred only in the nuclear-enriched or digitonin insoluble fraction (Figure 15B and 12B). Interestingly, this interaction can be

Figure 15: Digitonin-Soluble Fraction of eEF1A-^{252}DVY^{254}->AAA Binds to VHL and this Interaction is Abolished by GTP.

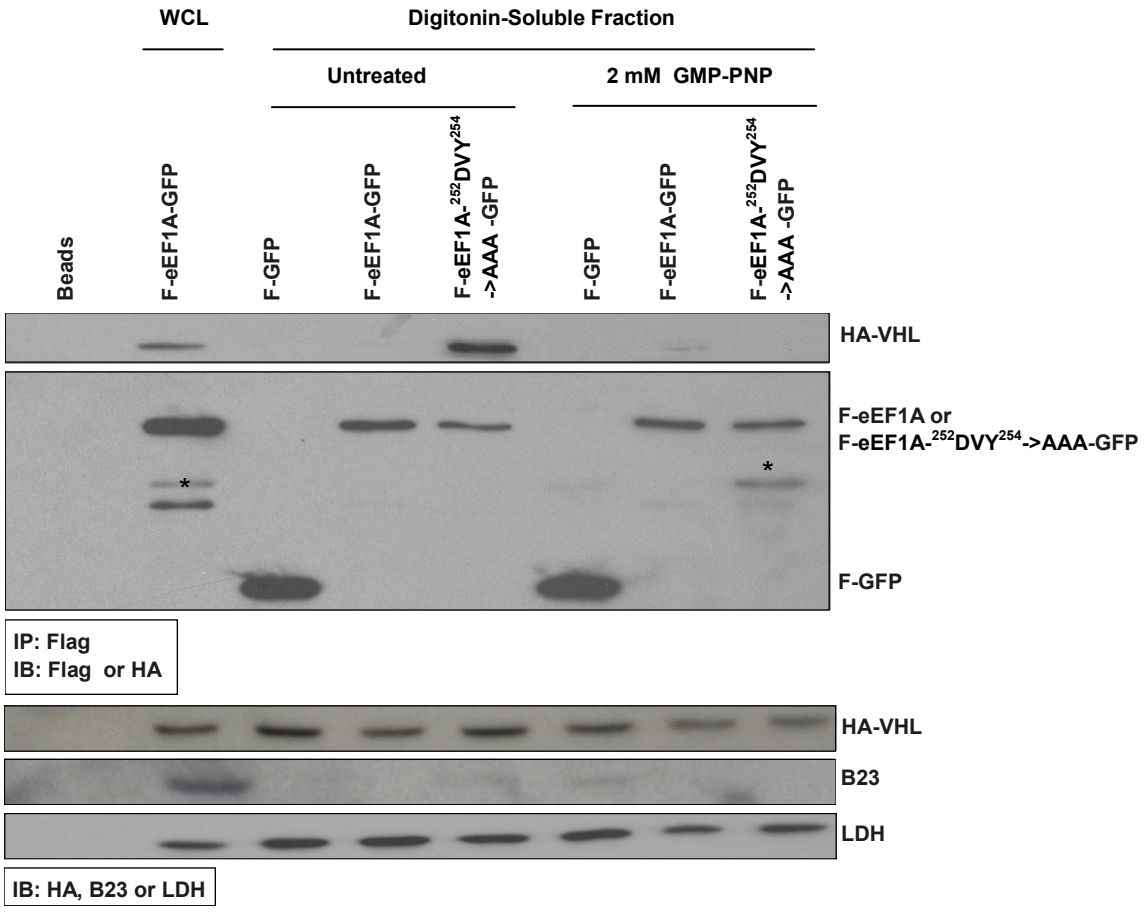
(A) VHL strongly interacts with eEF1A-^{252}DVY^{254}->AAA. MCF-7 cells transiently expressing HA-VHL with either F-GFP, F-eEF1A-GFP or F-eEF1A-^{252}DVY^{254}->AAA-GFP were immunoprecipitated and immunoblotted with anti-Flag beads. Flag or HA antibodies were used for immunoblotting. Asterisks indicate non-specific bands.

(B) Although the digitonin-soluble fraction of eEF1A does not interact with VHL, the cytosolic fraction of eEF1A-^{252}DVY^{254}->AAA binds to VHL. Furthermore, the interaction between VHL and eEF1A does not occur when eEF1A is bound to GTP. MCF-7 cells transiently expressing HA-VHL with either F-GFP, F-eEF1A-GFP or F-eEF1A-^{252}DVY^{254}->AAA-GFP were either left untreated or subjected to 2 mM GMP-PNP treatment before immunoprecipitation. The lysates were immunoprecipitated with anti-Flag beads and immunoblotted with either Flag or HA antibodies. Lysates were immunoblotted with HA to control for the presence of HA-VHL. Immunoblotting with B23 (nuclear marker) and LDH (cytoplasmic marker) served as controls for fractionation. Asterisks indicate non-specific bands.

A


Beads
F-GFP
F-EF1A-GFP
F-eEF1A-252DVY254 ->AAA -GFP
HA-VHL
FLAG
*
*
IP: Flag
IB: Flag or HA

B


WCL
Digitonin-Soluble Fraction
Untreated
2 mM GMP-PNP
Beads
F-eEF1A-GFP
F-GFP
F-eEF1A-GFP
F-eEF1A-252DVY254 ->AAA -GFP
F-GFP
F-eEF1A-GFP
F-eEF1A-252DVY254 ->AAA -GFP
HA-VHL
F-eEF1A or F-eEF1A-252DVY254->AAA-GFP
*
*
F-GFP
IP: Flag
IB: Flag or HA
HA-VHL
B23
LDH
IB: HA, B23 or LDH

