Nuclear import of baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)

by

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Abstract

Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the archetype of the *Baculoviridae* family, is an enveloped, rod-shaped, double-stranded DNA virus that replicates in the nucleus of its host cells. Baculoviruses have been used extensively as pesticides and in biological systems. Despite their importance, the mechanism by which baculovirus deliver its genome into the nucleus has been the subject of considerable debate. Molecules <39 nm in diameter enter the nucleus through nuclear pore complexes (NPCs) embedded within the nuclear envelope. Because the diameter of AcMNPV capsids (30 x 300 nm) falls below this limit, we hypothesize that AcMNPV capsids enter the nucleus via NPCs. In this thesis, we aim to visualize the mechanism of nuclear import used by the baculovirus AcMNPV capsid, to understand the role of cellular proteins facilitating viral capsid delivery into the nucleus, and to demonstrate the role of cellular actin in mediating nuclear import of the baculovirus capsid.

We found for the first time that an intact AcMNPV capsid is able to traverse the NPC, importing the entire capsid into the nucleoplasm. This transport occurs through the NPC central channel, which is able to open up completely to accommodate the AcMNPV capsid. Nuclear transport of these capsids was inhibited by physical blockade of NPCs, as well as low temperature conditions, which inhibits facilitated transport and dynamic structural changes of the NPC. We also showed that nuclear import of AcMNPV capsids occurred independently of importin-β and the Ran cycle, but required activation of actin nucleation. Finally, we demonstrated through targeted knockdown that Nup62 and Nup153 are not essential in mediating nuclear import of AcMNPV capsids. Conversely, these depletion studies showed that Nup358 enhances the nuclear import efficiency of AcMNPV capsids.

Our results support a model in which the intact baculovirus capsid, after being released into the cytoplasm during infection, enters the nucleus through the NPC, by a mechanism that does not require importin-β, Ran, and the three FG-Nups tested, but is dependent on actin nucleation. Because baculovirus capsids are among the largest cargos that translocation through the NPC, our results provide exciting new insights into how the NPC functions.

Preface

Sections of Chapter 1 have been published in peer-reviewed journals:

- **Au, S.**, Wu, W., and Panté, N. 2013. Baculovirus Nuclear Import: Open, Nuclear Pore Complex (NPC) Sesame. *Viruses*. 5(7): 1885-1900.
- **Au, S.**, and Panté, N. 2012. Nuclear transport of baculovirus: Revealing the nuclear pore complex passage. *Journal of Structural Biology.* 177(1): 90-98.
- Cohen, S.*, **Au, S.***, and Panté, N. 2010 How viruses access the nucleus. *BBA – Molecular Cell Research*. 1813(9):1634-1645. (* both authors contributed equally towards this publication).

Sections of Chapter 2 have been published in a peer-reviewed journal:

- **Au, S.***, Cohen, S.*, and Panté, N. 2010 Microinjection of *Xenopus laevis* oocytes as a system for studying nuclear transport of viruses. *Methods*, 51:114-120. (* both authors contributed equally towards this publication).

Sections of Chapter 3 have been published in a peer-reviewed journal:

- **Au, S.**, and Panté, N. 2012. Nuclear transport of baculovirus: Revealing the nuclear pore complex passage. *Journal of Structural Biology.* 177(1): 90-98.

Dr. Sarah Cohen and I contributed equally to the preparation of the two review articles indicated above where we are both co-authors. These were then revised together with Dr. Nelly Panté.

The 2013 review by Au, S., Wu, W., and Panté, N. was written with the assistance of Wei Wu who also created Figures 1-5, 1-6 and 1-7 in this thesis. Revisions were done by Dr. Nelly Panté.

I wrote the first drafts of the manuscript presented in Chapter 3, which was further revised by Dr. Nelly Panté. Chapters 4 and 5 contain work that I plan to submit in the near future. With the guidance of my supervisor Dr. Nelly Panté, I designed and performed all experiments, quantified and analyzed all data, presented in this thesis. The data presented is most up-to-date at the time of thesis completion.

The research presented in this thesis was approved by the UBC Animal Care Committee (Certificate A11-0321) and the UBC Bio-Safety Committee (Certificate B10-0057).

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List of Abbreviations

Amino acids and its corresponding 1-letter code:

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate/Aspartic Acid
E	Glu	Glutamate/Glutamic Acid
G	Gly	Glycine
K	Lys	Lysine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
T	Thr	Threonine
V	Val	Valine
Y	Tyr	Tyrosine

Other abbreviations:

ARM: armadillo repeats

˚C: degree Celsius

AcMNPV: *Autographa californica* multiple nucleopolyhedrovirus

BSA: bovine serum albumin

BV: budded virion

CAS: cellular apoptosis susceptibility

cNLS: classical nuclear localization sequence

CO_2: carbon dioxide

DMEM: Dulbecco's modified Eagle medium

DNA: deoxyribonucleic acid

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

EM: electron microscopy
ER: endoplasmic reticulum
ESCRT: endosomal sorting complex required for transport
FBS: fetal bovine serum
FG: phenylalanine glycine
FG-Nups: nucleoporins containing phenylalanine glycine repeats
GTP: guanine triphosphate
GV: granulovirus
HBV: hepatitis B virus
HEAT: helical-repeat motifs
HIV-1: human immunodeficiency virus-1
hnRNP: heterogeneous ribonucleoprotein particle
HSV-1: herpes simplex virus-1
INM: inner nuclear membrane
kbp: kilobase pairs
kDa: kilodalton
LSB: low-salt buffer
MBS: modified Barth's saline
MDa: megadalton
MLV: murine leukemia virus
MOI: multiplicity of infection
mRNA: messenger ribonucleic acid
NE: nuclear envelope
NLS: nuclear localization sequence
NP: nucleoprotein
NPC: nuclear pore complex
NPV: nucleopolyhedrovirus
NTC: nuclear transport complex
NTF2: nuclear transport factor 2
Nups: nucleoporins
OB: occlusion body
ODV: occlusion-derived virion
ONM: outer nuclear membrane

PBS: phosphate-buffered saline
PFA: paraformaldehyde
pH: potential hydrogen
PIC: pre-integration complex
POMs: pore membrane domains
RanBP1: Ran binding protein 1
RanBP2: Ran binding protein 2
RanGDP: Ran guanine diphosphate
RanGEF: Ran guanine exchange factor
RanGTP: Ran guanine triphosphate
RCC1: regulator of chromosome condensation 1
RNA: ribonucleic acid
RNPs: ribonucleoproteins
RRL: rabbit reticulocyte lysate
RT: room temperature
SDS: sodium dodecyl sulfate
Sf: *Spodoptera frugiperda*
SR: serine/arginine
SUMO: small ubiquitin-like modifier
SV40: simian virus 40
TB: transport buffer
TE: Tris-EDTA buffer
T.ni: *Trichoplusia ni*
TEM: transmission electron microscopy
TGF-β: transforming growth factor beta
tRNA: transfer ribonucleic acid
U snRNP: uridine-rich small nuclear ribonucleoprotein
vRNP: viral ribonucleoprotein
VSV: vesicular stomatitis virus
WGA: wheat germ agglutinin

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Thank you for teaching me since I was young to work hard for what you want. Nothing in life worth having comes easy.

I love you both.

Chapter 1

Introduction

Viruses are obligate intracellular pathogens that rely on the host cells that they infect to reproduce. Many viruses, in particular those with a DNA genome must enter the cell nucleus for viral replication. The host cells' nucleus contains cellular machineries necessary for viral DNA replication, transcription, and RNA processing. DNA viruses have developed strategies not only to enter the cytosol, but their genome must make its way into the nucleus to commence viral replication. My thesis will explore strategies the prototypical baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) developed for nuclear import of its viral capsid. This introduction will begin with background information about the structural and functional importance of cellular nucleocytoplasmic transport through nuclear pore complexes (NPCs), followed by a description of various mechanisms that have been exploited by viruses for nuclear entry. Finally, I will briefly introduce baculoviruses and will review the literature about baculovirus nuclear import, which has created some controversies about the mode of nuclear import used by baculoviruses.

1.1 Viral host interactions[1]

In order to establish a productive infection, viruses must overcome multiple barriers within the host cell. These barriers include the plasma membrane and the underlying cell cortex, an extremely dense cytoplasm through which molecular traffic is highly restricted, and any other

[1] A version of part of this section has been published: Cohen, S.*, Au, S.*, and Panté, N. 2011. How viruses access the nucleus. *Biochim Biophys Acta.* 1813(9): 1634-1645.

membranes that must be crossed in order to access the sites of viral replication or assembly. How different viruses accomplish these feats depends to a large degree on the size and structure of the virus. Viruses consist of an RNA or DNA genome surrounded by either multiple copies of capsid proteins (non-enveloped viruses) or both capsid proteins and a lipid membrane (enveloped viruses). The size of animal viruses ranges from approximately 25 nm to over 300 nm.

Viruses first attach to the host cell through interactions between viral membrane proteins (enveloped viruses) or three-dimensional structures on the capsid (non-enveloped viruses) and cell surface receptors; viruses are then internalized either by direct fusion of the viral envelope with the plasma membrane, or via one of the cell's many endocytic pathways (reviewed in Grove and Marsh, 2011; Marsh and Helenius, 2006; Mercer et al., 2010; Smith and Helenius, 2004). If entry is by endocytosis, then the virus escapes from the endocytic compartment to the cytosol. The escape strategy depends on the type of virus. For enveloped viruses, this involves fusion of the viral envelope with endosomal membranes. For non-enveloped viruses the endosomal escape process is less well understood, but can involve lysis of the endosomal membrane employing lytic peptides (reviewed in Smith and Helenius, 2004). The released viral capsid or nucleoprotein complex then traverses the cytoplasm, often by associating with cellular motor proteins which traffic along various cytoskeleton components (reviewed in Greber and Way, 2006; Radtke et al., 2006). Upon reaching the cellular compartment where viral replication occurs, the viral genome is released, often simultaneously with capsid disassembly. After using the cellular machinery for genome synthesis and production of new viral proteins, progeny virions are assembled, and then released from the cell. Release is usually through budding at the plasma membrane or into the endoplasmic reticulum (ER) followed by exocytosis for enveloped viruses; for non-enveloped

viruses, it is generally thought that virions are released during cell lysis, although some viruses may also be released by exocytosis (reviewed in Marsh and Helenius, 2006).

Many viruses, including most DNA viruses and some RNA viruses, depend on nuclear proteins for replication; therefore, their viral genome must enter the nucleus of the host cell (reviewed in Greber and Fornerod, 2005; Whittaker et al., 2000). Although there are numerous benefits, entry into the nucleus also poses a serious challenge for these viruses, since the nuclear envelope (NE) acts as a barrier between the cytoplasm and the nucleus, and transport of molecules into and out of the nucleus is tightly regulated.

1.2 Nucleocytoplasmic transport

Communication between the cytoplasm and the nucleus is mediated by NPCs embedded within the NE. The NE consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM) separated by the perinuclear space, a regular gap of about 30-50 nm (Figure 1-1). The nucleus, being a membrane-enclosed organelle, contributes significantly to the regulation of numerous cellular processes. For example, by controlling access of certain macromolecules to the nucleus, nuclear transport can regulate transcription, DNA replication, and the cell cycle. NPCs regulate the flow of proteins, RNAs, and RNA-protein complexes (such as ribosomal subunits, messenger ribonucleoproteins (RNPs) and splicesomal RNPs) into and out of the nucleus at a rate of up to 1500 molecules per second (Kowalczyk et al., 2011; Ribbeck and Gorlich, 2001; Strambio-De-Castillia et al., 2010; Wente and Rout, 2010).

1.2.1 Nuclear pore complex structure and composition

Being the largest protein complexes in eukaryotic cells (~60 MDa in yeast and ~125 MDa for vertebrates), the structure of the NPC is highly conserved among different species. Extensive transmission electron microscopy (TEM) (Akey, 1989; Akey and Radermacher, 1993; Hinshaw et al., 1992; Jarnik and Aebi, 1991; Ris and Malecki, 1993), scanning electron microscopy (SEM) (Goldberg and Allen, 1993, 1996; Ris, 1997; Ris and Malecki, 1993) and atomic force microscopy (Rakowska et al., 1998; Stoffler et al., 1999) studies have been carried out to elucidate the structure of the NPC (reviewed in Adams and Wente, 2013; Bilokapic and Schwartz, 2012; Elad et al., 2009; Fernandez-Martinez and Rout, 2012; Lim et al., 2008a; Lim et al., 2008b; Maco et al., 2006; Pante 2007; Rowat et al., 2008). According to studies using cryo-electron tomography, the overall NPC show an eightfold-rotation symmetry, and consists of both a cytoplasmic and a nucleoplasmic ring (Figure 1-1). Peripheral components extend from these membrane-embedded scaffold rings into the cytoplasm and nucleus. Eight 35-50 nm long filaments extend from the cytoplasmic domain and eight 50-100 nm long filaments extend into the nucleus to form the nuclear basket (Figure 1-1). The opening at the distal end of this nuclear basket is ~30 nm in diameter. The NE-embedded ring has a diameter of 120 nm, is 70 nm in height, and contains a large central channel of about 50 nm in diameter (Beck et al., 2004; Beck et al., 2007; Frenkiel-Krispin et al., 2010; Maimon et al., 2012; Stoffler et al., 1999) (Figure 1-1). The overall architecture of the NPC is relatively conserved among eukaryotic cells (reviewed in Brohawn et al., 2009) and many studies have shown the structural and functional similarities between yeast and mammalian NPCs, even though their sequences are not exactly conserved (Bapteste et al., 2005; Kiseleva et al., 2004). The human NPC is composed of ~34 proteins called nucleoporins (Nups) that form the stationary phase for nucleocytoplasmic exchange, whereas the

mobile phase is composed of transport receptors and their cargoes (reviewed in Wente and Rout, 2010). 30 of these Nups are soluble, and three are integral membrane proteins of the pore membrane domains (POMs). They are organized into several subcomplexes and in multiple copies, therefore a fully assembled NPC contains ~500-1000 Nups (reviewed in Hoelz et al., 2011).

Nups are classified into three categories based on where they reside within the NPC, and therefore based also on their role in nuclear transport (reviewed in Grossman et al., 2012). Transmembrane Nups reside in the membrane layer anchoring the NPC to the NE. The scaffold Nups are located between the membrane layer and the inner most layer of the NPC and they help form the structure of the NPC (reviewed in Lusk et al., 2007). Finally, the inner most layer of the NPC is composed of phenylalanine glycine (FG)-Nups that facilitate active transport of larger cargoes, and these make up about one-third of the total Nups (reviewed in Terry and Wente, 2009). Importantly, FG-Nups found in the peripheral region of NPCs (i.e. Nup358 and Nup153) are asymmetrically distributed while those in the central region (i.e. Nup62) of NPCs are symmetrically located (Figure 1-1). FG-Nups contain flexible structures that are responsible for the regulation of nucleocytoplasmic transport (Denning et al., 2003; Peleg and Lim, 2010; Zeitler and Weis, 2004). It is these Nups that ultimately function in nuclear transport (discussed in section 1.2.2.3) and therefore is often targeted by many viruses during nuclear entry (discussed in section 1.3.6).

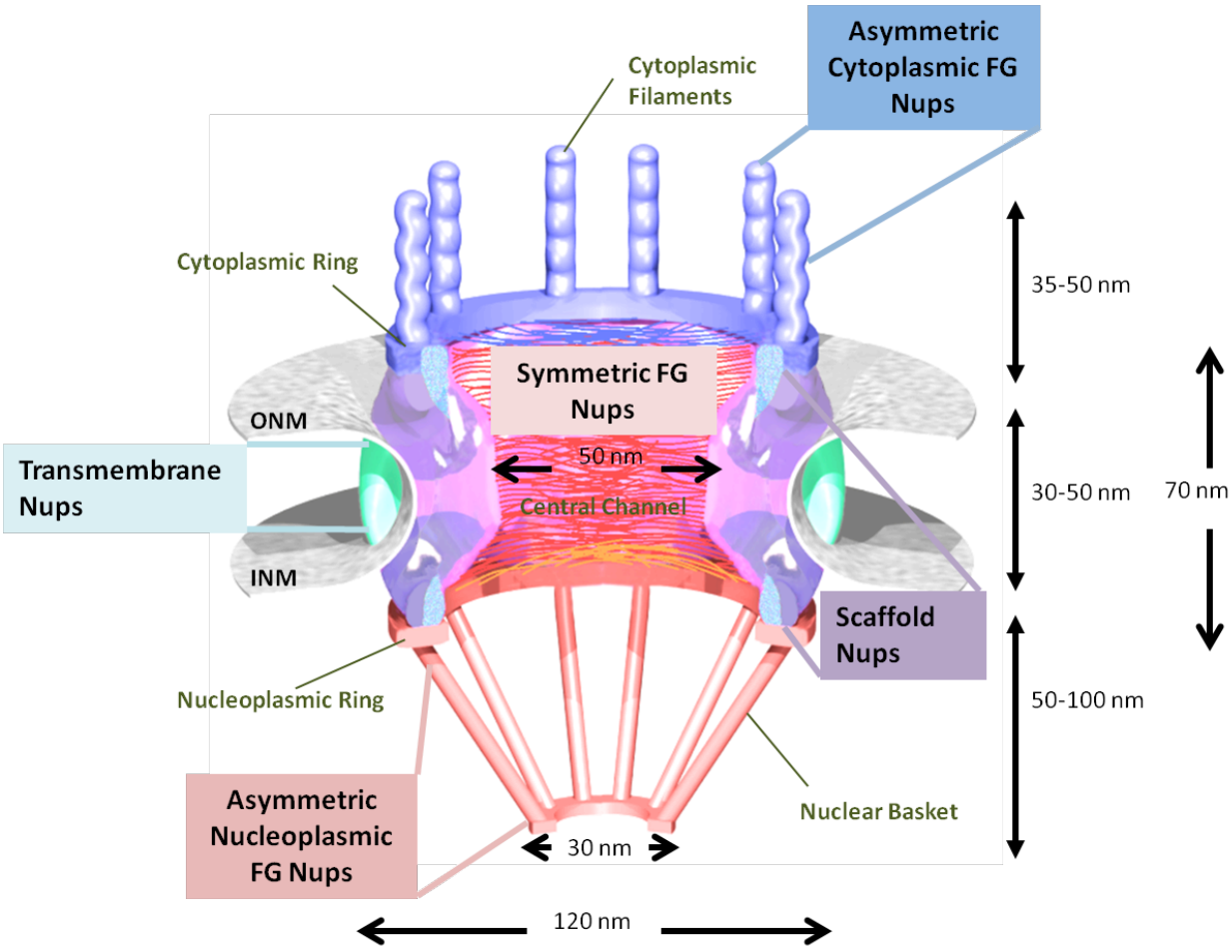

Figure 1-1: Structural components of the NPC. Schematic representation of the NPC with the position of the different classes of Nups. Names of the structural components and their dimensions are shown.

1.2.2 Key players involved in nuclear import

Two general mechanisms have been described for nuclear import: passive diffusion and facilitated translocation. Molecules < 9 nm in diameter or proteins < 40 kDa can passively diffuse into and out of the nucleus (Paine et al., 1975). However, most viruses are too large for passive diffusion and therefore must be actively transported into the nucleus. Facilitated nuclear import can accommodate the transport of molecules with diameters of up to 39 nm (Pante and Kann, 2002). This requires a set of signal sequence residing on the cargo to be transported and receptors referred to as karyopherins, that recognize a different set of signal sequence and mediate the translocation of the cargo through the NPC by binding to FG-Nups (reviewed in Chook and Suel, 2011; Fiserova and Goldberg, 2010; Jamali et al., 2011). A single translocation event during facilitated protein transport is reported to take 2-34 milliseconds (Yang and Musser, 2006), thus this process is highly regulated and efficient.

1.2.2.1 Nucleocytoplasmic transport receptors: Karyopherins

Karyopherins can be classified under two groups; those involved in nuclear import called importins and receptors for nuclear export referred to as exportins. Vertebrate karyopherins that were identified are listed in Table 1-1. Importins recognize nuclear localization sequence (NLS) residing within the cargo during nuclear import while exportins mediate the export of cargoes containing nuclear export signals from the nucleus. Currently, there are 20 known human karyopherins and 14 yeast karyopherins (Tran et al., 2007). Importin-β (importin-β1) is the most common karyopherin involved in nuclear import of cargoes. Importins appear to have higher affinity to binding sites within the nucleoplasmic face of NPCs, while export karyopherins

preferentially binds to the cytoplasmic face of NPCs (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003; Shah et al., 1998).

1.2.2.2 Nuclear localization signals

As mentioned in section 1.1.2.1, importins mediate nuclear import by binding to NLSs on large cargoes. The most studied NLS consists of one or two short stretches of basic amino acids, called the classical nuclear localization sequence (cNLS). Interestingly, the first identified cNLS was discovered in a viral protein, the large T antigen of simian virus 40 (SV40), recognized by importin-β and an adaptor protein importin-α (Kalderon et al., 1984; Lanford and Butel, 1984). cNLSs could also be composed of two clusters of positively charged residues separated by a spacer of 10-12 amino acids, similar to the NLS of the *Xenopus* protein nucleoplasmin (Dingwall et al., 1982, Dingwall et al., 1988). NLSs can also be relatively large, such as that of the M9 sequence in heterogeneous ribonucleoprotein particle (hnRNP) A1, which is 38 amino acids rich in glycine residues with a small number of residues being of positive charges (Pollard et al., 1996). Generally NLSs are difficult to identify by amino acid sequence alone, as they can be a set of positively charged amino acids or simply a region of arginine-glycine-rich residues. In addition to their function of mediating nuclear import, NLS activities can also be regulated by mechanisms such as protein modification (i.e. phosphorylation) or signal masking (reviewed in Pouton et al., 2007), thereby complicating the ability of identifying an active NLS in cargoes by simple sequence analysis.

Table 1-1: Vertebrate karyopherins involved in nuclear import and export

Vertebrate Karyopherins	Examples of Cargoes
Nuclear Import	
Importin-β1	Proteins containing cNLS using importin-α as adapter, U snRNP independent of importin-α, HIV Rev
Importin-4	Histones
Importin-5/Importin-β3	Histones, ribosomal proteins
Importin-7	Ribosomal proteins, mediator of TGF-β Smad
Importin-8	Mediator of TGF-β Smad
Importin-9	Histones, ribosomal proteins
Importin-11	SUMO E2-conjugating enzyme UBCM2
Transportin-1/Transportin-2	mRNA binding proteins, histones, ribosomal proteins
Transportin-3 (TRN-SR2)	HIV-1 integrase
Transportin SR1	Pre-mRNA splicing factor SR proteins
NTF2	RanGDP
Nuclear Import & Export	
Importin-13	SUMO E2-conjugating enzyme UBC9
Nuclear Export	
CAS	Importin-α
Exportin-t	tRNAs
CRM1/Exportin-1	NES containing proteins, ribosomal subunits
Exportin-4	Mediator of TGF-β Smad3
Exportin-5	Pre-mRNA
Exportin-6	Profilin, actin
TAP/NXT1(p15)	mRNA

1.2.2.3 Role of nucleoporins in nucleocytoplasmic transport

Experiments have demonstrated an increasing affinity gradient of Nups for importin-β from the cytoplasmic to nucleoplasmic side of the NPC (Ben-Efraim and Gerace, 2001). FG-Nups also serve as docking sites for transport receptors. Each FG-Nup is composed of 20-30 FG rich domains, such as FG, GLFG, FxFG (x could be any amino acid) (Frey and Gorlich, 2007, 2009). Studies have demonstrated that domains rich in FG-repeats do not form secondary or tertiary structures, but instead they bind to one another through hydrophobic interactions (Bayliss et al., 2000; Denning et al., 2003; Patel et al., 2007). Cargoes of similar size and shapes that bind to FG-Nups have a higher nuclear transport rate than those without the attachment to FG (Ribbeck and Gorlich, 2001). The NPC permeability barrier is disrupted in FG-domain deleted mutants, suggesting the necessity of FG-Nups in forming the selective gate of the NPC (Patel et al., 2007). However, half of the FG-repeats can be deleted from the NPC with little effect on nuclear transport, and FG-repeat domains can also be deleted without affecting the cells' viability (Strawn et al., 2004). Most recently, *in vitro* assays demonstrated that Nup98 is necessary for the formation of the NPC permeability barrier (Hulsmann et al., 2012). How a certain set of Nups can play such an important regulatory role in nucleocytoplasmic transport is still poorly understood, but many models discussed in section 1.2.3 have been proposed to explain this function.

1.2.2.4 Classic nuclear import pathway

The nuclear import of cNLS-bearing proteins is mediated by a heterodimer import receptor consisting of importin-α and importin-β (Figure 1-2). The N-terminus of importin-α contains an importin-β binding domain, while NLSs bind to the central region of importin-α. Therefore

importin-α, acting as an adaptor protein, binds to importin-β and in combination with the NLS containing cargo generates a nuclear transport complex (NTC). Importin-β of this complex interacts with FG-Nups and the driving force behind nuclear import is a gradient of the GTPase ras-related nuclear protein (Ran) across the NE. RanGTP is highly concentrated inside the nucleus, while RanGDP predominates within the cytoplasm (Kalab et al., 2002). Once the NTC is translocated into the nucleus, it attaches to the nuclear basket Nups such as Nup153 and Nup50 (also known as Npap60), which mediates the release of the NLS-cargo by breaking the interaction between the NLS and importin-α. RanGTP in the nucleus plays two distinct roles during the recycling process of nuclear transport receptors. It mediates the attachment of CAS (cellular apoptosis susceptibility) protein to importin-α, releasing it from Nup50, and recycles importin-α back into the cytoplasm (Pumroy et al., 2012) (i.e., CAS acts as the nuclear export receptor for importin-α). Subsequently, RanGTP also detaches importin-β from the NTC and mediates the export of importin-β back to the cytoplasm (reviewed in Jamali et al., 2011). A recent study discovered that two isoforms of the Nup50 protein, Npap60s and Npap60L, are able to control nuclear import efficiency. Npap60L promotes the dissociation of NLS-cargo from importin-α, while Npap60s stabilizes this interaction (Ogawa et al., 2010). This study suggests that both isoforms of Nup50 can regulate nuclear import of cNLS-containing cargoes.

1.2.2.5 Role of the Ran cycle in classical nuclear import

Ran is a 25-kDa GTPase, belonging to the Ras protein superfamily. Ran is a vital regulator of nuclear transport receptors (Moore, 1998) and its activity is regulated by the dynamic conversion between GTP- and GDP-bound states (Gorlich and Kutay, 1999). Ran-GTP predominately resides in the nucleus and is constantly exported from the nucleus (Figure 1-3), while Ran in its

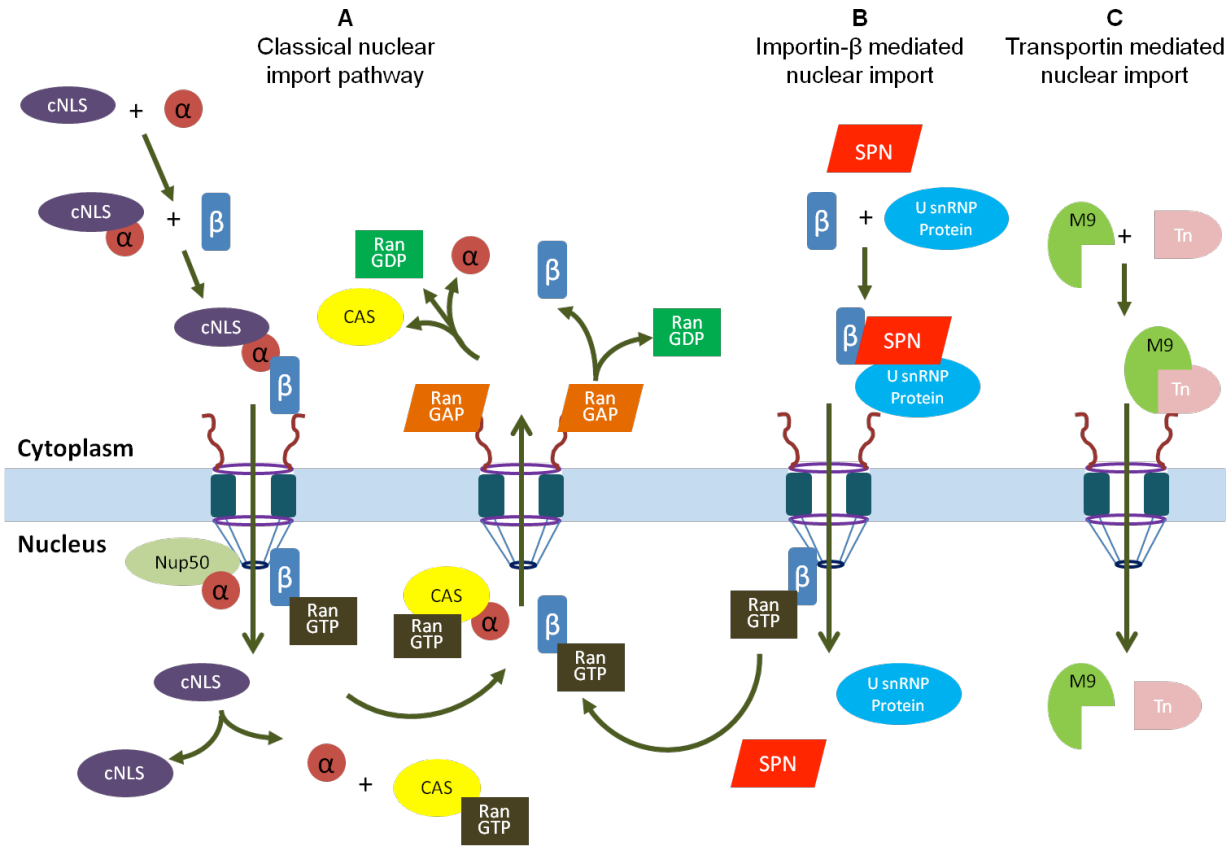

Figure 1-2: Nuclear import pathways. (A) During classical nuclear import a cNLS is recognized by importin-α, which then binds to importin-β. This nuclear transport complex is translocated into the nucleus through the NPC by successive docking of importin-β to Nups. The cNLS-containing cargo is released in the nucleus and importin-α and importin-β are recycled back into the cytoplasm. The recycling of these receptors is mediated by CAS and RanGTP. (B) Proteins like U snRNP are recognized by the adaptor protein snurportin (SPN) and bind to importin-β in the cytoplasm to deliver the cargo into the nucleus. Importin-β is then recycled back to the cytoplasm, a process mediated by RanGTP. (C) Cargoes containing the M9 signal sequence use transportin for nuclear import through the NPC, independently of the Ran cycle. The delivered cargo is be released into the nucleus and transportin is further recycled back to the cytoplasm.

GDP-bound form is found in the cytoplasm and shuttles into the nucleus by interacting with nuclear transport factor 2 (NTF2), a nuclear transport factor that interacts with FG-Nups (Moore and Blobel, 1994; Paschal and Gerace, 1995; Ribbeck et al., 1998; Smith et al., 1998). In the nucleus, the concentration of RanGTP is ~100-fold greater than in the cytoplasm due to the presence of a protein called regulator of chromosome condensation 1 (RCC1) found in the nucleus. RCC1 is a Ran guanine exchange factor (RanGEF) found in the nucleus bound to histones H2A and H2B (approximately one RanGEF molecule per nucleosome) responsible for the conversion of RanGDP to RanGTP (Bischoff and Ponstingl, 1991; Nemergut et al., 2001).

Alternatively, RanGTPase-activating protein (RanGAP) in the cytoplasm promotes RanGTP hydrolysis into RanGDP with the help of RanGTP-binding protein 1 (RanBP1) (Bischoff et al., 1995). Therefore, non-hydrolysable GTP analogs such as GTPγS inhibit classical nuclear transport and are often used in studies to determine the necessity of Ran in nuclear transport processes (Englmeier et al., 1999; Izaurralde et al., 1997). Karyopherins have different binding affinities for RanGTP. Interestingly, importins have a high binding affinity for RanGTP, which leads to the dissociation of the import cargoes, but exportins have a relatively low binding affinity to Ran in the absence of a cargo (reviewed in Weis, 2003). In the cytoplasm, RanGDP favors the binding of importins to cargo, while nuclear RanGTP interacts with importins, leading to the dissociation and subsequent release of the cargo from the importins into the nucleus (reviewed in Gorlich and Mattaj, 1996; Izaurralde et al., 1997). The bond created upon RanGTP and importin-β binding reduces the affinity of importin-β to FG-Nups (Otsuka et al., 2008). Therefore, the cellular levels of both forms of Ran must be controlled properly for nuclear transport efficiency and directionality.

1.2.2.6 Non-classical nuclear import pathways

Human cells contain at least 20 members of the karyopherin family of proteins involved in nucleocytoplasmic transport (reviewed in Mosammaparast and Pemberton, 2004) and 6 homologous members are found within the family of karyopherin-α (reviewed in Goldfarb et al., 2004), complicating the cargo/receptor combinations. However, as there are many more cargoes than karyopherins, each karyopherin can accommodate multiple cargoes, therefore many nuclear import pathways could co-exist to deliver cargos into the nucleus. Beyond the classical nuclear import mechanism, other pathways exist whereby variations of receptors or energy source are used. Non-classical nuclear import can involve an alternative adaptor protein, snurportin (Figure 1-2B). These processes can also occur independently of RanGTP, as demonstrated for the nuclear import of uridine-rich small nuclear RNP (U snRNP) (Palacios et al., 1997; Huber et al., 2002; Rollenhagen et al., 2003). Additionally, heterogeneous nuclear RNP (hnRNP) A1 protein has a M9 signal sequence that binds directly to transportin instead of importins during nuclear import (Pollard et al., 1996) (Figure 1-2C) . The 38-amino acid sequence M9 serves as both a nuclear import and export signal, using transportin (karyopherin β2) (Michael et al., 1995, Pollard et al., 1996). The M9 core, represented by the sequence SNFGPMKGGNFGGRSSGPY, is necessary and sufficient for protein nucleocytoplasmic shuttling (Michael et al., 1995, Pollard et al., 1996). This demonstrates the complexity in elucidating a nuclear import pathway pertaining to a certain cargo, as it could depend on the putative signal sequence it contains, whether RanGTP is involved, and whether a particular karyopherin mediates its nuclear import.

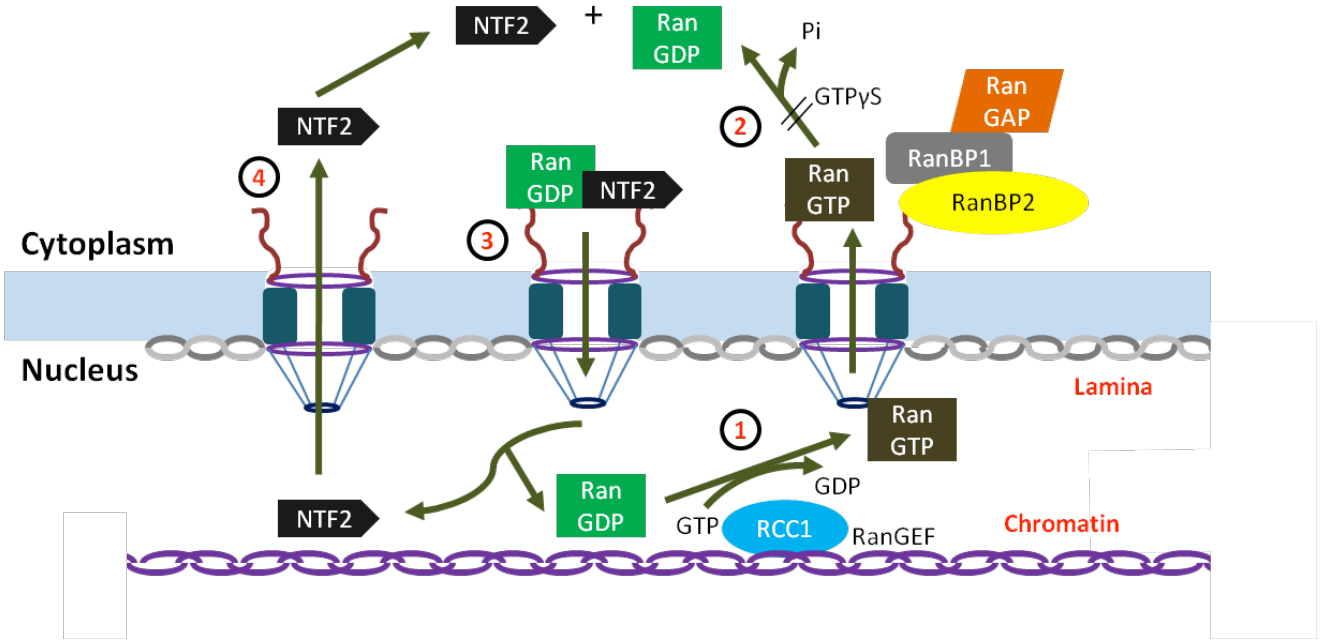

Figure 1-3: Scheme of the RanGTPase cycle. In the nucleus, RanGEF (RCC1) promotes the exchange of GDP to GTP on Ran (1). RanGTP is exported out into the cytoplasm and binds to RanBP1/RanBP2, where it gets hydrolysed from GTP to GDP by RanGAP (2). NTF2 acts as a nuclear import receptor for RanGDP (3). After nuclear import of the RanGDP-NTF2 complex, NTF2 returns to the cytoplasm (4) leaving RanGDP in the nucleus to start the cycle again.

1.2.3 Nuclear transport models[2]

Despite significant progress in identifying NLSs and their receptors (many of which have been crystallized and their structure solved; reviewed by Cook et al., 2007; Lange et al., 2007) and in characterizing the basis of the recognition of these molecules, the precise mechanism used by molecules to cross the NPC remains unknown. Several models have been proposed in recent years speculating on the mechanism for the facilitated movement of transport receptors and their cargo through the NPC. These include the affinity gradient, the virtual gating, the selective phase partition, the diffuse permeability and the oily spaghetti models (reviewed in Peleg and Lim, 2010; Peters, 2009; Wente and Rout, 2010). As their names suggest, these models explain some biophysical aspects of the movement of molecules through the central channel of the NPC. They take into account either interactions with or partitioning of transport receptors with FG Nups which occupy the NPC central channel (reviewed in Walde and Kehlenbach, 2010).

The idea of an *open* state of the NPC was originally proposed in earlier studies that suggested the concept of a central plug/transporter that resides within the NPC central channel (Akey, 1990, 1991; Akey and Radermacher, 1993). These studies hypothesized that the transporter remains in a closed position when cargos dock and further dilates when cargos are in transit through the NPC. The existence of an NPC central plug/transporter was supported by early structural analysis of the NPC that documented the presence of a massive particle in the central channel. Because the size, shape and position of this particle were highly variable, the central plug/transporter was later proposed to be molecules in transit (Beck et al., 2004; Elad et al., 2009; Stoffler et al., 2003).

[2] A version of part of this section has been published: Au, S., and Panté, N. 2012. Nuclear transport of baculovirus: Revealing the nuclear pore complex passage. *Journal of Structural Biology* 177: 90-98.

More recent models of NPC function propose that the central channel is filled by the FG-repeat domains of Nups. The Selective Phase model assumes that these domains are cross-linked, forming a hydrogel (Ribbeck and Gorlich, 2002). This model suggests an inverse relationship between cargo size and the rate of nuclear import (Ribbeck and Gorlich, 2001). In another proposed model for NPC function, the FG-repeat domains of Nups have been suggested to act as a *polymer brush* that sweeps away macromolecules from a large corona surrounding the entrance of the NPC central channel (Lim et al., 2007a; Lim et al., 2006; Lim et al., 2007b). The FG corona acts as a barrier to reject non-binding molecules, while molecules capable of binding to the FG corona cause local FG repeats to collapse, thereby allowing the molecule into the transport channel (Lim et al., 2006). A more recent proposed model, the Forest model, hypothesises that the central channel is separated into two zones - large molecules move through the inner part of the NPC channel, and smaller molecules travel along the outer part of this channel (Yamada et al., 2010). This model suggests that FG domains converge at the centre of the NPC, forming a central plug/transporter structure to allow the movement of larger cargos. Many models have been proposed aiming to understand the function of the NPC and DNA viruses that must replicate in the nucleus of their host cell, are great tools to use in determining the functional role of Nups and dynamic changes of NPCs during nucleocytoplasmic transport.

1.3 Nuclear entry of viruses[3]

The general current understanding is that nuclear-replicating viruses deliver their genome into the nucleus of their host cells by using the machinery that evolved for the nuclear import of cellular proteins (i.e., NPCs, NLSs, importins, GTP, and Ran). Because the size and structure of viruses

[3] A version of part of this section has been published: Cohen, S.*, Au, S.*, and Panté, N. 2011. How viruses access the nucleus. *Biochim Biophys Acta.* 1813(9): 1634-1645.

vary enormously (for example, herpes simplex virus is 120-nm in diameter (Roizman and Taddeo, 2007) but parvoviruses are 18 to 26-nm in diameter (Parrish & Berns, 2007)) and because there are several nuclear import pathways, each virus has evolved a unique strategy to deliver its genome into the nucleus. Two of the main factors affecting the nuclear entry strategy of a given virus are the size of the capsid, and the cellular location of genome release. As indicated in Figure 1-4, five general strategies have been identified, which we have ordered according to where in the cell uncoating of the viral genome occurs:

1) Some viruses, such as murine leukemia virus (MLV), gain access to the nucleus during mitosis, when the NE is temporarily disassembled.
2) Some viruses, such as human immunodeficiency virus 1 (HIV-1) and influenza A virus, undergo extensive disassembly in the cytoplasm. The components released into the cytoplasm contain NLSs and are thereby able to cross the NPC using the host transport machinery.
3) Some viral capsids use importins or viral proteins to attach to the cytoplasmic side of the NPC. Interaction with the NPC is then used as a cue for disassembly, and the viral genome crosses the NPC and is released into the nucleus, often as a complex with viral proteins. Viruses that use this strategy include herpesviruses (which bind to the NPC via importins) and adenoviruses (which bind directly to the NPC).
4) Some viral capsids, such as those of hepatitis B virus (HBV), are small enough to cross the NPC intact. Genome release then occurs at the nuclear side of the NPC or inside the nucleus.

5) Some viruses, such as parvoviruses, do not use the NPC to deliver their genome into the nucleus; rather, they transiently disrupt the NE and nuclear lamina, and enter the nucleus through the resulting gaps.

Although much progress has been made in characterizing general nuclear entry strategies used by different viruses, many of the molecular details remain obscure. The study of viral nuclear entry is complicated by the fact that viral proteins may enter the nucleus multiple times during the virus life-cycle: both as part of an incoming capsid or nucleoprotein, and perhaps also as a newly synthesized protein if assembly of progeny virions occurs in the nucleus. Thus, identification of NLSs and host factors involved in a particular viral nuclear import step can be challenging. Post-translational modifications such as phosphorylation of viral proteins can also play an important role in the exposure of NLSs (reviewed in Alvisi et al., 2008). This has been studied for HBV, but is probably true for other viruses as well. Viral transport into the nucleus, and genome release are both part of an intricate dance between the virus and host cell, many details of which remain to be elucidated. In the following sections, the five general strategies of nuclear import of viral genomes are discussed, with particular emphasis on the best-studied viruses.

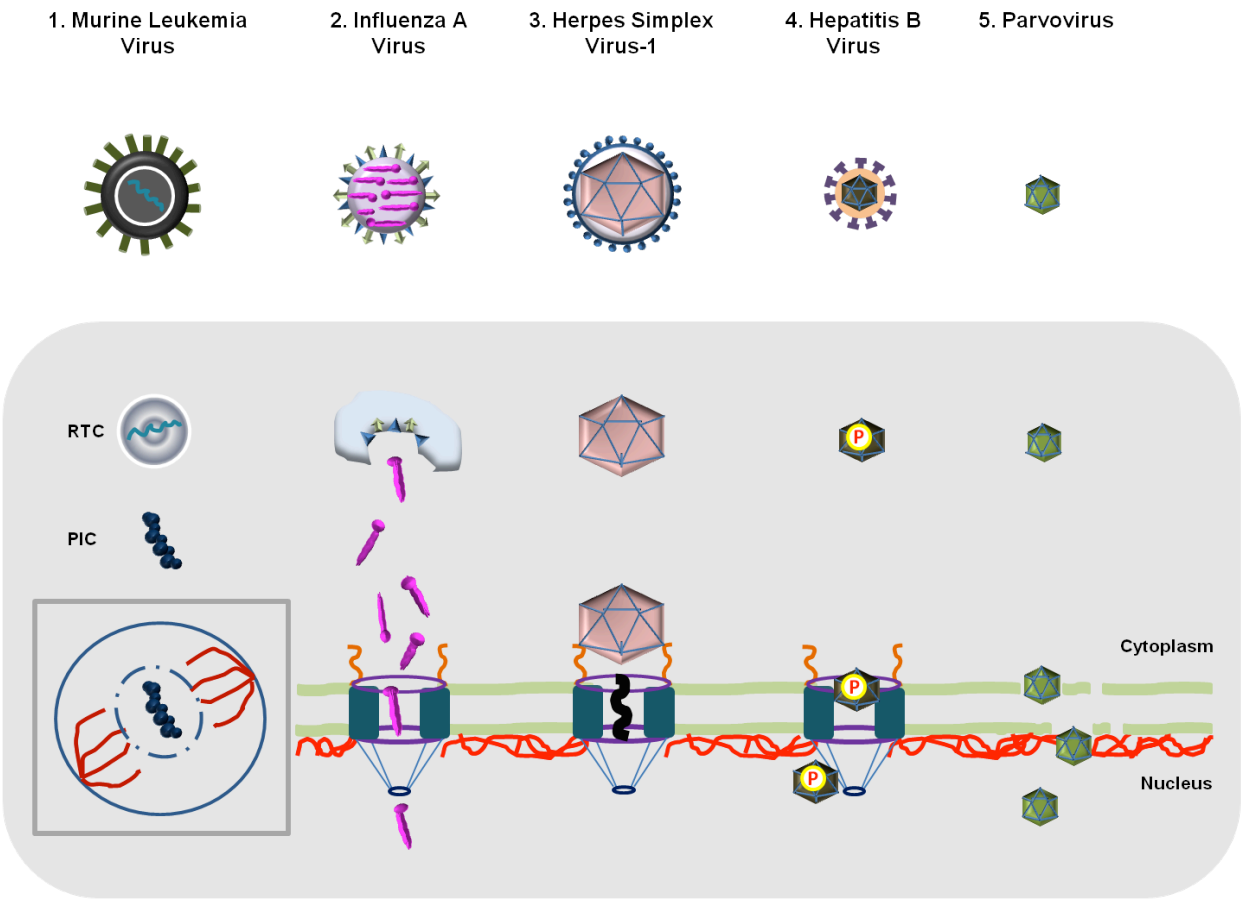

Figure 1-4: Five different strategies for nuclear entry of viral genomes. (1) MLV pre-integration complex (PIC) gains access to the nucleus during mitosis, when the NE is temporarily disassembled. (2) Influenza A virus undergoes extensive disassembly in the cytoplasm. Cytoplasmic released vRNPs containing NLSs are imported into the nucleus via NPCs. (3) HSV-1 capsids use importins to attach to the cytoplasmic side of the NPC. Interaction with the NPC then triggers the release of the viral genome, which then enters the nucleus through the NPC. (4) Phosphorylated form of HBV capsids cross the NPC intact followed by capsid disassembly at the nuclear basket. (5) Parvovirus transiently disrupts the NE and nuclear lamina, and enters the nucleus through the resulting gaps.

1.3.1 Nuclear entry during mitosis

Some viruses, such as the retrovirus MLV, can only access the nucleus of a host cell during mitosis, when the NE is temporarily disassembled (reviewed in Goff, 2007; Suzuki and Craigie, 2007). Retroviruses are RNA viruses which reverse transcribe their RNA genomes into DNA; the DNA is then integrated into the host genome, where it serves as a template for the synthesis of new RNA genomes. Retroviruses may enter the cell either by direct fusion of the viral envelope at the cell surface, or by fusion after internalization using an endocytic route (Goff, 2007). Fusion results in the release of the viral nucleoprotein core particle into the cytoplasm. This is followed by a poorly understood uncoating step and the formation of the reverse transcription complex, which for MLV includes the viral RNA genome, reverse transcriptase, integrase and the capsid protein (Fassati and Goff, 1999). Reverse transcription of RNA to DNA produces the pre-integration complex (PIC), which must then enter the nucleus to integrate into the host genome. The PIC of MLV is too large to enter the nucleus through the NPC by passive diffusion. Several lines of evidence indicate that MLV must wait for NE disassembly in order for the PIC to enter the nucleus (Harel et al., 1981; Miller et al., 1990; Roe et al., 1993).

1.3.2 Genome release in the cytoplasm, followed by entry through the nuclear pore complex

HIV-1 entry into cells is similar to the process described above for MLV, although the composition of the resulting PIC is somewhat different. While the MLV PIC includes reverse transcriptase, integrase and the capsid protein, the HIV-1 PIC is composed of reverse transcriptase, integrase, matrix protein, and the accessory protein Vpr, with the capsid protein largely dissociating prior to nuclear entry (reviewed in Suzuki and Craigie, 2007). It is generally

agreed that the HIV-1 PIC enters the nucleus by active transport through the NPC, but the molecular mechanism remains poorly understood (reviewed in Jayappa et al., 2012).

Every component of the HIV-1 PIC has been suggested to participate in mediating its nuclear entry (Suzuki and Craigie, 2007) by either binding directly to Nups or contain NLS-like properties and bind to receptors for nuclear import (Hearps and Jans, 2006; Ikeda et al., 2004; Piller et al., 2003; Woodward et al., 2009). Surprisingly, none of these viral components seems to be absolutely necessary or sufficient for nuclear entry of the PIC (Riviere et al., 2010; Yamashita and Emerman, 2005), signifying that viral components involved in nuclear transport of the HIV-1 PIC are highly redundant. Transport receptors involved in nuclear entry of the HIV-1 PIC also remains unclear. Members of the importin-α family (Gallay et al., 1997; Gallay et al., 1996; Vodicka et al., 1998), importin-β (Matreyek and Engelman, 2011; Popov et al., 1998), importin 7 (Ao et al., 2007; Fassati et al., 2003; Zaitseva et al., 2009), and transportin-3 (Brass et al., 2008; Christ et al., 2008; Matreyek and Engelman, 2011) have all been shown to be involved in the nuclear import of either individual viral proteins or of the PIC.

The influenza A virus is an enveloped virus, containing a segmented genome consisting of eight single-stranded negative-sense RNAs. While most RNA viruses replicate in the cytoplasm, influenza replication takes place in the nucleus, likely due to the requirement for cellular splicing machinery present there (reviewed in Engelhardt and Fodor, 2006). Each of the eight RNA segments is separately packed with several copies of the structural nucleoprotein (NP) and a single copy of a trimeric viral RNA polymerase into a viral ribonucleoprotein complex (vRNP) (Palese and Shaw, 2007). The influenza A virus is internalized into cells via the endocytic

pathway using either clathrin- or caveolae-dependent mechanisms, and the vRNPs are released into the cytoplasm upon endosomal acidification (Babcock et al., 2004; Martin and Helenius, 1991; Nunes-Correia et al., 2004; Roy et al., 2000; Sieczkarski and Whittaker, 2002; Skehel and Wiley, 2000). All four proteins (NP and the three RNA polymerases) of the vRNPs contain NLSs (Palese and Shaw, 2007). NP contains at least two NLSs that contribute to nuclear import of vRNPs (Cros et al., 2005; Neumann et al., 1997; Ozawa et al., 2007; Wang et al., 1997; Weber et al., 1998; Wu et al., 2007), and binds to a number of human importins α, both *in vitro* and *in vivo* (Melen et al., 2003; O'Neill et al., 1995; Wang et al., 1997). Thus, it is thought that vRNPs are transported into the nucleus using the classical importin-α/importin-β pathway. However, a recent genome-wide RNAi screen identified transportin 3 as a host factor required for influenza virus replication (Konig et al., 2010), indicating that other nuclear import pathways may play a role as well.

1.3.3 Genome release at the cytoplasmic side of the nuclear pore complex

Herpes viruses are enveloped viruses with an icosahedral capsid of 120-nm containing the viral double-stranded DNA, and a proteinaceous layer (called the tegument) between the capsid and the envelope (Roizman and Taddeo, 2007). The best-characterized herpesvirus in terms of nuclear import is the human herpes simplex virus-1 (HSV-1). Upon cellular entry, the capsid with its surrounding tegument is released into the peripheral cytoplasm. The tegument-capsid structure is then transported along microtubules to the NPC (Dohner et al., 2002; Sodeik et al., 1997). Electron microscopy (EM) studies using tissue-culture cells infected with HSV-1 (Sodeik et al., 1997), as well as *in vitro* binding studies of HSV-1 capsids with isolated nuclei from tissue-culture cells or *Xenopus* oocytes, demonstrated that the HSV-1 capsid binds to the

cytoplasmic side at a distance of ~50 nm away from the center of the NPC (Ojala et al., 2000; Shahin et al., 2006; Sodeik et al., 1997). Thus, the capsids are speculated to bind to Nup358 of the NPC cytoplasmic filaments. NPC-binding of the HSV-1 capsid is importinβ-dependent and requires RanGTPase (Ojala et al., 2000). After binding to the NPC, the HSV-1 capsid portal releases its DNA into the cell nucleus through the NPC (Cardone et al., 2007; Newcomb et al., 2001; Trus et al., 2004). This process leaves intact capsids devoid of the DNA (empty capsids) associated with the NPC (Ojala et al., 2000; Sodeik et al., 1997).

The 90-nm adenovirus capsid has also evolved a nuclear import mechanism in which its genome is released at the cytoplasmic side of the NPC. In contrast to HSV-1, however, the adenovirus capsid completely disassembles at the cytoplasmic side of the NPC. Adenoviruses are non-enveloped viruses composed of an icosahedral capsid surrounding an inner nucleoprotein core (Berk, 2007). A distinct structural feature of adenoviruses is the fibers projecting from the vertices of the capsid. Adenoviruses enter their host cells by receptor-mediated endocytosis and escape the endosome using a capsid component with membrane-lytic activity (Maier et al., 2010; Wiethoff et al., 2005). By the time it is delivered to the NPC via microtubule- and dynein mediated motility (Kelkar et al., 2004; Suomalainen et al., 1999), the virion has shed its fibers and several capsid-stabilizing proteins, and some of the remaining viral proteins have been proteolytically processed (Greber et al., 1996; Greber et al., 1993; Puntener et al., 2011). Upon binding to the cytoplasmic side of the NPC, in particular to Nup214, adenovirus capsids undergo complete disassembly resulting in the subsequent nuclear import of the viral genome and capsid proteins through the NPC (Greber et al., 1997; Strunze et al., 2011; Trotman et al., 2001; Wisnivesky et al., 1999). Strikingly, neither cytosol nor importins-α or -β are required for

binding of adenovirus capsids to isolated NE (Trotman et al., 2001). Protein VII, the most abundant core protein and the most tightly associated with the viral DNA, has been shown to contain NLSs (Wodrich et al., 2006) and to bind in vitro to several nuclear import receptors including importin-α, importin-β, importin-7 and transportin (Wodrich et al., 2006).

1.3.4 Nuclear entry of intact capsids through the NPC, followed by genome release

HBV is a small, enveloped virus that is able to cross the NPC intact. It has a diameter of 42 to 47-nm, containing a capsid with a single copy of partially double-stranded DNA genome (3.2 kilobase pairs (kbp)) (Seeger et al., 2007). The capsid is composed of 240 copies of a single type of protein (the core protein, 21 kDa), which is arranged into an icosahedral capsid of 36-nm in diameter (Vanlandschoot et al., 2003). A minor population of capsids with a diameter of 32-nm and composed of 180 copies of core protein also exist. The biological significance of the two different classes of capsids is not clear. HBV capsids are released in the cytoplasm after fusion of the viral envelope with the cellular membrane (Glebe and Urban, 2007), and are transported along microtubules towards the nucleus (Kann et al., 2007; Rabe et al., 2006). Studies with recombinant capsids (obtained by expressing the core protein in *Escherichia coli* (*E. coli*)), and semi-permeabilized cells first demonstrated that the HBV capsid binds to the NPC in a phosphorylation-and importin-α and -β-dependent manner (Kann et al., 1999). Phosphorylation of the C-terminus of the core protein is important to expose two cNLSs (Eckhardt et al., 1991; Kann et al., 1999; Yeh et al., 1990). Phosphorylated recombinant capsids binds to the NPC, crosses the NPC without disassembly and arrests and bind to Nup153 at the nuclear basket (Pante and Kann, 2002; Schmitz et al., 2010). Mature capsids containing the mature genome

disassemble at the nuclear basket, releasing their DNA into the nucleus, while those with an immature genome remain bound to Nup153 (Rabe et al., 2003b; Schmitz et al., 2010).

Another virus that likely enters the nucleus largely intact is the non-enveloped DNA polyomavirus SV40. SV40 enters the cell by an unusual mechanism: the virus is taken up by caveolar endocytosis, and then traffics to the ER (Pelkmans et al., 2001). It is unclear whether the virus escapes from the ER to the cytoplasm and then enters the nucleus through the NPC, or whether it enters the nucleus directly from the ER by penetrating the INM. When SV40 is micro-injected into cells, capsids can be seen traversing the NPCs (Yamada and Kasamatsu, 1993). However, because microinjection bypasses the normal entry route it is unclear whether this actually occurs during infection. It is also possible that SV40 can enter the nucleus via multiple routes.

1.3.5 Nuclear entry via disruption of the NE

While most viruses that replicate in the nucleus have been shown to make use of the host nuclear transport machinery, including the NPCs, another route is also possible: directly through the nuclear membranes. Parvoviruses enter the cell via receptor-mediated endocytosis, escape from endosomes and make their way towards the nucleus, possibly by microtubule-mediated transport (reviewed in Harbison et al., 2008). At only approximately 26-nm in diameter (Parrish and Berns, 2007), parvovirus capsids are small enough to enter the nucleus intact through the NPC, and it has been assumed that this is how the parvovirus genome accesses the nucleus. Several lines of evidence indicate that the parvoviral genome enters the nucleus in association with an intact capsid after escape from endosomes (Sonntag et al., 2006). However, the parvovirus minute

virus of mice (MVM) was shown to induce NE and nuclear lamina disruptions in microinjected *Xenopus laevis* oocytes or infected cells (Cohen et al., 2006; Cohen and Pante, 2005). In addition it has been shown that the parvovirus adeno-associated virus-2 enters purified nuclei independently of the NPC (Hansen et al., 2001), suggesting that this nuclear entry mechanism is a common feature of parvoviruses. While parvoviral capsid proteins do contain functional NLSs that are buried within the virion, it is unclear if they become exposed sufficiently to interact with importins (Agbandje-McKenna et al., 1998; Cotmore et al., 1999; Farr et al., 2005; Lombardo et al., 2000; Lombardo et al., 2002; Mani et al., 2006; Vihinen-Ranta et al., 1997).

In addition to parvoviruses, there are other viruses that may also use a similar strategy. As mentioned above, SV40 may be able to enter the nucleus from the ER by penetrating the INM. Lastly, it has been shown that overexpression of the HIV-1 protein Vpr induces ruptures of the NE (de Noronha et al., 2001). It has been suggested that these ruptures may mediate entry of the PIC into the nucleus (reviewed in Segura-Totten and Wilson, 2001). However, it is unclear whether Vpr-induced NE rupture actually occurs during infection of cells with HIV-1.

1.3.6 Role of nucleoporins during viral infection

Nuclear import of viruses often relies on the interaction with various Nups (Cohen et al., 2012). In some cases, Nups are also targeted by viruses to prevent host nucleocytoplasmic transport. For instance, the HSV-1 protein ICP27, which is important for the expression and nuclear export of the HSV-1 mRNA interacts directly with Nup62 during viral infection. This interaction is suggested to compete with the binding of host cell transport receptors to Nup62, thereby inhibiting host nuclear transport pathways (Malik et al., 2012).

In other cases, Nups are altered to assist in nuclear import of the virus itself. For example, a novel model proposed by Strunze and colleagues in 2011 suggest that kinesin-1, bound to the adenovirus capsid, and the NPC through the nucleoporins Nup214, and Nup358, is responsible for the disassembly of the adenovirus capsid and genome release (Strunze et al., 2011). In this case, Nup358, Nup214 and Nup62 were found mislocalized with disassembled viral particles in the cytoplasm in adenovirus-2 infected cells (Strunze et al., 2011). The disruption of Nups in these cells also increased the permeability barrier of the NPC, allowing for the increase in nuclear entry of the viral genome. Another example of altered Nups is during human rhinovirus and poliovirus infection, when a number of Nups, such as Nup62, Nup98, and Nup153 are degraded by proteases encoded by these viruses. This alteration to the NPC composition did not completely destroy the functionality of the NPC (Gustin and Sarnow, 2001, 2002; Park et al., 2008; Park et al., 2010), suggesting a redundancy in the function of Nups.

Nups commonly play an unidentified role in nuclear import of viral components. RNAi screens have identified host genes that are suggested to play a role in nuclear import of RNPs of the influenza virus, including importin-β1, Nup153, and Nup98 (Watanabe et al., 2010). These factors could be responsible for the initial nuclear import of viral RNPs, and or nuclear import of newly synthesized viral proteins. The Epstein-Barr virus (EBV) BGLP4 protein encodes for a protein kinase responsible for the phosphorylation of viral and cellular substrates necessary for DNA replication and nuclear egress of the viral capsid. This protein has been suggested to bind to Nup62 and Nup153, and nuclear translocation occurs independently of cytosolic factors (Chang et al., 2012). Knock down of Nup62 inhibited production of infectious vaccinia virus, no affect on nuclear entry, and minor affects on DNA replication and early and late viral gene

expression (Sivan et al., 2013). As NPCs are composed of Nups, it is inevitable that Nups play such important roles during the viral infection process.

1.3.7 General themes about viral nuclear import

Evidently, viruses have evolved a wide variety of strategies to invade the host cell nucleus. This allows the virus to make use of the cell's machinery for DNA replication and transcription. Viral trafficking and nuclear entry is also intimately linked with virion disassembly. In addition to using the cell's DNA replication machinery, viruses take advantage of compartmentalized cellular cues to ensure that genome release occurs at the correct time. Thus, in addition to cues such as acidification of endosomes, viruses also use binding to NPC proteins, importins or nuclear proteins to trigger genome release. Table 1-2 provides a summarized list of nuclear transport machinery exploited by viruses. The different nuclear entry strategies used by viruses depend largely on the size and structure of the virus, and have advantages and disadvantages. While significant progress has been made in understanding the general nuclear entry mechanisms used by viruses, much remains to be done. It has become evident that different viruses use different host nuclear import pathways, and viral genomes gain access to the nucleus of their host cells, not only by using the cellular nuclear import machinery, but also components of other cellular pathways. It is also evident that even viruses using the classical nuclear import receptors have evolved mechanisms to adjust the cell machinery for their needs. Although we now know more about how viruses access the nucleus, many molecular details, such as which viral NLSs are exposed at different times during infection, which viral protein interacts with cellular components, and which host transport factors are involved in each step, remain to be elucidated. A main determinant of how viral genomes gain access to the nucleus is the size of the capsid.

This thesis will examine the nuclear import mechanism of a unique rod-shaped virus: the archetype of baculoviruses, AcMNPV. While the diameter of the AcMNPV capsid is within the range of facilitated nuclear transport, the length of the capsid could be a limiting factor in differentiating where genome release occurs. Thus it is of significant interest to determine which one of the 5 modes of nuclear import, listed above, AcMNPV follows.

Table 1-2: Nuclear transport machinery exploited by nuclear replicating viruses

Protein	Alternative Name(s)	Virus
Importin-α	Karyopherin α	HIV-1
		Influenza A Virus
		Adenovirus
		HBV
Importin-β	Karyopherin β1 p97	HIV-1
		HSV-1
		Adenovirus
		HBV
		Influenza A Virus
Importin-7	IPO7 Ran binding protein 7 (RanBP7)	HIV-1
		Adenovirus
Transportin-1	TNPO1 Importin β2	Adenovirus
	Karyopherin β2	
Transportin-3	TNPO3 Importin 12	HIV-1
		Influenza A Virus
Nup62	p62	HIV-1
Nup98	Nup98-Nup96	HIV-1
Nup153		HIV-1
		HBV
Nup155		HIV-1
Nup214	CAN	HIV-1
		Adenovirus
Nup358	Ran binding protein 2 (RBP2)	HIV-1
		HSV-1

1.4 Baculoviruses[4]

1.4.1 Introduction to baculoviruses

Baculoviruses are a large and diverse group of rod-shaped (30 x 250 to 300-nm), enveloped viruses with circular double stranded-DNA genomes ranging from 80-180 kbp, encoding between 90-180 genes, that replicate in the nucleus of their host cells (reviewed in Rohrmann, 2011). They are pathogenic to arthropods, mainly insects, and are ubiquitously found in the environment. Members of the *Baculoviridae* family have been isolated from more than 700 host species. Baculoviruses play a role in the control of natural insect populations, and have long been used as bio-insecticides to control insect pests in agriculture and forestry (reviewed in Inceoglu et al., 2006).

1.4.2 Classification of baculoviruses

Most baculovirus isolates have been made from diseased caterpillars (Lepidoptera); some have been from sick sawflies (Hymenoptera) and very few from infected mosquito larvae (Diptera). Baculoviruses are recognized in their diseased hosts by the large proteinaceous bodies called occlusion bodies (OBs) they produce. Based on the distinct OB morphology, the family *Baculoviridae* was historically divided into two major genera: nucleopolyhedrovirus (NPV) and granulovirus (GV). NPV nucleocapsids are enclosed either singly (SNPV) or multiply (MNPV) within an envelope and embedded within a crystalline matrix of the protein polyhedrin, forming large (0.15 to 15-μm) polyhedral OBs. GVs contain a single enveloped nucleocapsid embedded within the protein granulin into a small (0.13 × 0.5-μm) oval-shaped OB (reviewed in Friesen,

[4] A version of part of this section has been published: Au, S., Wu, W., and Panté, N. 2013. Baculovirus Nuclear Import: Open, Nuclear Pore Complex (NPC) Sesame. *Viruses*. 5(7): 1885-1900.

2007). The advent of molecular technology enabled baculovirus classification to take a leap forward. By adding genome sequence information to existing morphological descriptive data, a better understanding of the evolutionary relatedness among the baculoviruses was obtained. Not surprisingly, the viral sequence data showed distinct clusters that co-aligned with the taxonomy of the hosts. This implied that viruses with lepidopteran hosts, for example, were more closely related to each other than they were to the viruses infecting dipteran or hymenopteran hosts, and *vice versa*. This observation is especially interesting when considering that the Lepidoptera are the most recent order of insects to have appeared (~232 mya), while the Diptera and Hymenoptera are older (~260 mya and 309 mya, respectively). Viruses isolated from lepidopteran hosts include GVs, MNPVs and SNPVs, while viruses from the two older orders are so far limited to SNPVs.

Genomic sequences of NPVs and GVs that infect Lepidoptera form two distinct clusters and represent the genera of Alpha- and Betabaculoviruses, respectively. Viruses isolated from saw flies constitute the Gammabaculovirus genus and those isolated from mosquito larvae are the Deltabaculoviruses (Figure 1-5) (reviewed in Jehle et al., 2006; Herniou et al., 2011). The Alphabaculoviruses are further divided into Group I and II; the two groups differ in gene content, but most noticeably in the fusion protein encoded by each group. Group I NPVs, such as the archetype AcMNPV, use a GP64 fusion protein, and Group II NPVs use an F-protein (Pearson and Rohrmann, 2002). Baculoviruses are normally named for the initial host from which they were first isolated. Thus, for example, the baculovirus that infect the alfalfa looper *Autographa californica* is named AcMNPV, and that from the spruce budworm *Choristoneura fumiferana* is named CfMNPV.

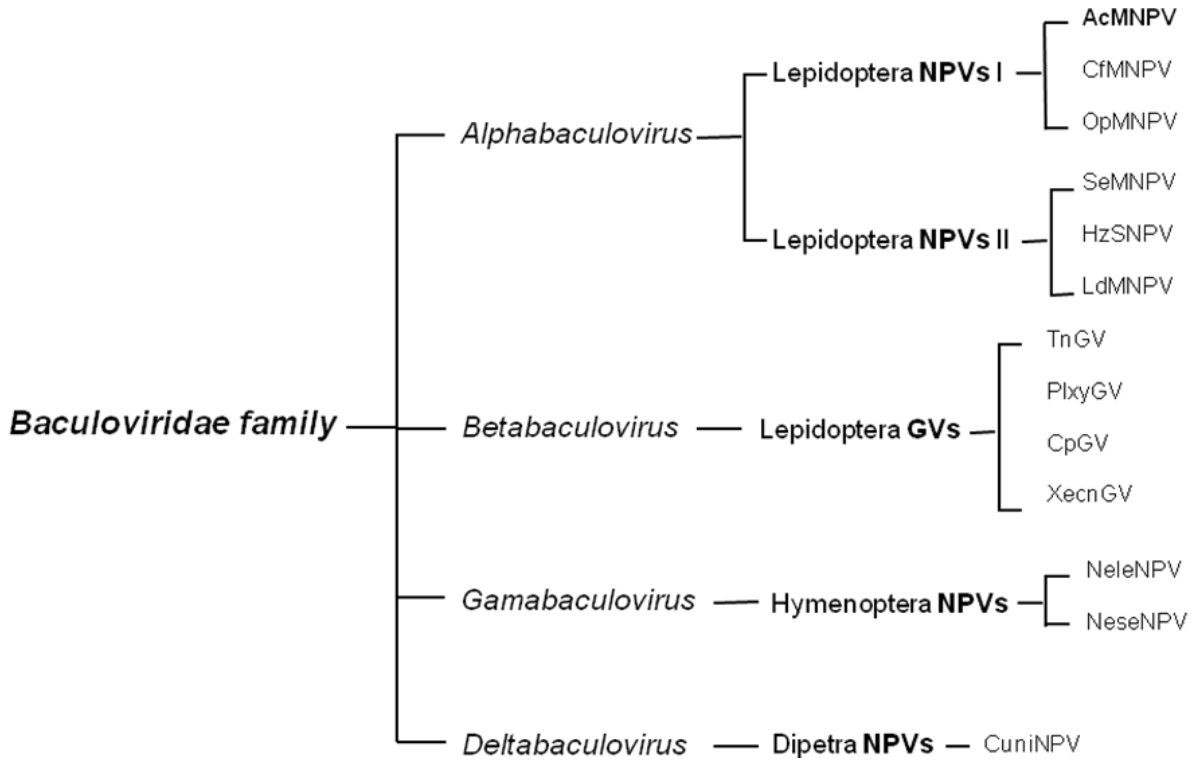

Figure 1-5: Classification of the Baculoviridae family. Only a small subset of the characterized species within each group is listed. This phylogram is for illustrative purposes only. AcMNPV, the most studied baculovirus and most commonly used viral vector for baculovirus expression vector systems, belongs to the type I Alphabaculovirus genus.

1.4.3 Baculovirus structure and composition

Most baculoviruses produce two types of infectious viral particles: Budded Virion (BV) and the Occlusion Derived Virion (ODV) (Figure 1-6). While all four genera of baculoviruses form ODVs, only Alpha-, Beta-, and Deltabaculoviruses generate BVs (Herniou, et al., 2011). Both forms of virions differ by the subcellular location and time they are produced in during the replication cycle. BVs are produced during the initial replication when capsids bud from infected cells and obtain their envelopes from the plasma membrane. Thus, BV contains a single capsid and a plasma membrane-derived envelope, which contains the viral fusion proteins (GP64 or F protein). ODVs are produced during the very late phase of replication and are formed in the nucleus by envelopement of a single or multiple capsids per virion, which then become incorporated within the protein matrix (polyhedrin for NPVs or granulin for GVs) forming OBs that are released into the environment upon death of the infected larva. While ODV is involved in virus transmission between insect larvae, BV is the infectious form responsible for cell-to-cell transmission within the host and in cell culture (reviewed in Rohrmann, 2011).

The capsid, which is the central component of both virion phenotypes, has a rod-shaped morphology with two distinct ends: apical cap end with a small protuberance and one end blunt (Figure 1-6). The baculovirus capsid contains over a dozen proteins (Braunagel et al., 2003; Wang et al., 2010), thus only the proteins pertinent to this thesis will be discussed here. The major capsid protein, VP39, is a 39-kDa protein that constitutes the barrel of the capsid encasing the viral genome (reviewed in Rohrmann, 2011). VP78/83 located at the blunt end of the capsid is involved in actin nucleation during viral infection (reviewed in Rohrmann, 2011).

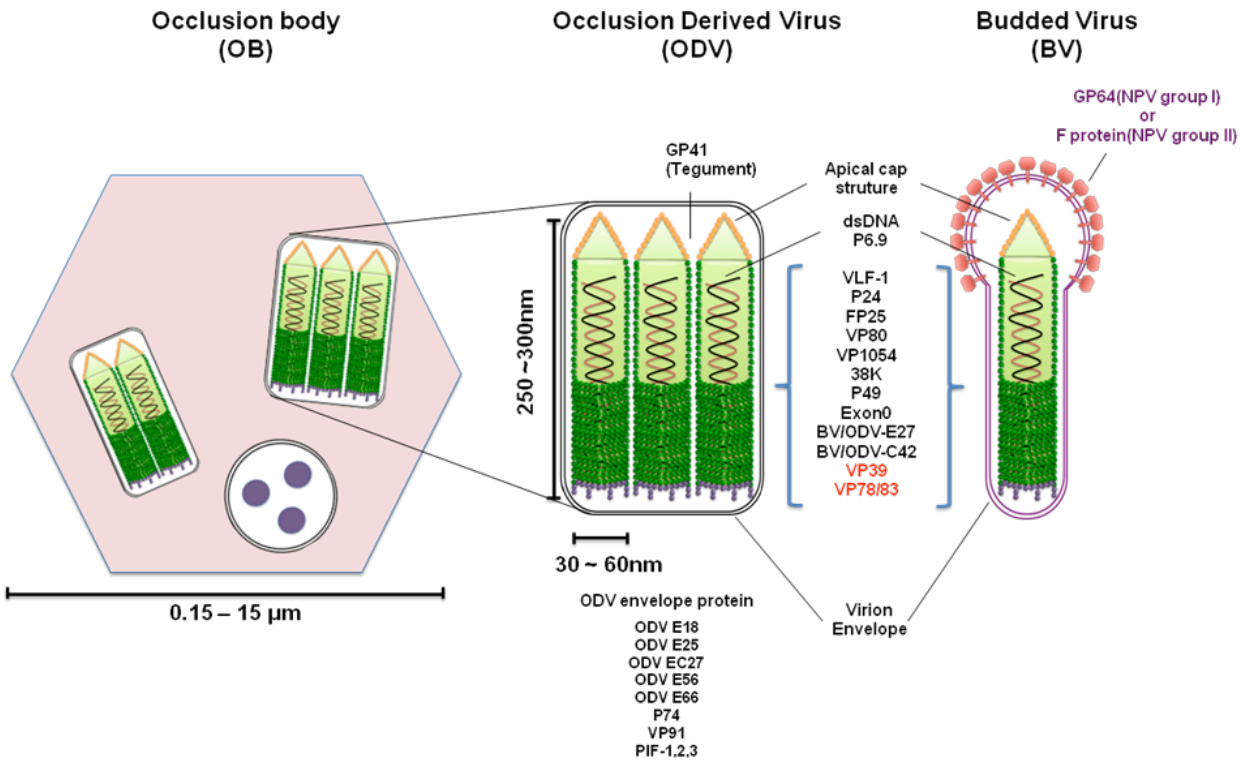

Figure 1-6: Schematic diagrams of the structure of baculovirus OB, ODV and BV. ODVs are embedded in a crystalline matrix of protein to form OBs. Shown here is the OB of NPVs. The ODV and BV envelope and their capsid(s) contain numerous proteins. Common proteins shared between the ODV and BV capsid are shown in between the ODV and BV diagram. The major capsid protein VP39 is present in the whole capsid, and VP78/83 is located at the opposite end to the apical cap. The fusion protein GP64 (NPV group I) or F protein (NPV group II) is found on one end of the BV envelope.

1.4.4 Baculovirus replication cycle

In its natural host, viral infection begins with larval ingestion of OBs where the alkaline pH in the columnar epithelial cells within the midgut causes dissolution of the OBs releasing ODV. The midgut is involved in enzyme secretion and absorption of digested food, where the entry and exit sites of the midgut has a pH near 7.0 but the central region can vary from pH 10 to 12 (Dow, 1992). A set of baculoviridae conserved proteins specific to ODV called per os infectivity factors (PIF) have been shown to be involved with virus binding to epithelial cells (Fang et al., 2009; Fang et al., 2006; Faulkner et al., 1997; Harrison et al., 2010; Kikhno et al., 2002), leading to the fusion of the virus with the cell membrane (Horton and Burand, 1993) (Figure 1-7 step 1). Once the virions pass the peritrophic membrane of the midgut epithelia, capsids in association with cellular actin migrate towards the nucleus of the cell. It is thought that an actin tail forms at one end of the capsid to help its migration towards the nucleus (Charlton and Volkman, 1991, 1993; Lanier and Volkman, 1998). The genome is released into the nucleus allowing for viral replication (Figure 1-7 steps 2 and 3) (reviewed in Passarelli, 2011).

During early phases of replication, the viral fusion protein GP64 is made, shuttled to sites on the cell membrane where newly formed capsids bud out of the cell by wrapping the GP64-studded cell membrane around a single capsid to generate BVs (Figure 1-7 step 4). Some capsids do not enter the nucleus after cell entry but instead they bud out of the cell to increase systemic infection (Monsma et al., 1996; Ohkawa et al., 2010; Washburn et al., 1999). Secondary infection occurs as BVs infect neighbouring cells. Baculovirus has adopted multiple mechanisms for BV cell entry, generating greater viral infection efficiency. GP64 is a pH-activated class III fusion protein of baculovirus that is essential for host cell receptor binding (Hefferon et al.,

1999). Structurally, GP64 is related to vesicular stomatitis virus (VSV) glycoprotein G (VSV G) and Herpes virus gB (Backovic and Jardetzky, 2011; Kadlec et al., 2008). It is a highly conserved protein and appears to contain amino acids similar to the thogotovirus, a subgroup of the Orthomyxoviridae, GP75 envelope glycoprotein (Morse et al., 1992). Attachment of GP64 to the cell surface leads to receptor-mediated endocytosis in insect cells (Figure 1-8 step 5) (Hefferon et al., 1999; Monsma and Blissard, 1995; Oomens and Blissard, 1999). Although the cell surface receptor has not been characterized in insect cells, the heparan sulfate subfamily syndecan-1 was shown to bind strongly to GP64 in a pH-dependent manner during viral transduction of mammalian cells only and not for infection of insect cells (Wu and Wang, 2012, Makkonen et al., 2013). In a study using VSV bearing GP64 of AcMNPV, researchers showed that inhibiting dynamin, clathrin, and macropinocytosis-mediated pathways impaired GP64-mediated cell entry (Kataoka et al., 2012). Viral fusion with the plasma membrane under low pH conditions, thereby bypassing the endocytosis pathway was recently shown to also occur (Dong et al., 2010). TEM evidence also showed multiple enveloped capsids within vesicles larger than clathrin-coated vesicles, suggesting that engulfment of the virus into macropinosomes during cell entry is also used in mammalian cells (Matilainen et al., 2005).

Once inside the endosome, GP64 mediates low-pH-triggered membrane fusion activity that's required for the release of capsids into the cytoplasm (Figure 1-7 step 6) (Blissard and Wenz, 1992; Kingsley et al., 1999; Leikina et al., 1992; Long et al., 2006; Markovic et al., 1998; Monsma and Blissard, 1995; Plonsky et al., 1999; Volkman and Goldsmith, 1985). A recent study showed the necessity of cellular VPS4, an ATPase of the endosomal sorting complex required for transport (ESCRT) pathway, in efficient entry of BVs in insect cells (Li and Blissard,

2012). Dominant negative VPS4 proteins in mammalian cells cause aberrant endosomes, affecting the trafficking of cargos from early endosomes to late endosomes and/or lysosomes. Upon viral infection in cells containing a dominant-negative construct of VPS4, virions were unable to traffic appropriately and capsids were not released from endosomes, thereby disrupting productive viral infection (Li and Blissard, 2012).

Upon capsid release into the cytoplasm, actin once again facilitates capsid transport towards the nucleus (Charlton and Volkman, 1991, 1993; Lanier and Volkman, 1998). However, microtubules are thought to impede infectivity as cells treated with microtubule depolymerizing drugs resulted in higher rates of nuclear accumulation of capsids, suggesting that microtubules act as a diffusion barrier for cytoplasmic trafficking of capsids (Salminen et al., 2005). The viral genome is further released into the nucleus. During very late stages of viral replication, capsids remaining in the nucleus become occluded and embedded within polyhedrin proteins to form OBs (Figure 1-7 steps 7 and 8). These OBs finally get released into the environment upon the death and disintegration of the larva (Figure 1-7 step 9).

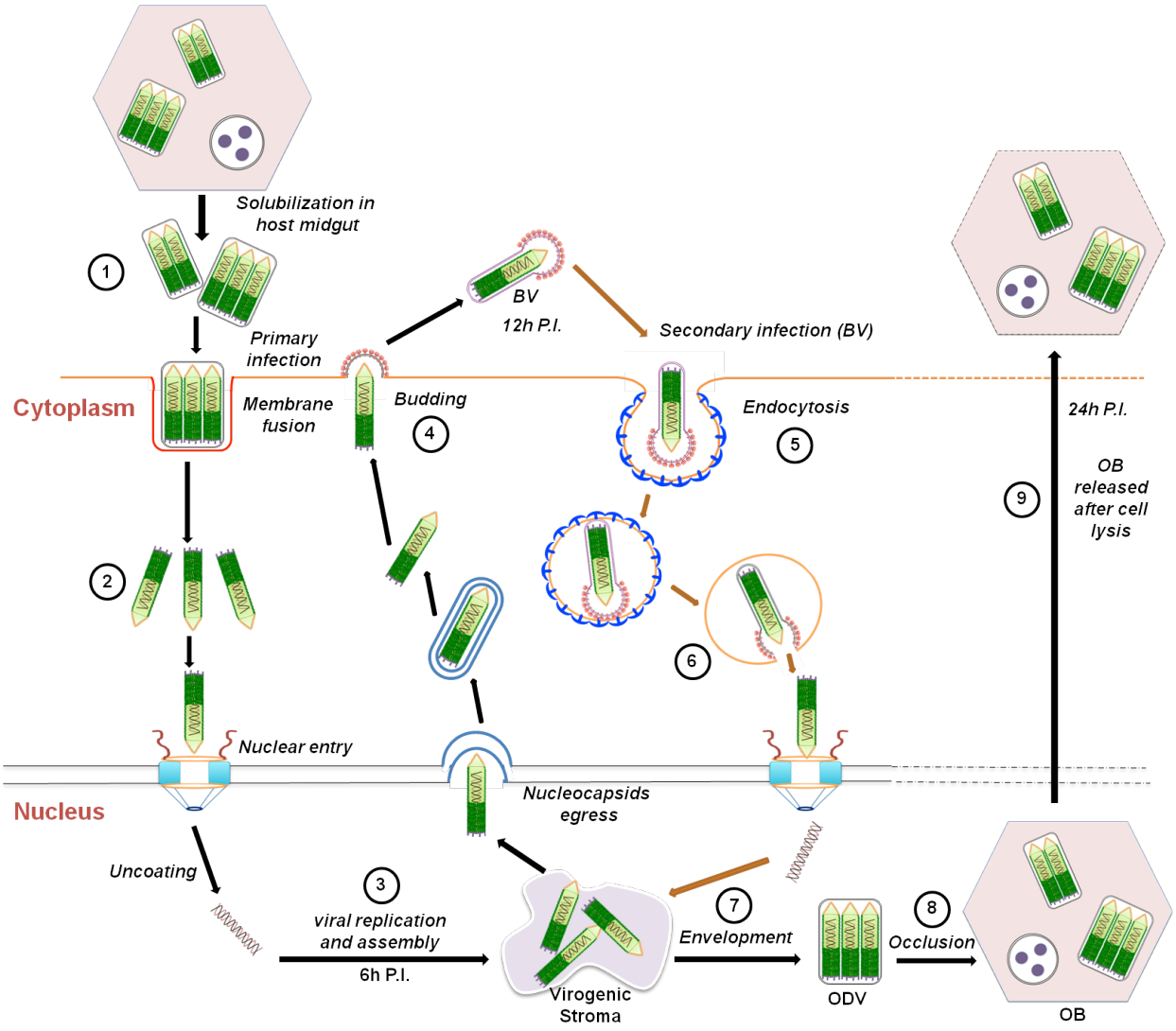

Figure 1-7: Baculovirus AcMNPV lifecycle. (1) OBs solubilise in the midgut of its host, releasing ODVs that enter the cell through membrane fusion. (2) Capsids released into the cytoplasm transport towards the nucleus and the genome is released into the nucleus. (3) Viral replication and capsid assembly occurs in the virogenic stroma during early stages of infection. (4) These capsids exit the nucleus and bud out of the cell generated BVs. (5) These BVs further infect neighbouring cells through endocytosis. (6) Capsids are release from endosomes and get transported towards the nucleus, allowing for genome release to occur. (7) During very late stages of infection, capsids remaining in the virogenic stroma obtains an envelope and (8) become occluded within a protein matrix to generate OBs. (9) These OBs are released into the environment when the host dies and cell lysis.