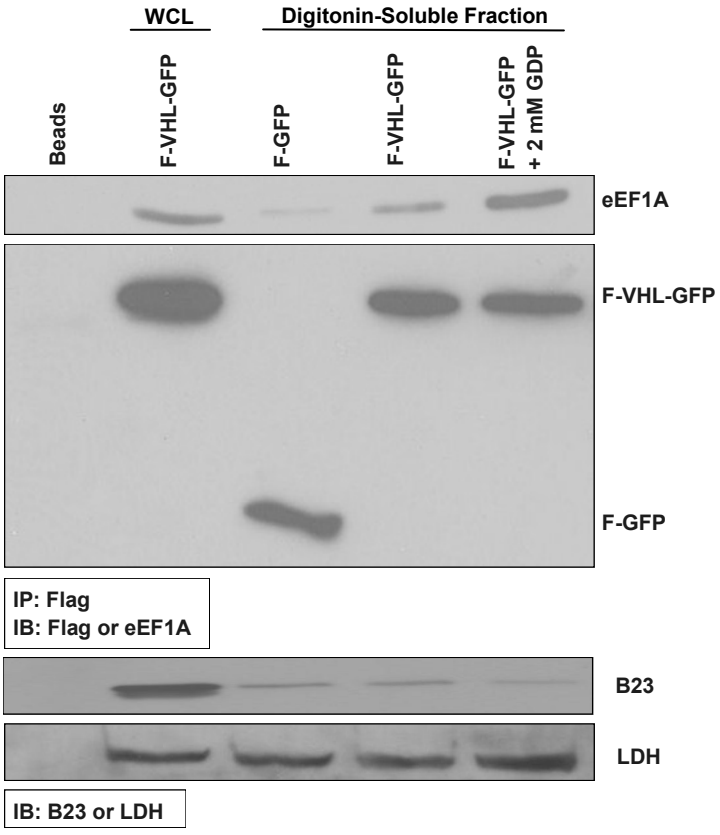
abolished when the lysates were incubated in the presence of GMP-PNP before being subjected to immunoprecipitation and immunoblotting (Figure 15B). By overwhelming the lysates with GMP-PNP, a considerable pool of eEF1A would be converted to eEF1A-GMP-PNP through nucleotide competition and through the utilization of the GTP/GDP exchange cycle. Taken together, these results support the notion that the interaction between eEF1A and VHL is dependent on the nucleotide status of eEF1A; particularly depicting that eEF1A-GTP is incapable of interacting with VHL (Figure 15B).

5.5 eEF1A-GDP Interacts with VHL.

The GTP/GDP cycle of eEF1A not only appears to be critical in protein synthesis but also in eEF1A's ability to mediate transcription-dependent nuclear export. Experiments conducted in this thesis revealed that eEF1A, when bound to GTP and is in its active form, cannot interact and bind to VHL (Figure 15B). This particular point is intriguing because the majority of eEF1A in the cell necessitates being in the active, GTP-bound state; to the point of employing a guanine nucleotide exchange factor (GEF) to accelerate the process of eEF1A-GTP regeneration. This set of observations may provide the explanation as why eEF1A and VHL do not interact in the cytosolic-enriched fraction, despite the abundance of both proteins in the cytoplasmic compartment (Figure 12B). It is plausible that VHL interacts with the less common variant of eEF1A: eEF1A-GDP. To test this hypothesis, digitonin-soluble lysates of MCF-7 transfected with F-VHL-GFP were incubated in the presence of GDP before being subjected to immunoprecipitation and immunoblotting. Evidently, the interaction between cytosolic VHL and endogenous eEF1A was restored in the presence of GDP (Figure 16). By overwhelming the lysates with GDP, a considerable pool of eEF1A would be bound to GDP and have the capability

Figure 16: Addition of GDP Restores the VHL-eEF1A Interaction.

The interaction between eEF1A and VHL was restored when eEF1A was incubated with GDP. Lysates of MCF-7 cells transiently expressing F-GFP or F-VHL-GFP were left untreated or treated with 2 mM GDP. The lysates were subsequently immunoprecipitated with anti-Flag beads and immunoblotted with either Flag or HA antibodies. Immunoblotting with B23 and LDH served as fractionation controls.


WCL
Digitonin-Soluble Fraction
Beads
F-VHL-GFP
F-GFP
F-VHL-GFP
F-VHL-GFP + 2 mM GDP
eEF1A
F-VHL-GFP
F-GFP
IP: Flag
IB: Flag or eEF1A
B23
LDH
IB: B23 or LDH

of interacting with VHL. Taken together, these results put forward the notion that the interaction between VHL and eEF1A can only occur when eEF1A is in the inactive, GDP-bound form (Figure 16).

5.6 eEF1A has the Ability to Access the Nuclear Compartment in Mammalian Cells.

Numerous observable properties of eEF1A-^{252}DVY^{254}->AAA have been altered or modified: 1.) its inability to interact with its GEF, eEF1Bα – likely shifting the steady-state nucleotide status of eEF1A to a more GDP-bound configuration (Figure 14B and 2.) its ability to interact with VHL with high affinity, even in the cytosolic-enriched or digitonin soluble fraction (Figure 15A-B). The next question we wanted to address was whether the predominantly cytoplasmic steady-state localization of eEF1A was altered in the eEF1A-^{252}DVY^{254}->AAA mutant construct. There is evidence in yeast cells that eEF1A can access the nuclear compartment and perhaps in mammalian cells, the ability of eEF1A to enter the nucleus is dependent on its nucleotide-bound state (Murthi et al., 2010). Experiments revealed that F-eEF1A-^{252}DVY^{254}->AAA-GFP depicted a nuclear-cytoplasmic localization, in stark contrast with F-eEF1A-GFP (Figure 17). This result suggests that eEF1A has the ability to access and import into the nuclear compartment. Furthermore, it appears that the nucleotide status of eEF1A not only dictates its ability to interact with VHL, but it also manages and directs the nuclear-cytoplasmic capabilities of eEF1A.

Figure 17: The Localization of eEF1A-^{252}DVY^{254}->AAA is Nuclear-Cytoplasmic.

The localization of eEF1A-^{252}DVY^{254}->AAA is nuclear cytoplasmic compared to the strictly cytoplasmic localization of wild-type eEF1A. MCF-7 cells transiently expressing F-eEF1A-GFP or F-eEF1A-^{252}DVY^{254}->AAA-GFP were observed and monitored for localization using a fluorescence microscope. Insets show Hoechst stained nuclei.

Eukaryotic Elongation Factor 1 Alpha (eEF1A) Amino Acid Sequence:

GTP/GDP

1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL

EF1B

61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV

GTP/GDP

121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKK

181 IGYNPDTVAFVPISGWNGDNMLEPSANMPWFKGWKVTRKDNASGTTLLEALDCILPPTR

EF1B EF1B

241 PTDKPLRLPLQ**DVY**KIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS

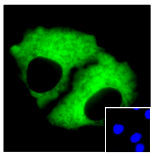
tRNA EF1B/tRNA

301 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPV

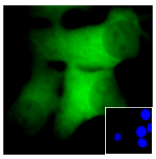
361 LDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPP

EF1B

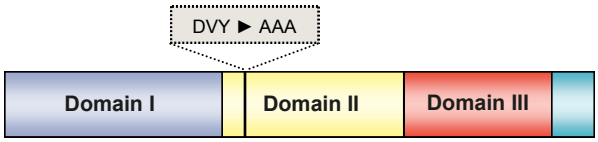
421 LGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK



F-eEF1A-GFP



F-eEF1A-^{252}DVY^{254}->AAA-GFP



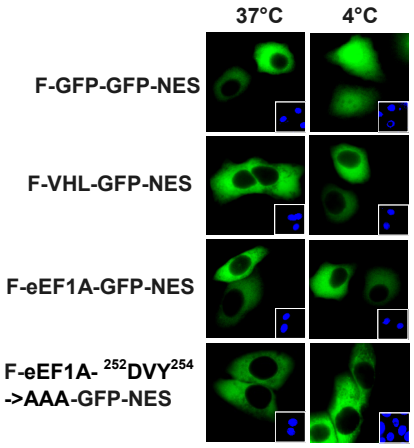
5.7 The Nuclear Import and Export of eEF1A-^{252}DVY^{254}->AAA is Energy Dependent.

The strictly cytoplasmic localization of eEF1A was altered when alanine substitution mutations were introduced (Figure 17). To address whether the detected nuclear signal of F-eEF1A-^{252}DVY^{254}->AAA-GFP was due to protein passive diffusion or if it is through an energy-dependent process, a live cell nuclear import assay was performed as developed in Bonicalzi et. al., 2001. Proteins were tagged with the energy-dependent human immunodeficiency virus REV NES and due to the strong nuclear export properties of the NES, a cytoplasmic steady-state localization was achieved (Figure 18A). Upon the inhibition of the NES function at 4˚C, GFP-GFP-NES accumulated in the nucleus, as expected, because this fusion protein is capable of passive diffusion (Figure 18A) (Bonicalzi et. al., 2001). As previously reported, VHL-GFP-NES remained cytoplasmic when incubated at 4˚C, thereby indicating that the fusion protein is incapable of passive diffusion (Figure 18A) (Bonicalzi et. al., 200; Groulx et. al., 2000). Furthermore, it can be observed that both the full length eEF1A and eEF1A-^{252}DVY^{254}->AAA remained cytoplasmic when incubated at 4˚C (Figure 18A). These results demonstrated that the detected nuclear signal of F-eEF1A-^{252}DVY^{254}->AAA-GFP was not due to protein passive diffusion and that the inhibition of eEF1Bα binding did not alter the properties of eEF1A which resulted in protein passive diffusion. It is also of importance to determine if eEF1A-^{252}DVY^{254}->AAA is capable of engaging in energy-dependent nuclear export. To test this, a strong NLS from the simian virus 40 large T antigen was fused to GFP-GFP, VHL, eEF1A and eEF1A-^{252}DVY^{254}->AAA to achieve a nuclear steady state localization (Figure 18B). The fusion proteins were subjected to cold (4˚C) treatment and as

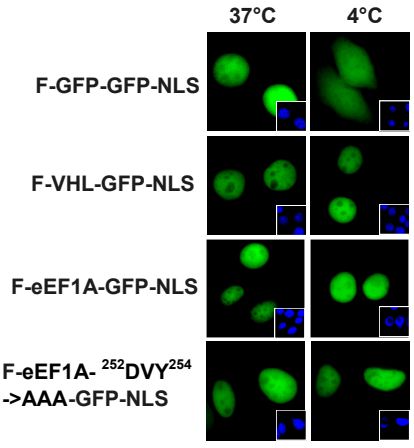
Figure 18: Energy-Dependent Nuclear Import and Export of the eEF1A-^{252}DVY^{254} ->AAA Mutant.

(A) Nuclear import of F-eEF1A-^{252}DVY^{254}->AAA-GFP is energy-dependent. MCF-7 cells transiently expressing F-GFP-GFP-NES, F-VHL-GFP-NES, F-eEF1A-GFP-NES or F-eEF1A-^{252}DVY^{254}->AAA-NES were either untreated or treated at 4˚C for 3 hours. Insets show Hoechst staining. **(B)** Energy-dependent nuclear export of F-eEF1A-^{252}DVY^{254}->AAA-GFP. MCF-7 cells transiently expressing F-GFP-GFP-NLS, F-VHL-GFP-NLS, F-eEF1A-GFP-NLS or F-eEF1A-^{252}DVY^{254}->AAA-NLS were subjected to cold treatment at 4˚C for 3 hours or left untreated. Insets show Hoechst stained nuclei. **(C)** F-eEF1A-^{252}DVY^{254}->AAA-GFP engages in energy-dependent nuclear export. MCF-7 cells transiently expressing F-pk-GFP-NLS, F-VHL-GFP-NLS, F-eEF1A-GFP-NLS or F-eEF1A-^{252}DVY^{254}->AAA-GFP-NLS were subjected to cytoplasmic fluorescence loss in photobleaching (FLIP). A small cytoplasmic region (white squares) was repeatedly bleached and images were taken in between pulses. The kinetics for the loss of nuclear fluorescence was calculated and plotted on a graph. Pseudocolored panels were included to illustrate the loss of fluorescence intensity over time.

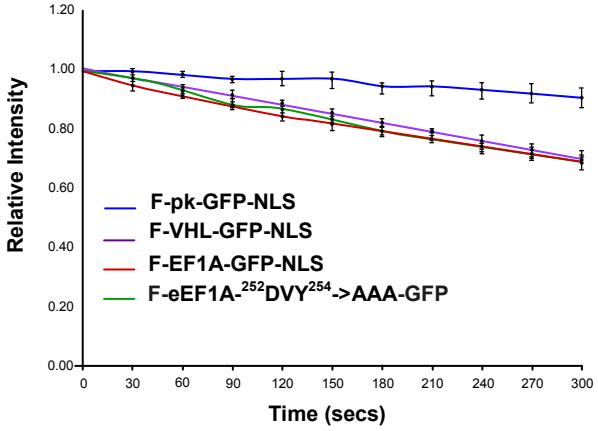
A

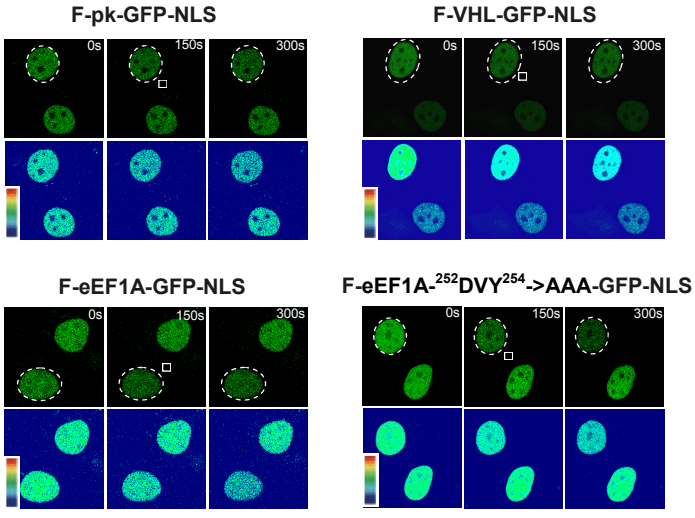

37°C
4°C
F-GFP-GFP-NES
F-VHL-GFP-NES
F-eEF1A-GFP-NES
F-eEF1A- 252DVY254
->AAA-GFP-NES

B


37°C
4°C
F-GFP-GFP-NLS
F-VHL-GFP-NLS
F-eEF1A-GFP-NLS
F-eEF1A- 252DVY254
->AAA-GFP-NLS

C


Relative Intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
150
180
210
240
270
300
Time (secs)
F-pk-GFP-NLS
F-VHL-GFP-NLS
F-EF1A-GFP-NLS
F-eEF1A-252DVY254->AAA-GFP