1.4.5 Ambiguities in baculovirus nuclear entry

The mechanism by which the genome of baculovirus enters the nucleus during infection has been rather confusing due to the number of species within the *Baculoviridae* family, structural complexities between genera, and variations between experimental techniques used in studies such as inoculation, infection, and transduction. Nuclear import of baculoviruses is an understudied topic as both viral and cellular proteins involved in this process are currently unknown. Initial research on the pathogenesis of baculoviruses focused on the inoculation of insect larvae or infection of insect cells with baculovirus.

Summers first demonstrated empty intact capsids docking at the NPC at 2 hours post-inoculation of *Trichoplusia ni (T. ni)* larvae with GV particles (Summers, 1969, 1971). These initial results suggested a mechanism of viral genome entry similar to that used by herpes simplex virus whereby the capsid docks at NPCs to inject its genome into the nucleus through NPCs (Ojala et al., 2000; Sodeik et al., 1997). On the contrary, a separate study using *Spodoptera frugiperda* (*Sf*) larvae inoculated with GV particles for 24 hours found capsids associating with NPCs within the nucleus (Walker et al., 1982). However, 24 hours post-inoculation is sufficient time for progeny capsids to be made, therefore GVs belonging to the genera of Betabaculovirus appear to dock at the cytoplasmic side of NPCs to inject the viral genome into the nucleus and progeny capsids dock at the nucleoplasmic side of NPCs, perhaps during nuclear egress of the capsid.

Subsequent studies of Alphabaculovirus NPVs demonstrated intact partially electron dense capsids attached to NPCs, implying a mechanism similar to that observed in Betabaculoviruses (Raghow and Grace, 1974). In support of this, a biochemical assay detected quick uncoating of

the viral capsid within the cytoplasm in *T.ni* infected *Sf* cells (Wang and Kelly, 1985). However, additional studies using a number of different Alphabaculovirus NPVs to inoculate larvae or infect insect cells demonstrated intact capsids inside the host cells' nucleus (Bassemir et al., 1983; Carstens et al., 1979; Kawanishi et al., 1972; Knudson and Harrap, 1976; Hirumi et al., 1975). Thus, it appears that uncoating of whole intact capsids occur in the nucleoplasm. This is supported by studies performed by Granados and colleagues where partially empty capsids were discovered in the nucleus of larvae infected with NPVs (Granados, 1978; Granados and Lawler, 1981).

Described above are two different modes of viral genome release into the nucleus exhibited by Alphabaculovirus NPVs; docking of capsids at the NPC to release the viral genome into the nucleus and viral uncoating occurs within the cytoplasm, or capsids enter the nucleus fully intact and viral uncoating occurs in the nucleus. Much of these earlier studies were unable to decipher if the capsids observed in the nucleus entered during mitosis in the absence of the nuclear membrane. Because capsids were not shown to cross NPCs, whether the capsids observed in the nucleus were imported through NPCs, entered through nuclear envelope breakages, or if they were newly generated during infection remained ambiguous.

1.4.6 Importance of baculovirus nuclear import

In 1983, Volkman and Goldsmith demonstrated that baculoviruses can enter mammalian cells without viral gene expression (Volkman and Goldsmith, 1983). Uptake of AcMNPV by mammalian cell lines was further demonstrated without expression of viral genes or viral replication (Carbonell et al., 1985; Groner et al., 1984; Hartig et al., 1991). More importantly,

recombinant baculoviruses can be delivered into human hepatocytes (Hofmann et al., 1995), which opened the prospect of using baculovirus for gene expression in both dividing and nondividing mammalian cells (Boyce and Bucher, 1996; Ojala et al., 2001; Pieroni et al., 2001; Shoji et al., 1997; Song et al., 2003). Boublik and colleagues showed in 1995 that a foreign protein can be fused to the GP64 envelope protein of AcMNPV, however this limits gene delivery to only cell entry into the target cell (Boublik et al., 1995). It was later discovered that foreign proteins can be fused to the N- and C-terminus of VP39 capsid protein and this allows gene delivery all the way into the nucleus (Kukkonen et al., 2003).

Baculovirus expression systems (BEVs) are extensively used for protein expression as baculovirus can be easily produced at high titres and are able to transduce a wide range of mammalian cells without cytotoxic effects (reviewed in Hitchman et al., 2011a; Lu et al., 2012; van Oers, 2011). These characteristics make them an excellent candidate as viral vectors for gene therapy and vaccine production (reviewed in Cox, 2012; Hitchman et al., 2011b; Hu, 2006; Lu et al., 2012; Rivera-Gonzalez et al., 2011; Rychlowska et al., 2011). For these reasons, it would be necessary to study the mechanism of baculovirus nuclear import and cellular factors that increase their transduction efficiency in mammalian cells.

1.5 Research objectives

Cellular entry and trafficking of viruses has been studied extensively, especially to enhance the understanding of viruses that are infectious and cause severe pathological symptoms in humans. In addition, nuclear transport of viruses that are used as vectors for gene delivery or protein expression (ie: adenovirus) have been of interest for use in designing efficient viral vectors.

However, nuclear transport of baculoviruses has been under-studied and is less well understood. In particular, the nuclear import mechanism of AcMNPV is still controversial and a NLS involved in nuclear transport has not been identified. The research described in this thesis will examine the nuclear import mechanism used by the rod-shaped capsid of AcMNPV. It will also attempt to identify cellular proteins that may play a role in providing efficient nuclear transport of this viral capsid. More specifically, I addressed the following questions:

1) Does the intact AcMNPV capsid enter the cell nucleus through the NPC? Or does the AcMNPV capsid eject its nucleic acid through the NPC leaving an intact empty capsid at the cytoplasmic side of the NPC, similar to Betabaculoviruses (GVs)?
2) What cellular proteins play a role in the nuclear entry mechanism of AcMNPV capsids?
3) What is the role of FG-Nups in the nuclear import of the rod-shaped AcMNPV capsid?

I have used cell biology and imaging techniques, such as EM and fluorescence microscopy in combination with biochemical assays to address these questions. The specific objectives of my Ph.D. thesis were the following:

1.5.1 Characterizing the nuclear import mechanism of baculovirus AcMNPV

Experiments described in Chapter 3 were set out to visualize cellular transport events of baculovirus AcMNPV leading up to genome release into the nucleus. Microinjection of *Xenopus laevis* oocytes followed by EM was used to elucidate whether intact capsids enter the nucleus through NPCs like the capsid of HBV or docks at the cytoplasmic face of NPCs and inject its genome, similar to the capsid of HSV-1. Electron micrographs provided visual evidence of

structural changes in the NPC, in addition to visualize potential interactions between the capsid and the NPC.

1.5.2 Determining cellular proteins essential in mediating nuclear import of AcMNPV capsids

I further investigated the necessity of various cellular proteins that allow for AcMNPV capsid nuclear import. Being a large rod-shaped virus, cellular proteins must be present to mediate active transport of the capsid towards and into the nucleus. Using the well-established digitonin-permeabilized cell assay for nuclear import (Adam et al., 1990), I tested whether cellular components involved in the classical nuclear import pathway are also essential in promoting nuclear import of the AcMNPV capsid. With the knowledge that actin is necessary for AcMNPV targeting to the NE, the role of cellular F-actin in combination with the ability of the capsid to activate Arp2/3 complex to mediate nuclear transport of the capsid was also tested. The results of these experiments are described in Chapter 4.

1.5.3 Elucidation of a potential role of FG-Nups in the nuclear import of AcMNPV capsids

Finally, besides determining cellular proteins that are necessary for nuclear import of AcMNPV capsids, I sought to determine whether FG-Nups may facilitate nuclear import of this viral component. This was characterized using siRNA to transiently deplete three FG-Nups that are often targeted by other viruses, and transducing the Nup-depleted cells or performing *in vitro* nuclear import assay with them. This allowed me to visualize the effect these Nups may have on the baculoviral nuclear import. The potential role of these Nups in the nuclear import of AcMNPV is described in Chapter 5.

Chapter 2

Materials and Methods[5]

2.1 Antibodies and reagents

To detect for baculovirus capsids, the antibody used was a rabbit polyclonal antibody against the VP39 capsid protein (provided by Dr. David Theilmann, Agriculture and Agri-Food Canada, Summerland, B.C., Canada), or a mouse monoclonal antibody against the VP39 capsid protein (provided by Dr. Robert Kotin, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA). Anti-Nup153 (SA1) against the nucleoporin Nup153 was a mouse monoclonal antibody (provided by Dr. Brian Burke, Institute of Medical Biology, Singapore). Anti-Nup358 antibodies against the nucleoporin Nup358 were rabbit polyclonal antibodies (provided by Dr. Mary Dasso, National Institute for Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA, and Dr. Frauke Melchior, Zentrum für Molekulare Biologie derUniversität Heidelberg, Heidelberg, Germany).

Commercial primary antibodies used were for beta-actin (Abcam, Cambridge, UK; Catalog number: ab6276,), Arp2 (Abcam; Catalog number: ab47654), Nup62 (Sigma-Aldrich, St. Louis, MO, USA; Catalog number: N1163), and the Nup-specific antibody QE5 (Abcam; Catalog number: ab24700).

[5] A version of part of this chapter has been published: Au, S.*, Cohen, S.*, and Panté, N. 2010. Microinjection of *Xenopus laevis* oocytes as a system for studying nuclear transport of viruses. *Methods* 51(11): 114-120.

Fluorophore-conjugated secondary antibodies for fluorescent microscopy were from Invitrogen (Grand Island, NY, USA). Peroxidase-conjuaged affinity purified secondary antibodies IgG (H+L) for Western blots were from Jackson ImmunoResearch (West Grove, PA, USA).

FITC-conjugated phalloidin was used to detect for F-actin (Sigma-Aldrich; Catalog number: P5282). Cytochalasin D (Sigma-Aldrich; Catalog number: C8273) was used to disrupt actin microfilaments and inhibit actin polymerization. CK666 was used to inhibit Arp2/3 activation, along with an inactive control, CK689 (EMD Millipore, Billerica, MA, USA; Catalog numbers: 182515 and 182517, respectively). Importazole is an inhibitor of importin-β transport receptors (Sigma-Aldrich; Catalog number: SML0341). GTPγS is a non-hydrolyzable G-protein-activating analog of GTP (EMD Millipore; Catalog number: 20-176). Rabbit reticulocyte lysate system was used as the standard cytosolic lysate for the digitonin-permeabilized cell import assay (Promega, Madison, WI, USA; Catalog number: L4960). G-actin/F-actin was separated in digitonin or cytochalasin D treated cells to determine changes in actin distribution (Cytoskeleton Inc., Denver, CO, USA; Catalog number: BK037). Wheat germ agglutinin (WGA) is a lectin that specifically binds to glycosylated Nups, acting as an inhibitor of receptor-mediated nuclear import (Sigma-Aldrich; Catalog number: L9640).

2.2 Cell culture and virus preparation

2.2.1 Cell lines

HeLa cells (American Type Culture Collection, Manasas, VA, USA) were grown on coverslips at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich; Catalog number: D5671) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich; Catalog

number: F1051), penicillin-streptomycin (Cellgro, Herndon, VA, USA; Catalog number: CO-002-CI) 1 mM sodium pyruvate (Gibco by Life Technologies; Catalog number 11360-070), and 2 mL L-glutamine (Cellgro; Catalog number: 25-005-Cl). *Spodoptera frugiperda* 9 (*Sf*9) cells were grown in Sf-900 II serum free medium (Invitrogen; Catalog number: 10902-088) supplemented with 2% FBS.

2.2.2 Maintenance of baculovirus AcMNPV

Recombinant AcMNPV, propagated in *E. coli* strain DH10B and amplified at a multiplicity of infection (MOI) of 1 in *Sf*9 cells were kindly provided by Dr. D. Theilmann. Virus was maintained by infecting *Sf9* cells at a MOI of 1 and harvested at day 5 post-infection by pelleting cells and debris at 1000 x g for 10 minutes at 4^{o}C. The titre of the supernatant was determined by endpoint dilution assay and was typically 10^{8} PFU/ml.

2.2.3 End point dilution assay

The original procedure described by Reed and Muench was modified for the use in determining baculovirus titres (Reed and Muench, 1938). Virus to be titred was diluted 10-fold from 10^{-1} to 10^{-8} in a final volume of 200 μL and an equal volume of resuspended *Sf*9 cells were added to each dilution. Cells were mixed and resuspended with the virus gently and 10 μL of each diluted mixture was added to each well of a 96 well plate (10 wells/dilution). Infected cells were incubated at 27^{o}C for 5 days and each well was scored for infected cells. Wells with infected cells were scored positive, while others remained negative. The median tissue culture infective dose ($TCID_{50}$) was determined using the Reed and Muench method, and the $TCID_{50}$ per mL was multiplied by 0.7 to generate PFU/mL.

2.2.4 Purification of baculovirus AcMNPV capsids:

A modified protocol based on those described by Obregon-Barboza et al., 2007 and Shoji et al., 1997 was used to purify baculovirus AcMNPV capsids from cultured cells. Cell debris was removed by centrifugation at 6000 x g for 15 minutes at 4°C. Baculovirus was pelleted by centrifugation at 75 600 x g at 4°C for 90 minutes and the pellet was resuspended in Tris-EDTA buffer (TE; which contains 50 mM Tris, 0.5 mM EDTA, pH 8.7). Baculoviruses were purified through a continuous 15%-60% (w/v) sucrose gradient and centrifuged at 77 000 x g for 90 minutes at 4°C. A visible band at about 2-cm from the bottom of the centrifuge tube was collected, washed in TE buffer, and centrifuged at 77 000 x g for 40 minutes at 4°C. This procedure was repeated twice to remove excess sucrose prior to treatment of these purified virions with 1% NP40 for one hour at 30°C to remove the viral envelope. De-enveloped capsids were washed by centrifugation and dialyzed for 24 hours to remove excess sucrose and NP40. The integrity of purified capsids was detected by negative staining followed by EM, a procedure described in section 2.6.1. These capsids were immediately used, or aliquoted and stored frozen at -80°C for later use.

2.3 *Xenopus laevis* oocyte isolation and microinjection

To isolate oocytes, a female *Xenopus laevis* frog was narcotized by immersion in a solution of 300 mg/L Tricaine methane sulfonate solution (MS222; 3-Aminobenzoic acid ethyl ester, Sigma-Aldrich; Catalog number: A5040) buffered to pH 7.5 with sodium bicarbonate. After 30-45 minutes, a 1-cm long incision through the skin was made using surgical scissors at about 1-cm above the leg fold of the frog and slightly offset from the ventral middle line of the stomach. A further incision of 6-mm long was made along the muscle under the skin, and a small portion of

the ovary (1 to 2-cm) was pulled out with sterilized forceps and removed with a pair of sterile scissors. The oocytes were placed in a solution of modified Barth's saline (MBS: 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 10 mM Hepes, 0.33 mM $Ca(NO_3)$, 0.41 mM CaCl, pH 7.5), the incision was sutured with sterilized medical surgical thread, and the frog was placed in fresh water for recovery.

Oocytes were then defolliculated in a 50 ml conical tube containing 20 ml collagenase solution (5 mg/ml collagenase (Sigma-Aldrich; Catalog number: 11 088 831 001) in calcium-free MBS) and placed on a shaker for approximately 40 minutes at 100 RPM. When oocytes were sufficiently de-folliculated, they were washed three times with MBS to remove excess collagenase. Stage VI oocytes, which are large with a distinct white rim separating the black animal hemisphere and the creamy colored vegetal hemisphere were selected and transferred into a multiwell dish (Nalge Nunc, Rochester, NY, USA, 10 µl well volume) for microinjection. Purified viral capsids were mixed with 1% bromophenol blue in a 10:1 ratio to aid in the visualization of the microinjection.

Injection needles were made by pulling a 6.6 µl Drummond micropipette with the Inject+Matic Puller and calibrated to 50 nl. For consistent cytoplasmic injection, the needle was inserted into the white rim separating both hemispheres at a 45-degree angle. A volume of 50 to 100 nl of the purified capsid solution was injected into the cytoplasm of each oocyte. Injected oocytes were placed in a small Petri dish filled with MBS, and incubated at room temperature for different time points. Experiments whereby nuclear import through the NPC was prohibited, oocytes were either pre-microinjected with WGA conjugated with gold particles (see section 2.4.1 for WGA-

conjugated gold preparation) prior to microinjection with purified capsids, or oocytes were incubated at 4°C instead of room temperature after microinjection with purified capsids. After this incubation time, the injected oocytes were transferred into a solution of 2% glutaraldehyde (Ted Pella, Redding, CA, USA; Catalog number: 18426) in MBS and fixed overnight at 4^{o}C.

2.3.1 Gold conjugation of WGA

Colloidal gold particles (8-nm) were prepared by reduction of tetrachloroauric acid with sodium citrate in the presence of tannic acid (as described in Slot and Geuze, 1985). Flocculation test was performed to determine the amount of protein needed to stabilize the gold particles. For this test, 5 µl of serially diluted WGA was mixed with 25 µl of colloidal gold particles. Twenty five µl of 10% NaCl was added to this mixture to visually check for changes in the color of the samples. The slightest change of color from red to blue indicates the instability of the gold complex, which suggests that twice that concentration of protein is need to stabilize 25 µl of colloidal gold particles. To form the WGA-conjugated gold complex, proteins, WGA and gold particles were stirred for 15 minutes at room temperature. BSA was added to this mixture to a final concentration of 0.1% and stirred for 10 minutes at room temperature. This entire mixture was centrifuged for 15 minutes at 32 000 RPM to obtain a pellet containing WGA-conjugated gold particles. These particles are ready to be microinjected into oocytes.

2.4 Baculovirus transduction of HeLa cells

HeLa cells were grown in monolayers on coverslips and then mock transduced or transduced with baculovirus at a MOI of 500 in Sf-900 medium supplemented with 2% FBS for 1 hour at 4^{o}C to allow for the virus to bind to the cell surface. The medium was then replaced with

phosphate buffered saline (PBS), pH 4.7 for 5 minutes, because washing cells with low pH increases transductivity (Dong et al. 2010). Finally cells were incubated with fresh DMEM and placed in an incubator at 37^{o}C and 5% CO_2 to begin synchronized transduction. For some experiments, HeLa cells were pretreated for 1 hour and incubated during transduction with 40 μM of Importazole to inhibit importin-β mediated nuclear import. CK666 and CK689 were used at 1 mM during the pretreatment period of 1 hour and transduction of HeLa cells was carried out as indicated above, in the presence of 1 mM of each drug.

2.5 Electron microscopy

All samples were visualized using a Hitachi-7600 TEM operated at an accelerated voltage of 80 kilovolts (kV), with the exception of samples prepared for electron tomography (section 2.6.3).

2.5.1 Negative staining followed by electron microscopy of purified de-enveloped viral capsids

To confirm the purity and integrity of the viral capsids, a 10 μL drop of purified capsids was placed on top of a parlodion/carbon coated copper EM grid that was glow discharged for 30 seconds. After 8 minutes, the grid was washed 4 times in drops of distilled water, and then negatively stained in 2% uranyl acetate for 1 minute.

2.5.2 Preparation of microinjected oocytes for electron microscopy

Fixed oocytes as described in section 2.3 were washed in MBS and the black animal hemisphere containing the nucleus were dissected using tweezers and fixed with 2% glutaraldehyde in low-salt buffer (LSB: 1 mM KCl, 0.5 mM MgCl2, 10 mM HEPES, pH 7.5) for one hour at room

temperature. A light blue color (from the bromophenol blue) in the cytosol was used as an indication of oocytes that were successfully microinjected. After fixation, samples were washed three times in LSB for 5 minutes each, embedded in 2% low-melting agarose, and post-fixed with 1% osmium-tetroxide in LSB for one hour. Samples were washed three times in LSB for 5 minutes each, and then sequentially dehydrated in 50%, 70%, and 90% ethanol for 20 minutes each. To complete the dehydration steps, samples were incubated in 100% ethanol for 15 minutes twice, followed by 15 minute incubation in acetone. Fixed and dehydrated samples were sequentially infiltrated by incubation in a mixture of Epon 812 Fluka (Sigma-Aldrich; Catalog number: 45345) and acetone at a 1:1 ratio for 1 hour, followed by a 2:1 ratio for 2 hours, and finally in pure Epon for 8 hours. Epon-infiltrated samples were placed into flat embedding molds (Ted Pella) containing pure Epon, and allowed to polymerize at 60°C for two days.

After polymerization, 50-nm-thin sections through the NE from Epon-embedded oocytes were cut on a Leica Ultracut Ultramicrotome (Leica Microsystems, Wetzlar, Germany, Leica Ultracut T) using a diamond knife (Diatome, Hatfield, PA, USA). Sections were collected on parlodion/carbon-coated EM copper grids, stained with 2% uranyl acetate for 15 minutes, and 2% lead citrate for 5 minutes.

2.5.3 Electron tomography

For electron tomography, 200-nm thick sections through the NE were made, and sections were placed on slot grids coated with 1% formvar. Single axis tilt series of 200-nm samples were recorded automatically over tilt angles ranging from −30° to +30° in two degree increments, and then from −70° to +70° every degree on a FEI Tecnai G2 F20 TEM (Hillsboro, OR, USA)

operated at an accelerated voltage of 200 kV. Tomograms were acquired using FEI's TEM tomography software and reconstructed using FEI's Inspect3D software.

2.5.4 Embedding and electron microscopy of baculovirus transduced HeLa cells

HeLa cells were grown on glass coverslips and transduced for 6 hours as described above (section 2.5.1). Transduced cells were scraped off the coverslip, washed with PBS pH 7.4, and centrifuged for 15 seconds at 15 000 x g to obtain a pellet. Cells were fixed in 2% glutaraldehyde (Ted Pella) in PBS for 1 hour, followed by 4 washes in PBS pH 7.4 by centrifuging for 15 seconds at 15 000 x g, again to obtain a pellet. After fixation, samples were embedded in 2% low-melting agarose and post-fixed in 4% osmium tetroxide in PBS for 1 hour. Samples were further processed and embedded into Epon as described above for oocytes (see section 2.6.2)

2.6 Fluorescence microscopy

2.6.1 Immunofluorescence microscopy of baculovirus transduced HeLa cells

HeLa cells were grown on glass coverslips and transduced as described in section 2.4.4. For immunostaining cells were fixed with 3% paraformaldehyde (PFA) in PBS for 10 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes, blocked with PBS containing 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) and 10% Goat Serum for 30 minutes at 37^{o}C, and labelled with primary antibodies for Nup62 (1:200), SA1 (1:100), Nup358 (1:500) and/or VP39 (1:500) for 1 hour at 37^{o}C. Secondary antibodies containing fluorophores were used at a 1:1000 dilution for 45 minutes at 37^{o}C, followed by mounting the coverslips with Prolong Gold antifade reagent containing DAPI (Invitrogen). Samples were visualized using an Olympus Fluoview FV1000 laser scanning microscope (Shinjuku, Tokyo, Japan).

2.6.2 Cell permeabilization and *in vitro* nuclear import assay

Adherent HeLa cells were permeabilized at room temperature (RT) with 20 μg/ml digitonin (Sigma-Aldrich) in transport buffer (TB; which contains 20 mM HEPES, pH 7.4, 110 mM potassium acetate, 1 mM EGTA (Ethylene Glycol Tetraacetic Acid), 5 mM sodium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol) for 4 minutes. Permeabilized cells were washed with TB and incubated with TB containing 70 kDa dextran Texas Red (Invitrogen), Cy3-labeled cNLS-BSA, or de-enveloped capsids for 60 minutes at 37°C in the presence or absence of soluble import factors (20% rabbit reticulocyte lysate (RRL), Promega (Madison, WI, USA)) and ATP regenerating system (0.4 mM ATP, 0.45 mM GTP, 4.5 mM phosphocreatine and 18 U/mL phosphocreatine kinase; Sigma-Aldrich) as a source of energy, and Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Grenzacherstrasse, Basel, Switzerland) at 10 μg/mL. In addition, 1.6 mg/mL of BSA was added to prevent nonspecific binding of the transport cargo to the cells. For WGA treatment, 0.5 mg/ml WGA was added for 30 minutes prior to the addition of import substrates. 60 minutes after nuclear import assays, cells were washed with TB three times to stop the reaction, fixed with 3% PFA in PBS, subjected to immunofluorescence staining as described in section 2.6.1 using VP39 antibody (1:500), and coverslips were mounted onto slides with DAPI. Samples were visualized using an Olympus Fluoview FV1000 laser scanning microscope.

To test the necessity of the Ran cycle for nuclear import of baculovirus capsids, 200 μM of GTPγS was added to the import mixture. Similarly, experiments were performed with the importin-β inhibitor importazole. In this case, the permeabilized cells were pretreated with 40-μM of importazole for 1 hour, and 40 μM of importazole was also added to the import mixture.

CK666 and CK689 were used at 1 mM during the pretreatment period of 1 hour and 1 mM was also added to the import mixture.

2.6.3 Cellular detection of Arp2/3 and F-actin

To detect the presence of Arp2/3 present in cells untreated or treated with digitonin, HeLa cells were grown on coverslips, left untreated or treated with 20 μg/mL of digitonin, and washed 3X in TB prior to fixing in 3% PFA in PBS for 10 minutes. The same immunofluorescence assay as described in section 2.6.1 was performed and an antibody against Arp2 was used at a 1:300 dilution. A Zeiss (Oberkochen, Germany) Axioplan 2 fluorescence microscope was used to visualize the overall distribution of Arp2/3.

The abundance of F-actin present in untreated, digitonin- or cytochalasin D-treated cells was detected by immunofluoresence microscopy. For the digitonin treatment, experiments were performed similar to those for Arp2/3 described above. For cytochalasin D treatment, the cells were incubated with 0.5 μM cytochalasin D for 1 hour at 37^{o}C prior to performing the immunofluorescence assay described in section 2.6.1. Fluorescein isothiocyanate (FITC)-phalloidin was used at a 1:100 dilution to detect for F-actin under each condition.

2.6.4 Conjugation of import substrate with fluorophores

BSA covalently attached to the NLS of SV40 T antigen (CGGGPKKKRKVED) at a ratio of 5:1 of NLS:BSA was custom made (Sigma Genosys, Woodlands, TX, USA). NLS-BSA was labelled with Cy3 fluorophore (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacture's protocol. Briefly, NLS-BSA was washed with 4 ml of 0.1 M sodium bicarbonate,

pH 9.3 in a 30K Amicon Ultra-4 filtration device and centrifuged at 3000 x g for 10 minutes. NLS-BSA was further incubated with the Cy3 fluorophore for 1 hour at room temperature, shielded from light. Labelled NLS-BSA was separated from excess unconjugated dye by 4 sequential washes in PBS, pH 7.4, in the Amicon filter. Successfully conjugated Cy3-NLS-BSA was aliquoted and stored at -20^{o}C shielded from light.

2.7 Silencing of nucleoporins

HeLa cells were seeded 24 hours prior to siRNA treatment. For controls, cells were either mock transfected, or transfected with a nonsilencing control (Qiagen, Hilden, Germany) at a final concentration of 10 nM. siRNA against Nup62 (Qiagen) was used at a final concentration of 20 nM using HiPerfect Transfection Reagent (Qiagen) according to the manufactures' instructions. The siRNA targeting Nup62 (5′-GCAACTGCTCCAACCTCAT-3′) was purchased from Qiagen. siRNA against Nup153 was used at a final concentration of 10 nM using lipofectamine RNAiMAX (Invitrogen) according to the manufactures' instructions. The sequence used was purchased from Dharmacon (Lafayette, CO, USA) and corresponds to nucleotide 2593-2615 of human Nup153 (AAGGCAGACUCUACCAAAUGUTT). siRNA against Nup358 (5′-CACAGACAAAGCCGUUGAA-3′) corresponding to nucleotides 351-369 was purchased from Qiagen and used at a final concentration of 25 nM using lipofectamine RNAiMAX (Invitrogen) according to manufactures' instructions. Expression of Nup62, Nup153, and Nup358 was assessed by Western blot and immunofluorescence microscopy two, and three days after transfection. Digitonin-permeabilized HeLa cell import assay and transduction of HeLa cells with baculovirus AcMNPV was performed in Nup depleted cells following protocols described in sections 2.7.2 and 2.5, respectively.

2.8 Biochemistry

2.8.1 Isolation of G- and F-actin

To detect for the abundance of G- and F-actin in digitonin- or cytochalasin D-treated cells, HeLa cells in suspension were pretreated with 20 μg/mL digitonin for 1 minute or 0.5 μM cytochalasin D for 1 hour, and lysed using a G-actin/F-actin *in vivo* assay kit (Cytoskeleton) following manufacture's instruction. Cells were lysed in F-actin stabilization buffer at 30^{o}C for 10 minutes, and cell lysates centrifuged at 100 000 x g for 1 hour at 37^{o}C to separate the F-actin pool from G-actin pool. The resulting F-actin pellet was resuspended with 1 μM cytochalasin D to the same volume as the supernatant, placed on ice for 1 hour with gentle mixing every 15 minutes, and equal amounts of samples were loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel for analysis by Western blotting.

2.8.2 Western Blots

The success of Nup knockdown was detected via Western blots. HeLa cells were grown, transfected with siRNA as described in section 2.8.1, and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% NP-40, 10 mM phenylmethylsulfonyl fluoride (PMSF), 1 μm pepstatin, 10 μg/ml aprotinin, and 2 mg/ml leupeptin (Roche)) on ice for 1 hour. Lysates were cleared by centrifugation at 15 000 x g for 10 minutes at 4^{o}C. The supernatants were mixed with a 5X Laemmli sample buffer and aliquots with equal amounts of protein were loaded on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane, and the success of each knockdown were detected by Western blot using the antibody QE5 (1:500) which

recognizes Nup62 and Nup153, or with other antibodies for Nup358 (1:1000), while using Beta-actin (1:10000) as a loading control. Western blot was also performed to quantify the total amount of G- and F-actin in cells untreated, or treated with either digitonin or cytochalasin D. Soluble and insoluble forms of actin was isolated according to section 2.9.1 and 20 μL of 5X Laemmli Sample buffer was added to each fraction prior to protein boiling and loading on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and the Beta-actin (1:10000) antibody was used to detect for actin.

2.9 Statistical analyses

Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). All data are represented as standard error of the mean (SEM). All comparisons employed two-tailed unpaired Student's t-test. The t-test investigates the likelihood that the difference between the means of the two groups could have been caused by chance. $P<0.01$ was used as a threshold of significance. All data sets represent results of at least 200 cells from 3 separate experiments.

Chapter 3

Mechanism of AcMNPV Nuclear Import[6]

3.1 Introduction

Initial studies of the nuclear import of baculovirus have been rather confusing and have generated some apparent contradictions in the literature (discussed in section 1.4.5). This is in part due to the fact that different studies used different baculovirus genera, which as explained above have obvious structural differences (described in section 1.4.3). In addition, variations among experimental techniques used in these studies, the viral phenotype used (e.g. ODV or BV) and the type of experimental host used (e.g. larvae, insect cells or mammalian cells in culture) contributed to the different findings.

In this chapter, we used capsids of the insect virus baculovirus AcMNPV to characterize its mechanism of nuclear import. We first confirmed the ability of AcMNPV to transduce mammalian cells and traced its mode of cellular entry leading up to nuclear import of the viral capsid in these cells by EM. We also used the non-dividing *Xenopus laevis* oocytes, containing abundant NPCs, for microinjection studies followed by EM of thin-sections to visualize whether the AcMNPV capsid enters the nucleus through the NPC. Microinjection of *Xenopus laevis* oocytes is a good system for studying nuclear transport because a mature oocyte contains numerous NPCs and both the NE and NPCs are well preserved in EM studies. As described in

[6] A version of part of this chapter has been published: Au, S., and Panté, N. 2012. Nuclear transport of baculovirus: Revealing the nuclear pore complex passage. *Journal of Structural Biology* 177: 90-98.

section 1.3.4, intact HBV capsids are seen to cross the NPC central channel in *Xenopus laevis* oocytes using this microinjection system (Rabe et al., 2003b). This suggests that microinjection of oocytes with viral capsids followed by processing of oocytes for TEM is a viable assay to visualize nuclear import processes. Electron tomography was also used to provide enhanced structural information about the NPC, especially during translocation of the large baculovirus capsid. In addition, we also performed microinjection experiment under conditions that would inhibit nuclear import of the capsid to determine the necessity of NPCs for nuclear entry. With the combination of all these experimental strategies, we found that the intact AcMNPV capsid (dimensions ~30 to 300-nm) enters the nucleus through the NPC. Our results demonstrate that the NPC must undergo big structural changes to accomodate the transport of this large capsid, which indicates that the NPC is a very flexible structure.

3.2 Results

3.2.1 Baculovirus capsids enter nuclei of HeLa cells

In order to determine the route of nuclear entry for baculovirus AcMNPV, HeLa cells were mock transduced or transduced with AcMNPV BVs (as described in section 2.5), and processed for embedding thin sectioning TEM (as described in section 2.5.4) to visualize cellular entry leading to nuclear transport.

Although transduction was synchronized, capsids were found at various subcellular locations 6 hours post-transduction (Figure 3-1). Virions were seen at the periphery of the cell or being engulfed within plasma membrane protrusions that lead to macropinocytosis (Figures 3-1, D and E). Single virions were found in vesicles that resemble endosomes as well as multiple virions in

what appears to be macropinosomes (Figures 3-1F and 3-2A). At this time, some virions were seen in compartments juxtapose to the nucleus, presumably late endosomes or lysosomes (Figure 3-2B), while de-enveloped capsids were seen docked at the NPC (Figure 3-2C). This indicates that cytoplasmically released capsids were able to travel towards the nucleus. Intact capsids were also found inside the nucleus, presumably where capsid uncoating occurs (Figures 3-2, D-F). Our EM analysis of HeLa cells transduced with AcMNPV confirms previous published data that AcMNPV is able to enter HeLa cells (van Loo et al., 2001, Shoji et al., 1997, Hofmann et al., 1995). In addition, we show here that AcMNPV capsids are able to enter the nuclei of a human cell line.

3.2.2 Monitoring nuclear import of AcMNPV capsids in *Xenopus laevis* oocytes

Xenopus laevis oocytes were used to maximize the chances of visualizing nuclear import through NPCs. A mature oocyte contains $5x10^7$ NPCs (~60 NPCs/μm) while a human cell has only 2000-5000 NPCs (10-20 NPCs/μm) (Grossman et al., 2012). To mimic the state of the virion during normal infection in insect cells or transduction in mammalian cells, where capsids released from endosomes are devoid of an envelope, we microinjected de-enveloped capsids into oocytes. This highlights additional advantages of the oocyte microinjection system in that the oocyte does not have to deal with the entry of the virus into the cell (because it is microinjected), nor with the removal of any envelope (since we are microinjecting purified virions). These capsids were purified from *Sf*9 cells infected with WT E2 strain of AcMNPV (as described in sections 2.2.2 and 2.2.4) and examined by EM after negative staining with uranyl acetate to evaluate the purity and integrity of the capsids (as described in section 2.5.1).

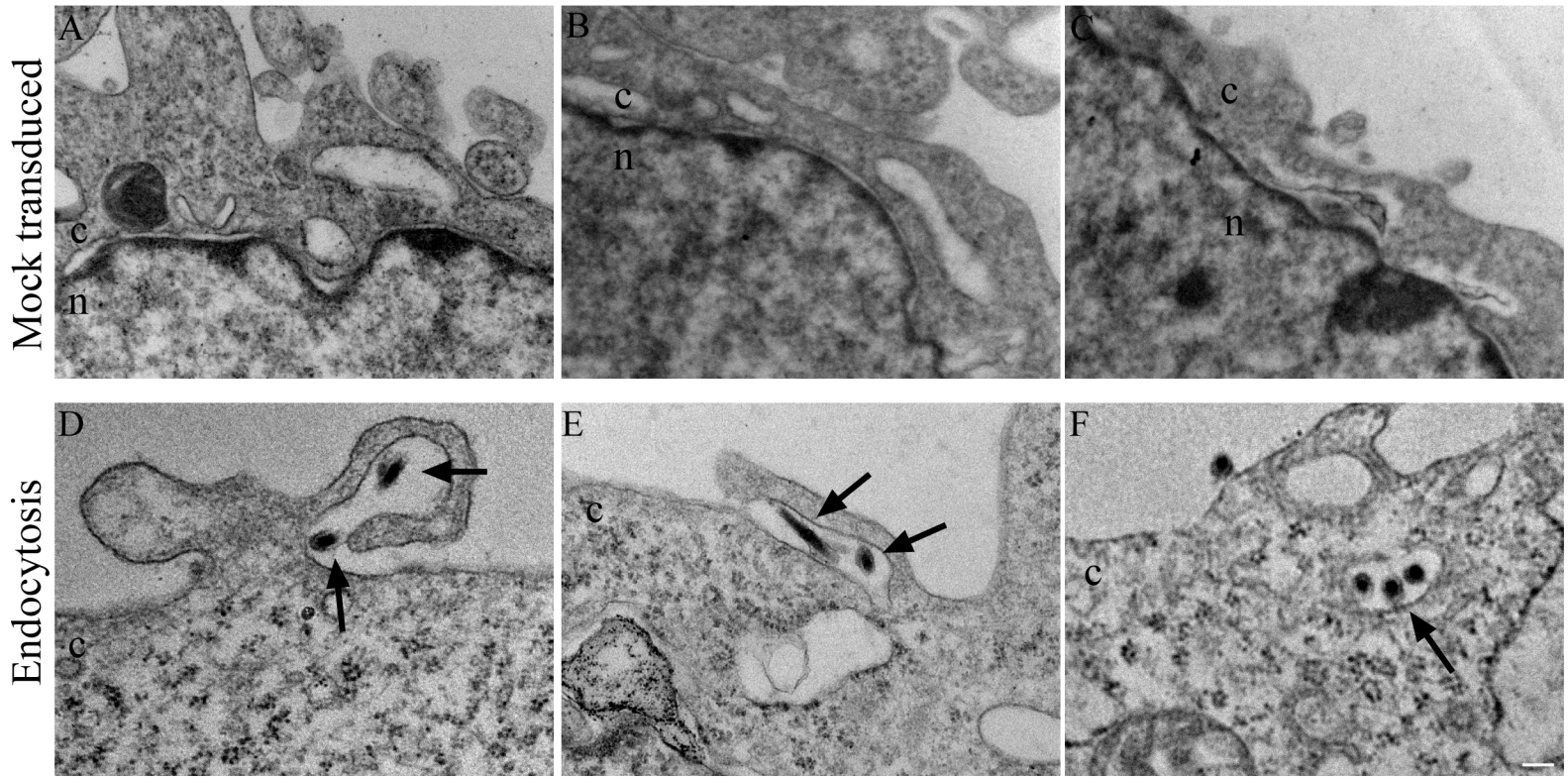

Figure 3-1: Baculovirus AcMNPV transduction in HeLa cells. (A-C) HeLa cells mock transduced for 6 hours. (D-E) Selected micrographs showing AcMNPV capsids entering HeLa cells by macropinocytosis after the cells have been transduced with AcMNPV for 6 hours. (F) After viral entry into the cell, several capsids were found within large vesicles with the appearance of macropinosomes. Scale bar, 100 nm. n, nucleus; c, cytoplasm. Arrows point to capsids.

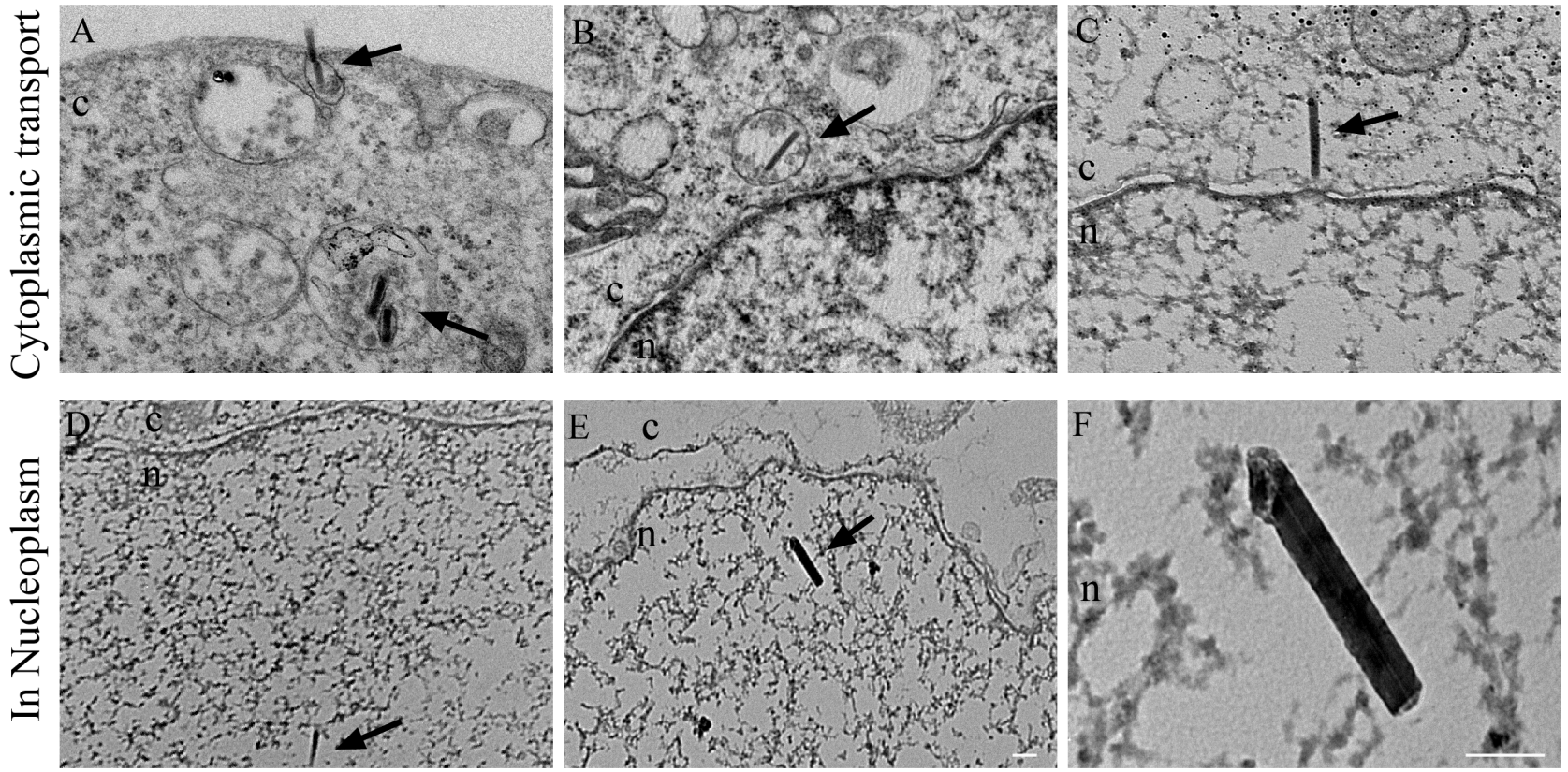

Figure 3-2: AcMNPV capsids enter nuclei of HeLa cells. Selected micrographs showing that after transduction of HeLa cells with AcMNPV for 6 hours, baculovirus virions were endocytosed and transported towards the nucleus of HeLa cells. Some virions remained in late endosomes or lysosomes (A and B). De-enveloped capsids were seen docked at the NPC prior to nuclear entry (C). Intact capsids were found inside the nucleus (D and E). The capsid in figure E is enlarged in panel F. Scale bars, 100 nm. n, nucleus; c, cytoplasm. Arrows point to capsids.