F-pk-GFP-NLS
F-VHL-GFP-NLS
F-eEF1A-GFP-NLS
F-eEF1A-252DVY254->AAA-GFP-NLS
0s
150s
300s

expected, GFP-GFP-NLS diffused into the cytoplasm (Figure 18B). However, VHL-GFP-NLS, eEF1A-GFP-NLS and eEF1A-^{252}DVY^{254}->AAA-NLS remained nuclear, suggesting that if these fusion proteins were capable of nuclear export, it will do so in a manner that requires energy. To further study the nuclear export capabilities of eEF1A-^{252}DVY^{254}->AAA, fluorescence loss in photobleaching (FLIP) experiments were performed as described in Khacho et. al., 2008a. As previously described and observed in this thesis, pk-GFP-NLS localized to the nucleus and did not undergo significant loss in nuclear fluorescence, illustrating that it is incapable of nuclear export (Figure 18C) (Khacho et. al., 2008a). It can be noted that VHL-GFP-NLS, eEF1A-GFP-NLS and eEF1A-^{252}DVY^{254}->AAA-GFP-NLS lost nuclear fluorescence over time (Figure 18C). Interestingly, the rate of the nuclear export of eEF1A and eEF1A-^{252}DVY^{254}->AAA was comparable, therefore demonstrating that the detected nuclear signal of F-eEF1A-^{252}DVY^{254}->AAA-GFP was not due to a decrease in nuclear export but due to an increase in nuclear import (Figure 18C). Taken together, these results demonstrate that eEF1A-^{252}DVY^{254}->AAA is capable of engaging in energy-dependent nuclear import and export (Figure 18A-C).

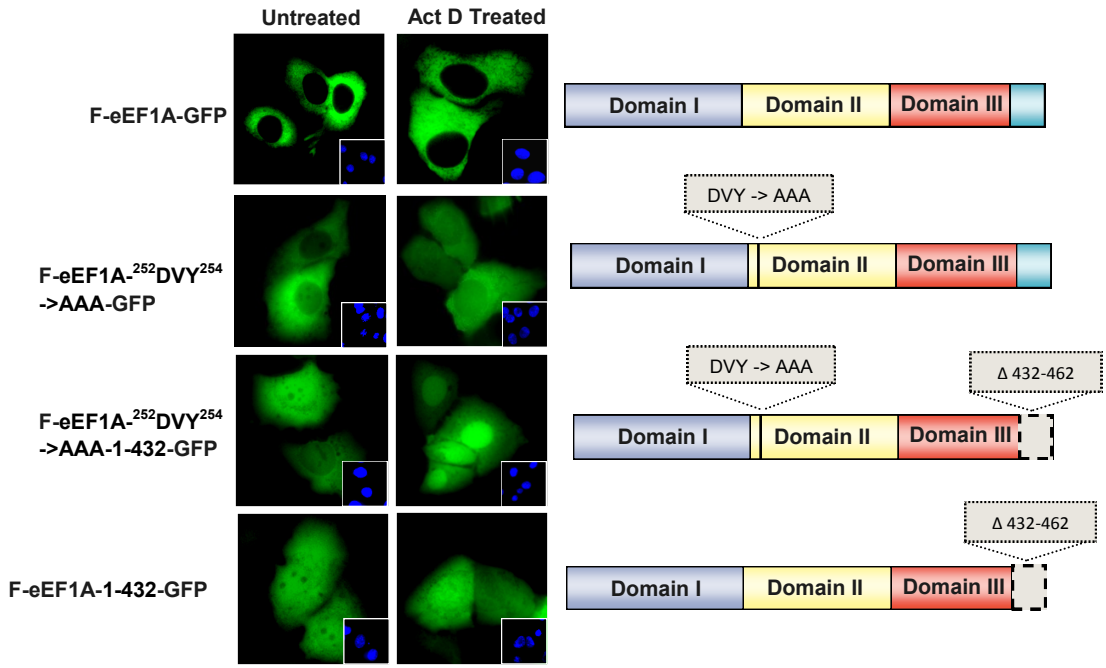
5.8 eEF1A Harbours a Domain that is Sensitive to Ongoing Transcription.

This study suggests that eEF1A has the ability to engage in nuclear-cytoplasmic trafficking (Figure 18A-C). The ability of eEF1A to bind to its substrates and import into the nuclear compartment appears to be dependent, at least in part, on eEF1A being bound to GDP (Figure 15B, 16 and 18A-C). However, the requirement of ongoing transcription for the TD-NEM mediated nuclear export of proteins remains to be further explored. MCF-7 cells transiently transfected with F-eEF1A-GFP and F-eEF1A-^{252}DVY^{254}->AAA-

GFP were treated with Act D, a transcriptional inhibitor, and their respective protein localization were monitored for any changes. Results showed that the inhibition of transcription did not alter the steady state localization of F-eEF1A-GFP and F-eEF1A-^{252}DVY^{254}->AAA GFP (Figure 19). Through mutational analysis, it was discovered that the deletion of the last 30 amino acids from eEF1A-^{252}DVY^{254}->AAA resulted in a nuclear accumulation of the protein when subjected to Act D treatment (Figure 19). Interestingly, this nuclear accumulation only occurred when the last 30 amino acids were removed from eEF1A-^{252}DVY^{254}->AAA and not from wild-type eEF1A (eEF1A 1-432) (Figure 19). These results suggest the possibility that eEF1A may participate in multiple nuclear export pathways; the transcription-dependent pathway may be the pathway utilized by eEF1A to export its substrates.

Figure 19: Variant of eEF1A is Sensitive to Ongoing Transcription.

Inhibition of transcription resulted in a nuclear accumulation of F-eEF1A-^{252}DVY^{254}->AAA-1-432-GFP. MCF-7 cells transiently expressing F-eEF1A-GFP, F-eEF1A-^{252}DVY^{254}->AAA-GFP, F-eEF1A-^{252}DVY^{254}->AAA-1-432-GFP or F-eEF1A-1-432-GFP were left untreated or subjected to 6 hours treatment with 8 μM Act D and monitored using a fluorescence microscope. Schematic diagram demonstrates deletions sites. Insets demonstrate the Hoechst staining of the cells.