As documented in Figure 3-3A, the purification of the capsid by our protocol was effective, yielding typical rod-shaped capsids that had a diameter of ~30-nm and variable length of 250 to 300-nm. In this preparation, the virus was completely devoid of the envelope and the capsids appeared electron dense, which is an indication that they contain the viral genetic material. These capsids had the expected morphology with two distinct ends: one end blunt and one end conical with a small protuberance (arrows in Figure 3-3B).

We then microinjected these capsids into the cytoplasm of *Xenopus laevis* oocytes and followed their fate by EM after the injected oocytes were embedded in Epon and thin sectioned (as described in section 2.5.2). To allow observation of the large capsids at NPCs, oocytes were incubated at room temperature at different times. Our time-course experiment showed that at two hours post-microinjection, about a quarter of the capsids were in the cytoplasm, away from the NE (Figures 3-4, A and E), while the remaining capsids were already at the NPC (Figures 3-4, B and E). After four hours, however, almost all of the capsids were seen docked at the NPC or very close to the NPC (within a distance of 100-nm from the NPC; Figure 3-4, C and F). In the micrographs showing capsids at the NPC, the capsids were interacting vertically with the NPC, and in most of the micrographs we were able to distinguish the conical end of the capsid at the NPC and the blunt end away from the NPC. Some capsids were also found inside the nucleus after four hours post-microinjection (Figure 3-4D); however, capsids devoid of DNA (empty capsids, which are not electron dense) or disassembled capsids were not observed. Eight hours post microinjection was a sufficient amount of time for capsids to enter the nucleus, as both the cytoplasm and nucleus were capsid-free (data not shown). This also suggests that by eight hours,

capsid disassembly has occurred and the DNA genome has been released into the nucleus. Unfortunately, we were unable to detect the order in which these events occurred.

3.2.3 Cellular transport of capsids is delayed at low temperature

Biochemical and physiological inhibitors of nuclear transport have been used extensively to arrest imported molecules at intermediate stages of its passage into the nucleus. Accumulation of cargos at the NPC cytoplasmic filaments and at the cytoplasmic entrance of the NPC central channel have been observed by EM when nuclear import is inhibited at 4°C (Pante and Aebi, 1996; Rollenhagen et al., 2003; Rollenhagen and Pante, 2006; Pante, 2007). Low temperature inhibits the translocation of cargo through the NPC but does not inhibit its initial docking at the NPC. It is surmised that this condition yielded an increased amount of capsids at the cytoplasmic face of the NPC; however, we found that in oocytes incubated for two hours at 4°C post-microinjection, 88% of the capsids remained in the cytoplasm far from the nucleus (Figure 3-5, A and C), in contrast to the 23% observed when oocytes were incubated for two hours at room temperature (Figure 3-4, A and E). Similarly, significantly more capsids were found docked at the NPC after four hours incubation at room temperature (Figure 3-4, D and F) than after four hours incubation at 4°C (Figure 3-5, B and D). The delayed progress of capsids transiting towards the nucleus suggests that metabolic energy is the driving force that allows the capsid to move within the cytoplasm. Our data is in agreement with the previous findings that actin-based motility drives the capsid towards the nucleus (Charlton and Volkman, 1993; Ohkawa and Volkman, 1999; Ohkawa et al., 2010).

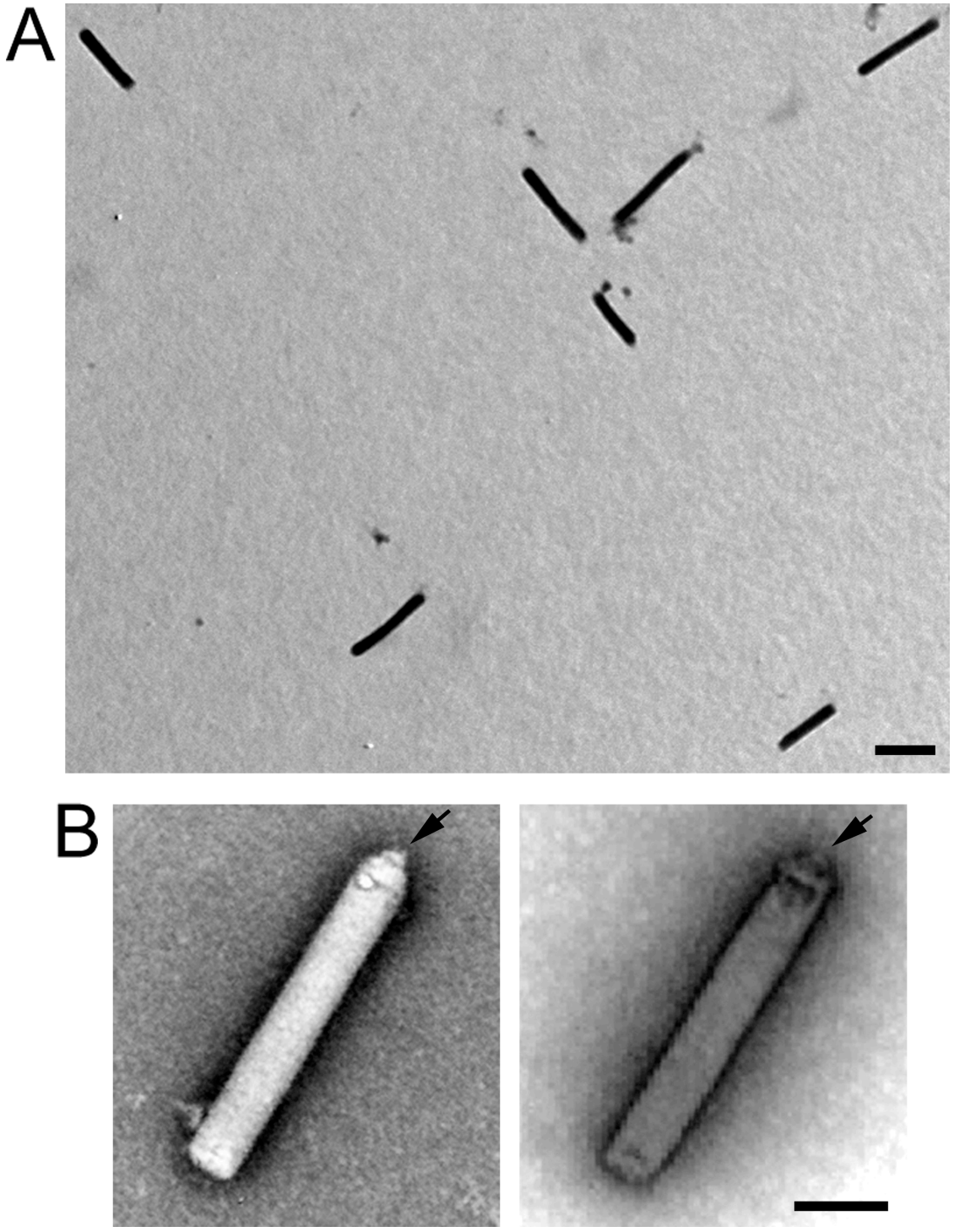

Figure 3-3: Electron micrograph of purified AcMNPV capsids negatively stained with uranyl acetate. The micrograph in (A) documents that the purified capsids were variable in length. The micrographs in (B) document the morphology of the capsid with its two distinct ends; a blunt end and a conical end with a small protuberance (arrows). Scale bars, 200 nm in (A) and 50 nm in (B).

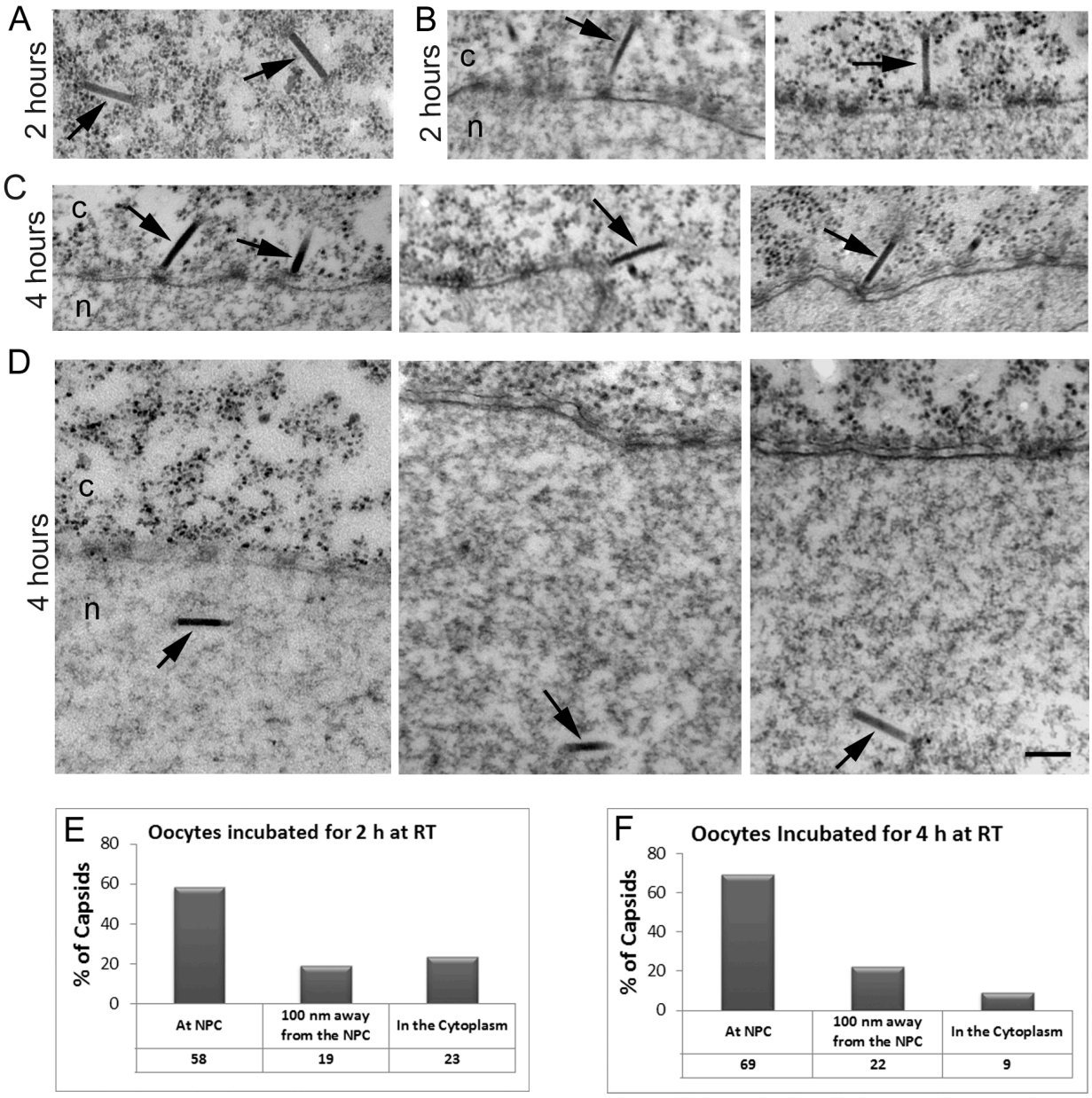

Figure 3-4: *Xenopus laevis* oocytes microinjected with baculovirus capsids and incubated at room temperature. Oocytes were incubated for 2 (A and B) or 4 h (C and D) at RT. Bar graphs (E and F) show the percentage of capsids found associated with the NPC, 100-nm away from the NPC, and in the cytoplasm from experiments performed as indicated above. A total of 150 capsids were scored for each condition from three different experiments. Capsids were found in the cytoplasm (A), at the NPC or at 100-nm from the NPC by 2 hours post-microinjection (B). Most capsids were docked at the NPC (C) by 4 hours post-microinjection, and some were inside the nucleus (D) by this time. Scale bar, 200-nm. n, nucleus; c, cytoplasm. Arrows point to capsids.

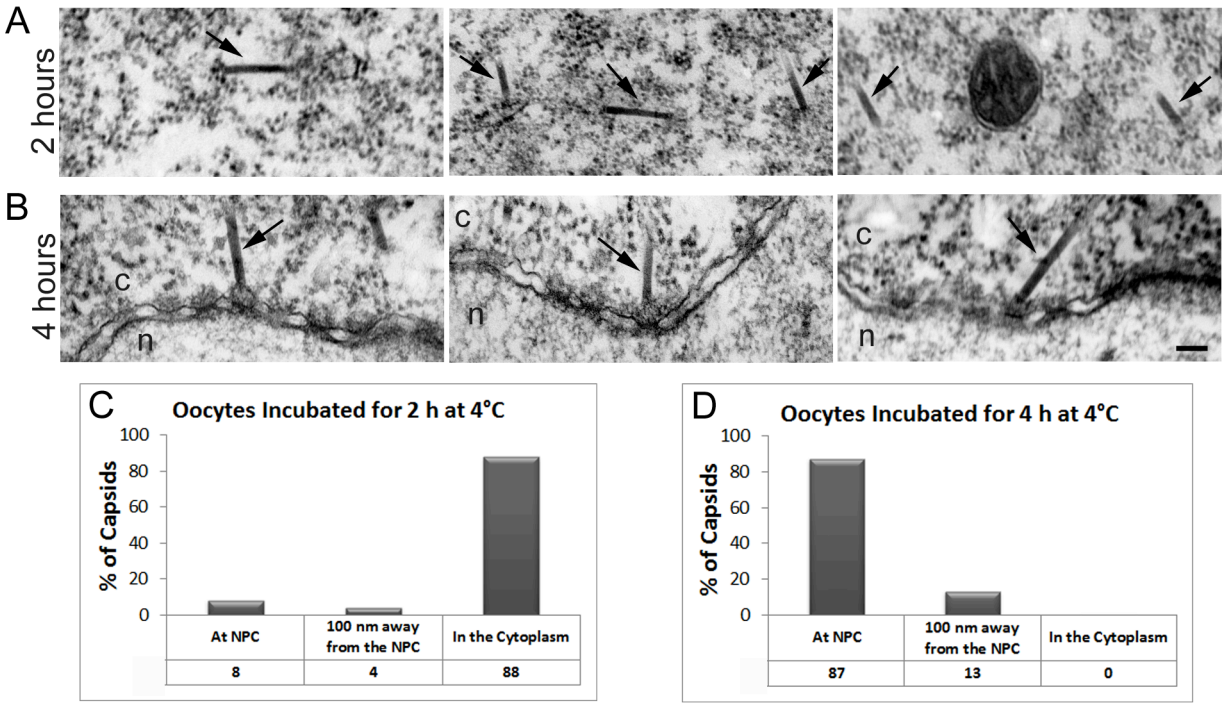

Figure 3-5: *Xenopus laevis* oocytes microinjected with baculovirus capsids and incubated at 4°C. Oocytes were incubated for 2 hours (A) or 4 hours (B) at 4°C. Bar graphs (C and D) show the percentage of capsids found associated with the NPC, 100-nm away from the NPC, and in the cytoplasm from experiments performed as indicated above. A total of 150 capsids were scored for each condition from three different experiments. Most capsids were found in the cytoplasm at 2 hours post-microinjection (A), while the majority of capsids were docked at the NPC at 4 hours post-microinjection (B). No capsids were found inside the nucleus under these conditions. Scale bar, 200-nm. n, nucleus; c,cytoplasm. Arrows point to capsids.

3.2.4 Capsids remain intact while vertically traversing the NPC

Electron micrographs from oocytes that were incubated for 3.5 hours post microinjection at room temperature showed the capsid vertically traversing the NPC and some of them midway through the NPC (Figure 3-6). Remarkably, the NPCs containing capsids in transit appeared less electron-dense than neighbouring NPCs not engaged in capsid nuclear transport. In particular, an area of 8 to 10-nm in diameter surrounding the capsid appeared completely empty, as if all the material normally filling the NPC central channel had retracted, presumably to allow movement of the capsid across the NPC.

In order to compensate for the fact that thin sections of 50-nm through the NE may not be representative of the NPC as a whole, we obtained thicker sections of 200-nm to generate tomograms of capsids in the midst of being imported into the nucleus through the NPCs. EM tomograms showed that both the capsid and NPC remain intact during this translocation event (Figure 3-7). Similarly, an unfilled area around the capsid while traveling through the central channel can be seen in Figure 3-7. Via the tomographic reconstruction 3-D view, we observed intact capsids in transit through the NPCs, documenting that, in fact, the intact capsid crosses the NPC (Figure 3-8). Likewise, the NPC was seen to wrap around the capsid, further demonstrating the movement of the capsid within the central channel of the NPC.

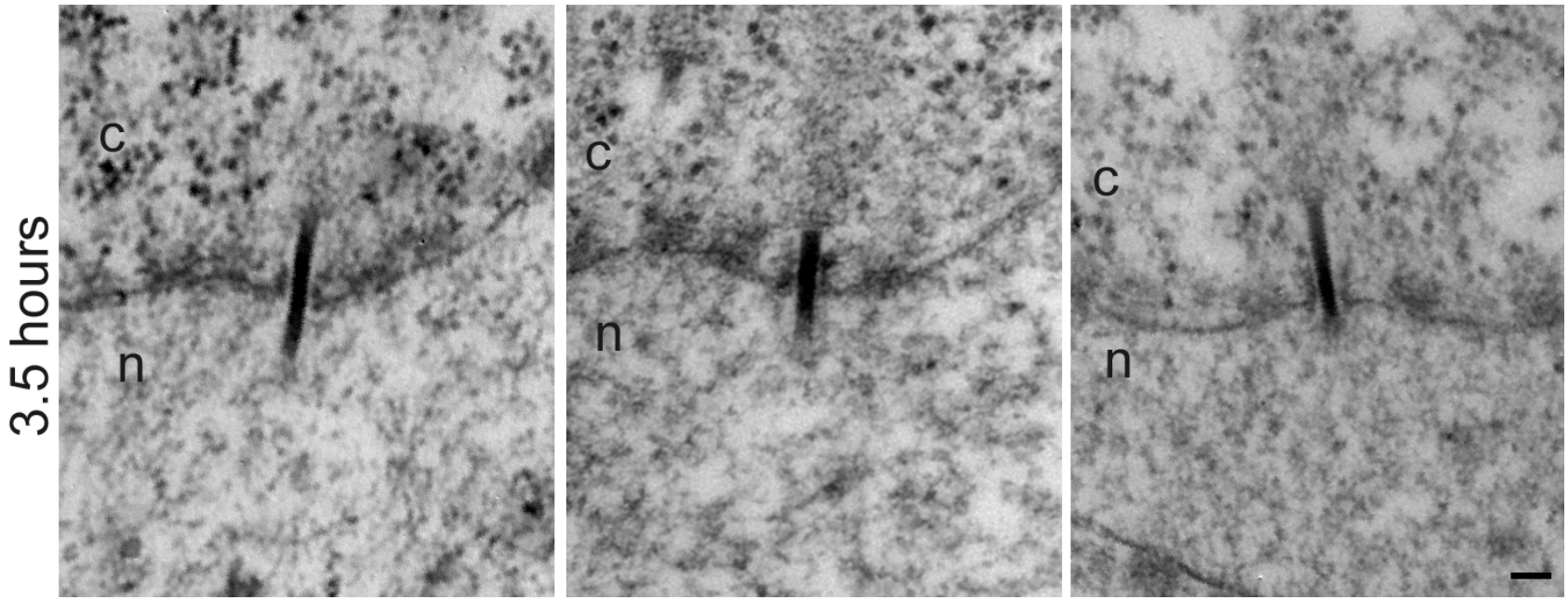

Figure 3-6: Intact AcMNPV capsids traverse the NPC. Electron micrographs of NPC cross-sections from *Xenopus laevis* oocytes that have been microinjected with baculovirus AcMNPV capsid and incubated at room temperature for 3.5 hours. Capsids of 250 to 300-nm in length are seen traversing the NPCs. Capsids appear fully intact in its native conformation while crossing the NPC. Note the capsid in the middle panel appears shorter due to the variability in the length of these capsids, as documented in Figure 3-3. Scale bar, 100-nm. n, nucleus; c, cytoplasm.

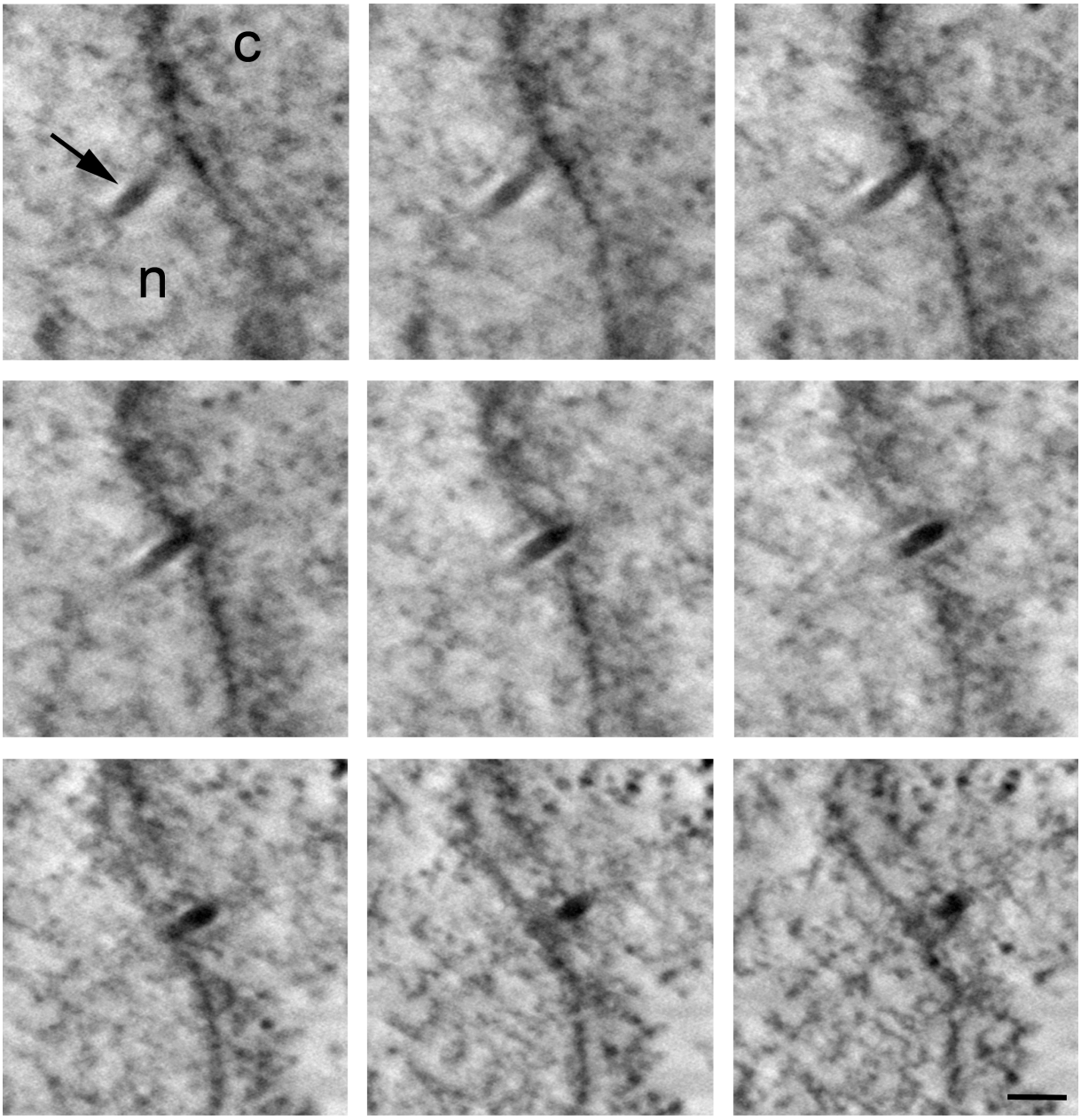

Figure 3-7: Tomographic x-y slices of an intact AcMNPV capsid being transported through the NPC. These slices are spaced approximately 20-nm through the 3D volume of the tomographic tilt series of a capsid in the midst of being imported into the nucleus through the NPCs. Scale bar, 200-nm. n, nucleus; c, cytoplasm. Arrow points to the capsids.

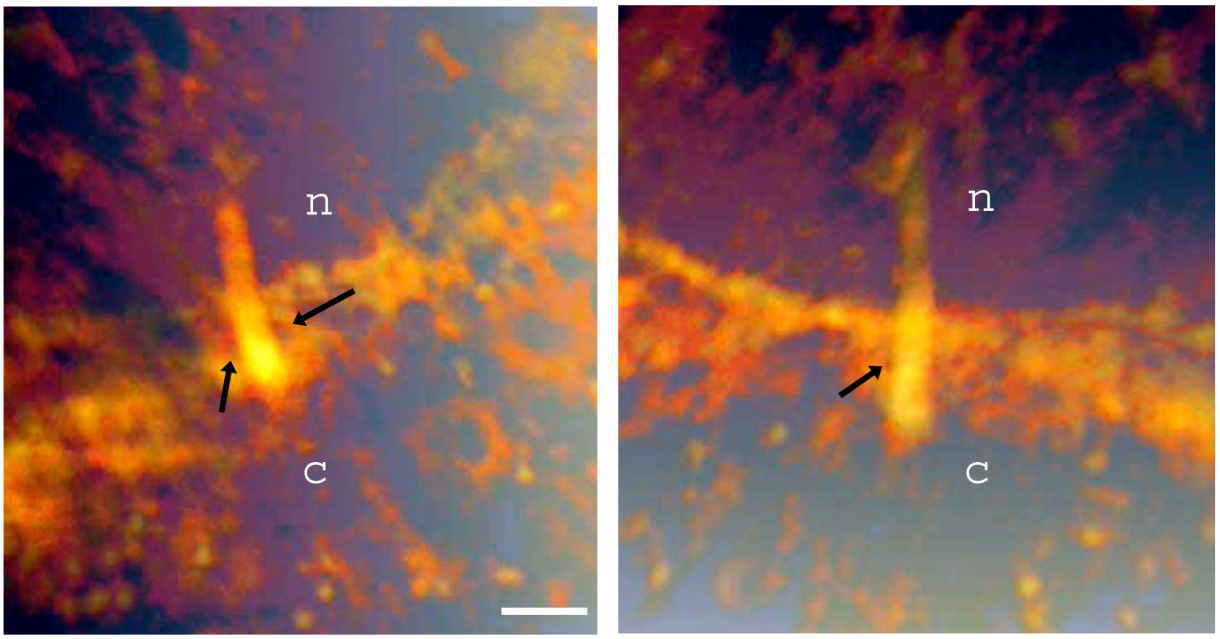

Figure 3-8: Tomographic reconstruction of an intact capsid traversing the NPC. A capsid of ~250-nm can be seen going through the central channel of the NPC, leaving a spacer on each side of the capsid (black arrow). Scale bar, 200-nm. n, nucleus; c, cytoplasm.

3.2.5 Initial docking of the capsid occurs at the cytoplasmic filaments of NPCs

The low temperature experiments also demonstrated that the NPC cytoplasmic filaments act as the first binding sites for the capsid prior to capsid translocation through the NPC. Oocytes that were incubated for four hours at 4°C yielded 87% of capsid at the cytoplasmic face of the NPC at about 100-nm from the center of the NPC, and in most of the micrographs the cytoplasmic filaments were clearly depicted (Figure 3-5). In most of the micrographs, we were also able to visualize the conical end of the capsid at the NPC, and the blunt end of the capsid away from the NPC (see for example, Figure 3-5 left panel).

To confirm our results of the initial binding of the capsid to the NPC cytoplasmic filaments under conditions that do not delay the targeting of the capsid to the NPC, we used WGA, a well characterized inhibitor of nuclear import that binds O-linked N-acetyl glucosamine residues on glycosylated Nups, thereby blocking the interaction between nuclear transport receptors and Nups and inhibiting nuclear transport (Finlay et al., 1987; Newmeyer and Forbes, 1988). For these experiments, we conjugated 10-nm gold particles to WGA (to visualize the binding of WGA to the NPC) and the WGA-gold complexes were pre-microinjected into the cytoplasm of the oocytes. After two hours of incubation at room temperature, the oocytes were again microinjected into their cytoplasm with capsids and incubated for eight hours at room temperature. After eight hours, WGA-gold particles had accumulated at the entrance of the NPC central channel, blocking the translocation of the capsid through the NPC, and the capsids remained at the NPC cytoplasmic face at a distance of about 100 nm from the centre of the NPC (Figure 3-9).

We also attempted to block the NPC from its nuclear side by pre-microinjecting WGA-gold into the nucleus of the oocytes. Similar to the cytoplasmic injection, nuclear injected oocytes were incubated for two hours, post-microinjected with capsids, and further incubated for eight hours at room temperature. Under these conditions, the WGA-gold particles were within the NPC central channel, while the capsid remained on the cytoplasmic face of the NPC (Figure 3-9). Furthermore, at eight hours post-microinjection we observed capsids in the cytoplasm when the NPCs were blocked by WGA, an occurrence that was not observed when NPCs were uninhibited. Our data demonstrates that the NPC cytoplasmic filaments are the initial docking sites for the capsids**.**

3.3 Discussion

With a diameter of ~30-nm, the baculovirus capsid is small enough to cross the NPC without apparent deformation; however, direct demonstration of the actual translocation of the capsid through the NPC has not been previously reported. In baculovirus transduced mammalian cells, we observed intact capsids in both the cytoplasm and nucleus. Similar to previous studies using NPVs, AcMNPV capsids are able to enter the nucleus fully intact (Bassemir et al., 1983; Carstens et al., 1979; Granados, 1978; Granados et al., 1981; Knudson and Harrap, 1976, Hirumi et al., 1975). Upon microinjection of baculovirus capsids into the *Xenopus laevis* oocyte cytoplasm and analysis of the oocytes by EM, we observed capsids docking at the cytoplasmic side of the NPC in what appears to be NPC cytoplasmic filaments (Figures 3-4, B and C, and 3-5 B). Capsid interaction with the NPC appears to be with the conical end, and not with the blunt end.

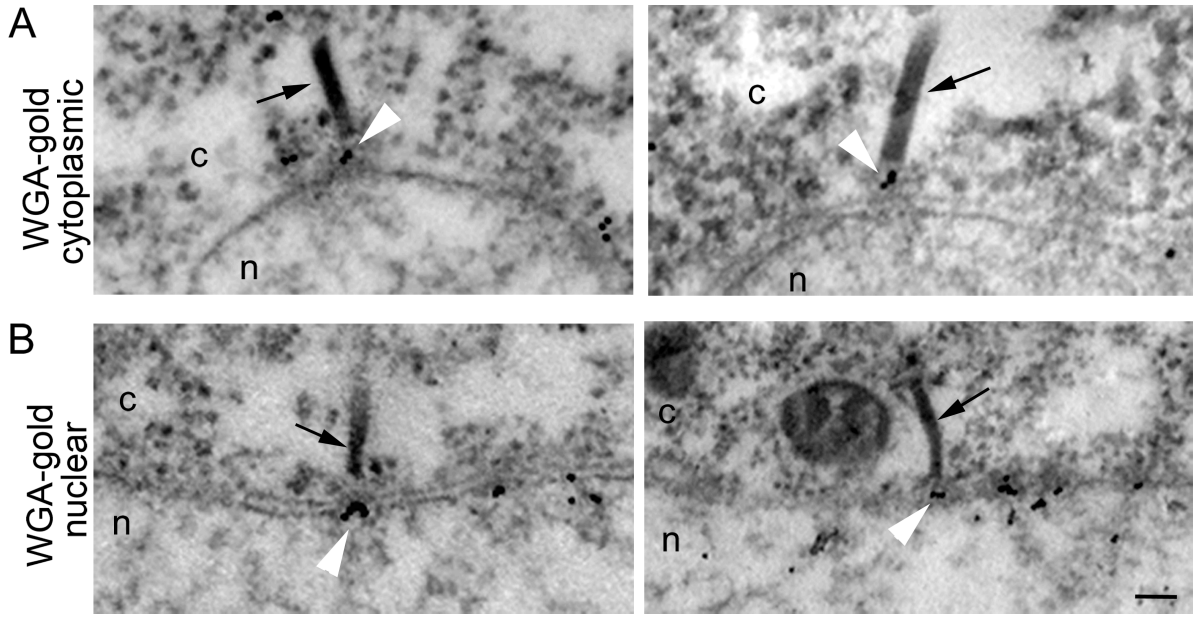

Figure 3-9: WGA blocked nuclear import of baculovirus capsids. Electron micrograph of *Xenopus laevis* oocytes that were microinjected with WGA-gold into either the cytoplasm (A) or nucleus (B) incubated at room temperature for 2 hours, followed by cytoplasmic injection of baculovirus AcMNPV capsids and further incubated for 8 hours. When NPCs were inhibited by WGA-gold particles, capsids remained interacting with the NPC cytoplasmic filaments 8 hours post-microinjection. No capsids were found inside the nucleus when NPCs were inhibited with WGA. Scale bar, 200-nm. n, nucleus; c, cytoplasm. Black arrows point to capsids and white arrowheads point to WGA-gold particles at NPCs.

In addition, we also observed capsids in the nucleus of the injected oocytes (Figure 3-4D). As baculoviruses do not replicate in *Xenopus laevis* oocytes, the capsids found inside the nucleus must have been imported from the cytoplasm through the NPC. Consistent with this explanation, we depicted the capsid midway through the NPC (Figure 3-6), and demonstrated by electron tomography (Figures 3-7 and 3-8) that the capsid crosses the NPC intact. Thus, our data supports a nuclear entry mode for AcMNPV from the Alphabaculoviruses group I NPV that involves translocation of the intact capsid through the NPC, similar to a DNA virus of similar diameter, the HBV capsid (Pante and Kann, 2002; Rabe et al., 2003a).

The delayed progress of capsids transiting through the cytoplasm towards NPCs when oocytes were incubated at 4°C suggests that active transport within the cytoplasm was also hindered. VP78/83 capsid protein of AcMNPV has been shown to associate with actin-like structures in the cytoplasm (Charlton and Volkman, 1993). More recently, it was demonstrated that when the Arp2/3 complex binding region in VP78/83 was mutated, viral motility of the capsid within the cytoplasm, as well as viral gene expression was delayed (Ohkawa et al., 2010). Therefore, incubating oocytes at low temperature could have impeded active transport of the capsid via actin within the cytoplasm.

The NPC cytoplasmic filaments are the initial docking sites for several molecules undergoing nuclear import. Using WGA as an inhibitor of nuclear import, by both cytoplasmic and nuclear injection of this inhibitor into *Xenopus laevis* oocytes, we demonstrated that capsids remained at the NPC cytoplasmic filaments when WGA-gold particles impeded transport through the NPC central channel (Figure 3-9). Capsids were also observed at the NPC cytoplasmic filaments when the oocytes were incubated at 4°C (Figure 3-5). This finding is in contrast to studies using 14-nm

nucleoplasmin-conjugated gold (Pante and Aebi, 1996), in which gold particles were documented to locate at the central channel of the NPC when microinjected into *Xenopus laevis* oocytes at 4°C. This difference may be due to the size of the cargo, and supports the idea that different mechanisms of nuclear entry exist and size of the cargo could be the main determinant. Consistent with this, splicesomal RNPs conjugated with gold particles were observed at the NPC cytoplasmic filaments (like the baculovirus capsid) and not at the NPC central channel (reviewed in Rollenhagen and Pante, 2006). This spatial difference could also be the result of a large cargo, such as the viral capsid or the splicesomal RNPs with discrete sites for nuclear transport receptor binding, as opposed to a single gold particle containing numerous copies of the same small protein and multiple sites for nuclear transport receptor binding.

Noticeably NPCs not engaged in capsid nuclear transport or those with capsids docked to their cytoplasmic filaments appear electron-dense (Figure 3-4C), as if nucleoporins residing within this gate prohibits the passage of the capsid. Moreover, such an unlocked mechanism seems to completely open up the NPC central gate so that an apparent space of about 10-nm was detected around the capsids in the electron micrographs and electron tomograms of capsids that were in the middle of the NPC. Even the NPC cytoplasmic filaments, which usually have a kinky appearance in the electron micrographs (Pante and Aebi, 1996), appear straightened out (compare the short filaments emanating from the NPC into the cytoplasm in Figures 3-6 right panel and 3-5B). It appears that the NPC cytoplasmic filaments and nucleoporins residing in the NPC central channel re-localize to clear out the entire passageway for the rod-shaped capsid to traverse the NPC and enter the nucleus.

In summary, our findings indicate for the very first time that the baculovirus capsid, ~30 x 300-nm in size, is able to enter the nucleus fully intact through the NPC and changes of the NPC central channel accommodate this nuclear entry event by dynamically relocalizing Nups within this gated channel.

Chapter 4

Cellular Proteins Essential in Mediating Nuclear Import of AcMNPV

4.1 Introduction

In Chapter 3, we found that the intact AcMNPV capsid crosses the NPC for nuclear import, releasing viral capsids into the nucleus. Most large cargoes undergo facilitated nuclear import that requires cytosolic proteins such as importin-β and Ran. As the baculovirus falls into the category of facilitated nuclear transport, we next investigated the necessity of cellular proteins that allow for this event to occur. Due to the size of the baculovirus capsid, we hypothesize that nuclear import receptors are necessary in mediating capsid entry into the nucleus. This chapter describes an investigation using the well-established digitonin-permabilized cell import assay (Adam et al., 1990) to determine cytosolic proteins that may be involved in nuclear import of the AcMNPV capsid. This system allows us to manipulate cytosolic proteins that may be required for nuclear transport of the viral capsid, leaving the structure of the nucleus fully intact and functional. Because a functional NLS has not been elucidated in the baculovirus capsid, we wish to determine if it uses the classical nuclear import pathway whereby importin-β and RanGTP mediates nuclear import of the capsid. Alternatively, it may use a Ran-independent mechanism.

In addition to soluble proteins within the cell, the cellular cytoskeleton acts as a trafficking support for viral movement within the cell. Most commonly, microtubules are often used as tracks for viruses like HSV and adenovirus, to move towards the nucleus. Filamentous actin (F-

actin) on the other hand is formed from globular actin (G-actin) monomers, and generally acts to direct cytoplasmic transport of organelles and secretory vesicles via actin nucleation or by motor proteins, myosin V and VII (Mooseker and Cheney, 1995; Pantaloni et al., 2001), rarely exploited by viruses for cellular transport. For baculovirus, actin nucleation was previously shown to occur upon capsid release from endosomes from the VP78/83 protein located at one end of the capsid, and is involved in cytoplasmic trafficking of the viral capsid towards the nucleus (Charlton and Volkman, 1993; Goley et al., 2006; Ohkawa et al., 2010). We therefore hypothesize that cellular actin plays a crucial role during capsid entry into the nucleus. In this chapter we also aim to determine the role of cellular F-actin and actin nucleation in mediating nuclear import of the AcMNPV capsid by performing experiments with inhibitors of the actin cytoskeleton and Arp2/3 complex.

4.2 Results

4.2.1 Baculovirus capsid follows an unconventional nuclear import mechanism and does not require an energy regenerating source

In order to determine the cytosolic components necessary for the nuclear import of AcMNPV capsids, we used the well-established digitonin-permeabilized cell system (Adam et al., 1990), that is commonly used in the field of nuclear transport, which allows me to control various components to be added to cells. In this system, once the cell membrane is permeabilized with digitonin, contents in the cytoplasm are washed away leaving the nucleus and nuclear envelope intact. we first optimized this protocol for HeLa cells by trying different concentrations and incubation times of digitonin. The success of the protocol was evaluated by incubating the cells with a 70 kDa Dextran Texas Red that does not diffuse into the nucleus (Figure 4-1A). We then

incubated permeabilized cells with or without a cytosolic extract (rabbit reticulocyte lysate (RRL)), an energy-regenerating system (E), and AcMNPV capsids (purified as described in section 2.2.4). As shown in Figure 4-1B (bottom), viral capsids detected using an antibody against the VP39 capsid protein (which are visualized as distinct fluorescent red dots) were seen inside the nucleus of HeLa cells when the import assay was performed with or without the cytosolic extract and the energy-regenerating system. Upon quantification, there was no observable difference in the import efficiency under permissive and non-permissive conditions of nuclear import (Figure 4-1C). To verify that these cells allowed active transport of molecules into the nucleus under permissive conditions (presence of cytosolic factors and energy) but prohibited nuclear import in the absence of all cytosolic factors, we performed a control experiment using NLS-BSA conjugated with Cy3, which as expected, remained in the cytoplasm in the absence of rabbit reticulolysate (RRL) and energy but was able to enter the nucleus when both factors were included (Figure 4-1B, top). In addition, nuclear import of Cy3-NLS-BSA occurred in 100% of the cells treated with digitonin, as oppose to ~50% for capsid nuclear import (Figure 4-1C), which exemplifies the inefficiency of nuclear import of the baculovirus capsid in digitonin permeabilized HeLa cells.

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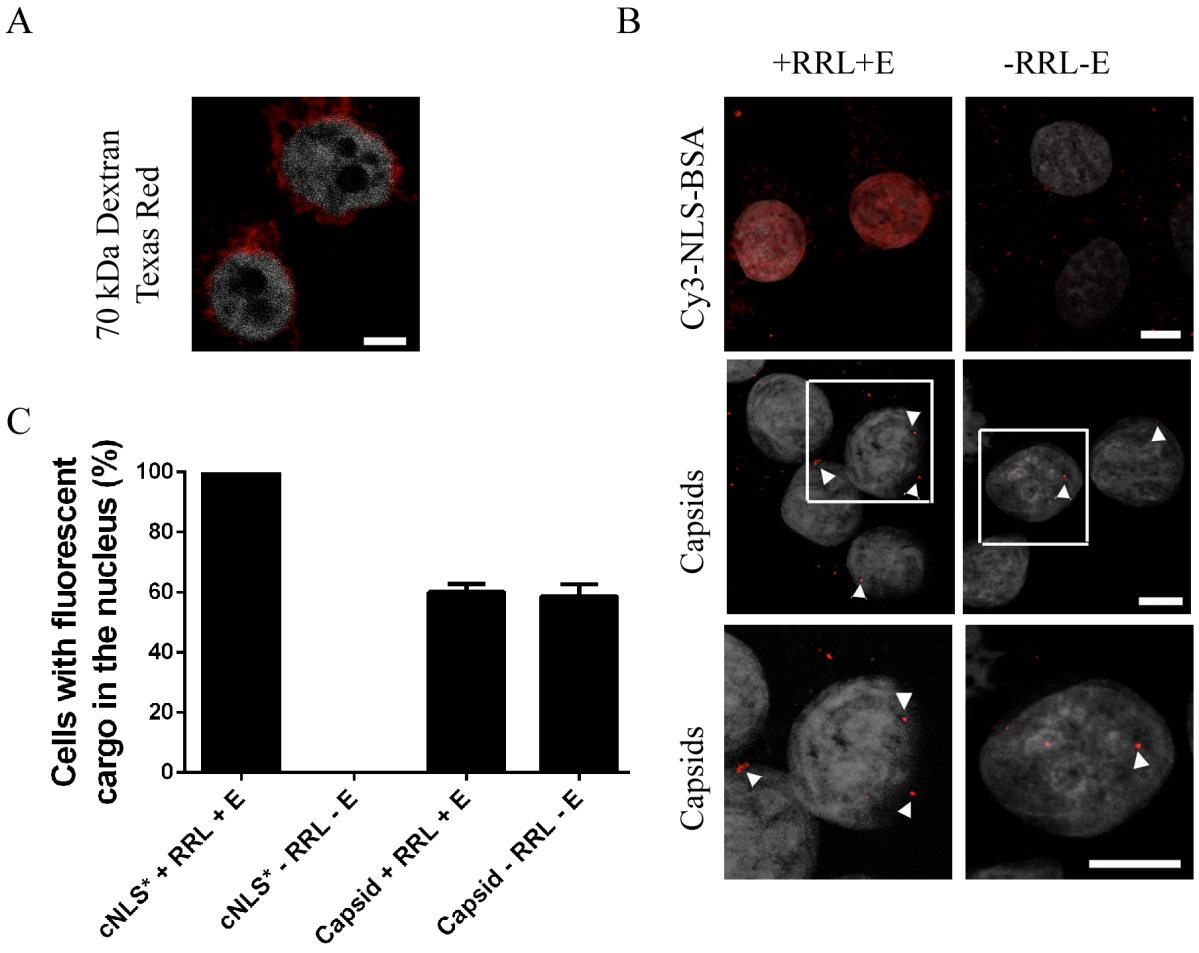


Figure 4-1: Nuclear import of baculovirus capsids occurs in the absence of cytosolic factors and an energy source in digitonin permeabilized HeLa cells. Digitonin-permeabilized HeLa cells were incubated with (A) 70 kDa Dextran fluorescently-labeled with Texas Red, (B) Cy3-labeled BSA carrying a classical NLS (Cy3-NLS-BSA) or baculovirus capsids in the presence or absence of cytosolic factors of rabbit reticulolysate (RRL) and an energy regenerating system (E). Portions or regions of cells within the white boxed areas are magnified in the lower panels. (C) Quantification of the number of cells with fluorescent cargo in the nucleus for all conditions. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-µm. DAPI, grey; Dextran (A), Cy3-NLS-BSA or capsids (B), red. White arrowheads point to capsids.

4.2.2 Nuclear entry of the baculovirus capsid in the absence of soluble factors is not due to disruption of the nuclear envelope

Viruses have been shown to enter the nucleus through NE disruptions as demonstrated with parvovirus MVM (Cohen et al., 2006; Cohen et al., 2012; Cohen et al., 2011; Cohen and Pante, 2005) and SV40 (Butin-Israeli et al., 2011). To validate the ability of AcMNPV capsids to translocate into the nucleus in the absence of cytosolic factors and an energy regenerating system, we used WGA to block the NPCs and thereby impede nuclear import of the capsid via NPCs in digitonin-permeabilized HeLa cells depleted of essential nuclear import factors. Under this condition, viral nuclecapsids were seen to remain in the cytoplasm (Figure 4-2B, right panel), suggesting that the mode of nuclear entry of viral capsids seen in Figure 4-1B was not the result of NE damages that may have occurred during sample processing or breakages caused by the viral capsid. Cy3-NLS-BSA was further used as a control (Figure 4-2A) to verify that NPCs were sufficiently blocked by WGA as Cy3-NLS-BSA was seen to reside in the cytoplasm when all essential nuclear import factors were present.

4.2.3 Inhibitor of importin-β-mediated nuclear entry did not affect nuclear import efficiency of baculovirus capsids

To further confirm the ability of AcMNPV capsids to enter the nucleus following a non-classical mechanism, we further pursued to determine more specifically if importin-β receptors help mediate nuclear import of the baculovirus capsid. Therefore we used an inhibitor of importin-β mediated import in two experimental systems: (1) digitonin-permeabilized cells and (2) cells transduced with AcMNPV. Importazole was recently discovered using a high-throughput screen as a compound that is able to inhibit the binding between importin-β and RanGTP, therefore

blocking importin-β-mediated nuclear import without affecting transportin-mediated nuclear import and CRM1-mediated nuclear export (Soderholm et al., 2011). First, we used Cy3-NLS-BSA as a control to test the concentration of importazole needed to inhibit nuclear import in digitonin-permeabilized cells. As shown in Figure 4-3A, Cy3-NLS-BSA remained cytoplasmic under permissive conditions which contained all necessary components for nuclear import to occur, due to the inhibition of importin-β-mediated nuclear import by importazole. However, using the same concentration of importazole in cells incubated with the viral capsids instead of Cy3-NLS-BSA, capsids were seen in the nucleus at a similar efficiency compared to untreated cells (Figure 4-3, B and C). This confirms that nuclear import of the baculovirus capsid follows a non-classical nuclear import pathway that does not rely on importin-β.

Similarly in transduced cells, importazole treatment did not impede the ability of capsid import into the nucleus, supporting the idea that import occurs independently of importin-β receptors (Figure 4-4A). We also treated cells with 4% DMSO to test that drug resuspension using DMSO did not affect our results. Nuclear import efficiency also did not significantly decrease due to the addition of importazole during transduction (Figure 4-4B), therefore confirming that classical importin-β mechanism is not used in this case. Electron micrographs also show that baculovirus is able to transduce HeLa cells in the presence of importazole, and intact capsids were imported into the nucleus of HeLa cells transduced with baculovirus AcMNPV even when importin-β mediated nuclear import was blocked (Figure 4-5). This suggests that baculovirus AcMNPV capsids do not use the classical importin-β-mediated nuclear import mechanism.

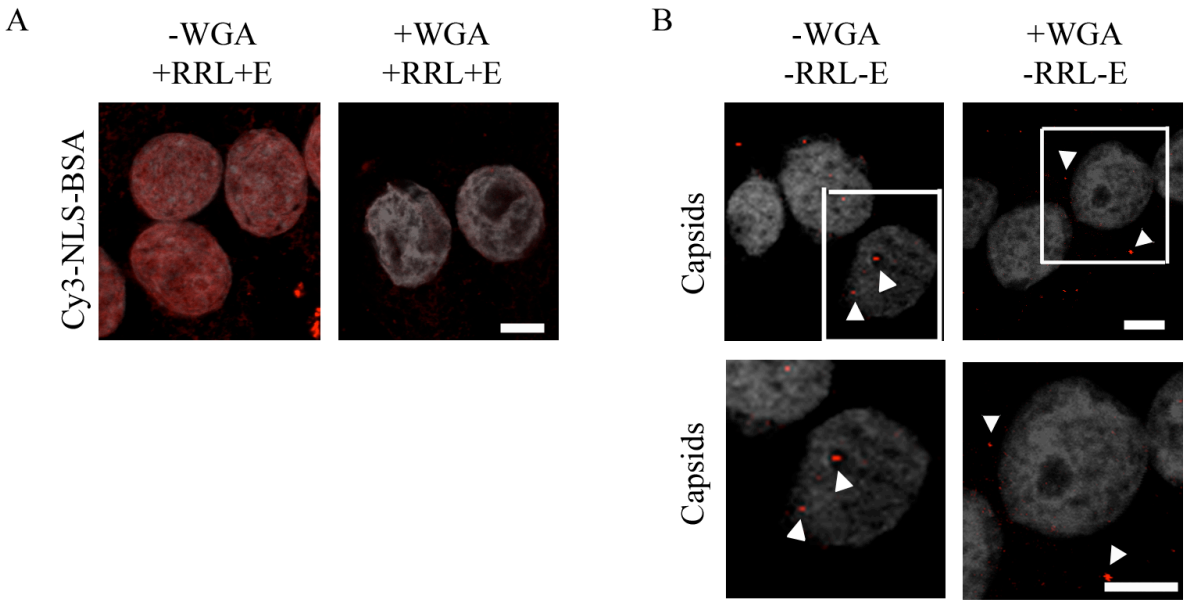
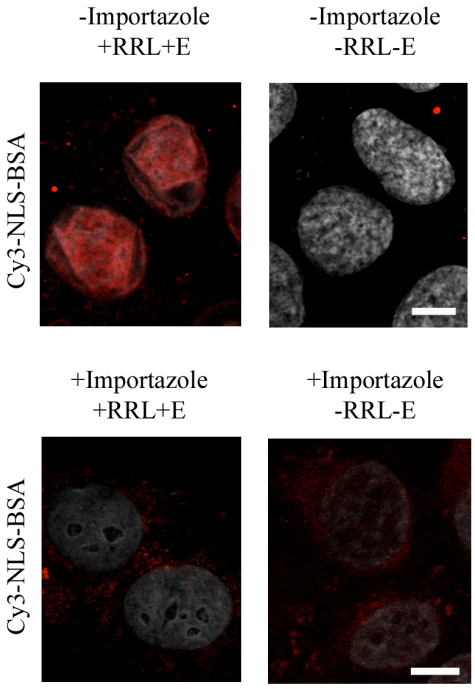
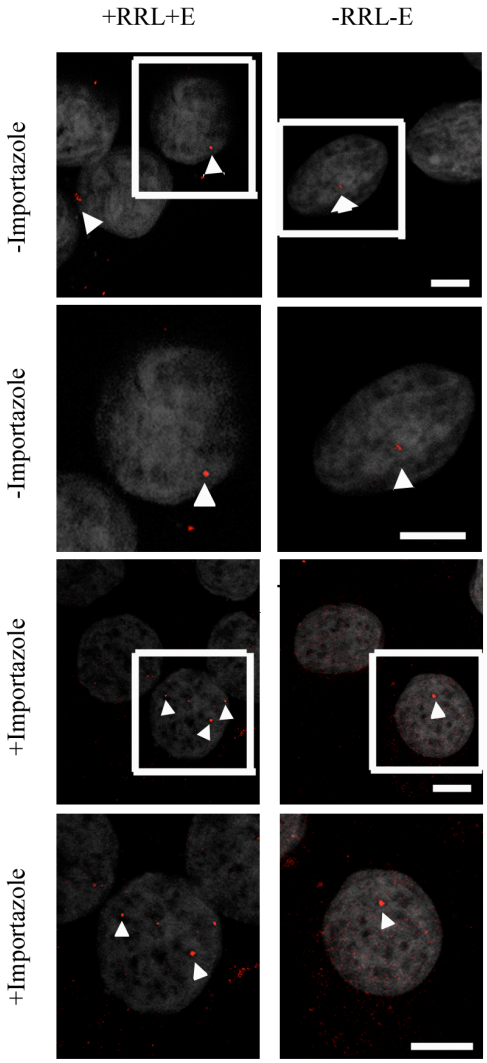


Figure 4-2: Inhibiting nuclear import of viral capsids through NPCs using WGA. Digitonin-permeabilized HeLa cells were incubated with (A) Cy3-NLS-BSA or (B) capsids in the presence or absence of WGA to block NPCs prior to performing the import assay under (A) permissive (presence of cytosolic factors (RRL) and an energy regenerating system (E)) and (B) non-permissive conditions (absence of RRL and E). (A) Nuclear import of Cy3-NLS-BSA was inhibited in the presence of WGA even in the presence of RRL and E. (B) Capsids were able to enter the nucleus in the absence of RRL and E, but entry into the nucleus was inhibited in the presence of WGA. Images were obtained using an Olympus Confocal Microscope. Cell within the white boxed area is magnified in the lower panels. Scale bars, 10-µm. DAPI, grey; Cy3-NLS-BSA or capsids, red. White arrowheads point to capsids.

A


-Importazole
+RRL+E
-Importazole
-RRL-E
Cy3-NLS-BSA
+Importazole
+RRL+E
+Importazole
-RRL-E
Cy3-NLS-BSA

B


+RRL+E
-RRL-E
-Importazole
-Importazole
+Importazole
+Importazole

C

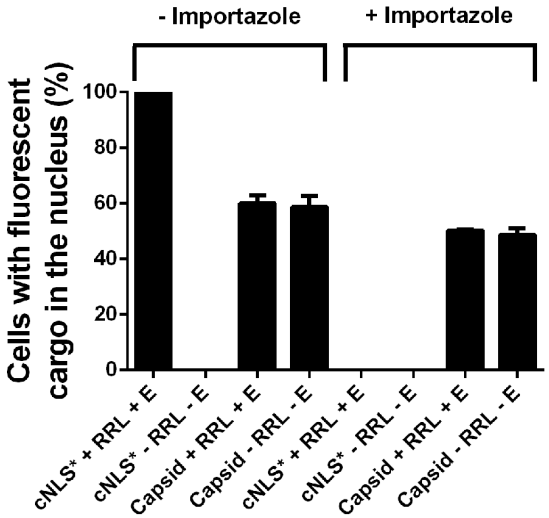

- Importazole
+ Importazole
Cells with fluorescent cargo in the nucleus (%)
100
80
60
40
20
0
cNLS* + RRL + E
cNLS* - RRL - E
Capsid + RRL + E
Capsid - RRL - E
cNLS* + RRL + E
cNLS* - RRL - E
Capsid + RRL + E
Capsid - RRL - E

Figure 4-3: Nuclear import of AcMNPV capsids occurs independently of importin-β. Digitonin-permeabilized HeLa cell import assay was performed in the presence or absence importazole. Import substrates (A) Cy3-NLS-BSA and (B) viral capsids were assayed in their ability to enter the nucleus using confocal microscopy. Portions or regions of cells within the white boxed areas are magnified in the lower panels. (C) Quantification of the number of cells with fluorescent cargo in the nucleus for all conditions. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; Cy3-NLS-BSA (A) or capsids (B), red. White arrowheads point to capsids.

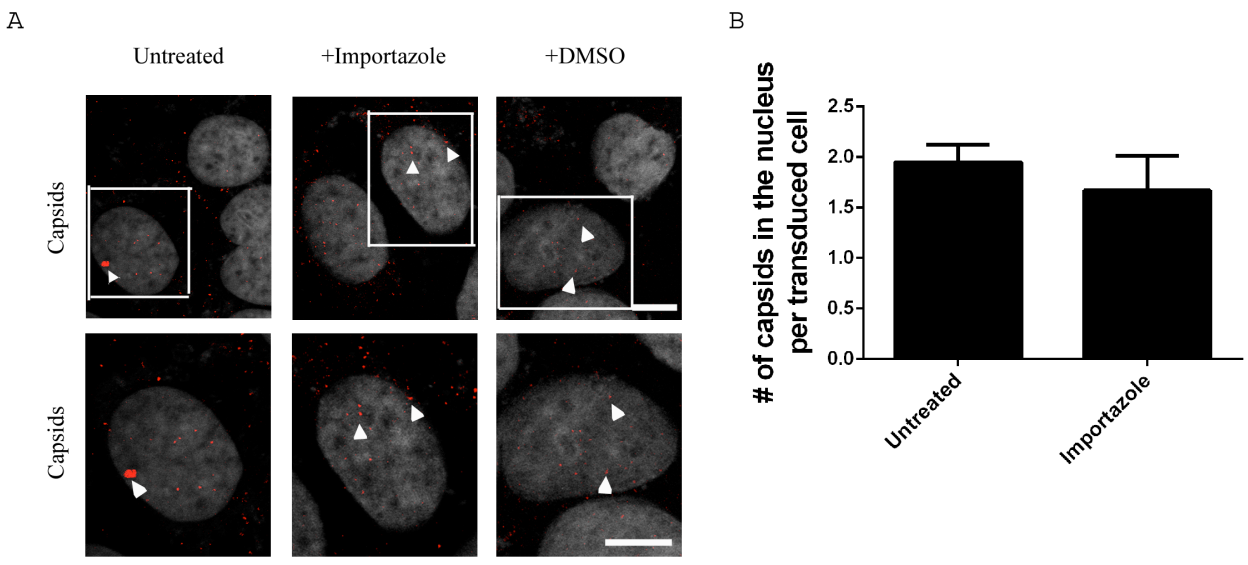


Figure 4-4: Importazole did not affect baculovirus capsid nuclear entry during transduction. (A) HeLa cells were transduced in the presence or absence of 40 μM of importazole, or DMSO as a control. Viral capsids were assayed in their ability to enter the nucleus using confocal microscopy 6 hours post-transduction. Portions or regions of cells within the white boxed areas are magnified in the lower panels. (B) Quantification of the number of capsids successfully imported into the nucleus in the presence or absence of importazole. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bar, 10 μm. DAPI, grey; capsids, red. White arrowheads point to capsids.

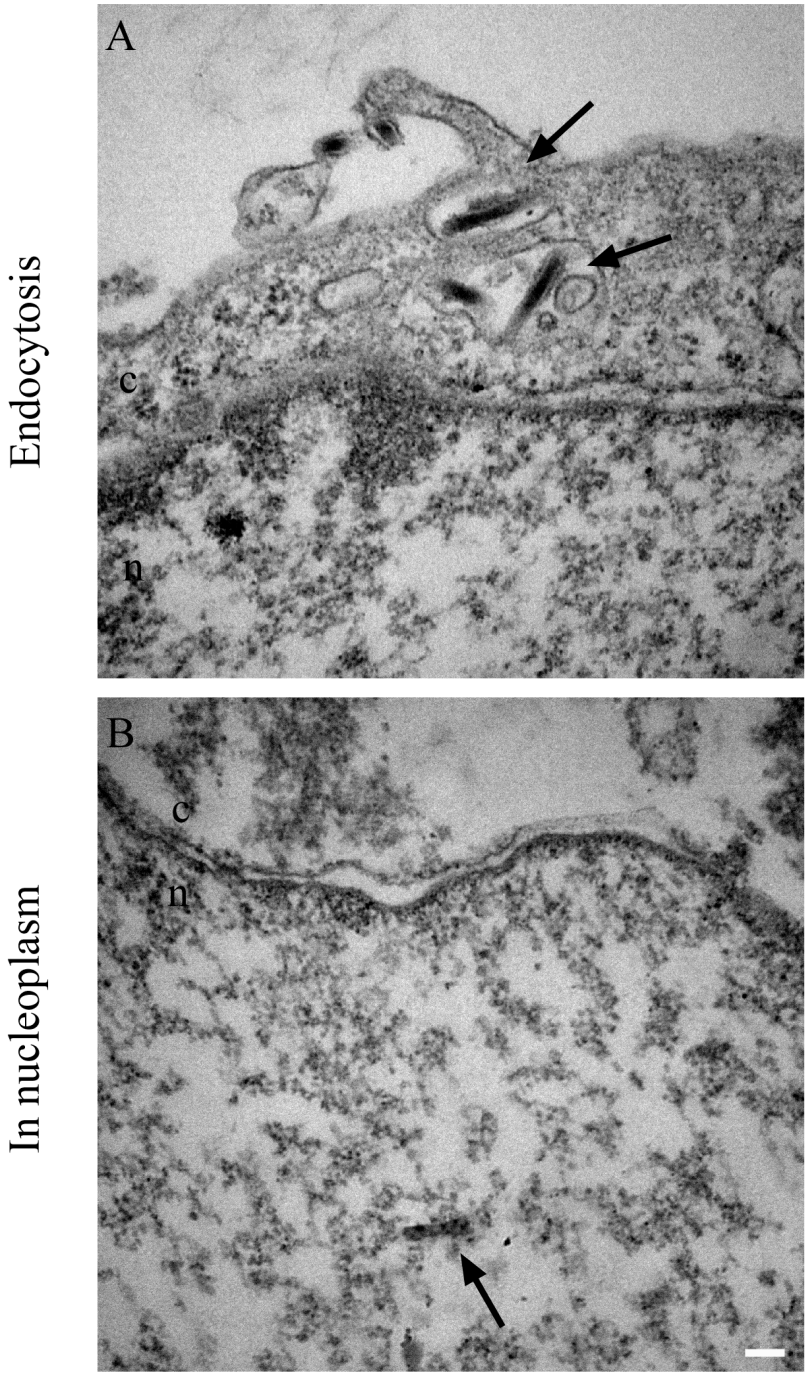


Figure 4-5: Electron micrograph of baculovirus transduced HeLa cells in the presence of importazole. TEM demonstrating that baculovirus enter the cells and the capsid enter the nucleus of HeLa cells transduced with baculovirus in the presence of importazole. Scale bar, 100-nm. n, nucleus; c, cytoplasm. Arrows point to capsids.

4.2.4 GTP hydrolysis by Ran is not required for capsid nuclear import

Classical pathway of nucleocytoplasmic transport has been characterized to use the small GTPase Ran (Melchior et al., 1993; Moore and Blobel, 1993). Ran is thought to bind the NPC at its cytoplasmic periphery, close to the site where NLS-protein-receptor complex binds to the NPC, further acting as a switch to activate transport across the NPC (Melchior and Gerace, 1995). We used GTPγS to determine the necessity of the RanGTPase in the process of nuclear import of baculovirus capsids in digitonin-permeabilized cells. Standard transport reactions were set up in either the presence or absence of GTPγS. As observed in Figure 4-6A, nuclear import assays in the presence of GTPγS resulted in Cy3-NLS-BSA remaining in the cytoplasm even when cytosolic factors and energy was available, confirming the need for the RanGTPase for classical nuclear import. However, viral capsids were detected inside the nucleus under the same conditions (Figure 4-6B), and the nuclear import efficiency was similar in the presence or absence of GTPγS (Figure 4-6C). This further suggests that nuclear import of baculovirus AcMNPV capsids do not follow the conventional nuclear transport mechanism, and RanGTPase is not essential for nuclear entry of this capsid. Ran in its GTP form is necessary for the dissociation of importin-β from the incoming NLS-containing cargo (reviewed in Jamali et al., 2011). Our data further supports the notion that nuclear import of the baculovirus capsid occurs independently of importin-β as RanGTPase is not a necessary component. This nuclear import strategy is similar to that used by β-catenin whereby the protein is able to shuttle between the nucleus and cytoplasm independently of Ran and transport receptors (Sharma et al., 2012).

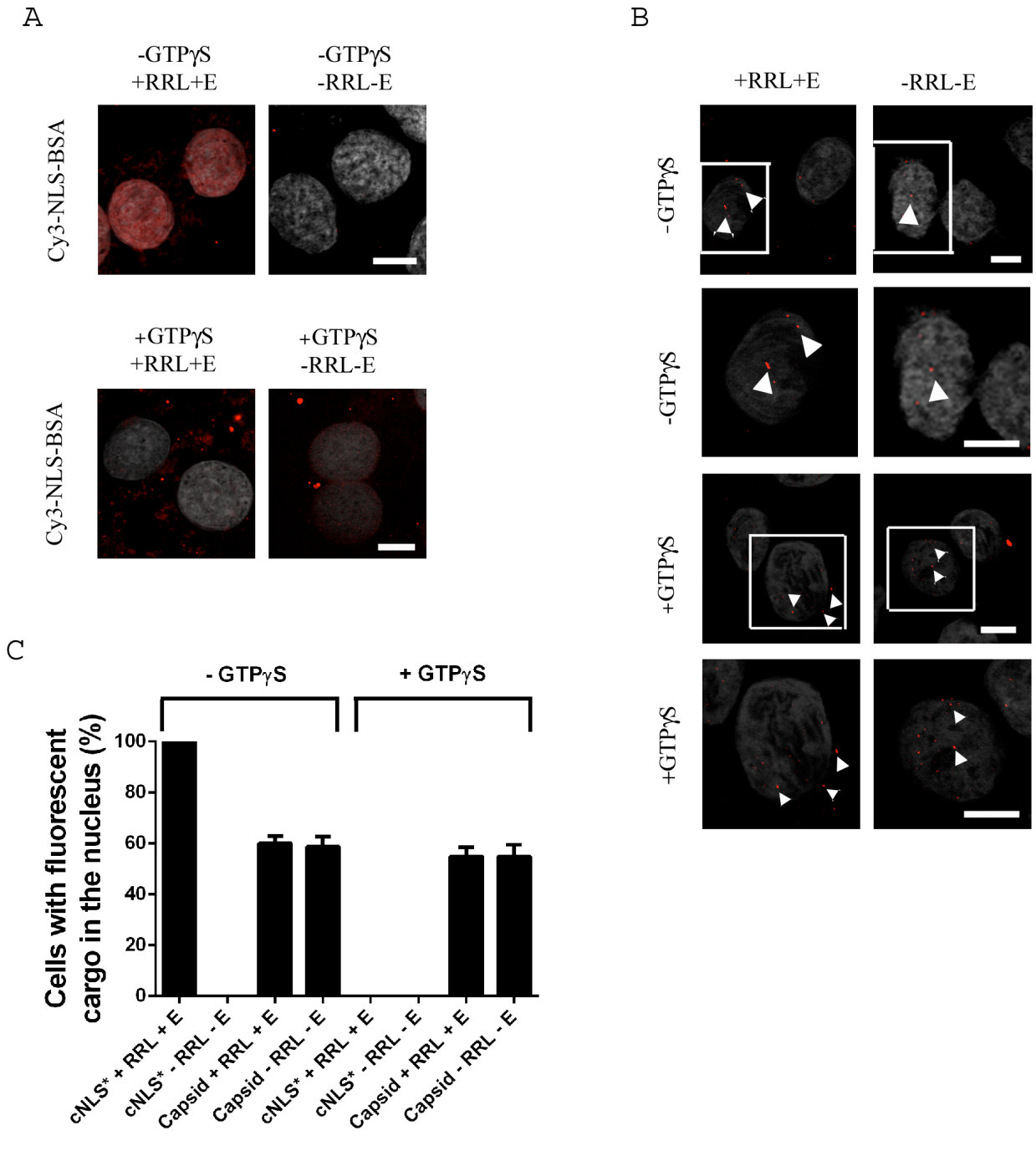
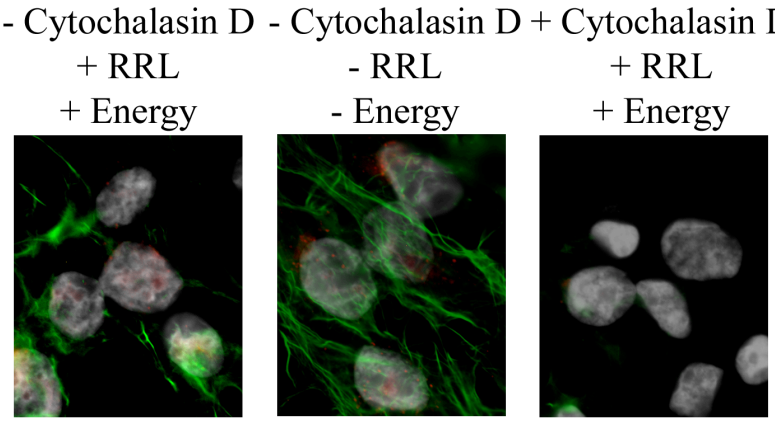

A
-GTPγS
+RRL+E
-GTPγS
-RRL-E
Cy3-NLS-BSA
+GTPγS
+RRL+E
+GTPγS
-RRL-E
Cy3-NLS-BSA
B
+RRL+E
-RRL-E
-GTPγS
-GTPγS
+GTPγS
+GTPγS
C
- GTPγS
+ GTPγS
Cells with fluorescent cargo in the nucleus (%)
100
80
60
40
20
0
cNLS* + RRL + E
cNLS* - RRL - E
Capsid + RRL + E
Capsid - RRL - E
cNLS* + RRL + E
cNLS* - RRL - E
Capsid + RRL + E
Capsid - RRL - E

Figure 4-6: Nuclear import of baculovirus capsid occurs independently of Ran. Digitonin permeabilized HeLa cell import assay was performed in the presence or absence of GTPγS. Import substrates Cy3-NLS-BSA (A) and viral capsids (B) were assayed using confocal microscopy for their ability to enter the nucleus of digitonin-permeabilized HeLa cells using confocal microscopy. Portions or regions of cells within the white boxed areas are magnified in the lower panels. (C) Quantification of the number of cells with fluorescent cargoes in the nucleus for all conditions. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; Cy3-NLS-BSA (A) or capsids (B), red. White arrowheads point to capsids.

4.2.5 Intact F-actin is necessary for nuclear import of the viral capsid

It is a well-known concept that baculovirus uses actin-nucleation for intra-cellular mobility. Actin nucleation relies heavily on the availability of host F-actin and Arp2/3 complex. When we attempted to disrupt cellular F-actin by the addition of cytochalasin D in digitonin-permeabilized HeLa cells, nuclear import of the viral capsid did not occur, in the presence or absence of cytosolic factors and an energy source (Figure 4-7A). We further wanted to determine how much F-actin remained in cells treated with digitonin or cytochalasin D, as the abundance of F-actin in these cells may affect the ability for viral nuclear import. We used untreated, digitonin treated, and cytochalasin D treated cells, and observed the dynamic changes to cellular F-actin by taking serial section images from the top to the bottom of cell that were immunostained with FITC-phalloidin. We noticed a decrease and change in distribution of cellular F-actin in both digitonin-permeabilized and cytochalasin D treated HeLa cells (Figure 4-7B), which led us to hypothesize that digitonin treatment could be disrupting F-actin. We next isolated cellular G- and F-actin when cells were untreated, treated with digitonin, or treated with cytochalasin D to determine if there are changes in the abundance of both forms of actin in each condition. As shown in Figure 4-7C, treating cells with digitonin did in fact increase the amount of G-actin available in the cell, while cytochalasin D treatment drastically increased the amount of soluble G-actin, suggesting that both treatments were able to disrupt cellular F-actin. This leads us to infer that there could be an effect of digitonin in the efficiency of capsid nuclear import if digitonin mildly disrupts F-actin because baculovirus uses actin-nucleation for cell motility.

A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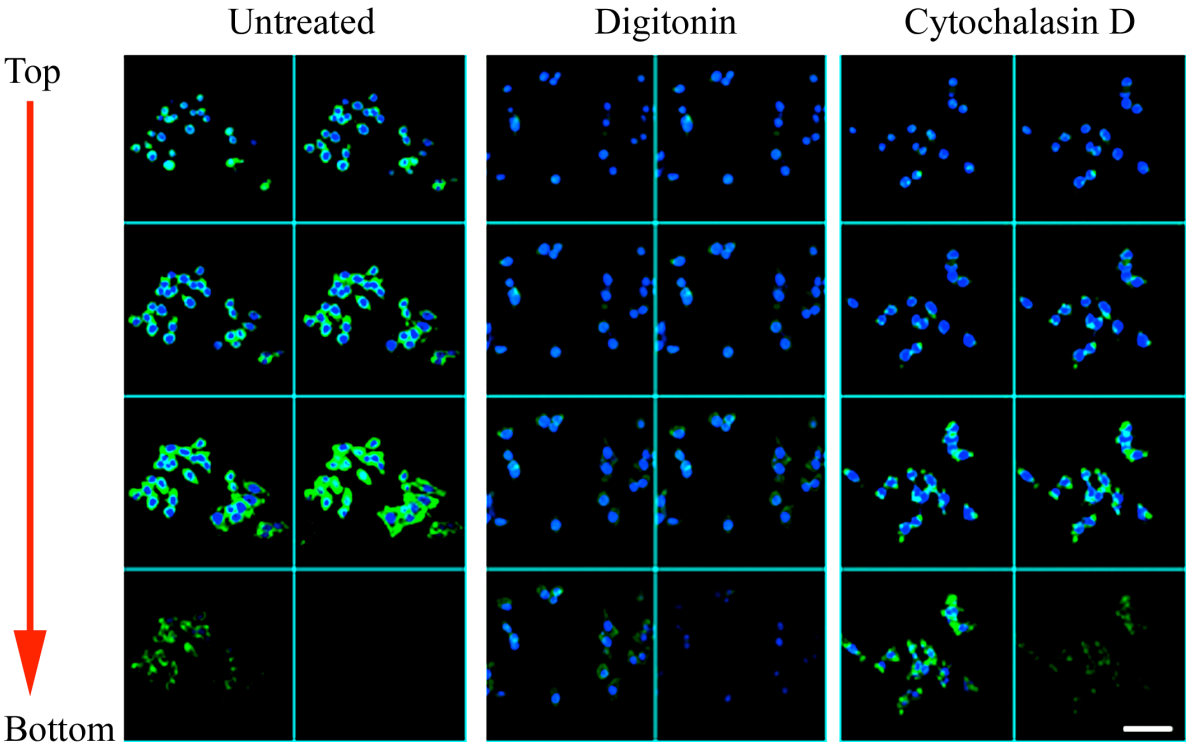
+ RRL
+ Energy
- Cytochalasin D
- RRL
- Energy
+ Cytochalasin D
+ RRL
+ Energy

B


Untreated
Digitonin
Cytochalasin D
Top
Bottom

C

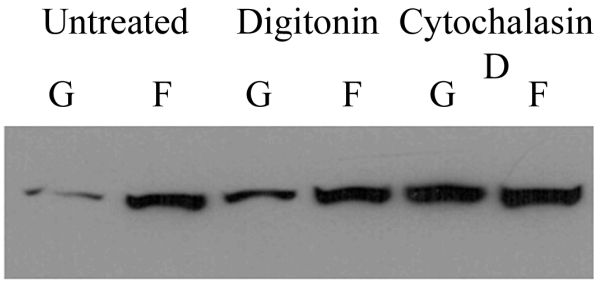

Untreated
Digitonin
Cytochalasin D
G
F
G
F
G
F

Figure 4-7: Cellular F-actin is a necessary component to mediate nuclear import of AcMNPV capsids, and is disrupted in digitonin permeabilized HeLa cells. (A) Digitonin-permeabilized HeLa cells import assay in cells untreated or treated with cytochalasin D. Import assays were performed with AcMNPV capsids in the presence or absence of RRL and energy. FITC-phalloidin was used for actin staining. Disrupting F-actin inhibits nuclear import of AcMNPV capsids. (B) Confocal microscopy images displaying serial sections of HeLa cells, from the top to the bottom, of HeLa-cells untreated, treated with digitonin or cytochalasin D. F-actin appears rearranged and disrupted when cells were treated with digitonin and cytochalasin D. (C) Abundance of actin was detected in HeLa cells untreated, or treated with digitonin or cytochalasin D. Soluble and insoluble fractions containing G- and F-actin, respectively, were analyzed by immunoblotting with a beta-actin antibody. Scale bars, 10-μm. DAPI, grey (A) and blue (B); FITC-phalloidin (A and B), green; capsid (A), red.

4.2.6 Arp2/3 complex is reduced in digitonin-permeabilized HeLa cells

Besides cellular F-actin, actin nucleation relies on the Arp2/3 complex to generate the daughter actin filament. We set out to demonstrate the abundance of Arp2/3 in untreated cells and cells treated with digitonin. Since the digitonin-permeabilized cell import assay removes all cytosolic soluble proteins, we wanted to determine how much Arp2/3 remains in these digitonin treated cells. As shown in Figure 4-8, HeLa cells treated with digitonin still contained Arp2/3 complexes, although less when compared to cells untreated. This suggests that a portion of the Arp2/3 could also be binding to the remaining F-actin in the cell and be used by the baculovirus capsid in the digitonin-permeabilized cell import assay. The amount of Arp2/3 contained within the digitonin treated cells would therefore allow actin nucleation to still occur, however potentially at a lower efficiency.

4.2.7 Inhibiting activation of the Arp2/3 complex impedes nuclear entry of AcMNPV capsids

To determine the necessity of actin nucleation using host Arp2/3 in mediating nuclear import of baculovirus capsids, we used a cell-permeable compound CK666 that selectively inhibits actin nucleation mediated by Arp2/3 complex. CK666 binds to a pocket between Arp2 and Arp3 preventing Arp2/3 from shifting into an active conformation upon WASP binding (Nolen et al., 2009). Many small-molecule inhibitors of Arp2/3 have been discovered recently and caution is warranted in determining specificity of these drugs and ensuring there are no off-target effects. CK666 is notably a more reliable inhibitor as no additional phenotypes are observed when used in Arp2/3 depleted fibroblasts, compared to blebbings that are found when using CK869. The irregular protrusions at the plasma membrane suggest that CK869 could cause

possible off-target effects (Rotty et al., 2013). For these reasons, we chose to use the Arp2/3 inhibitor CK666 instead of CK869, which is also commercially available.

In baculovirus transduced HeLa cells treated with CK666, we observed a significant decrease in nuclear accumulation of viral capsids, compared to untreated cells or cells treated with the cell-permeable inactive control that exhibits no Arp2/3 inhibitory activity, CK689 (Figure 4-9A). Far fewer cells displayed viral capsids in the nucleus when treated with CK666, and statistical analysis using GraphPad Prism software's unpaired Student's t-test showed a significant decrease of nuclear import of the capsid in the presence of CK666 (Figure 4-9B). Using electron microscopy, capsids released from endosomes can be seen in the cytoplasm and close to the nuclear periphery, but not inside the nucleus of infected cells treated with CK666 (Figure 4-10). This suggests that CK666 does not interfere with cellular entry of baculovirus or endosomal release of capsids, but instead Arp2/3 activation is necessary for capsid entry into the nucleus. However, actin polymerization by Arp2/3 could also be involved in other cellular processes such as endocytosis, or the driving of vesicles away from the plasma membrane, therefore diminishing the amount of capsids available for nuclear entry (Kaksonen et al., 2006; Rotty et al., 2013). Taking these limitations into consideration, we further performed a digitonin-permeabilized HeLa cell import assay in the presence or absence of CK666. As a control, Cy3-NLS-BSA was able to enter the nucleus in the presence of cytosolic factors and an energy regenerating source, but remained cytoplasmic in its absence (Figure 4-11B). In this case, we also detected a significant decrease in nuclear accumulation of viral capsids when cells were treated with CK666, compared to cells untreated or cells treated with CK689 (Figures 4-11, A and C). Therefore these results suggest a necessity of actin nucleation promoted by Arp2/3 complex for nuclear import of baculovirus capsids.

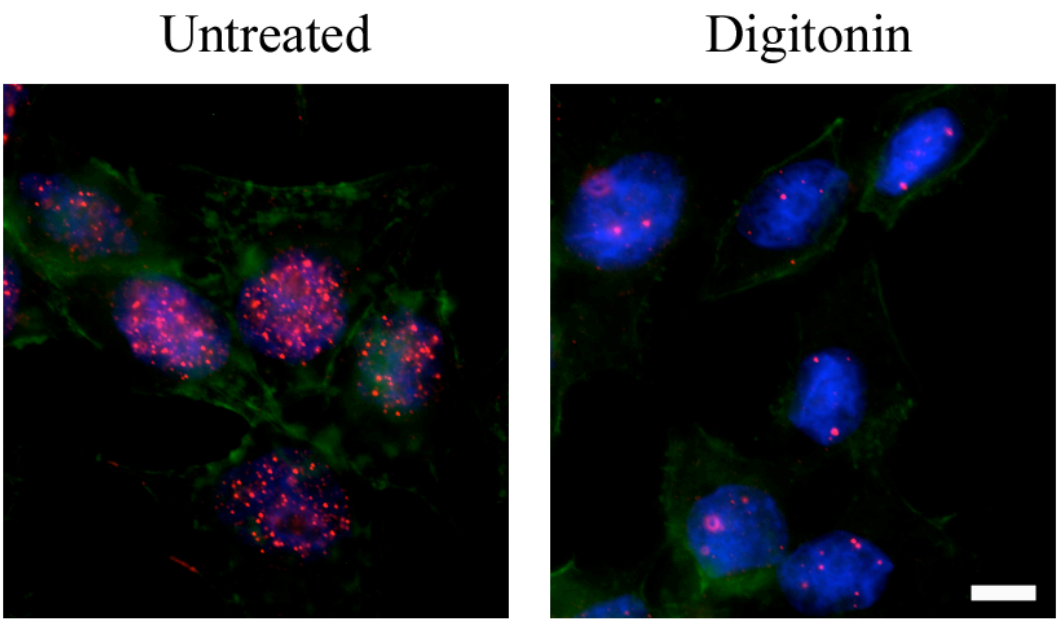


Figure 4-8: Abundance of Arp2/3 is significantly reduced in digitonin treated cells. HeLa cells were untreated, or treated with digitonin to visualize the distribution and abundance of cellular Arp2/3 using indirect immunofluorescence microscopy. Scale bar, 10-µm. DAPI, blue; FITC-phalloidin, green; Arp2/3, red.