Untreated
Act D Treated
F-eEF1A-GFP
F-eEF1A-252DVY254 ->AAA-GFP
F-eEF1A-252DVY254 ->AAA-1-432-GFP
F-eEF1A-1-432-GFP
Domain I
Domain II
Domain III
DVY -> AAA
Δ 432-462

6.0 DISCUSSION

6.1 Summary of Major Findings.

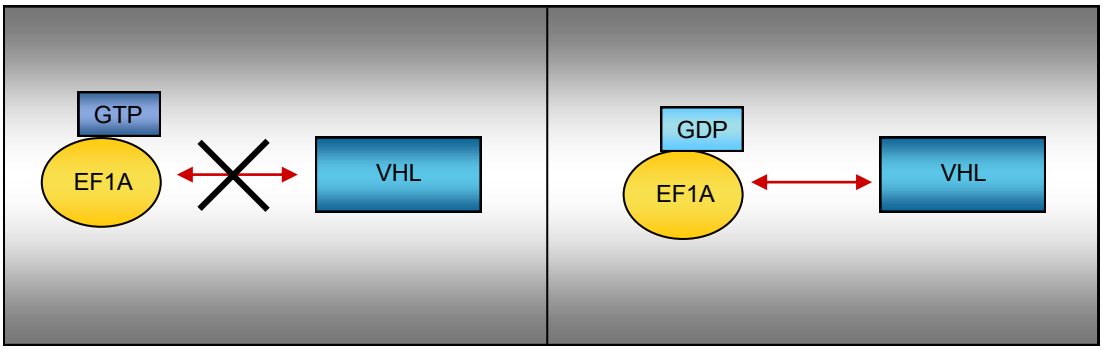
In this thesis, we provide evidence that eEF1A mediates the nuclear export of proteins encoding a TD-NEM by engaging in energy-dependent nuclear-cytoplasmic shuttling in a manner that is dependent on its nucleotide-bound state. Evidence which supports our model includes subcellular fractionation, immunoprecipitation, protein mutational analysis and FLIP experiments; all of which will be discussed in detail later on in this thesis. Taken together, our experimental results suggest that eEF1A in the GDP-bound form can enter the nuclear compartment, bind to TD-NEM encoding proteins and effectively mediate protein nuclear export (Figure 20A-B).

Fractionation experiments revealed that the interaction between VHL and eEF1A appears to be regulated, at least in part, by the eEF1A protein. A point mutant of eEF1A (eEF1A-^{252}DVY^{254}->AAA) uncovered that the nucleotide status of eEF1A is particularly crucial in its ability to interact with VHL. The eEF1A-^{252}DVY^{254}->AAA point mutant was incapable of binding to EF1Bα; theoretically creating a mutant form of eEF1A which would spend a greater amount of time bound to GDP at steady-state. Experiments conducted in this thesis show that eEF1A in its active, GTP-bound configuration does not interact with VHL and that the interaction between eEF1A and VHL appear to require eEF1A to be in the inactive, GDP-bound form (Figure 20A). Due to the strong interaction between eEF1A-^{252}DVY^{254}->AAA and VHL, it is of interest to note that the steady-state localization of eEF1A-^{252}DVY^{254}->AAA was observed to be nuclear-cytoplasmic, in stark contrast to the strictly cytoplasmic localization of wild-type eEF1A, as observed in this study and in other publications. The ability of eEF1A-^{252}DVY^{254}

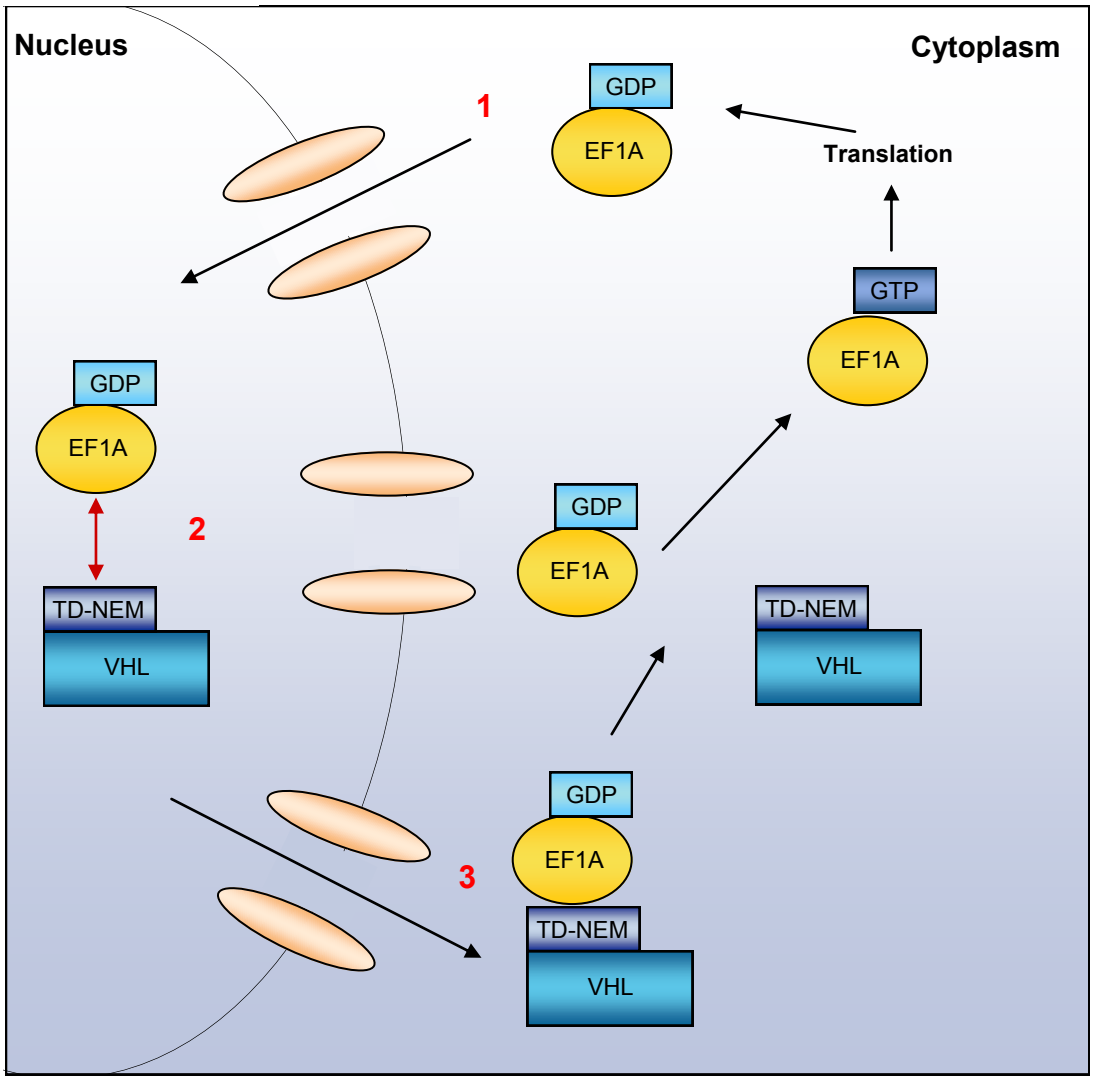
Figure 20: The Mechanism of the Nuclear-Cytoplasmic Shuttling of eEF1A Required for the Transcription-Dependent Nuclear Export of Proteins Containing a TD-NEM.