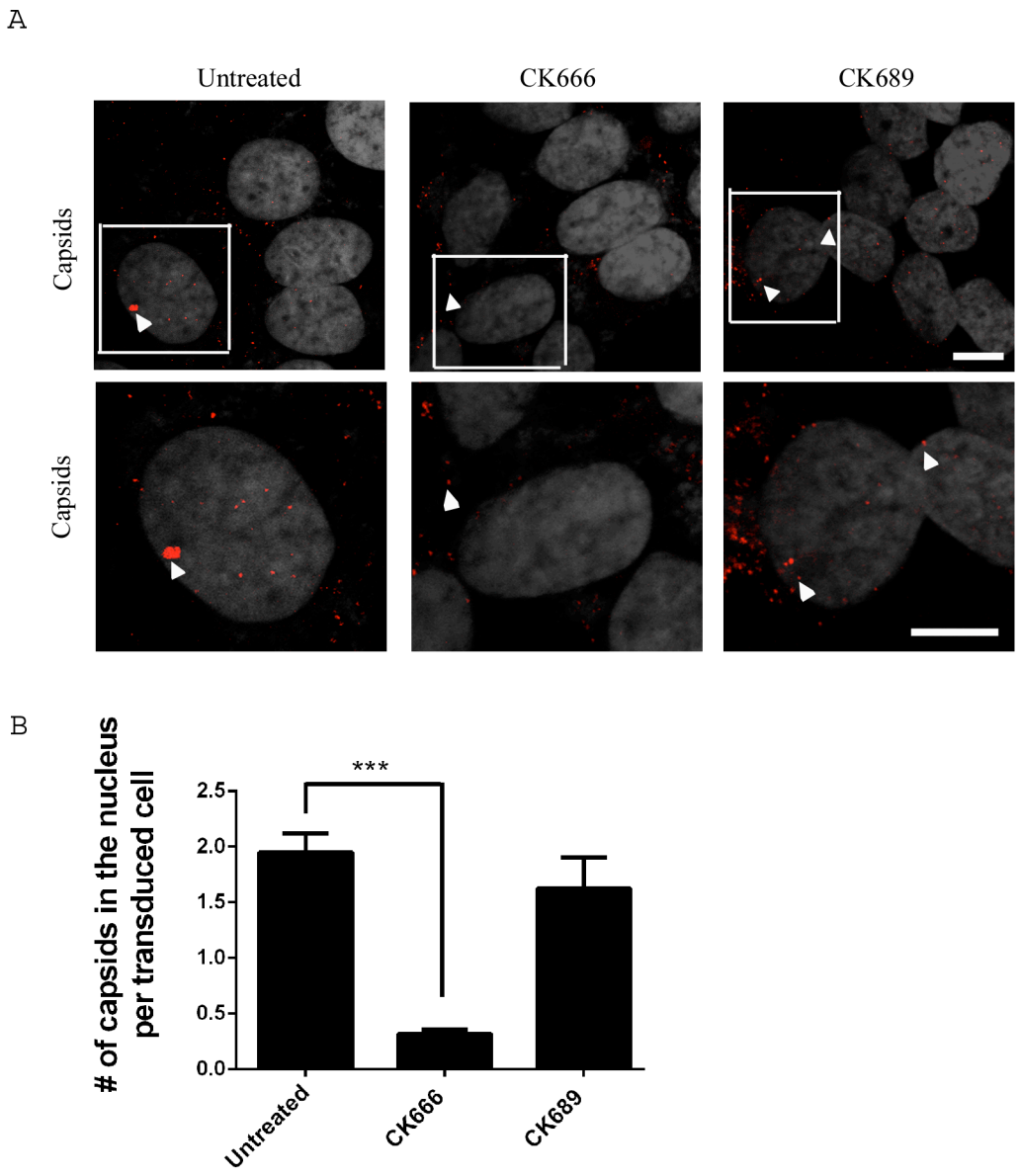


Figure 4-9: CK666 significantly reduced viral capsid nuclear import during transduction. (A) HeLa cells were untreated, treated with CK666, or the control CK689 and transduced with baculovirus. The ability for viral capsids to enter the nucleus was assayed using confocal microscopy. Portions or regions of cells within the white boxed areas are magnified below in the lower panels. (B) Quantification of the number of capsids in the nucleus of transduced cells. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bar, 10-µm. DAPI, grey; capsids,red. ***P=0.0007 (unpaired Student's t-test). White arrowheads point to capsids.

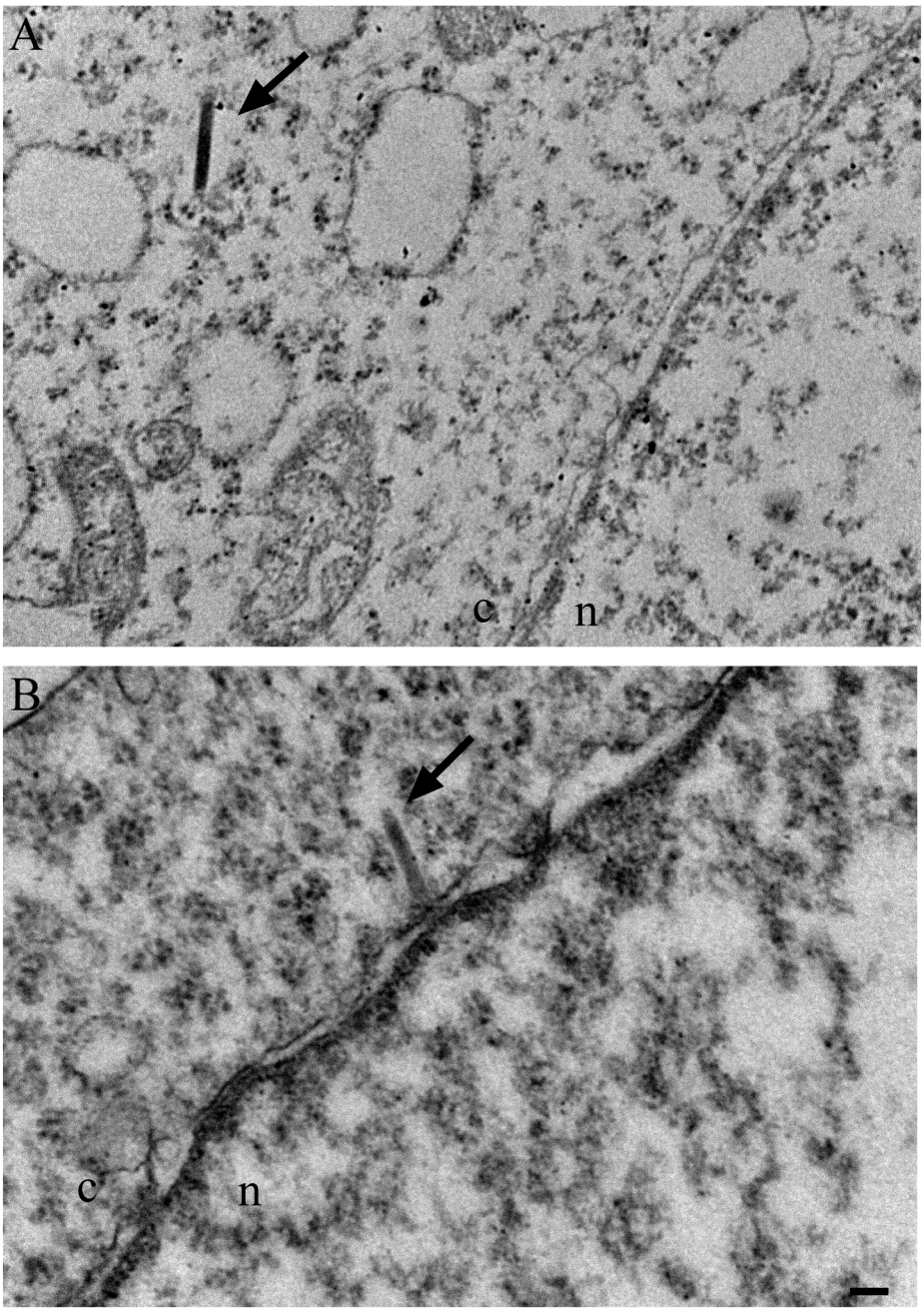


Figure 4-10: Electron micrographs of baculovirus transduced HeLa cells in the presence of CK666. TEM demonstrating that baculovirus is able to transduce and enter HeLa cells in the presence of CK666 (A), but capsids were unable to enter the nucleus (B). Scale bar, 100-nm. n, nucleus; c, cytoplasm. Arrows point to capsids.

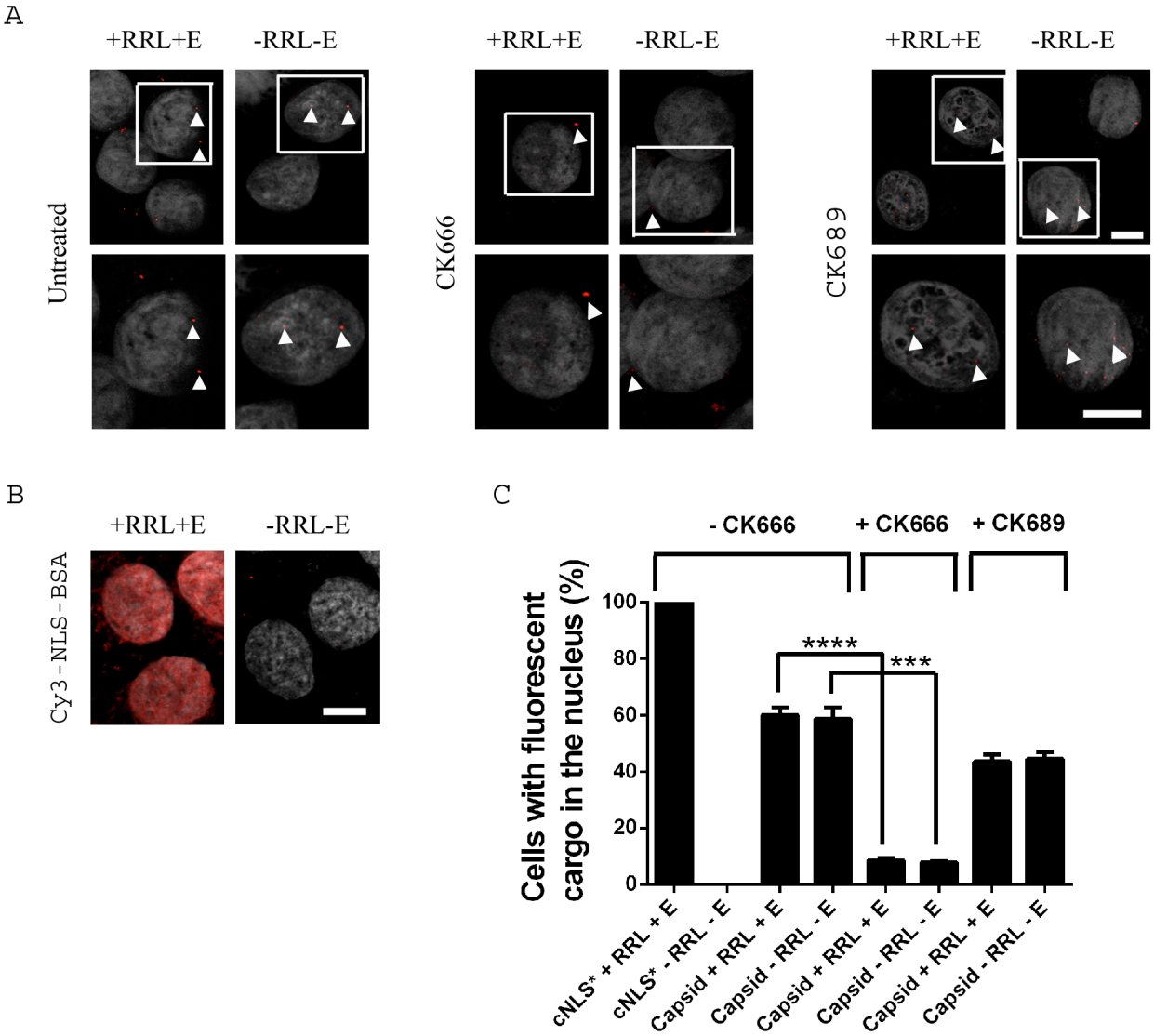

A
+RRL+E
-RRL-E
Untreated
CK666
CK689
B
Cy3-NLS-BSA
C
- CK666
+ CK666
+ CK689
Cells with fluorescent cargo in the nucleus (%)
100
80
60
40
20
0
****
***
cNLS* + RRL + E
cNLS* - RRL - E
Capsid + RRL + E
Capsid - RRL - E

Figure 4-11: Inhibitor of Arp2/3 significantly reduced nuclear import efficiency of baculovirus capsids. (A) Digitonin-permeabilized HeLa cells were untreated, treated with CK666, or the control CK689 and incubated with viral capsids in the presence or absence of cytosolic factors (RRL) and an energy regenerating system (E). Viral capsids were assayed using confocal microscopy for their ability to enter the nucleus of digitonin-permeabilized HeLa cells under these conditions using confocal microscopy. The portions or regions of cells within the white boxed areas are magnified in the lower panels. (B) Cy3-NLS-BSA was a control to ensure that HeLa cells were permissive (presence of RRL and E) and non-permissive (absence of RRL and E) for nuclear import. (C) Quantification of the number of nuclei with fluorescent cargoes. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Statistical analyses were performed using GraphPad Prism Software. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; capsids, red. ***P=0.0002, ****P=.0001 (unpaired Student's t-test). White arrowheads point to capsids.

4.3 Discussion

In this chapter, we identified cellular factors required for nuclear import of baculovirus AcMNPV capsids. Surprisingly, nuclear import of the baculovirus capsid occurred in the digitonin-permeabilized import assay in the presence and absence of cytosolic factors and energy (Figure 4-1). In these permeabilized HeLa cells, digitonin mildly permeabilized the plasma membrane, leaving the nucleus fully intact and fully able to import any cargo that enters the nucleus through the NPC when supplied with nuclear import receptors, Ran, and energy. This was confirmed with control experiments using 70 kDa Dextran Texas Red and Cy3-NLS-BSA. Dextran remained in the cytoplasm in digitonin-permabilized cells, demonstrating that digitonin did not disrupt the nuclear membranes (Figure 4-1A). As the digitonin-permeabilized cell import assay does not necessarily deplete all remnants of cytosolic proteins, we were reassured that cytosolic factors mediating nuclear import were completely depleted in these cells as Cy3-NLS-BSA remained cytoplasmic (Figure 4-1B) under conditions where nuclear import is not permitted.

Our result that AcMNPV capsids were able to enter nuclei of digitonin-permeabilized cells under conditions that inhibited nuclear import of Cy3-NLS-BSA suggests that nuclear entry of AcMNPV capsids does not require the classical NLS conventional nuclear import strategy of using cellular receptors and an energy source. Nuclear import of the capsid in the absence of cytosolic factors was inhibited when cells were pretreated with WGA, further suggesting that baculovirus capsids only enter the nucleus through NPCs, unlike parvoviruses that cause brief NE disruption for viral entry into the nucleus (Cohen et al., 2006). Our results presented in this chapter are consistent with those obtained through microinjection of *Xenopus laevis* oocytes

presented in Chapter 3, and the ability of AcMNPV capsids to enter the nucleus even when importin-β-mediated nuclear import is inhibited (Chapter 4). As NLSs have not been identified or characterized in the baculovirus capsid, it is not too surprising that the baculovirus capsid is able to enter the nucleus under conditions that prohibit nuclear entry of Cy3-NLS-BSA.

The ability of viral capsids or viral proteins to get imported into the nucleus in the absence of cytosolic factors is not uncommon. Nuclear import of the HIV-1 integrase protein was previously proposed to occur independently of members of the karyopherin β family (Woodward et al., 2009). In addition, baculovirus capsids were found inside the nucleus when cells were treated with a non-hydrolyzable form of Ran (Figure 4-6) and an inhibitor of importin-β (Figures 4-3, 4-4, and 4-5), further confirming that nuclear import of the AcMNPV capsid follows a non-classical mechanism. However, digitonin permeabilized cells should also be depleted of receptors from other nuclear import pathways, such as transportin. This suggests that the baculovirus capsid itself may have properties of nuclear import receptors that allow nuclear import of the AcMNPV capsid to occur in the absence of cytosolic receptors, similar to that of HIV-1.

In contrast to the successful nuclear import of baculovirus capsids when the import assay was performed under conditions that inhibited the classical nuclear import pathway, disrupting F-actin and inhibiting the Arp2/3 complex did block nuclear import of the capsid (Figures 4-7A, 4-9, 4-10, and 4-11). As seen in Figure 4-10, baculovirus capsids were found close to the NE when HeLa cells were transduced with baculovirus in the presence of the Arp2/3 inhibitor, suggesting that baculovirus can diffuse through the cytoplasm towards the nucleus, but cannot enter the nucleus when Arp2/3 activation is inhibited. This further suggests the need for actin nucleation

to occur for capsid delivery into the nucleus. Even though much of the F-actin is disrupted and Arp2/3 is washed away in digitonin-permeabilized HeLa cells (Figures 4-7, A and C, and Figure 4-8), the amount of F-actin and Arp2/3 in these cells are sufficient to mediate nuclear import of the AcMNPV capsid. Based on these results we speculate that in addition to the role of actin polymerization for baculovirus cellular transport, actin nucleation mediated by Arp2/3 facilitates baculovirus capsid translocation through the NPC.

We propose that upon baculovirus capsid binding to the NPC, cell signalling events may occur independently of nuclear transport receptors that cause dynamic changes to the structure of the NPC, while actin nucleation acts as an extra energy source to push the viral capsid through the central channel into the nucleus. In summary, the data presented in this chapter suggests that the baculovirus capsid enter the nucleus using a mechanism different than the classical nuclear import pathway, and that F-actin and Arp2/3 play an important role in this mechanism.

Chapter 5

Studying the Possible Role of FG-Nups in the Nuclear Import of AcMNPV Capsids

5.1 Introduction

Selective nuclear transport of large macromolecules involves Nups containing phenylalanine and glycine repeats (FG-Nups), which act as binding sites for nuclear import receptors. As described in section 1.1.2.3, FG-Nups regulate the permeability barrier of the NPC. Nup358, Nup62 and Nup153, positioned at the cytoplasmic filaments, within the central channel, and the nuclear basket of NPCs, respectively, are FG-nups that commonly act as binding sites for receptor-mediated nuclear import (i.e., importin-β). Each of these Nups contains domains within their N- and C-terminus for binding to Ran and importin-β that are essential for active nuclear transport through the central channel. Viruses have been shown to target these Nups for rearrangement and degradation during the viral infection and replication cycle (described in section 1.3.6), and most often changes to these Nups result in blocking nuclear import of proteins involved in activating the host cells' immune response. Even though baculovirus has not previously been directly linked to these FG-Nups, the role of these Nups during baculovirus AcMNPV nuclear entry may provide additional information about the function of these FG-Nups during translocation of a large cargo. As demonstrated in Chapter 4, baculovirus AcMNPV does not follow the classical nuclear import pathway, therefore we hypothesize that FG-Nups such as Nup62, Nup153, and Nup358 are dispensable during this process. In addition, because these Nups are in the path that the baculovirus capsid must cross while transiting through the NPC, these Nups may play a role

in baculovirus nuclear import. This chapter will focus on investigating the role of these three FG-Nups in nuclear import of baculovirus capsids. We used siRNA to transiently deplete these proteins and performed *in vitro* nuclear import assays with these cells, as well as baculovirus transduction to visualize the ability of baculoviral capsids to enter the nucleus of these Nup depleted cells.

5.2 Results

5.2.1 Nup62 is not necessary for efficient nuclear import of AcMNPV capsids

Nup62 residing within the NPC central channel was successfully knocked down in HeLa cells treated with siRNA for 72 hours. As shown in Figure 5-1A, high efficiency of knockdown was achieved with 20 nM of the siRNA. Under this condition, the NPC did not become more permeable as 70 kDa Dextran Texas Red remained cytoplasmic in digitonin-permeabilized cells (Figure 5-1B). In addition, Nup62 siRNA treated cells did not show a decrease in Cy3-NLS-BSA nuclear import in the presence of cytosolic factors and energy (Figure 5-1C, top panel). Nuclear import of Cy3-NLS-BSA occurred under permissive conditions, and the protein remained cytoplasmic in the absence of cytosolic factors and an energy regenerating source. Proteins in the central channel of the NPC have been suggested to undergo conformational changes and regulate the movement of molecules into and out of the nucleus. However, we did not see an influx of Cy3-NLS-BSA in Nup62 depleted cells. This may suggest that Nup62 alone is not an essential protein in importin-β mediated nuclear import. Interestingly, baculovirus capsids were observed in the nucleus of Nup62-depleted cells under both permissive and non-permissive conditions (Figure 5-1C, lower panel). Our quantified data (Figure 5-1D) did not show a significant difference in the amount of capsids found within the nucleus between both conditions.

We further transduced Nup62-depleted cells with baculovirus to determine if nuclear import efficiency of capsids will differ in transduced cells compared with the *in vitro* import assay. Similar to our results with digitonin-permeabilized HeLa cells (Figure 5-1), depletion of Nup62 showed no significant impediment on nuclear import of the baculovirus capsid compared to cells treated with transfection reagent alone (Figure 5-2, A and B). This indicates that Nup62 does not mediate the nuclear import of baculovirus capsids.

5.2.2 Depletion of Nup153 does not alter the nuclear import efficiency of AcMNPV capsids

Nup153 is the main component of the nuclear baskets of NPCs. Viruses or their components that are able to cross the NPC often interact with Nup153, as described in section 1.3.2 for HIV-1 and 1.3.4 for HBV. Nup153 interacts with Nup50 within the nucleus and both of these Nups are highly dynamic, as they constantly shuttle into and out of the nucleus (Rabut et al., 2004). This interaction results in efficient importin-β mediated nuclear import (Makise et al., 2012). We transfected Nup153 siRNA into HeLa cells to determine its role in mediating nuclear import of AcMNPV capsids. Nup153 was successfully knockdown in HeLa cells treated with 10 nM of siRNA for 72 hours (Figure 5-3A). Depletion of Nup153 also did not alter the permeability barrier of the NPC, as 70 kDa Dextra Texas Red remained cytoplasmic in digitonin-permeabilized cells (Figure 5-3B). However, nuclear import of Cy3-NLS-BSA was reduced in these Nup153 knockdown cells, even in the presence of cytosolic factors and energy (Figure 5-3C, top panel). These results are consistent with previously published data showing a 40% reduction in nuclear import efficiency of Cy3-NLS-BSA in Nup153 knockdown cells (Zhou and Pante, 2010). Depleting Nup153 had no affect on the ability of AcMNPV capsids to enter the nucleus. VP39 antibody detection of the capsids was seen in the nucleus, at similar efficiency between permissive and non-permissive conditions (Figure5-3C, bottom panel, and D).

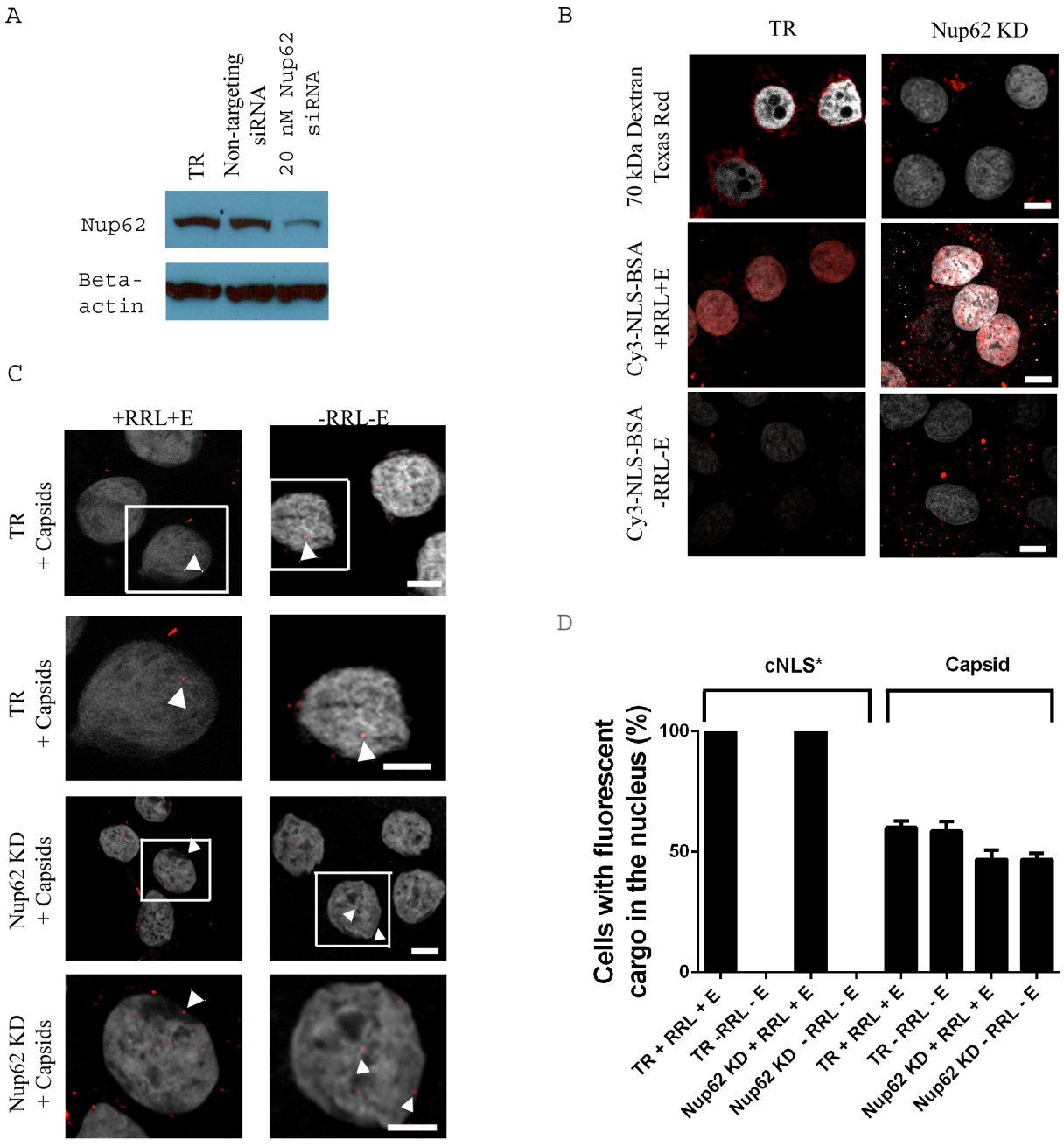

A
TR
Non-targeting siRNA
20 nM Nup62 siRNA
Nup62
Beta-actin
B
TR
Nup62 KD
70 kDa Dextran Texas Red
Cy3-NLS-BSA +RRL+E
Cy3-NLS-BSA -RRL-E
C
+RRL+E
-RRL-E
TR + Capsids
TR + Capsids
Nup62 KD + Capsids
Nup62 KD + Capsids
D
cNLS*
Capsid
Cells with fluorescent cargo in the nucleus (%)
100
50
0
TR + RRL + E
TR -RRL - E
Nup62 KD + RRL + E
Nup62 KD - RRL - E
TR + RRL + E
TR - RRL - E
Nup62 KD + RRL + E
Nup62 KD - RRL - E

Figure 5-1: Depletion of Nup62 by RNAi did not affect the nuclear import efficiency of baculovirus capsids. (A) Nup62 knockdown was assayed by Western blotting using specific antibodies against Nup62s in transfection reagent (TR) treated, non-targeting siRNA treated, or Nup62-siRNA treated cells. Beta-actin labelling was used as a loading control. (B and C) Digitonin-permeabilized HeLa cell import assay was performed in both non-transfected (TR) HeLa cells and Nup62-depleted cells with (B) 70 kDa Dextran fluorescently-labeled with Texas Red (as a permeabilization control), Cy3-NLS-BSA, or (C) baculovirus capsids. The portions or regions of cells within the white boxed areas are magnified in the lower panels. Localization of the fluorescence substrate was assayed by confocal microscopy. (D) Quantification of the number of cells with fluorescent cargoes in the nucleus. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; 70 kDa Dextran and Cy3-NLS-BSA (B), or capsids (C), red. White arrowheads point to capsids.

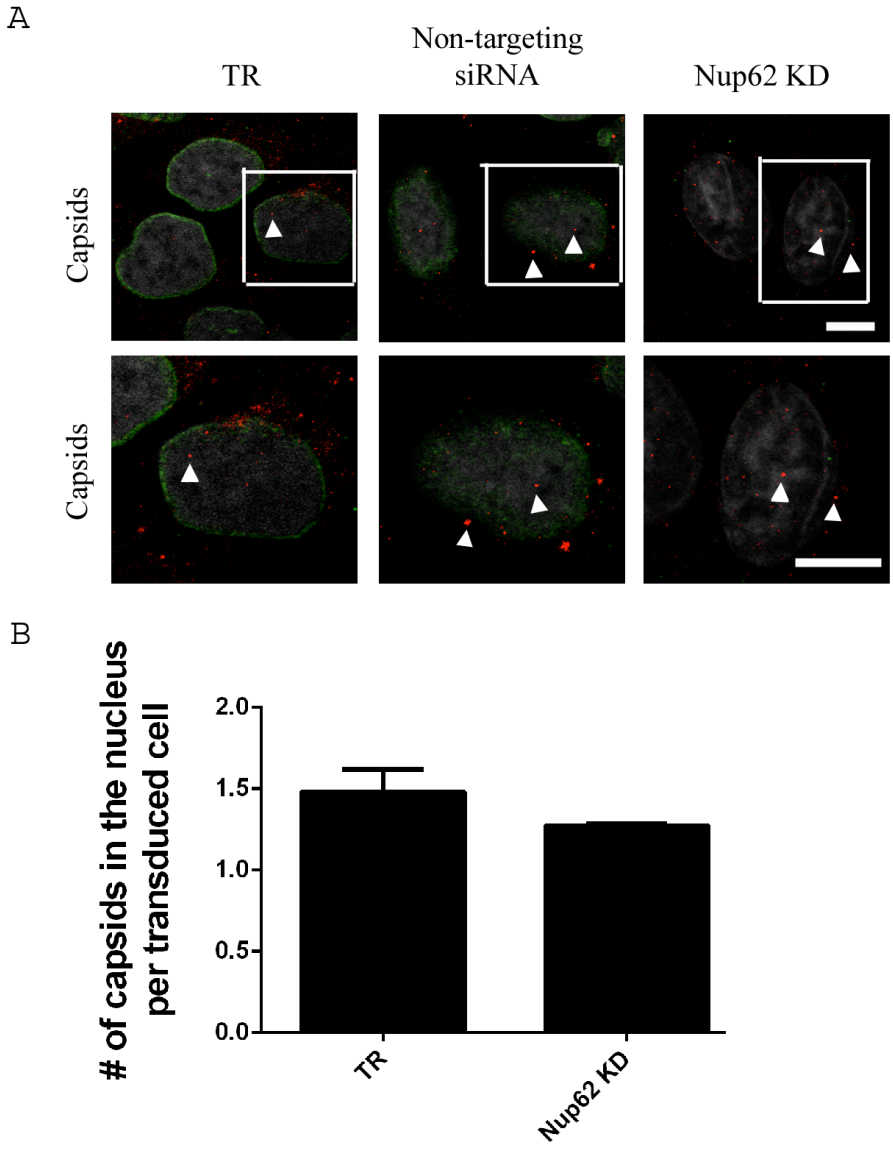


Figure 5-2: Nup62 is not necessary for nuclear import of baculovirus capsids during transduction. (A) HeLa cells treated with transfection reagent (TR), non-targeting siRNA, or depleted of Nup62 by RNAi, transduced with baculovirus and assayed for indirect immunofluorescence confocal microscopy using antibodies against Nup62 and the viral capsid protein VP39. The portions or regions of cells within the white boxed areas are magnified in the lower panels. (B) Quantification of the number of capsids in the nuclei of transduced cells. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; Nup62, green; capsids, red. White arrowheads point to capsids.

Similarly to the nuclear import assay with Nup153 knockdown, in transduced cells, capsids were found in the nucleus (Figure 5-4). Because Nup153 is necessary in mediating efficient importin-β mediating nuclear import, our findings are consistent with our previous data showing that baculovirus capsids can enter the nucleus independently of importin-β receptors.

5.2.3 Nup358 is necessary for efficient nuclear import

Microinjection of *Xenopus laevis* oocyte experiments showed viral capsids docking at the cytoplasmic periphery of the NPC prior to nuclear import. The cytoplasmic filament protein Nup358, often referred to as RanBP2 interchangeably, is located at the distal end of the cytoplasmic filament. It has been shown to interact with single-stranded RNA and components of the nucleocytoplasmic transport machinery, such as Ran, SUMOylated RanGAP1, and CRM1 (Cook et al., 2007; Hoelz et al., 2011; Kassube et al., 2012). To test the necessity of this protein as an initial binding site for AcMNPV capsids prior to nuclear import, HeLa cells were treated with 25 nM of Nup358 siRNA for 72 hours (Figure5-5A). 70 kDa Dextran Texas Red also did not freely diffuse into the nucleus in nuclear import assays performed with cells depleted of Nup358, hence the NPC diffusion barrier was not disrupted (Figure 5-5B). Similar to our observed results in Nup153-depleted cells, Cy3-NLS-BSA entered the nucleus at a lower efficiency in Nup358 knockdown cells compared to cells untreated with the siRNA, even in the presence of cytosolic factors and energy (Figure 5-5C, top panel). This phenomenon is consistent with the results obtained by Hutten and colleagues suggesting that the Nup358-RanGAP complex is required for efficient importin-α/β-dependent nuclear import (Hutten et al., 2009). In digitonin-permeabilized HeLa cells depleted of Nup358, viral capsids were seen in the nucleus when the assay was performed with or without cytosolic factors and energy (Figure 5-5C, bottom panel).

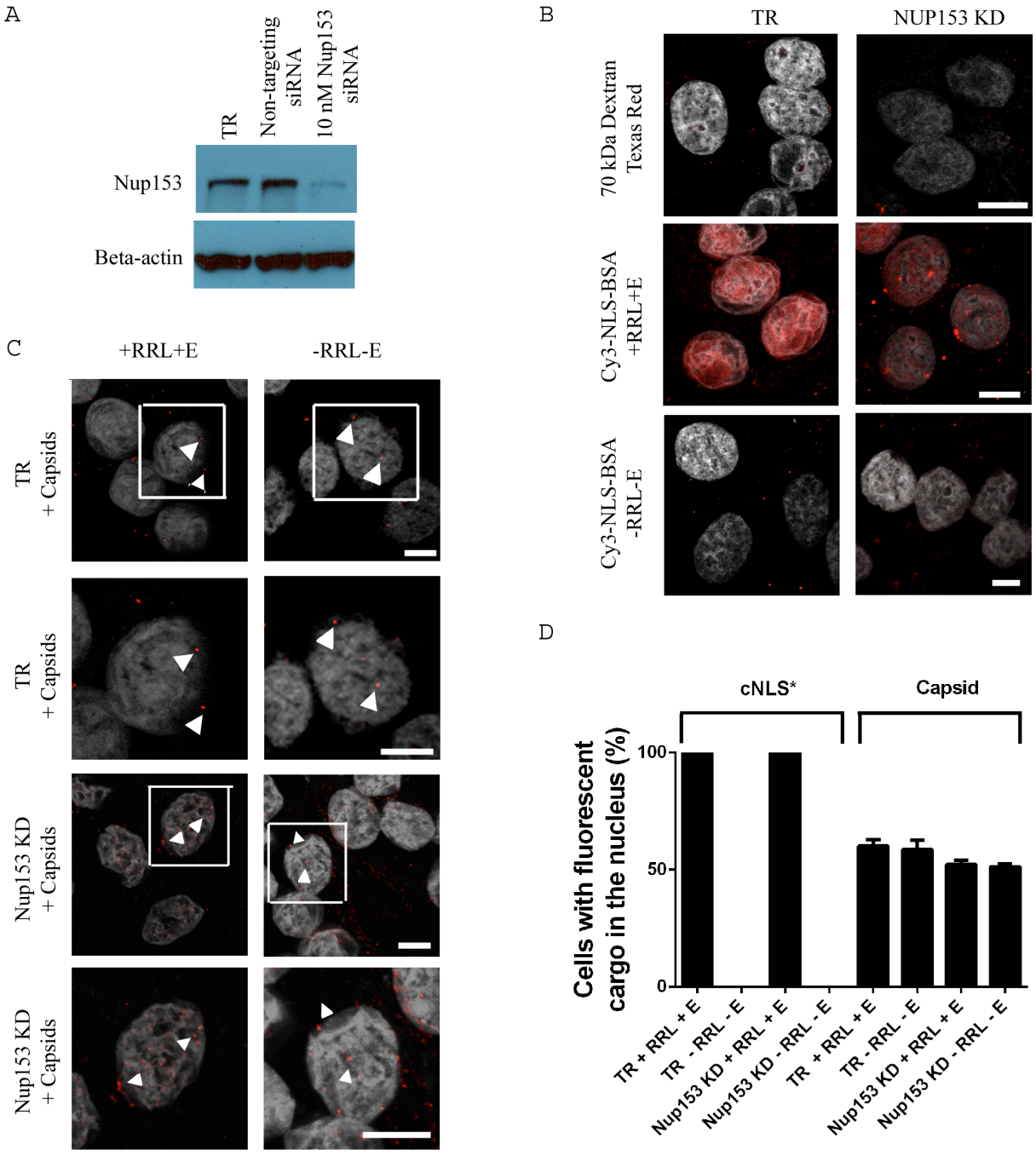

A
TR
Non-targeting siRNA
10 nM Nup153 siRNA
Nup153
Beta-actin
B
TR
NUP153 KD
70 kDa Dextran Texas Red
Cy3-NLS-BSA +RRL+E
Cy3-NLS-BSA -RRL-E
C
+RRL+E
-RRL-E
TR + Capsids
TR + Capsids
Nup153 KD + Capsids
Nup153 KD + Capsids
D
cNLS*
Capsid
Cells with fluorescent cargo in the nucleus (%)
100
50
0
TR + RRL + E
TR - RRL - E
Nup153 KD + RRL + E
Nup153 KD - RRL - E
TR + RRL + E
TR - RRL - E
Nup153 KD + RRL + E
Nup153 KD - RRL - E

Figure 5-3: Depletion of Nup153 by RNAi did not affect the nuclear import efficiency of baculovirus capsids. (A) Nup153 knockdown was assayed by Western blotting using specific antibodies against Nup153s in transfection reagent (TR) treated, non-targeting siRNA treated, or Nup153-siRNA treated cells. Beta-actin labelling was used as a loading control. (B and C) Digitonin-permeabilized HeLa cell import assay was performed in both non-transfected (TR) HeLa cells and Nup153-depleted cells with (B) 70 kDa Dextran fluorescently-labeled with Texas Red (as a permeabilization control), Cy3-NLS-BSA, or (C) baculovirus capsids. The portions or regions of cells within the white boxed areas are magnified in the lower panels. Localization of the fluorescence substrate was assayed by confocal microscopy. (D) Quantification of the number of cells with fluorescent cargoes in the nucleus. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; 70 kDa Dextran and Cy3-NLS-BSA (B), or capsids (C), red. White arrowheads point to capsids.

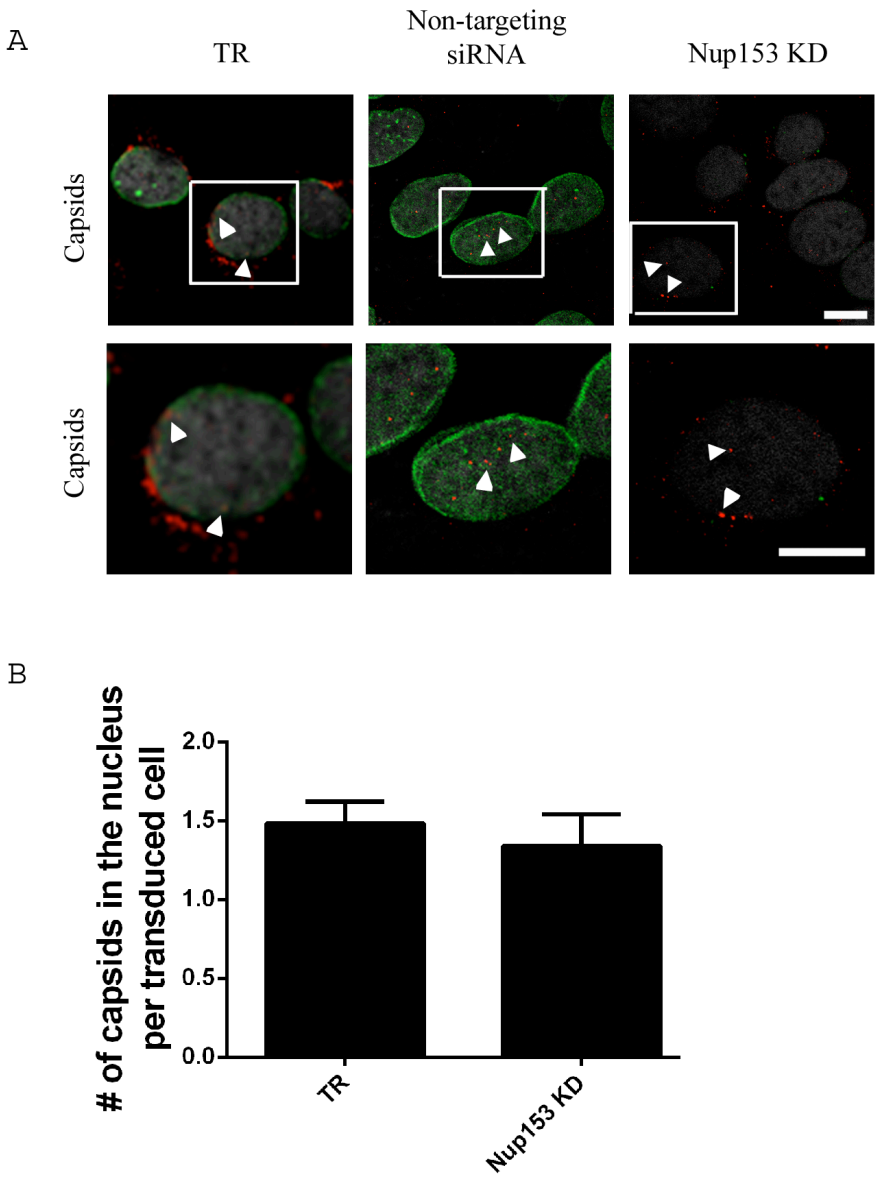


Figure 5-4: Nup153 is not necessary for nuclear import of baculovirus capsids in transduced cells. (A) HeLa cells treated with transfection reagent (TR), non-targeting siRNA, or depleted of Nup153, transduced with baculovirus and assayed for indirect immunofluorescence confocal microscopy. The portions or regions of cells within the white boxed areas are magnified in the lower panels. (B) Quantification of the number of viral capsids in the nuclei of transduced cells. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; Nup153, green; capsids, red. White arrowheads point to capsids.

However, our quantified data suggests a significant difference in the nuclear import efficiency of viral capsids between untreated cells and cells depleted of Nup358 (Figure 5-5D). Nuclear import of viral capsids was more efficient in wild-type untreated cells containing Nup358. The observed decrease in the amount of capsids found inside the nucleus of Nup358 depleted cells suggests the necessity of this Nup, residing within the cytoplasmic filaments, for efficient nuclear import of the viral capsid, but capsid nuclear import can still occur without Nup358.

Similarly to the nuclear import assay with Nup153-depleted HeLa cells, in cells transduced with baculovirus, capsids were equally able to enter the nucleus in Nup358 knockdown cells, although at a lower efficiency (Figure 5-6A). However, this decrease in efficiency was not statistically significant (Figure 5-6B) suggesting that Nup358 mediates efficient nuclear import of baculovirus capsids.

5.3 Discussion

The data in this chapter suggests that the three FG-Nups we tested do not play an important role in nuclear import of baculovirus capsids. All three Nups that we investigated here are FG-containing Nups that play a role in various nuclear transport events. As Nups are highly redundant and are divided into different categories based on their function and location (described in section 1.2.1), depletion of a single Nup individually in our assays may not fully affect the nuclear import efficiency of baculovirus capsids. For instance, Nup62 within the central channel forms a complex of ~235 kDa with Nup58, Nup54, and Nup45. While the transport channel is formed of only Nup62, Nup54, and Nup58, studies have shown that an NPC consists of 128, 64, and 32 molecules of each respective Nup (Solmaz et al., 2011).

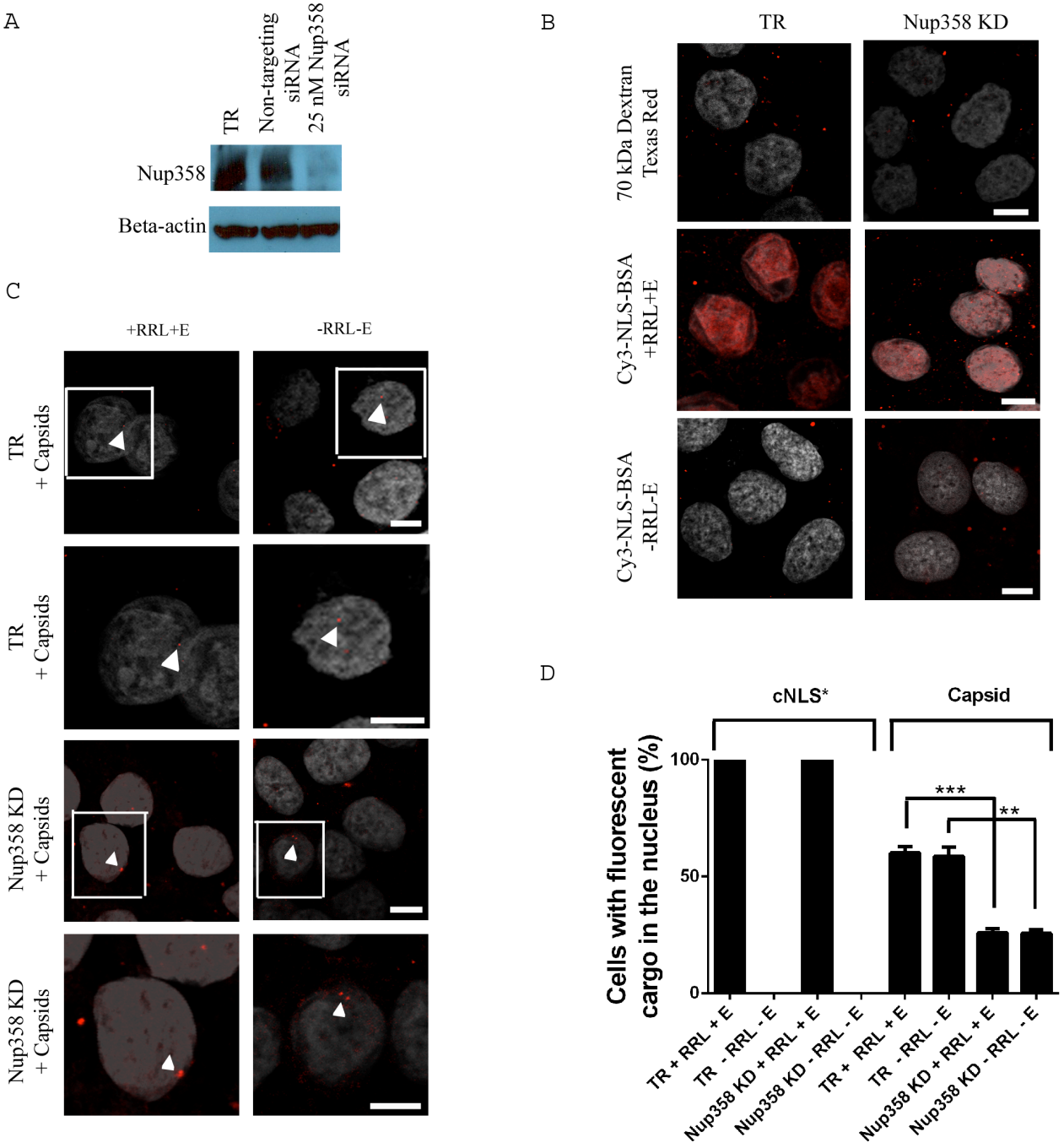

A
TR
Non-targeting siRNA
25 nM Nup358 siRNA
Nup358
Beta-actin
B
TR
Nup358 KD
70 kDa Dextran Texas Red
Cy3-NLS-BSA +RRL+E
Cy3-NLS-BSA -RRL-E
C
+RRL+E
-RRL-E
TR + Capsids
TR + Capsids
Nup358 KD + Capsids
Nup358 KD + Capsids
D
cNLS*
Capsid
Cells with fluorescent cargo in the nucleus (%)
100
50
0
***
**
TR + RRL + E
TR - RRL - E
Nup358 KD + RRL + E
Nup358 KD - RRL - E
TR + RRL + E
TR - RRL - E
Nup358 KD + RRL + E
Nup358 KD - RRL - E

Figure 5-5: Depletion of Nup358 by RNAi reduced the nuclear import efficiency of baculovirus capsids. (A) Nup358 knockdown was assayed by Western blotting using specific antibodies against Nup358s in transfection reagent (TR) treated, non-targeting siRNA treated, or Nup358-siRNA treated cells. Beta-actin labelling was used as a loading control. (B and C) Digitonin-permeabilized HeLa cell import assay was performed in both non-transfected (TR) HeLa cells and Nup358-depleted cells with (B) 70 kDa Dextran fluorescently-labeled with Texas Red (as a permeabilization control), Cy3-NLS-BSA, or (C) baculovirus capsids. The portions or regions of cells within the white boxed areas are magnified in the lower panels. Localization of the fluorescence substrate was assayed by confocal microscopy. (D) Quantification of the number of cells with fluorescent cargoes in the nucleus. cNLS* represents Cy3-NLS-BSA, which contains the classical NLS. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; 70 kDa Dextran and Cy3-NLS-BSA (B), or capsids (C), red. White arrowheads point to capsids. **P=0.0016, ***P=0.0004 (unpaired Student's t-test). White arrowheads point to capsids.

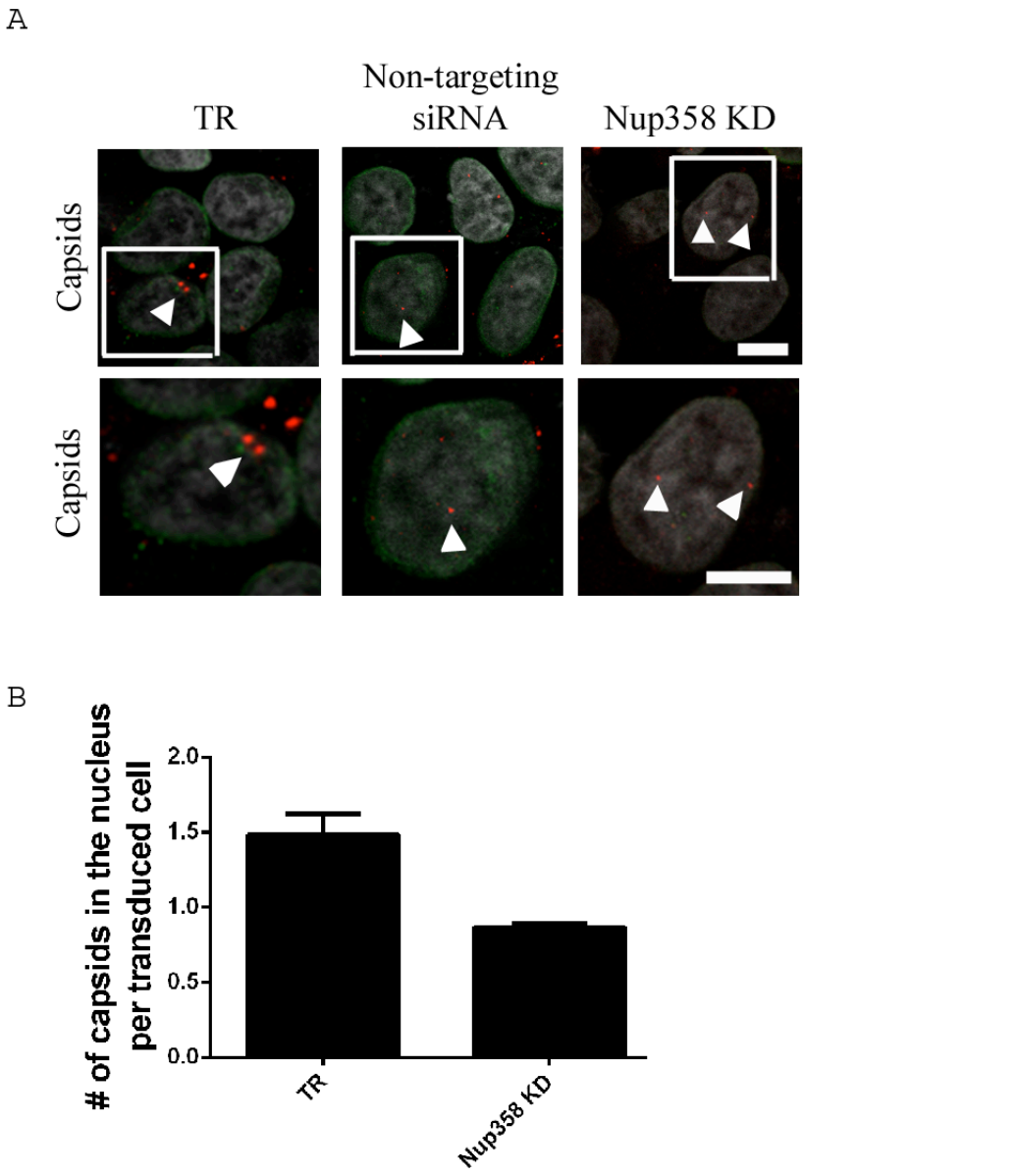


Figure 5-6: Nuclear import of baculovirus capsids is less efficient in baculovirus transduced cells depleted of Nup358. (A)HeLa cells treated with transfection reagent (TR), non-targeting siRNA, or depleted of Nup358, transduced with baculovirus and assayed for indirect immunofluorescence confocal microscopy. The portions or regions of cells within the white boxed areas are magnified in the lower panels. (B) Quantification of the number of nuclei with viral capsids. Images were obtained using an Olympus Confocal Microscope, and quantification was performed using confocal images. Shown are the mean value and standard error of the mean (SEM) scored from 200 cells for each condition from three different experiments. Scale bars, 10-μm. DAPI, grey; Nup358, green; capsids, red. White arrowheads point to capsids.

Because the central channel is formed through a complex with other Nups, it is very plausible that depleting Nup62 alone will not be sufficient in blocking nuclear import of the baculovirus capsid.

The Nup62 complex within the central channel of the NPC encompassing Nup58 contain amphipathic α-helical regions that slide against each other (Hoelz et al., 2011). This dynamic movement could potentially allow the central channel to dilate in response to cargo translocation, a phenomenon observed in electron micrographs (Akey, 1990, 1995; Beck et al., 2004; Solmaz et al., 2011). This could explain the empty space we observed during baculovirus translocation into the nucleus through the central channel in our EM data. However baculovirus capsid nuclear import efficiency did not change between cells containing Nup62 compared to those without, suggesting that the sliding motion does not increase or decrease NPC permeability. This can also be observed in the nuclear import of Cy3-NLS-BSA in Nup62 depleted cells, as Cy3-NLS-BSA was able to enter the nucleus (Figure 5-1) but interestingly at a lower efficiency than in untreated cells (Figure 3-1). The N-terminal FG region of Nup62 serves as a docking site for NTF2 (nuclear transport factor 2), the receptor for RanGTPase (Clarkson et al., 1996; Paschal and Gerace, 1995), while the C-terminus has been shown to interact with importin-β *in vitro* (Percipalle et al., 1997). In this case, the ability of the viral capsid to enter the nucleus independently of the Ran cycle and importin-receptors is consistent with our data that Nup62 within the central channel is dispensible.

The C-terminus of Nup153 is necessary for importin-α/β-mediated nuclear import, but not transportin mediated nuclear import (Shah et al., 1998; Walther et al., 2001). However because nuclear import of the baculovirus capsid, as shown in Chapter 4, occurs in a non-classical

mechanism (in the absence of importin-α/β), our data here supports the idea that Nup153 is important for importin-β mediated nuclear import, but not for baculovirus nuclear import. As decribed in section 1.3.4, Nup153 acts as a docking site for HBV capsids, triggering capsid disassembly and genome release into the nucleus even though HBV has been shown to enter the nucleus via classical nuclear import pathway of importin-α/β (Schmitz et al., 2010). We suggest that the nuclear basket may open up to allow the intact baculovirus capsid to pass through, and in doing so, Nup153 may also dynamically relocate and not interact with the capsid. If this model is true, it would explain why depleting Nup153 did not affect the nuclear import efficiency of baculovirus capsids.

Nup358 at the periphery of cytoplasmic filaments provide binding sites for transport factors, Ran, and RanGAP1 and these binding events are essential in the formation of the nuclear transport complex. Both Nup358 and Nup153 contain zinc finger domains that act as a binding site for Ran, thereby increasing the efficiency of nucleocytoplasmic transport (Yaseen and Blobel, 1999). Consistent with our data in Chapter 4 where nuclear import of baculovirus capsids occur independently of the Ran cycle, it is not surprising that capsids still localized in the nucleus in Nup358 and Nup153 depleted cells. It remains intriguing to see a significant decrease in capsid nuclear import efficiency only when Nup358 is depleted. Nup358 has been shown to be dispensable in *Xenopus laevis* oocytes for importin-α/β-dependent nuclear import. However, a reduced nuclear import rate was observed in *Drosophila* cells depleted of Nup358 (Sabri et al., 2007). Therefore, the dominant role of Nup358 may not be to form a permeability barrier for nuclear import, but to act only as a docking site (Walther et al., 2002). Because nuclear import of baculovirus capsids occurred more efficiently in the presence of Nup358, our data cannot rule

out the possibility that docking of the viral capsid to Nup358 is an important step. Future studies are needed to demonstrate the potential interaction between Nup358 and the baculovirus capsid.

Chapter 6

General Discussion and Future Perspective

Nuclear import of baculovirus is an under-studied topic despite the virus' popular use in agriculture and research. I have shown for the first time that AcMNPV capsids are able to traverse the NPC completely intact, further releasing capsids into the nucleoplasm. There are a number of novel findings to highlight. First, the NPC appears to undergo dynamic rearrangement to accommodate such a large cargo through the central channel. Second, interestingly, import of the baculovirus capsid does not follow the classical nuclear import pathway of using importin-β and RanGTPase. Third, the role of three FG-Nups was investigated and only one of these proteins was found to play a role in the nuclear import efficiency of baculovirus capsids. Fourth, cellular F-actin and the availability of Arp2/3 to promote actin nucleation are critical for nuclear entry of the baculovirus capsid. I propose that nuclear import of baculovirus AcMNPV capsids occurs via a unique and previously undefined mechanism that is importin-β and Ran-independent, and requires actin filaments to facilitate its translocation into the nucleus.

The baculovirus AcMNPV capsid is among the largest cargoes that translocate through the NPC, therefore studies on nuclear import of baculovirus might provide important information that can be used to uncover new modes of NPC translocation. Below I will first discuss the dynamic flexibility of the NPC that enables successful translocation of the baculovirus capsid, then the properties of the baculovirus capsid that assist in its nuclear import, the cellular proteins essential for mediating nuclear import of AcMNPV capsids, and finally using baculovirus as an example to understand nuclear transport models.

6.1 Dynamic flexibility of the NPC

Electron microscopy studies presented in Chapter 3 demonstrate that the NPC is extremely flexible and must undergo a large scale of rearrangement to allow such a large capsid to occupy its central channel. Considering the width of the capsid (~30-nm) and our measurements of the empty space surrounding the NPC-crossing capsid (about 10-nm from each side of the capsid), the NPC central channel expanded to ~50-nm to allow the capsid to pass through it. This is the same value for the dimension of the NPC central channel that was deduced from 3-D reconstructions of cryo-EM of NPCs (Beck et al., 2004; Beck et al., 2007; Frenkiel-Krispin et al., 2010; Stoffler et al., 2003). Our finding indicates that whatever is normally filling the NPC central channel must undergo conformational changes, leaving the NPC in an 'open state' that allows the capsid to travel across it.

As illustrated in Figure 3-5, under conditions where transport across the NPC is not occurring, cytoplasmic filaments bend inward into the NPC central channel (Pante and Aebi, 1996), while FG-repeat domains of Nups within this channel are natively unfolded and dispersed throughout this channel creating the typical electron-dense appearance of the center of the NPC in electron micrographs of NPC cross-sections (Figure 6-1). This 'closed state' does not allow the passage of large cargoes. The electron micrographs of AcMNPV capsids caught in the middle of the NPC (Figure 3-6) indicate that the FG-Nups in the central channel changes into the open conformation, like an elevator door- or an iris-mechanism (Akey, 1990) that completely opens up, leaving the 50-nm in diameter opening of the NPC central channel (Figure 6-1). Presumably cellular receptors or signaling events that are yet unknown function as the magical phrase 'Open, Sesame' that notifies nucleoporins in the central channel to move towards the body of the NPC

unlocking this gate and leaving the NPC in a 'open state' that allows the capsid to get across it (Figure 6-1). It is possible that such a mechanism is only for large cargo, like the baculovirus capsid, while other mechanisms may work only for small cargos.

The ability for the central channel of the NPC to accommodate a large intact capsid ~250 to 300-nm in length is rather unique. A similar situation was observed for the NPC translocation of the Balbiani ring granule, a premessenger RNP complex of very large size synthesized in the larval salivary glands of *Chironomus tentans*. These granules are 50-nm in diameter and consist of an RNP ribbon bent into a ring-like structure. As the granule is exported from the nucleus through the NPC, the ribbon (25-nm in diameter by 135-nm in length) straightens out and has been shown to occupy the central channel of the NPC (Mehlin and Daneholt, 1993; Mehlin et al., 1992, 1995). Both our data with the baculovirus capsid and the published results for the Balbiani ring granules clearly illustrate the flexibility of the NPC central channel.

6.2 Properties of the baculovirus capsid that assist in nuclear import

6.2.1 Endocytic modifications do not mediate nuclear import of AcMNPV capsids

The exposure to conditions along the endocytic pathway is often necessary for viruses to become competent for nuclear import. For example the acidic environment of the endosome could trigger exposure of NLSs due to conformational changes caused by the change in pH. Given that baculovirus capsids were microinjected into the cytoplasm of *Xenopus laevis* oocytes, we bypassed the endocytic route.

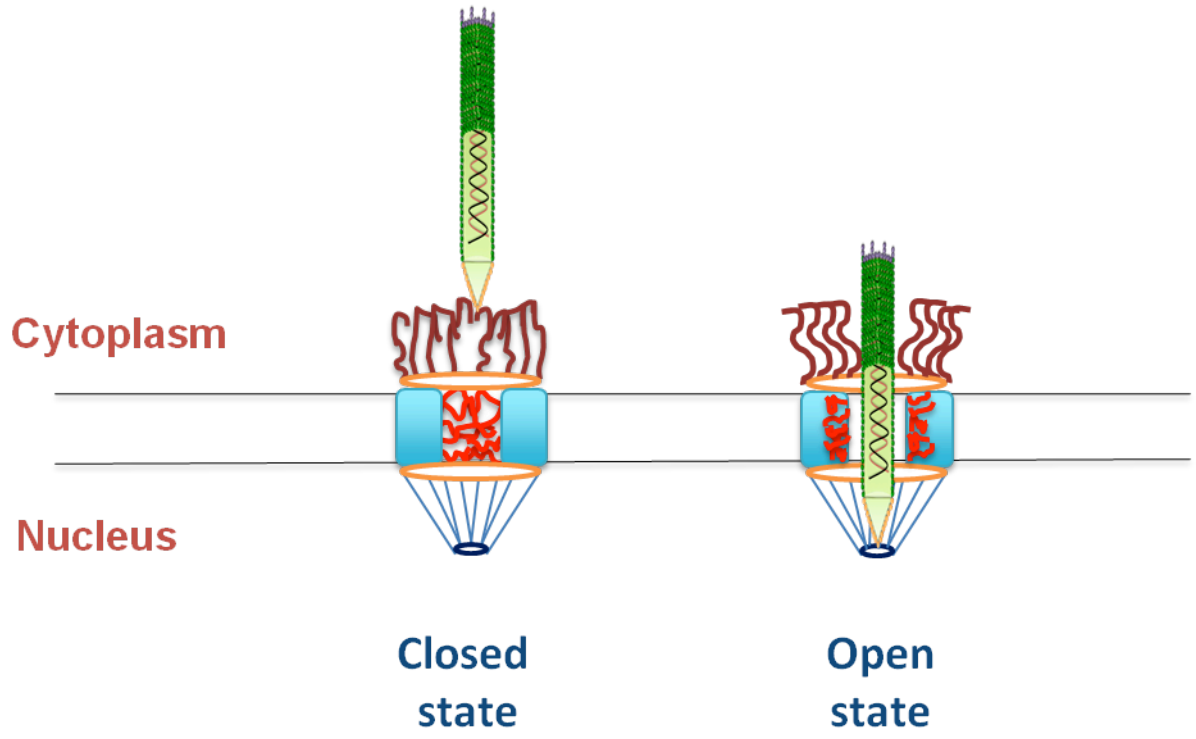


Figure 6-1: Dynamic changes of the NPC. Schematic diagram illustrating the 'closed' and 'open' states of the NPC central channel. In the closed state, the NPC cytoplasmic filaments and nucleoporins within the central channel prevent the passage of the capsid. The open state consists of proteins with different conformations as the cytoplasmic filaments straighten out and nucleoporins retract within the central channel towards the body of the NPC to open up the passageway, allowing for translocation of the capsid through the central channel.

Since we still observed nuclear import of the injected capsids, our data suggests that either the NLSs were already exposed on the surface of the capsid, or modifications of the capsid in the cytoplasm facilitated this exposure. However, this is difficult to test since putative NLSs on the capsid and the nuclear transport receptors recognizing these remain largely undefined. Our data further confirmed previous suggestions that endocytic conditions are not necessary for the exposure of NLSs for nuclear import of baculovirus capsids (Salminen et al., 2005). More recently, baculovirions were shown to be able to enter and infect *Sf*9 insect cells through a non-endocytic pathway, further supporting the idea that putative NLSs, if any, are within the capsid itself and do not require modifications triggered by the acidic environment of the endosome (Dong et al., 2010).

6.2.2 Unidentified putative NLSs on the baculovirus capsid

In most cases, facilitated nuclear import requires the presence of an NLS. Because the endocytic route is not necessary for mediating nuclear import, baculoviral capsid proteins may contain putative NLSs. Of all 12 known capsid proteins shown in Figure 1-6, only VP80 has been suggested to contain a bipartite set of putative cNLS (aa 424-439), similar to the nucleoplasmin protein. This protein is located at one end of the capsid, but whether it is the apical end or the blunt end of the capsid remains unknown (Marek et al., 2011). In addition, the function of this putative cNLS in nuclear import of the entire intact capsid has not been examined. Loss of function and gain of function experiments must be performed to fully conclude that the putative bipartite cNLS of VP80 functions in nuclear import of the entire baculovirus capsid. If this protein is located at the apical end of the capsid, it would be interesting to determine the functional role of that putative cNLS in nuclear import of the baculovirus capsid. However, if the protein is located at the blunt end of the capsid, it would be physically impossible to function in

mediating nuclear import of the capsid as the apical end of the capsid interacts with and enters the NPC first.

NLSs may also exist on other capsid proteins but have yet to be proven to be bona fide NLSs as they are instrinsically difficult to identify. For example, a monopartite cNLS is comprised of either a stretch of 4 or 7 amino acid residues in defined sets of patterns. However, there is variability in these patterns which can be a stretch of 4 basic amino acids (K or R), or 3 basic amino acids (K or R), with the fourth amino acid being an H or P or a stretch of 7 amino acids starting with P and followed within 3 residues by 3 K/R residues out of 4 (Ketha and Atreya, 2008). We performed sequence alignment of the prototypical amino acid sequence PKKKRKV of the cNLS of SV40 large T-antigen with the 12 AcMNPV capsid proteins and did not find any of these capsid proteins to contain the exact SV40 large T-antigen cNLS. In addition, the nucleoplasmin bipartite sequence KR[PAATKKAGQA]KKKK could not be aligned with any of the 12 AcMNPV capsid proteins. This eliminates the possibility that proteins residing on the baculovirus capsid contain these two particular cNLSs. In addition, this data is in agreement with the results presented in Chapter 4 that the baculovirus capsid does not use the classical nuclear import pathway. Future studies using bioinformatic analysis tools could be performed to elucidate putative cNLSs or non-classical NLSs on the currently known 12 AcMNPV capsid proteins, but their function in mediating nuclear import of the entire capsid must be validated further. Since our data rules out the use of the classical nuclear import pathway for the nuclear import of AcMNPV capsids, it further suggests that the putative cNLS on VP80 (Marek et al., 2011) is not involved in nuclear import of the intact capsid, but could likely be used during nuclear entry of the protein itself which is needed for capsid assembly in the nucleus.

Interestingly, proteins may contain unexposed NLSs that may not be recognized by import receptors, and therefore are not sufficient in driving/supporting nuclear import. For instance, signal transducers and activators of transcription (STATs) that are activated by cytokines and growth factors, contain a non-functional cNLS. However, upon dimerization of homodimeric STAT1 or heterodimeric STAT1/STAT2, these complexes are able to bind with importin-α, leading to nuclear import of the entire complex (Fagerlund et al., 2002). Thus, it is possible that even though the baculovirus capsid may contain a NLS, it may not be fully functional to mediate nuclear import of the entire capsid unless it oligomerizes with other proteins. The NLS may need to become exposed upon dimerization or binding of a second protein to mediate nuclear import of the baculovirus capsid.

Besides the classical nuclear import pathway using the prototypical cNLS, other pathways and NLSs exist as well (described in section 1.2.2.6). It has been shown that the prevalence of classical nuclear import in yeast *S.cerevisiae* is ~55%, meaning that ~45% of the nuclear residing proteins use alternative pathways (Lange et al., 2007). This suggests that although the classical nuclear import pathway is predominantly used, other mechanisms are just as prevalent and cannot be disregarded. An alternative NLS referred to as the M9 sequence binds to transportin for nuclear import, and was initially identified by a span of 30-40 basic residues rich in glycine and serine (reviewed in Xu et al., 2010). Recently, cargoes using the receptor transportin (including those containing the previously suggested M9 sequence) have been re-defined to contain PY-NLS. In this classification, the cargoes are described by its physical properties and requirement for intrinsic structural disorder, as well as the overall basic set of sequence (Lee et al., 2006). The N-terminus is hydrophobic or consists of a basic motif while the C-terminus contains an $RX_{2-5}PY$ motif (Lee et al., 2006; Xu et al., 2010). Following these parameters, future

studies on the physical properties of the 12 baculovirus capsid proteins will help elucidate putative NLSs that may use transportin instead of classical nuclear import receptors.

6.3 Cellular proteins essential in mediating nuclear import of AcMNPV

6.3.1 Proteins that follow the non-classical nuclear import pathways

As outlined in section 1.2.2, many nuclear import pathways exist within the cell. For this reason, different cellular proteins in various combinations may also play a role in mediating nuclear import. Our data in Chapter 4 suggests that baculovirus nuclear import occurs independently of the classical nuclear import pathway as we show that nuclear import of the capsid can occur in the absence of key cytosolic proteins. As a consequence, inhibitors of importin-β and the addition of a non-hydrolyzable form of GTP did not hinder the ability for baculovirus capsids to enter the nucleus.

Studies dissecting pathways for nucleocytoplasmic transport have shown that these processes are much more elaborate than 'one size fits all' (reviewed in Moore, 1998). For instance, nuclear import of human cyclin-B1 Cdc-2, a regulatory protein involved in mitosis, is Ran-independent but importin-β-dependent (Takizawa et al., 1999). This is similar to the nuclear import of U snRNPs as described in section 1.2.2.6 whereby nuclear entry requires importin-β. In this case, the protein snurportin-1 behaves like importin-α by mediating the binding between the NLS on U snRNP and importin-β (Huber et al., 2002; Rollenhagen et al., 2003). Proteins such as U1a and U2b spliceosomal proteins are also able to enter the nucleus without cytosol and do not require RanGTPase activity. But instead, these proteins rely on ATP (Hetzer and Mattaj, 2000), indicating that nuclear import of these proteins do not occur by diffusion but is an active process,

requiring energy, that does not involve the classical nuclear import pathway of importin receptors and RanGTPase. In addition, nuclear import of U1a and U2b spliceosomal proteins was reduced in the presence of importin-β, suggesting that there are common binding sites among the proteins (Hetzer and Mattaj, 2000).

Another example of an unconventional nuclear import mechanism is the latency-associated nuclear antigen (LANA), a nuclear targeted protein which contains a bi-functional nuclear localization sequence, and is involved in tumorigenesis and is evolutionarily conserved between Karposi's sarcoma-associated herpesvirus (KSHV) and retroperitoneal fibromatosis herpesvirus (RFHV). LANA contains a set of classical bipartite NLS embedded within a non-classical NLS. This is advantageous for the virus as it suggests that the protein may localize to distinct subnuclear compartments, presumably interacting with different nuclear components to maintain viral latency (Cherezova et al., 2011). NP1 of human bocavirus (HBoV) is a nuclear protein involved in DNA replication and in the inhibition of IFN-β (Li et al., 2013). Similar to LANA, this protein also contains a set of cNLSs embedded within a non-cNLS, perhaps to increase infection efficiency. These examples demonstrate the complexity of identifying a region within protein sequences that could potentially function as an NLS. As proteins may contain more than a single NLS, loss of function experiments would need to be performed to determine the role of each NLS.

Instead of NLSs, proteins themselves may contain properties that allow them to facilitate nuclear import. Large proteins such as actinin-4, β1-spectrin, as well as β-catenin, containing amphipathic motifs, have been shown to overcome the selectivity barrier of the NPC created by FG-Nups. Actinin-4 and β1-spectrin contain multiple spectrin repeats (SRs) that are sufficient in

mediating nuclear import (Kumeta et al., 2012). A recent study showed that conformational changes to these large proteins, exposing hydrophobic amino acid residues, results in overcoming the permeability barrier of the NPC (Kumeta et al., 2012). The baculovirus capsid could contain amphipathic motifs and thus, capsid proteins can be examined to determine if these motifs exists. Once again, loss of function experiments must be performed to validate the role of those particular motifs in mediating nuclear import of the entire baculovirus capsid. However, in our EM results in Chapter 3, structural distortion of the capsid during nuclear translocation was not observed, suggesting that conformational changes did not occur to expose hydrophobic amino acid residues. This eliminates the potential role of putative amphipathic (armadillo) ARM repeats, a characteristic of importin-α, in mediating nuclear import.

Currently, our results show that nuclear import of the baculovirus capsid occurs in a non-classical Ran-independent manner, but can be inhibited using WGA. This suggests that binding site on the NPC is necessary during capsid translocation and this process does not rely on Ran. It remains possible that the viral capsid requires hydrolysable ATP instead of GTP, similar to spliceosomal proteins U1a and U2b. Since capsids can be seen in the nucleus when a non-hydrolyzable form of Ran is added or in the absence of an energy regenerating system, it seems probable that energy is not a requirement for translocation of the capsid through the NPC. This is similar to nuclear translocation of β-catenin, a Wingless/Wnt signal transduction pathway protein, which can accumulate in the nucleus in a temperature-dependent and WGA-sensitive manner, in the absence of cytosol, an energy regenerating system, and RanGTPase (Fagotto et al., 1998; Yokoya et al., 1999). β-catenin possesses 12 ARM repeating motifs (Huber et al., 1997; Peifer et al., 1994), a common characteristic found in importin-α. These ARM repeats have been shown to be fundamentally similar to HEAT motifs in importin-β responsible for interaction with Nups

(Malik et al., 1997), suggesting that β-catenin competes for these same Nup binding sites. Furthermore it was recently shown that these ARM repeats in β-catenin do indeed facilitate nuclear import via binding to nucleoporins Nup62, Nup153, and Nup358 (Sharma et al., 2012). We also compared these ARM repeat sequences to the 12 capsid protein sequences and observed no similarity, implying that the baculovirus capsid does not follow the same nuclear import mechanism exploited by β-catenin. As our data suggest that the baculovirus capsid does not use the classical nuclear import mechanism involving importin-β, it is therefore not surprising that the capsid itself would not possess ARM repeats as importin-β is an essential receptor involved in nuclear import of cargoes that require importin-α.

We contemplated the possibility of baculovirus capsid proteins possessing importin-β like properties, thereby not requiring addition cytosolic factors provided in digitonin-permeabilized cell assays. Importin-β is characterized by containing a set of 19 helical-repeat motifs (HEAT repeats (the binding site for Nups is located between HEAT repeats 4-8)), an importin-α binding domain, and a GTP binding domain (Strom and Weis, 2001). We compared the sequence of these three importin-β-like features to the amino acid sequence of all 12 AcMNPV capsid proteins using the online program Clustal Omega and observed no sequence similarities (see Appendix A). Similar sequences were seen scattered throughout the alignment and no obvious trends can be seen. This suggests that the baculovirus capsid itself does not substitute the role of importin-β by binding to Nups during nuclear translocation.

In summary, our data suggests that baculovirus uniquely uses a nuclear import mechanism that is different than those previously studied for other large cargoes that enter the nucleus through

NPCs. Properties pertaining to the baculovirus capsid itself also unraveled no identifiable importin-α/β-like characteristics that may be used to directly bind to components of the NPC.

6.3.2 Role of cytoskeletal structures in mediating nuclear import of baculovirus capsids

Viruses often make use of cellular cytoskeletal components of the cell during viral infection or cellular transport. The use of cell culture media RPMI 1640 was recently shown to alter the activity of protein kinase C subtypes, α and ε, which resulted in the relocalization of vimentin in mammalian cells (Mahonen et al., 2010; Turkki et al., 2013). Coincidently, transduction of baculovirus in mammalian cells was more efficient in RPMI 1640 media treated cells, further showing an increase in the nuclear import efficiency of baculovirus capsids (Mahonen et al., 2010). This suggests that vimentin plays a role in the transduction of baculovirus. Similarly, hepatocytes, which contain low levels of vimentin, have been shown to be more susceptible to baculovirus transduction compared to other mammalian cell lines (Bilello et al., 2001; Hofmann et al., 1995). Perhaps for this reason, baculovirus infection efficiency is highest in insect cells, which do not contain intermediate filaments (Volkman and Zaal, 1990). In addition, depolymerization of microtubules also occurs during baculovirus infection of *Sf* cells. Drugs used to stabilize microtubules enhance the ability of these cytoskeletal structures to act as barriers during baculovirus transport towards the nucleus, thereby interfering with viral replication (Salminen et al., 2005; Volkman and Zaal, 1990). These data suggests that removal of the diffusion or transport constraints within the cytoplasm is important for the nuclear entry of the baculovirus capsid.

The baculovirus capsid itself contains a WASP-like protein, VP78/83, located at one end of the capsid, involved in promoting actin nucleation (reviewed in Rohrmann, 2011). This facilitates the movement of baculovirus capsids towards the nucleus. This is similar to bacterial pathogens such as *Listeria, Shigella, Rickettsia, Mycobacterium marinum* and *Burkholderia pseudomallei* that are able to induce actin polymerization during cellular entry into its host, these pathogens have all adapted different strategies for interacting with the host Arp2/3 complex (reviewed in Gouin et al., 2005). From the data presented in Chapter 4, we demonstrated that polymerization of cellular F-actin upon binding of the Arp2/3 complex is needed to deliver the baculoviral capsid into the nucleus. Once VP78/83 on the viral capsid binds to F-actin, the Arp2/3 complex promotes actin nucleation from the mother filament to generate daughter filaments, and this is necessary for the delivery of viral capsids into the nucleus. Similarly in oocytes microinjected with purified baculovirus capsids and incubated at low temperature, nuclear import of the baculovirus was inhibited. The low temperature inhibits actin polymerization and could possibly provide structural changes to the NPC that do not facilitate the opening up of the central channel for cargoes to translocate into. This conclusion is supported by the results of the experiments with the Arp2/3 inhibitor CK666, which was able to prevent activation of Arp2/3 complex, thereby preventing nuclear import of baculovirus capsids. Additionally, the actin depolymerisation drug, cytochalasin D, also inhibited nuclear import of the baculovirus capsid, therefore it appears that unlike vimentin and microtubules, cellular F-actin does not act as an impediment but is necessary for mediating nuclear import of the baculovirus capsid. To test if F-actin and Arp2/3 complex are sufficient in driving the baculovirus capsid into the nucleus without other cytosolic factors, experiments could be performed using purified nuclei incubated with baculovirus capsids in the presence of actin filaments and Arp2/3 complex, creating an

artificial environment composed of actin and Arp2/3. It would be interesting to see if actin and Arp2/3 alone are sufficient in driving the baculovirus capsid into the nucleus.

6.3.3 Role of FG-Nups during nuclear import of the baculovirus capsid

In Chapter 5 we investigated the necessity of three FG-Nups in mediating nuclear import of baculovirus capsids. As the capsid is able to traverse the NPC, we chose to investigate Nup358 found at the cytoplasmic filaments, Nup153 residing within the nuclear basket, and Nup62 in the central channel of the NPC. Our results indicate that both Nup62 and Nup153 are not essential proteins for nuclear entry of the baculovirus capsid, as depleting both of these proteins made no significant changes to the ability for the capsid to enter the nucleus. As we noticed using EM in Chapter 3 that capsids dock at the cytoplasmic filaments prior to entering the nucleus, it is not to our surprise that depleting Nup358 significantly decreased the nuclear import efficiency of the baculovirus capsid.

Nup358 within the cytoplasmic filaments of NPCs was recently shown to promote binding and nuclear import of two proteins; DBC-1 (deleted in breast cancer 1) and DMAP-1 (DNA methyltransferase 1 associated protein 1) (Walde et al., 2012). Binding of both DBC-1 and DMAP-1 to Nup358 occurred independently of transport receptors, and distinct regions within Nup358 act to increase the efficiency of nuclear import of both proteins (Walde et al., 2012). The role of Nup358 for baculovirus nuclear import may be similar to that of DBC-1 and DMAP-1 as our data suggests that nuclear import of baculovirus capsids can occur independently of transport receptors and the efficiency of nuclear import was decreased in Nup358-depleted HeLa cells. In addition, Nup98, Nup358 and Nup153 have been identified as host factors involved in HIV-1 infection (Brass et al., 2008; Di Nunzio et al., 2013; Konig et al., 2008, Monette et al., 2011).

The cyclophilin-homology domain of Nup358 mediates HIV-1 core docking at the NPC (Di Nunzio et al., 2012). Studies have showed that a single point mutation in the HIV-1 capsid protein could change the nuclear transport requirements of the virus, and cyclophilin domain of Nup358/RanBP2 determines the nuclear import pathway of the HIV-1 capsid (Lee et al., 2010; Schaller et al., 2011). A cyclophilin A-binding loop on the HIV-1 capsid has been suggested to interact with the cyclophylin domain of Nup358/RanBP2, which may lead to capsid uncoating during nuclear entry of the HIV-1 core for efficient infection (Bichel et al., 2013). Similarly, baculovirus capsid proteins may contain properties that mediate capsid binding to the cyclophilin-homology domain of Nup358. Therefore, depletion of Nup358 created an impediment on the nuclear import efficiency of baculovirus capsids. Future experiments can be performed mutating the cyclophilin domain of Nup358 to determine its role in nuclear import of the baculovirus capsid. Additional experiments could also be necessary to determine which baculovirus capsid protein mediates the binding of the capsid to Nup358.

Depletion of Nup153 has been suggested to interfere with nuclear import of viruses such as HIV-1 and HBV (Di Nunzio et al., 2012; Koh et al., 2013, Lee et al., 2010). For instance, Nup153 is essential in efficient nuclear import of HBV, which similar to baculovirus is able to translocate through the central channel of the NPC. However, contrary to baculovirus capsids, HBV capsids dock and disassemble at the nuclear basket in an importin-α and importin-β-dependent mechanism, releasing their viral genome into the nucleus (Schmitz et al., 2010). In the case of baculovirus, docking of the capsid to Nup153 at the nuclear basket did not appear to be necessary for capsid entry into the nucleus as capsids were found inside the nucleoplasm in Nup153-depleted HeLa cells. The baculovirus capsid was also able to enter the nucleus independently of nuclear import receptors, suggesting that baculovirus AcMNPV undergoes a

nuclear import mechanism that is different than that of HBV. Because Nup153 has been shown to interact with numerous proteins involved in the classical nuclear transport pathway (reviewed in Ball and Ullman, 2005) and our results indicate that depleting Nup153 did not alter the nuclear import efficiency of the baculovirus capsid, our findings in Chapter 4 supports the idea that nuclear import of baculovirus capsids occur independently of the classical import pathway.

Nup62 in the central channel of the NPC is often targeted for degradation or relocalization by viruses during viral infection (described in section 1.3.6). For example, during the late stages of HIV-1 replication, the abundance and localization of Nup62 and a number of other FG-Nups are altered (Monette et al., 2009; Monette et al., 2011). Similar to Nup358 and Nup153, these studies indicate that Nups may play important roles during HIV-1 infection at steps other than nuclear entry of the PIC. Often this includes inhibiting nuclear import of host cellular proteins, and therefore eliminating the host cellular immune response. Nup62 may act as a binding site for nuclear transport receptors, as molecules move through the central channel. However, if nuclear import of baculovirus capsids occur independently of the classical nuclear import pathway, as suggested by our data, then binding of Nup62 may not be necessary. Therefore depleting Nup62 will not play a role in nuclear import of the baculovirus capsid. Depleting Nup62 did not increase the nuclear import efficiency of baculovirus capsids, and did not result in an influx of Cy3-NLS-BSA. This suggests that Nup62 is not involved in the regulation of baculovirus capsid nuclear transport and even more importantly, Nup62 alone is not sufficient in the regulation of nuclear import. From EM images, we observe changes and relocalization of proteins that normally reside within the central channel upon capsid translocation. Nups within the central channel, in its 'closed' state appears electron dense as molecules are unable to enter the nucleus. In its 'open' state, Nups appears retracted and therefore must relocalize to provide a passageway for the viral

capsid. In theory, the absence of Nup62 should provide an open gate for capsids to enter the nucleus, which however was not the case. This suggests that Nup62 alone does not regulate the permeability barrier of nucleocytoplasmic transport of macromolecules, but may need to work together with Nup54 and Nup58 (Solmaz et al., 2011). As Nup54 and Nup58 together form the ring within the midplane of the transport channel that may undergo conformational changes to regulate nucleocytoplasmic transport, depletion of Nup62 itself may not have a drastic effect on the opening of the central channel. In addition to the Nup62 complex, other FG-Nups, such as the cytoplasmic and nucleoplasmic FG-Nups, may be able to extend into the central channel of the NPC, therefore acting as a regulator of nucleocytoplasmic transport.