(A) eEF1A binds to VHL in the inactive, GDP bound form. Experiments suggest that eEF1A in the active, GTP-bound configuration is incompetent to interact with VHL whereas the GDP bound form of eEF1A is a requirement for interaction with VHL. **(B)** Proposed model of the nuclear-cytoplasmic shuttling of eEF1A. eEF1A-GDP enters the nuclear compartment and binds to proteins encoding a TD-NEM. This complex exports out of the nucleus in an energy-dependent manner that is sensitive to ongoing RNA Pol-II transcription. In the cytoplasm, the VHL-eEF1A-GDP complex dissociates and eEF1A is either converted to eEF1A-GTP to function in translation or can remain in the GDP bound form to once again function in mediating the export of proteins encoding a TD-NEM.

A


GTP
EF1A
VHL
GDP
EF1A
VHL

B


Nucleus
Cytoplasm
1
GDP
EF1A
Translation
GTP
EF1A
GDP
EF1A
2
TD-NEM
VHL
GDP
EF1A
TD-NEM
VHL
GDP
EF1A
3
TD-NEM
VHL

->AAA to access the nuclear compartment was determined to be through an energy-dependent process and not merely through protein passive diffusion (Figure 20B). Through FLIP analysis, it was revealed that eEF1A-^{252}DVY^{254}->AAA is capable of engaging in nuclear egression and that a certain variant of eEF1A was particularly sensitive to RNA Pol-II transcriptional inhibitors. Consequently, these results suggest that the GDP-bound population of eEF1A mediates the nuclear export of proteins harbouring a TD-NEM by accessing the nuclear compartment and engaging in nuclear export that is dependent on ongoing transcription (Figure 20B).

6.2 The Interaction between VHL and eEF1A.

The ability of VHL to engage in nuclear export is dependent on the interplay between ongoing transcription and the interaction of eEF1A (Khacho et. al., 2008a, Khacho et. al., 2008b; Lee et. al., 1999). Through mutational analysis, eEF1A was determined to interact specifically with the TD-NEM of VHL and that this interaction can be abolished by RNA Pol-II inhibitors (Khacho et. al., 2008b). To better understand the ability of a cytoplasmic protein in mediating the nuclear export of proteins encoding a TD-NEM, we decided to conduct fractionation experiments using digitonin to decipher where the interaction between VHL and eEF1A occurred. Digitonin is a steroidal compound that selectively permeabilizes cholesterol-rich membranes, resulting in the release of cytosolic soluble components and leaving behind the nuclear insoluble components (Fiskum et al., 1980; Mackall et al., 1979; Scallen and Dietert, 1969; Weigel et al., 1983; Zuurendonk and Tager, 1974). Fractionation utilizing digitonin was chosen instead of a mechanical method of fractionation due to the purity of the fractionation and the reproducibility of the results. Despite the abundance of eEF1A and VHL in the cytoplasmic compartment,

fractionation experiments revealed that eEF1A interacts with VHL only in the nuclear insoluble fraction. To control for the possibility that digitonin interferes with the interaction between cytosolic VHL and eEF1A, the digitonin insoluble fraction was lysed in the presence of digitonin (data not shown). Results showed that eEF1A and VHL were still capable of interacting, thereby eliminating the possibility that digitonin affects the interaction between VHL and eEF1A (data not shown). These results suggest that the interaction between VHL and eEF1A occurs within the nuclear compartment.

6.3 The Interaction Between VHL and eEF1A is Dependent on eEF1A.

The interaction between eEF1A and VHL appear to occur within the nuclear compartment; a surprising result considering the abundance of eEF1A and VHL in the cytoplasmic compartment and the miniscule amount of eEF1A detected within the nuclear compartment through immunloblotting. There are several possible interpretations for this result: 1.) VHL in the nuclear insoluble fraction contains some form of modification that renders it competent to interact with eEF1A or 2.) some variant of eEF1A is required for interaction with VHL. To address these possibilities, lysates of F-VHL-GFP were incubated with additional lysates of either the cytosolic soluble fraction or the nuclear insoluble fraction. We have uncovered that the modifier appears to be within eEF1A due to the ability of the cytosolic soluble fraction of VHL to bind to eEF1A only when incubated with additional nuclear insoluble fraction of eEF1A. These results show that cytosolic and nuclear VHL are both competent to bind to eEF1A, however the requirement for interaction appears to be regulated by the eEF1A protein.

6.4 Nucleotide-Bound State of eEF1A.

As a member of the translation factor family of GTPases, eEF1A is a known molecular switch and can either be in the active, GTP-bound configuration or in the GDP-inactive bound state. The ability of eEF1A to cycle between the GTP and GDP bound state has been particularly important in its canonical role in translation. In fact, eEF1A in the cytoplasmic compartment is constantly being regenerated to the GTP bound state by the alpha subunit of the GEF, EF1B (Perez et al., 1998; van Damme et al., 1990; Gromadski et al., 2007). Studies have demonstrated that eEF1Bα has the ability to accelerate GDP dissociation by up to 700-fold (Pittman et al., 2006). We hypothesized that perhaps the nucleotide status of eEF1A may be essential in its ability to interact with VHL. Interestingly, lysates of F-VHL-GFP incubated in the presence of GMP-PNP, a non-hydrolysable analog of GTP, resulted in a decrease of eEF1A binding to VHL. These observations suggest that eEF1A-GTP is incapable or has a reduced affinity for VHL, a potential reason as to why eEF1A and VHL does not bind in the cytosolic soluble fraction. Perhaps, within the nuclear insoluble fraction, there is a pool of eEF1A in the GDP bound state that is competent to bind to VHL.

To further test the premise that the nucleotide-bound state of eEF1A may be a determining factor in its ability to interact with VHL, we decided to create a mutant form of eEF1A which would theoretically remain in the GDP-bound configuration for a greater period of time at steady-state compared to wild-type eEF1A. To accomplish this, we created a mutant form of eEF1A that is unable to bind eEF1Bα, through point-directed mutagenesis of a key eEF1Bα binding site, as indicated in yeast models which shares an 81% sequence identity with human eEF1A (Soares et al., 2009). Although there are

several eEF1Bα binding sites located on eEF1A, it appears that the disruption of one key eEF1Bα binding site is sufficient in preventing the binding of eEF1Bα. This point mutant, referred to as eEF1A-^{252}DVY^{254}->AAA, should remain bound to GDP for a greater period of time compared to wild-type eEF1A, due to the inability of eEF1Bα in catalyzing the release of GDP. However, in this study, we cannot formally exclude the possible role of eEF1A monophosphate (GMP) or nucleotide-free eEF1A, though it appears that eEF1A and other G-proteins are unstable when unbound to nucleotide(s) and exchange factors, prompting the question as to whether nucleotide-free eEF1A is still functional within the cell (Ferguson et al., 1986; Lapadat and Spremulli, 1989; Lutz et al., 2002). Furthermore, the predicted ability of eEF1A-^{252}DVY^{254}->AAA to remain bound to GDP for a greater period of time remains to be validated. Thus, experiments using radioactivity (orthophosphate) and thin layer chromatrography would specifically address the nucleotide status of the eEF1A-^{252}DVY^{254}->AAA mutant. Nevertheless, it appears that the nucleotide cycle of eEF1A may not only be important in its role in translation, but could also be the basis in the transcription-dependent nuclear export machinery.

6.5 The Interaction Between VHL and eEF1A is Dependent on the Nucleotide Status of eEF1A.

Fractionation experiments utilizing eEF1A-^{252}DVY^{254}->AAA proved necessary in further deciphering the nucleotide status of eEF1A required for its interaction with VHL. As expected, wild-type eEF1A in the cytosolic soluble fraction was unable to bind to VHL; presumably due to the predominantly GTP-bound population of eEF1A in the cytoplasmic compartment. EF1Bα facilitates the nucleotide exchange of eEF1A and due to the higher intracellular concentration of GTP compared to GDP, it is

thermodynamically favoured for eEF1A to re-bind to GTP (Andersen et al., 2001; Gromadski et al., 2007). Furthermore, due to the high cellular concentration of aa-tRNA, studies have demonstrated that the equilibrium furthermore shifts towards the active, GTP-bound state due to the formation of EF-Tu (prokaryotic homolog of eEF1A) -GTP:aa-tRNA (Gromadski et al., 2007). Immunoprecipitation of whole cell lysates (WCL) of eEF1A and eEF1A-^{252}DVY^{254}->AAA showed that eEF1A-^{252}DVY^{254}->AAA has an increased affinity for VHL in comparison to wild-type eEF1A. Interestingly, the cytosolic soluble fraction of eEF1A-^{252}DVY^{254}->AAA was capable of interacting with VHL. We suggest that this interaction occurs due to the presumed ability of eEF1A-^{252}DVY^{254}->AAA to remain bound to GDP for a greater period of time compared to wild-type eEF1A. In the presence of GMP-PNP, the interaction between eEF1A-^{252}DVY^{254} ->AAA and VHL in the cytosolic soluble fraction was abolished, further demonstrating that eEF1A bound to GTP is incapable of interacting with VHL. We then wanted to test whether an interaction between the cytosolic soluble fraction of eEF1A and VHL could be restored in the presence of GDP. Results indicated that the increased presence of GDP in the cytosolic soluble lysates facilitated the interaction between eEF1A and VHL, thereby implying that the interaction between VHL and eEF1A can only occur when eEF1A is bound to GDP. Taken together, these results depict that the population of eEF1A competent to bind to VHL is the inactive, GDP-bound form of eEF1A.

6.6 eEF1A May Shuttle Between the Nucleus and Cytoplasm.

The ability of eEF1A to mediate the nuclear export of proteins and RNA species in yeast has been an intriguing observation due to its apparently exclusive cytoplasmic localization at steady state. In yeast, studies have demonstrated that the nuclear export of

tRNA occurs by two pathways: 1.) the exportin Los1p pathway and 2.) the aminoacylation and eEF1A-dependent pathway (Grosshans et al., 2000). Notably, a recent study showed that endogenous eEF1A-GFP can be detected by confocal microscopy in the nuclear compartment of yeast cells (Murthi et. al., 2010). In mammalian cells, studies thus far have failed to demonstrate eEF1A, both endogenously and GFP-tagged, within the nucleus (Bohnsack et al., 2002; Calado et al., 2002). Experiments revealed that Exp-5 mediates the nuclear export of eEF1A and tRNA; however, due to the inability to detect eEF1A within the nucleus, it has been proposed that Exp-5 expels the nucleus of unwanted eEF1A that may have entered the nuclear compartment during cell division (Calado et al., 2002).

Despite the abundance of both VHL and eEF1A in the cytoplasm, subcellular fractionation experiments conducted in this thesis suggests that the interaction between VHL and eEF1A may occur within the nucleus. Furthermore, experiments revealed that the interaction between VHL and eEF1A requires eEF1A to be bound to GDP. We therefore hypothesized that perhaps eEF1A can access the nuclear compartment in a manner that is regulated, at least in part, by its nucleotide-bound status. Furthermore, it was observed that whole cell lysates of eEF1A-^{252}DVY^{254}->AAA bound much more strongly to VHL compared to wild-type eEF1A. Due to eEF1A-^{252}DVY^{254}->AAA's strong affinity for VHL, we predicted that the steady-state localization of eEF1A-^{252}DVY^{254}->AAA may be altered. In fact, eEF1A-^{252}DVY^{254}->AAA transiently transfected into MCF-7 cells displayed a nuclear-cytoplasmic localization. This set of observations suggests that although wild-type eEF1A at steady-state has only been detected in the cytoplasmic compartment, it has the ability to enter the nucleus,

presumably in the GDP-bound form (Figure 20). Furthermore, the nucleotide status of eEF1A not only regulates its ability to bind to substrates but also appears to regulate localization. The nucleotide exchange mechanism of eEF1A may be utilized by cells to decipher whether eEF1A has the right to access the nucleus or whether it should remain in the cytosol to participate in protein synthesis. In this thesis, we cannot formally exclude the possible role of the EF1B complex in maintaining the predominantly cytoplasmic localization of eEF1A. Furthermore, the percentage of eEF1A cycling between the nuclear and cytoplasmic compartments remains to be quantified. Moreover, eEF1A does not contain a classical NLS and has not been reported to contain a non-classical NLS, which prompts further questions as to how eEF1A can be imported in the nucleus, as well as the precise mechanism of translocation through the NPC.

6.7 Energy-Dependent Nuclear-Cytoplasmic Shuttling.

Numerous properties of eEF1A-^{252}DVY^{254}->AAA have been identified, such as its nuclear-cytoplasmic steady-state localization, its inability to bind to eEF1Bα and its strong capability of interacting with VHL. The detected nuclear signal of eEF1A-^{252}DVY^{254}->AAA demonstrates that eEF1A harbours the ability to enter the nuclear compartment. Based on the molecular weight of eEF1A, which is approximately 50 kDA, it is highly unlikely that eEF1A diffuses across the nuclear pore complex; nevertheless, because multiple properties of eEF1A-^{252}DVY^{254}->AAA have been distorted, its mode of action in entering the nuclear compartment remains unknown. Utilizing the strong HIV Rev NES and the live cell nuclear import assay developed in Bonicalzi et. al., 2001, the nuclear import of eEF1A-^{252}DVY^{254}->AAA was confirmed to be through an energy-dependent process. It is also of importance to determine if the detected nuclear signal of

eEF1A-^{252}DVY^{254}->AAA is an interplay between active import and nuclear export. Utilizing FLIP and the simian virus large T antigen SV40 NLS/live cell nuclear export assay, results demonstrated that eEF1A-^{252}DVY^{254}->AAA has the ability to engage in nuclear export in an energy-dependent manner. Interestingly, the observed rate of the nuclear export of wild-type eEF1A and eEF1A-^{252}DVY^{254}->AAA were similar, suggesting that the observed nuclear signal of eEF1A-^{252}DVY^{254}->AAA was not due to an inability or decreased ability to engage in nuclear export, but that the nuclear import properties of eEF1A-^{252}DVY^{254}->AAA have been altered, resulting in an increased import rate. Taken together, these results demonstrate that eEF1A has the ability to engage in nuclear-cytoplasmic shuttling and that the nucleotide status, at least in part, may regulate the cycling process (Figure 20).

6.8 eEF1A Shuttling is Sensitive to Ongoing Transcription.

The nuclear export of VHL and other proteins encoding a TD-NEM, such as PABP1 and Cyclin C, require ongoing transcription (Groulx and Lee, 2002; Khacho et al., 2008a; Lee et al., 1999). The inhibition of transcription resulted in a decreased export rate of these proteins, as was measured through FLIP analysis (Khacho et al., 2008a). Based on the requirement of ongoing transcription for nuclear export, we initially hypothesized the involvement of RNA. However, despite utilizing multiple techniques (urea and denaturing agarose gels, as well as radioactivity), we were unable to detect RNA within the VHL-eEF1A complex (date not shown). Despite remaining undetected with the current methods utilized, the presence of RNA is still plausible and the advent of future technologies and techniques may enhance RNA detection. Temporarily ruling out the involvement of RNA, the requirement of ongoing transcription required for nuclear

export remained to be addressed and further explored. Based on the observation that eEF1A-^{252}DVY^{254}->AAA can engage in nuclear-cytoplasmic shuttling, we hypothesized that perhaps the sensitivity to transcriptional inhibitors, such as Act D, may be embedded within eEF1A itself. Interestingly, eEF1A and eEF1A-^{252}DVY^{254}->AAA were insensitive to Act D treatments and the localization of these proteins were not altered. However, through mutational analysis of wild-type eEF1A, it was determined that eEF1A is sensitive to Act D treatment only when the last 30 amino acids of eEF1A were removed. Furthermore, this sensitivity to Act D was only manifested when the last 30 amino acids were removed from eEF1A-^{252}DVY^{254}->AAA and not from wild-type eEF1A. These results suggest that eEF1A may export out of the nucleus utilizing multiple pathways. It has already been reported that eEF1A utilizes the Exp-5 pathway to export out of the nucleus through its interaction with tRNA (Bohnsack et al., 2002; Calado et al., 2002). It is therefore possible that the last 30 amino acids of eEF1A could be involved in the Exp-5 pathway and the removal of these amino acids has revealed the alternative export pathway of eEF1A, one which requires ongoing transcription. However, at this time, the possibility of a third or multiple other nuclear export pathways cannot be excluded. It is interesting that the nuclear accumulation and sensitivity to Act D treatment was only observed with eEF1A-^{252}DVY^{254}->AAA-1-432 and not with wild-type eEF1A-1-432. Previous work has shown that Exp-5 exports eEF1A out of the nuclear compartment and this mechanism was proposed as a method of eliminating eEF1A from the nucleus (Calado et. al., 2002). However, our group has previously demonstrated that silencing Exp-5 had no effect on the nuclear export rate of VHL, consequently revealing that the nuclear export of eEF1A's substrates is independent of the Exp-5 pathway (Khacho et al.,

2008b). Therefore, eEF1A may engage in nuclear export utilizing Exp-5, but does not mediate the export of proteins encoding a TD-NEM. On the other hand, transcription-dependent nuclear export of eEF1A may be the pathway utilized by eEF1A to export TD-NEM containing proteins. These findings are significant in illustrating the ability of eEF1A in utilizing multiple export pathways and effectively discriminating which particular pathway can be exploited for the export of proteins encoding a TD-NEM.

6.9 Future Directions

6.9.1 Deciphering the Nucleotide Status of eEF1A-^{252}DVY^{254}->AAA.

The involvement of an abundant cytoplasmic protein in mediating nuclear export of proteins has been a fascinating and baffling query. In this thesis, we have uncovered the ability of eEF1A to access the nuclear compartment and to engage in nuclear-cytoplasmic trafficking. It appears that the ability of eEF1A to enter the nucleus may be dependent on its nucleotide status. We hypothesize that due to the inability of eEF1A-^{252}DVY^{254} ->AAA to interact with eEF1Bα, this eEF1A mutant will remain in the GDP-bound state for a greater period of time compared to its wild-type counterpart. However, the nucleotide status of eEF1A-^{252}DVY^{254}->AAA remains to be confirmed through the use of radioactivity and thin layer chromatography. As well, the rate of GTP/GDP exchange for eEF1A-^{252}DVY^{254}->AAA remains to be quantified and compared to wild-type eEF1A.

6.9.2 eEF1A – An Ancient Protein.

The eEF1A protein is remarkably conserved throughout evolution. Numerous other proteins may have changed, adapted or even emerged in response to the ever changing condition of the cell, yet eEF1A remained relatively unaltered. With this in mind, the proteins comprising the NPC evolved long after the eEF1A protein. Work conducted in

this thesis suggests that eEF1A is a nuclear-cytoplasmic shuttling protein and preliminary experiments have failed to show associate proteins within the VHL-eEF1A complex. Due to the conserved nature of eEF1A, it is plausible that the nucleoporins have developed to accommodate eEF1A. Therefore, eEF1A may have the necessary qualities required to enter the NPC without the aid of transport proteins commonly required for the nuclear import and export of other proteins. Thus, experiments comparing the structure of eEF1A with other transport proteins, such as proteins from members of the karyopherin β family, may shed light on eEF1A's ability to navigate through the meshwork of nucleoporins located in the NPC.

7.0 CONCLUSION

The goal of this thesis was to understand eEF1A's role in the transcription-dependent nuclear export of proteins encoding a TD-NEM. We put forward the model that eEF1A is a nuclear-cytoplasmic shuttling protein and its ability to access the nuclear compartment is dependent on its nucleotide status. Furthermore, I have presented evidence that the inactive, GDP-bound form of eEF1A binds to VHL in the nuclear compartment and mediates protein nuclear export in a manner that requires ongoing transcription. Consequently, additional factors may be involved within the TD-NEM mediated nuclear export machinery that remains to be uncovered and furthermore explored.

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