FG-Nups individually may not play an important role in the translocation of baculovirus capsids through the central channel of the NPC; this is due to the redundancy of Nups that NPCs are composed of. The loss of one FG-Nup functioning as a regulator of active nuclear import can be compensated by the role of other FG-Nups within the NPC. This scenario is supported by data presented in Chapter 4 where Cy3-NLS-BSA is still capable of entering the nucleus in Nup-depleted HeLa cells. As baculovirus enters the nucleus independently of the classical nuclear import pathway, our results support previous findings that FG-Nups are crucial in mediating nuclear import of cargoes that require cellular receptors such as importin-β (reviewed in Wente and Rout 2010). In addition, our data supports the notion from previous studies that cells remain viable upon depletion of FG-Nups due to the redundancy of Nups in the NPC.

6.4 Using baculovirus to understand nuclear transport models

Despite advances in microscopic techniques to help correlate the structure of the NPC with its function, the molecular mechanism of translocation through the NPC remains elusive. Although several models have been proposed in recent years to explain this mechanism, due to the lack of *in vivo* experimental setups to test these models, they remain controversial and are a major topic of debate. One feasible strategy to test these models focuses on the visualization of large cargos crossing the NPC.

Recent models of NPC function propose that the central channel is filled by the FG-repeat domains of Nups. Our results of the nuclear import of that intact baculovirus seem to fit with some of these models, but not all. For example, the Selective Phase model must demonstrate how the hydrogels within the central channel could reform after being significantly disrupted, leaving an empty space of 50-nm in diameter by 70-nm in height (the entire dimensions of the NPC central channel) that allows the passage of the baculovirus capsid. Nevertheless, this model suggests an inverse relationship between cargo size and the rate of nuclear import (Ribbeck and Gorlich, 2001); thus, in order to test this model it will be valuable to measure the rate of nuclear import of the baculovirus capsid using live-cell imaging. The Polymer Brush model where molecules that bind to FG repeats in the Nups, causing FG-nups to collapse and allowing molecules into the transport channel would seem feasible. It is possible to imagine that as soon as the conical end of the capsid interacts with the large FG corona surrounding the cytoplasmic entrance of the NPC, the FG motifs in the central channel are swept away, leaving the central channel empty and ready to be transited by the capsid. To demonstrate that this model is applicable for nuclear translocation of the baculovirus capsid, it would be worthwhile to perform

immuno-EM of FG-Nups and see their distribution on NPC-containing capsids. The more recent Forest model suggesting that a central plug/transporter is formed by FG domains must consider if such a central plug/transporter exists, its fate when large cargos cross the NPC central channel remains to be explained.

Our results indicate that whatever is normally filling the NPC central channel must be remarkably flexible, and is able to retract itself into the membrane-embedded scaffold ring of the NPC, leaving the entire central channel open for the passage of the large baculovirus capsid. Although our data does not seem to fit with some of the proposed models for NPC function, we do not discard the possibility that the NPC may use different modes of translocation depending on the size of the cargo being translocated. Thus, some of these models may be valid for cargos smaller than the baculovirus capsid. Nevertheless, the translocation of a long cargo, such as the baculovirus capsid, supports a model in which the total length and width of the central channel is completely emptied to accommodate the intact capsid.

6.5 Concluding remarks

Prior to this study, the cellular mechanisms exploited by baculovirus capsids to enter the nucleus were poorly understood. We are the first to demonstrate that intact baculovirus AcMNPV capsids are able to traverse the NPC during nuclear import. This process involves dynamic relocalization of proteins residing within the central channel of the NPC, as well as structural changes to the NPC cytoplasmic filaments. Nuclear import of such a long cargo demonstrated the ability for NPCs to remain intact and not disrupt the selective barrier during this process.

In addition, we also demonstrated that nuclear import of baculovirus capsids occur independently of RanGTPase and importin-β, and that depleting FG-Nups individually did not completely block the nuclear translocation of the baculovirus AcMNPV capsid (summarized in Figure 6-2A). Moreover, cellular F-actin and soluble Arp2/3 complex is necessary for the migration of baculovirus AcMNPV capsids towards the nucleus, as well as during translocation of the viral capsid into the nucleus (summarized in Figure 6-2B). Without F-actin and Arp2/3, nuclear import of baculovirus capsid was inhibited even in the presence of cellular receptors and energy. Once the baculovirus capsid is docked at the cytoplasmic face of the NPC, we envision that actin polymerization occurring at one end of the baculovirus capsid could potentially assist in pushing the capsid through the NPC, which could explain how the baculovirus capsid can enter the nucleus in the absence of energy.

This work provides more insight into the baculovirus life cycle, and opens up the opportunity to address many more questions that remain unanswered. For example, although we demonstrated that the baculovirus AcMNPV capsid crosses the NPC, it is unclear exactly which nuclear import pathway baculovirus uses, even though our data rules out the classical nuclear import pathway. Another question deals with putative NLSs on the viral capsid, which remain unknown. Characterization of these potential NLSs will be crucial to the development of strategies that make nuclear import of baculovirus more efficient in mammalian cells. In addition, even though our data suggest that baculovirus does not follow the classical nuclear import pathway, it is interesting to determine potential cellular binding partners of the baculovirus capsid. *In vitro* immunoprecipitation studies as well as *in vivo* binding assays followed by mass spectrometry can help elucidate these binding partners.

Because baculoviruses are one of the largest viruses that have been observed to cross the NPC, using baculovirus in future studies of the NPC gated passageway will provide a better understanding of the nuclear transport models, especially in determining how large molecules cross the NPC. As baculoviruses are not a serious threat to humans and do not replicate in mammalian cells, they remain a very useful tool for biomedical applications. Our results suggest that baculovirus AcMNPV uses an unconventional mechanism to deliver its viral genome into the nucleus. Understanding this nuclear import process and cellular proteins involved is crucial in the development of baculovirus as an efficient viral vector for use in gene therapy and protein expression. As cellular actin and the Arp2/3 complex appears to be necessary in baculovirus capsid delivery into the nucleus, it may be beneficial to incorporate exogenous amounts of F-actin and the Arp2/3 complex into a biological system to increase nuclear import efficiency of the baculovirus capsid. Additionally, this knowledge can assist in generating a highly pathogenic pesticide that can more effectively control agricultural pests.

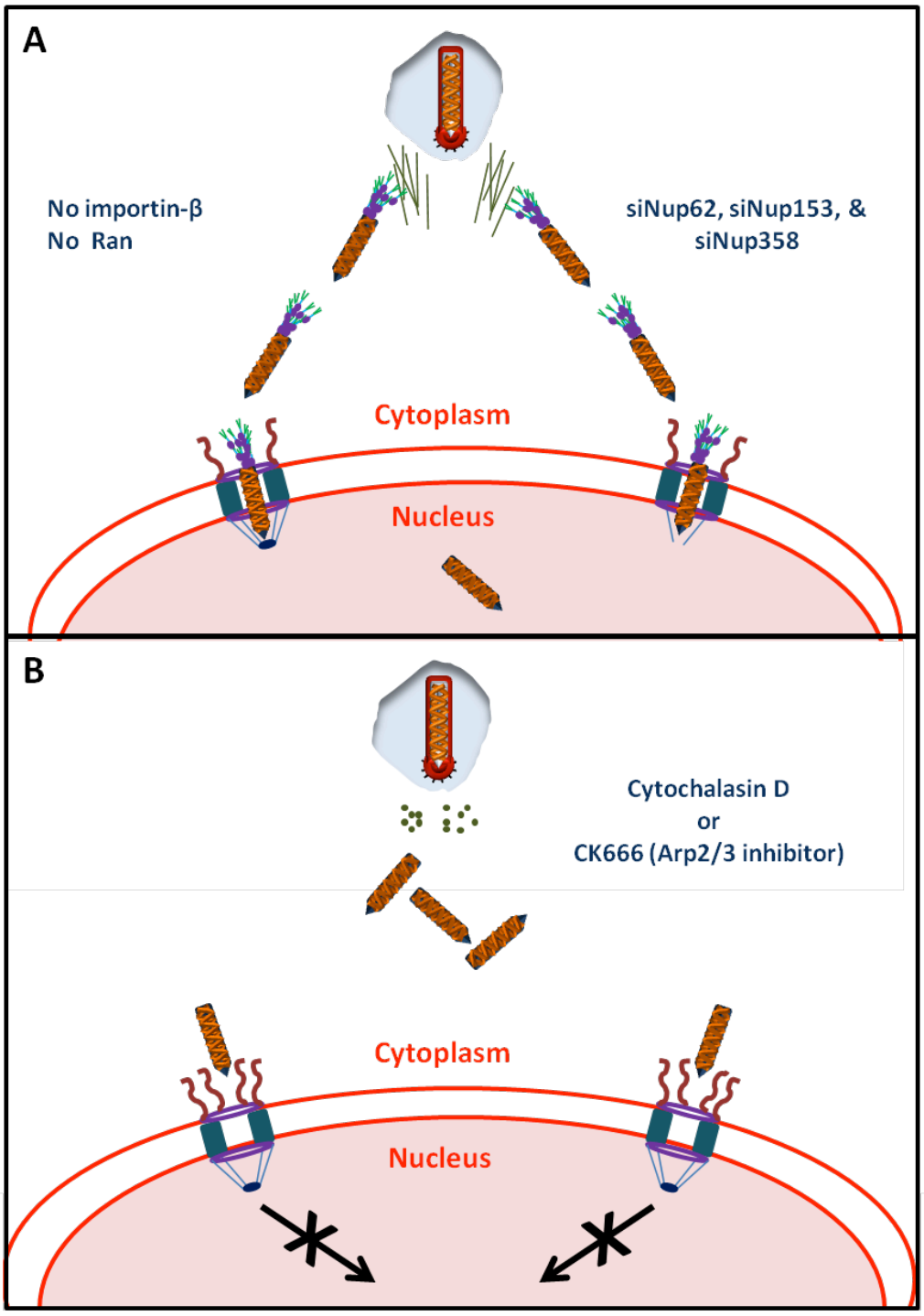


Figure 6-2: Proposed scheme of nuclear import of the baculovirus AcMNPV capsid. (A) Baculovirus capsids can enter the nuclear through NPCs in the absence of cytosolic factors and in Nup62, Nup153, and Nup358 depleted cells. (B) Capsids remain in the cytoplasm, docked at NPCS in F-actin depleted and Arp2/3 inhibited cells, suggesting an important role for these cytoskeletal components in mediating nuclear import of the baculovirus capsid.

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Appendix A

Sequence Alignment was performed using ClustalW2 online program to compare the structural relatedness between 12 AcMNPV capsid proteins (VP39, VP78/83, P24, VP80, VP1054, Exon0, FP25, VLF-1, P49, E27, 38K, and C42) and properties of importin-β (HEAT1, HEAT2, HEAT3, HEAT4, HEAT6, HEAT7, HEAT8, importin-α, and GTP binding domain). Highlighted in yellow are regions of sequence similarity between the aligned roteins. However sequence alignment did not show structural relatedness between these 12 capsid proteins and any of the importin-β domains, suggesting that the viral capsid does not contain importin-β-like properties.

```
Symbols:

  regions of sequence similarity
* identical
: conserved substitutions
. semi-conserved substitutions
```

Sequence alignment of VP39 and HEAT1 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat1       ------------------------------------------------------------
VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat1       ------------------------------------------------------------
VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat1       ------------------------------------------------------------
VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat1       ------------------------------------------------------------
VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat1       ------------------------------------------------------------
VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat1       ------------------------------------------------------------
VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat1       ------------------------------------------------------------
VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat1       ------------------------------------------------------------
VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat1       --AACCAGTGGCCAGAACTCATT----------------------------------- 21
              .**** :..**.****:*.**
VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat1       ----------------------CCTC-------AGCTGGTGGCCAAT-------------- 39
                                   * **       :***** *. ****
VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat1       ------------------------------------------------------------GTC 42
                                                                           *:*
VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat1       AC-------------------------------------------------AAAC---------- 48
            **                                                  ****
VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat1       ---------CCCAACAGCACAGAG--------CAC---ATGAAGGAGT------------ 76
                     ***.**.**.*.***         *.*   *:**** ***
VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat1       ------------CG----------------------ACATTGGAAG-------------- 88
                        **                      *.*** ****
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat1       ----------------------------------CCATCG--------GTTATATTTG--- 104
                                               **.:**        * *.*:****
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat1       ------------------------------------------------CCAAGATATAGA 116
                                                             ****** .:*.:
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat1       CCCA---------------------------------------------------------- 120
            ** *
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat1       -------------------------
```

Sequence alignment of VP39 and HEAT2 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat2       ------------------------------GATAAAT---------------------- 7
                                          **  ****

VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat2       ----CCAATGAG--------------------ATTCTGACTGCCATAATC---------- 33
                ***:  *:*                    **:*:.*  ***.*.:**

VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat2       --------CAGGGGATG---------------------------------------------- 42
                    *..  *****

VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat2       ------------------------------------------------------------AGG 45
                                                                       ***

VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat2       ----------------AAAGAAGAG--------------------CCTAGT--------- 60
                            **..*****                    **:**

VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat2       ----------------------AATAATGTGAAGCTAGCTG---------------------- 79
                                   **  *.*.***:**:***  *

VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat2       --------CTACGAATGCACT---------------------------------------- 92
                    *:*****:.*.*:

VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat2       ------------------------------------------------------------
VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat2       -----------CCTGAACTCATT---------GGAG-------------------TTC--- 111
                       **  ****:*.**         **:*                   ***

VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat2       ------ACCAAAGCA---------------------------------------------- 120
                   *****.**.

VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat2       ------------------------------------------------------------
VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat2       ------------------------------------------------------------
VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat2       ------------------------------------------------------------
VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat2       ------------------------------------------------------------
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat2       ------------------------------------------------------------
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat2       ------------------------------------------------------------
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat2       ------------------------------------------------------------
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat2       -------------------------
```

Sequence alignment of VP39 and HEAT3 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat3       ------------------------------------------------------------
VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat3       ------------------------------------------------------------
VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat3       ------------------------------------------------------------
VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat3       ------------------------------------------------------------
VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat3       ------------------------------------------------------------
VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat3       ----------------TCTGAAAGG----------------------------------- 9
                            ***.**..*
VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat3       -------------------------CACTTTAT--------------TATGCAGGTGGT 29
                                     ****:***              ***.*.*.:***
VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat3       CTGTGAAGCCACAC----------------AGTGT-------------------------- 48
             * : ****.****                ** **
VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat3       -----------CCAGA----------TACGAGGGT--------ACGAGTGGC--------- 71
                       **.**          *:**.****        ****.* **
VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat3       ---------------------------------TGCT--------------------TTAC 79
                                              ****                    ****
VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat3       AG-----------------------------------------------------AATC--- 85
            **                                                     ****
VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat3       ------------TGGTGAAGATAATGT---CCTTATATTAT--------CAG--------- 114
                       :***  :***:*:**    .*  *.***:**        *.*
VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat3       ------------------------------------------------------------
VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat3       ------------------------------------------------------------
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat3       ------------------------------------------------------------
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat3       ------------------------------------------------------------
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat3       ------------------------------------------------------------
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat3       -------------------------
```

Sequence alignment of VP39 and HEAT4 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat4       ------------------------------------------------------------
VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat4       ------------------------------------------------------------
VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat4       ------------------------------------------------------------
VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat4       ------------------------GGAG--------CACTACAGTAT--------CTGG- 19
                                    *.**        **.*:**.:**        **.*

VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat4       ----------------------------------------TTCCAATCCTCACACAGACACT 41
                                                    **  .*:***:..*.**..**.*

VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat4       ----------------AACTAAACAG---------------------------------- 51
                            *:********

VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat4       ---------GACGAAAATGATG----------------------------ATGACGATG-- 73
                     .*******  *.:*                            **..***:*

VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat4       ------------------------------------------------------ACTG 77
                                                                   :***

VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat4       G--------AACCC---------------------------------------------- 83
            *        *****

VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat4       ----------------------------------CTG----------------------- 86
                                               ***

VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat4       ------------------------------------------------CAAAGCAGC 95
                                                             ****  *..*

VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat4       AGGGG-------------------TGTGCCTCATG-------------------------- 111
            *  **.                  **  ***:*.**

VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat4       -CTTCTG-GCCA-----CC------------------------------------------ 123
             *  ****  ***.     **

VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat4       ------------------------------------------------------------
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat4       ------------------------------------------------------------
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat4       ------------------------------------------------------------
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat4       ------------------------------------------------------------
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat4       -------------------------
```

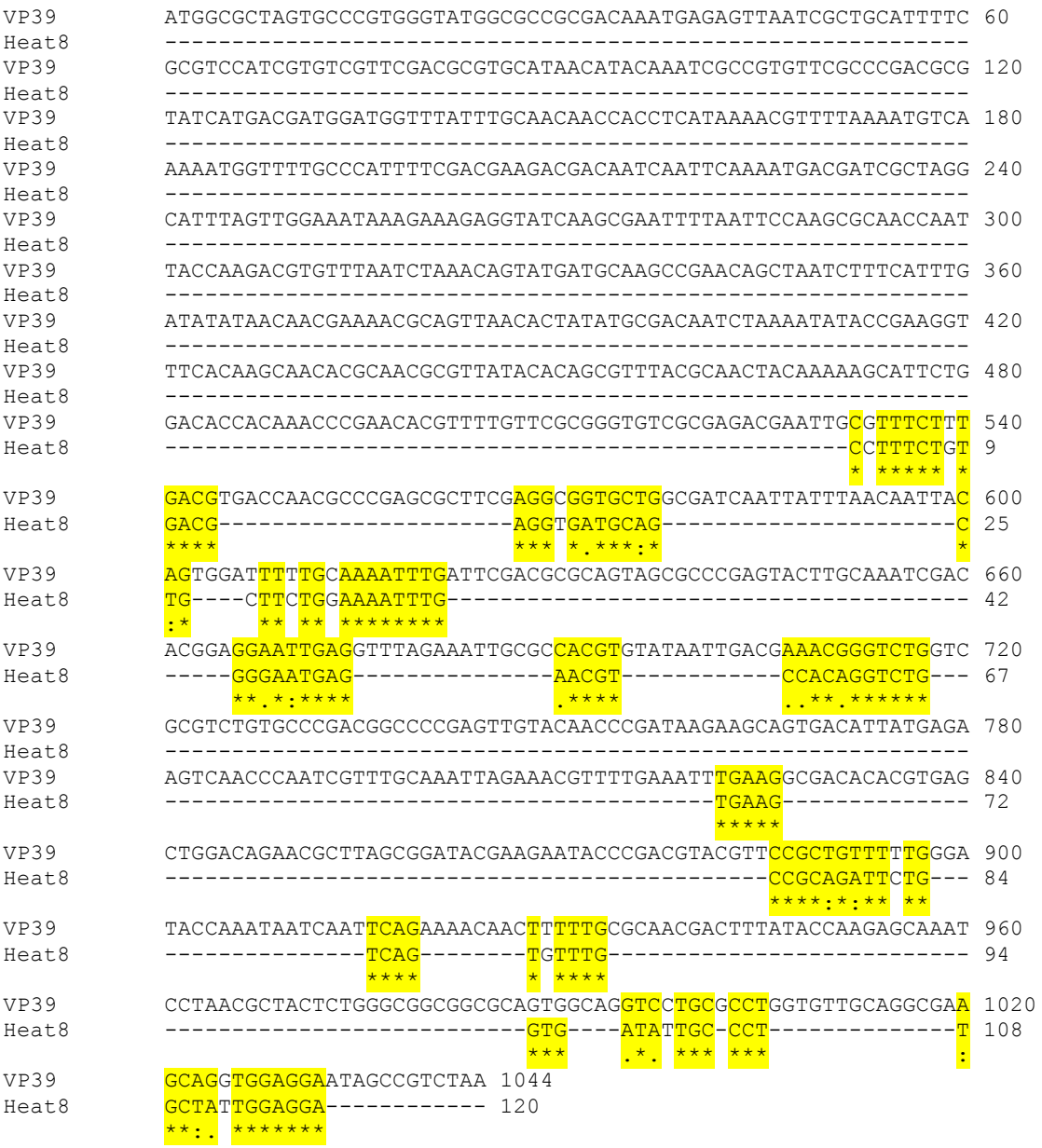
Sequence alignment of VP39 and HEAT6 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat6       ------------------------------------------------------------
VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat6       ------------------------------------------------------------
VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat6       ------------------------------------------------------------
VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat6       ------------------------------------------------------------
VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat6       ------------------------------------------------------------
VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat6       ------------------------------------------------------------
VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat6       ------------------------------------------------------------
VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat6       ------------------------------------------------------------
VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat6       ------------------------------------------------------------
VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat6       ------------------------------------------------------------
VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat6       ------------------------------------------------------------
VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat6       ----------------------------CCAC-----TAGTT----ATACAGG------- 16
                                         ****     **.**    *:**.**
VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat6       ----CTATGCCC-------------------ACCCTAATAGAA--------TTAATGAAA 45
                **.*****                   ****  *::****        :*:****.*
VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat6       G---ACCCCAG-------------------------------TGTAG-------------- 58
            .   *.****.                               **:**
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat6       ------------------------------------------------------TTG--- 61
                                                                     ***
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat6       --------------TTCGAGATACAGCT------GCATGGACTGTAGGCAGAAT------ 95
                          ***...*:***.**      ***: **** ** .*..*.:
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat6       ----------TTGTGAGCTGCT-------------TCCTGAA------------------ 114
                      * **.**  **               *****..
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat6       -------------------------
```

Sequence alignment of VP39 and HEAT7 domain

```
VP39        ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Heat7       ------------------------------------------------------------
VP39        GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Heat7       ------------------------------------------------------------
VP39        TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Heat7       ------------------------------------------------------------
VP39        AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Heat7       ------------------------------------------------------------
VP39        CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Heat7       ------------------------------------------------------------
VP39        TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Heat7       ------------------------------------------------------------
VP39        ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Heat7       ------------------------------------------------------------
VP39        TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Heat7       ------------------------------------------------------------
VP39        GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Heat7       ------------------------------------------------------------
VP39        GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Heat7       --------------CAGATCTCT--GATGTGGTTATGGC----------CTCCCTGTTAA 34
                          *.**  *  **   **  *  ***  .****           *..*:.***.
VP39        AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Heat7       GG----------------ATGTTCCAAAGCACAG-------------CT----------- 54
            .*                :**:*  *.*.**.***             **
VP39        ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Heat7       ----------------------------------------------GGGTCTG--- 61
                                                           *******
VP39        GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Heat7       ---------------------GGGGAGTACAA---------------------------- 72
                                 *.*  :******
VP39        AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Heat7       ------------------------------------------------------------
VP39        CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Heat7       ----------------GAGGATGC--------CCTGATG--------GCAG--------- 91
                            *.****.*        **  **  *        **:*
VP39        TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Heat7       ----------------------------TTAGC---------------------------- 96
                                        **:**
VP39        CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Heat7       ---------ACACTGG------------TGGAAG-------------TGTTG-------- 114
                     **:****             ***.**             *****
VP39        GCAGGTGGAGGAATAGCCGTCTAA 1044
Heat7       ---GGTGGT--------------- 120
               *****:
```

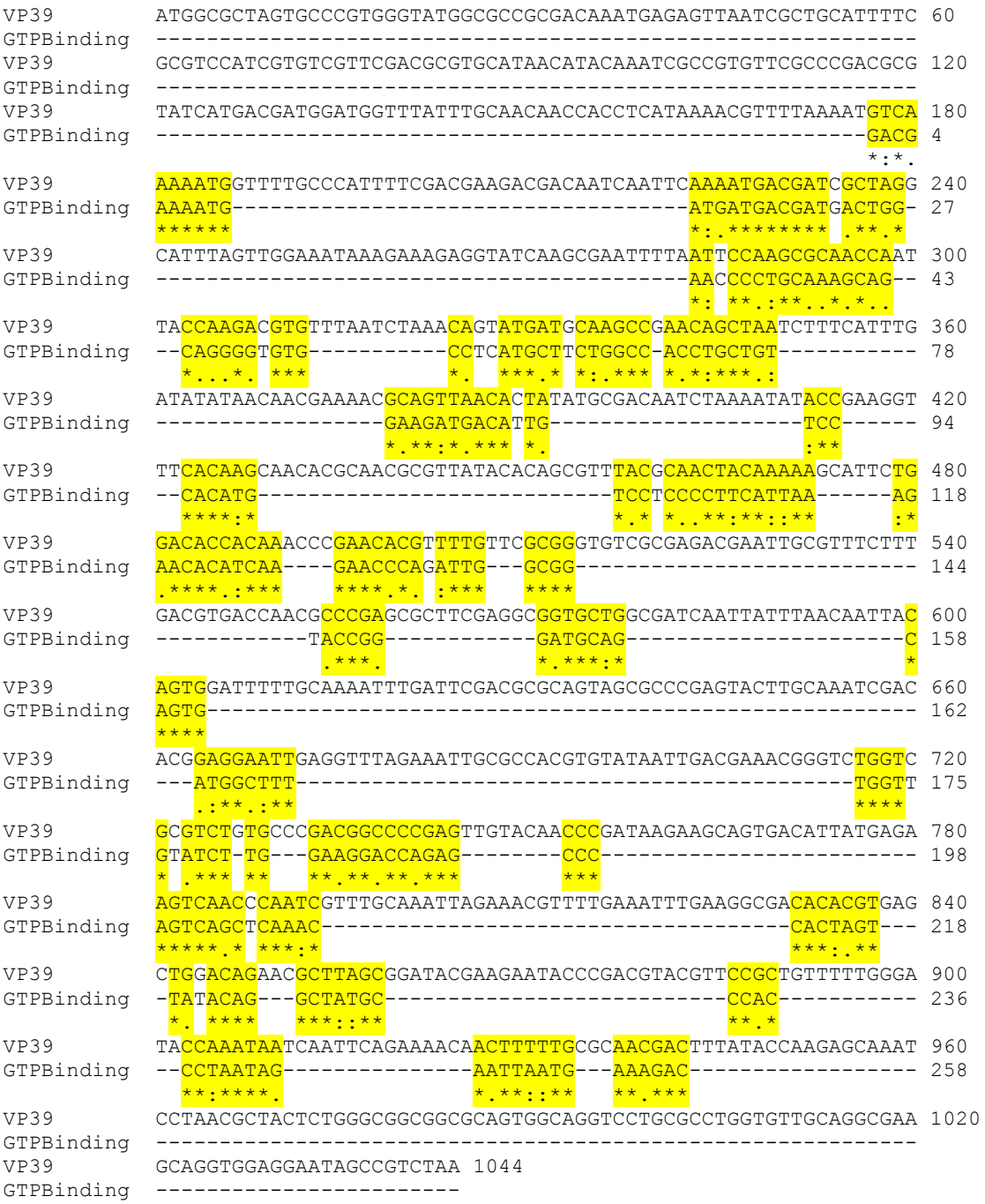
Sequence alignment of VP39 and HEAT8 domain



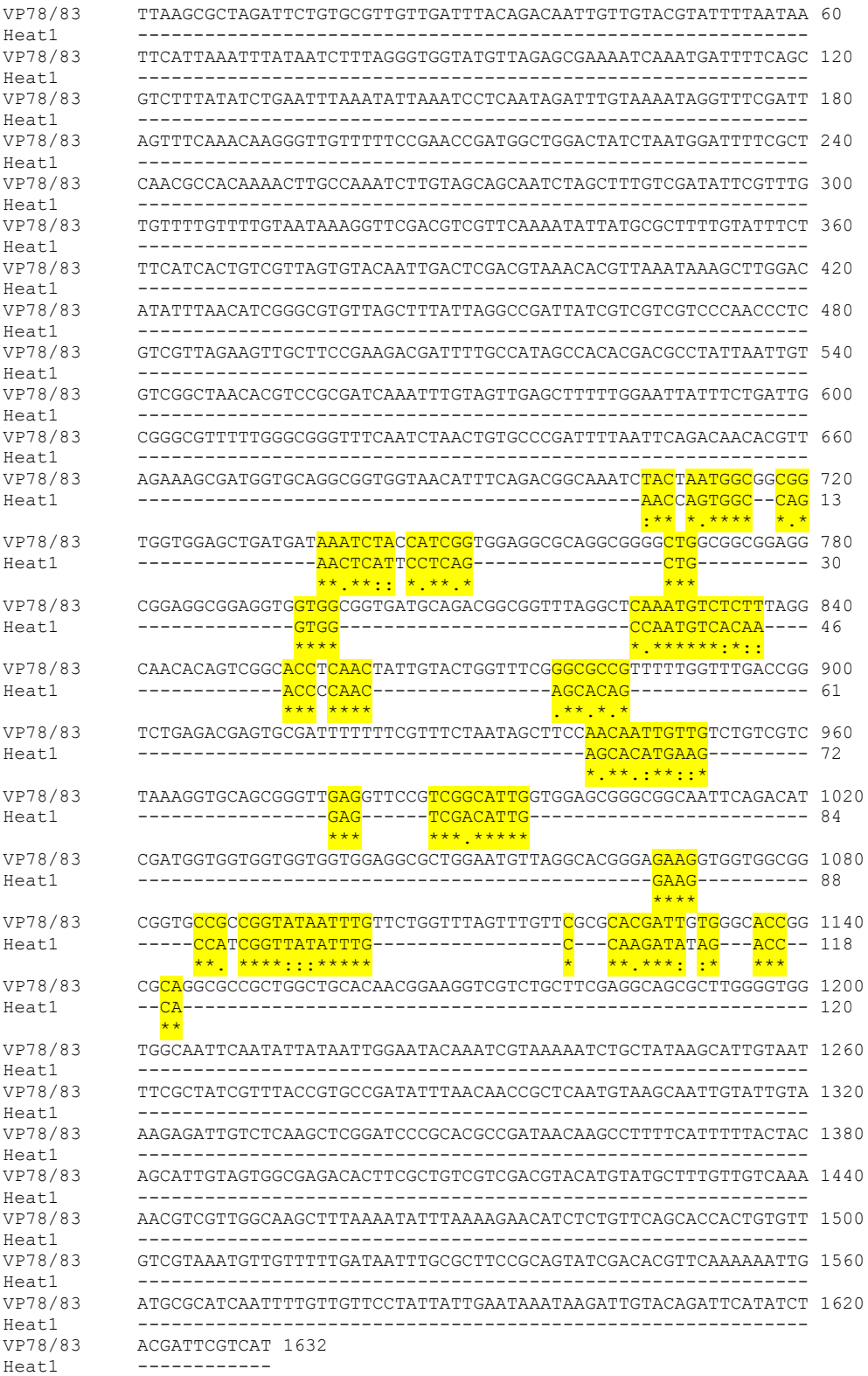
Sequence alignment of VP39 and importin-α binding domain

```
VP39         ATGGCGCTAGTGCCCGTGGGTATGGCGCCGCGACAAATGAGAGTTAATCGCTGCATTTTC 60
Importin-α   ------------------------------------------------------------
VP39         GCGTCCATCGTGTCGTTCGACGCGTGCATAACATACAAATCGCCGTGTTCGCCCGACGCG 120
Importin-α   ------------------AC---------ACTAACTAAAC------------------- 13
                               **         **::**:**:*
VP39         TATCATGACGATGGATGGTTTATTTGCAACAACCACCTCATAAAACGTTTTAAAATGTCA 180
Importin-α   ----AGGACG----------------------------------------AAAATG--- 25
                 * ****                                        ******
VP39         AAAATGGTTTTGCCCATTTTCGACGAAGACGACAATCAATTCAAAATGACGATCGCTAGG 240
Importin-α   ---ATG----------------------------------------ATGACGATGACTGG- 42
                ***                                        ******** .**.*
VP39         CATTTAGTTGGAAATAAAGAAAGAGGTATCAAGCGAATTTTAATTCCAAGCGCAACCAAT 300
Importin-α   ------------------------------------------------------------
VP39         TACCAAGACGTGTTTAATCTAAACAGTATGATGCAAGCCGAACAGCTAATCTTTCATTTG 360
Importin-α   ------------------------------------------------------------
VP39         ATATATAACAACGAAAACGCAGTTAACACTATATGCGACAATCTAAAATATACCGAAGGT 420
Importin-α   ------------------------------------------------------------
VP39         TTCACAAGCAACACGCAACGCGTTATACACAGCGTTTACGCAACTACAAAAAGCATTCTG 480
Importin-α   ------------------------------------------------------------
VP39         GACACCACAAACCCGAACACGTTTTGTTCGCGGGTGTCGCGAGACGAATTGCGTTTCTTT 540
Importin-α   ------------------------------------------------------------
VP39         GACGTGACCAACGCCCGAGCGCTTCGAGGCGGTGCTGGCGATCAATTATTTAACAATTAC 600
Importin-α   ------------------------------------------------------------
VP39         AGTGGATTTTTGCAAAATTTGATTCGACGCGCAGTAGCGCCCGAGTACTTGCAAATCGAC 660
Importin-α   ------------------------------------------------------------
VP39         ACGGAGGAATTGAGGTTTAGAAATTGCGCCACGTGTATAATTGACGAAACGGGTCTGGTC 720
Importin-α   ------------------------------------------------------------
VP39         GCGTCTGTGCCCGACGGCCCCGAGTTGTACAACCCGATAAGAAGCAGTGACATTATGAGA 780
Importin-α   ------------------------------------------------------------
VP39         AGTCAACCCAATCGTTTGCAAATTAGAAACGTTTTGAAATTTGAAGGCGACACACGTGAG 840
Importin-α   ------------------------------------------------------------
VP39         CTGGACAGAACGCTTAGCGGATACGAAGAATACCCGACGTACGTTCCGCTGTTTTTGGGA 900
Importin-α   ------------------------------------------------------------
VP39         TACCAAATAATCAATTCAGAAAACAACTTTTTGCGCAACGACTTTATACCAAGAGCAAAT 960
Importin-α   ------------------------------------------------------------
VP39         CCTAACGCTACTCTGGGCGGCGGCGCAGTGGCAGGTCCTGCGCCTGGTGTTGCAGGCGAA 1020
Importin-α   ------------------------------------------------------------
VP39         GCAGGTGGAGGAATAGCCGTCTAA 1044
Importin-α   ------------------------
```

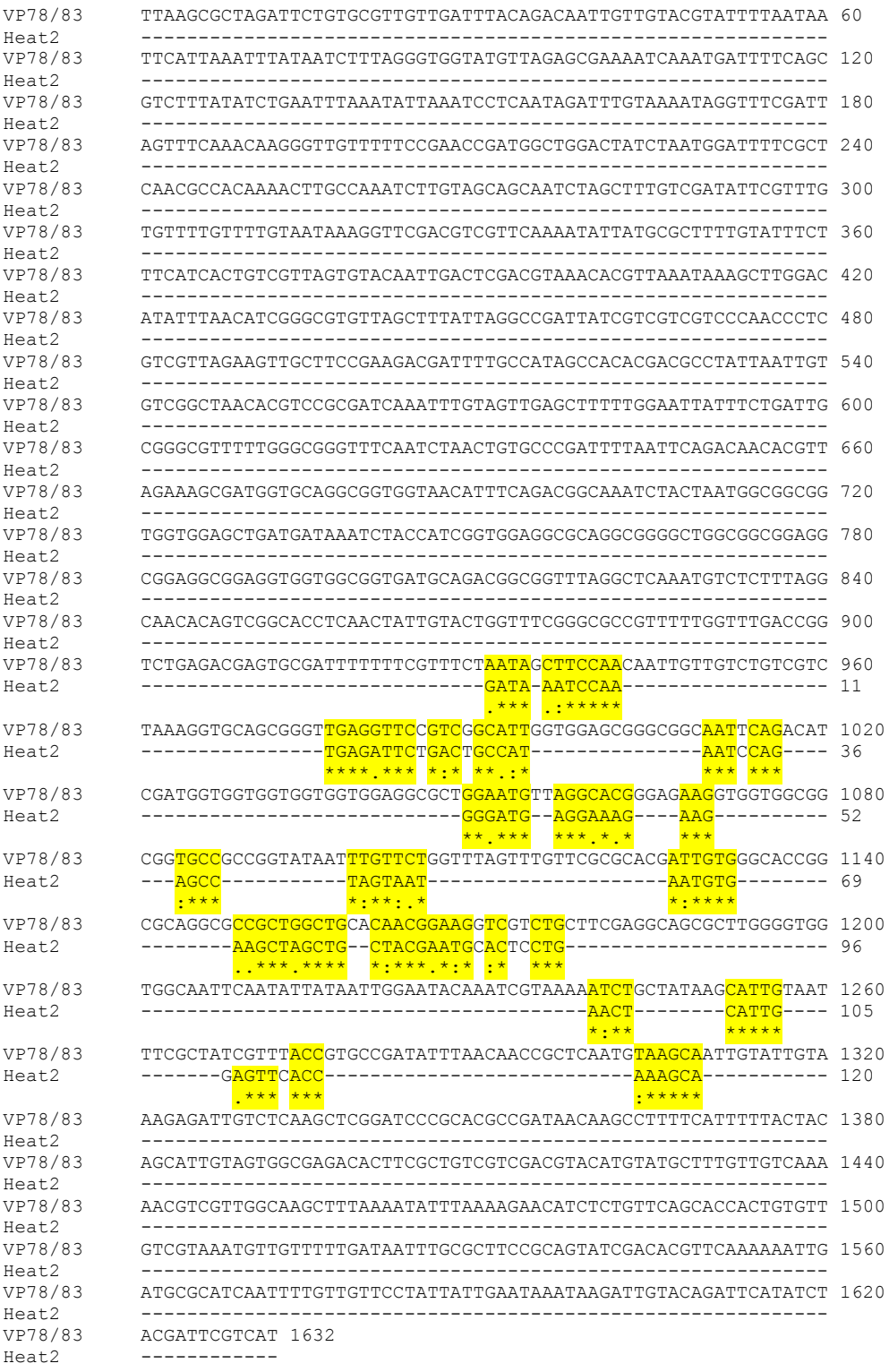
Sequence alignment of VP39 and Ran GTP Binding domain



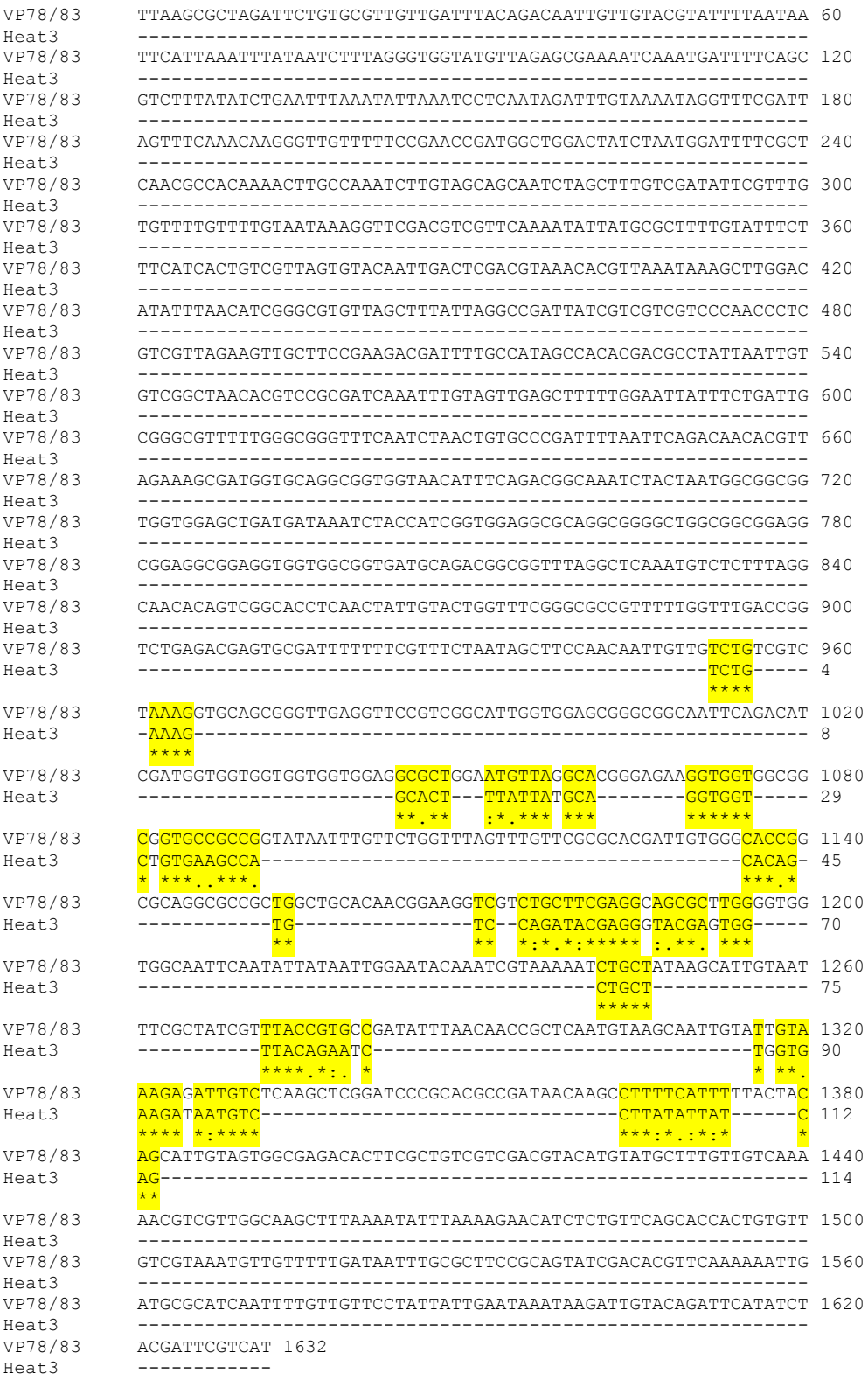
Sequence alignment of VP78/83 and HEAT1 domain



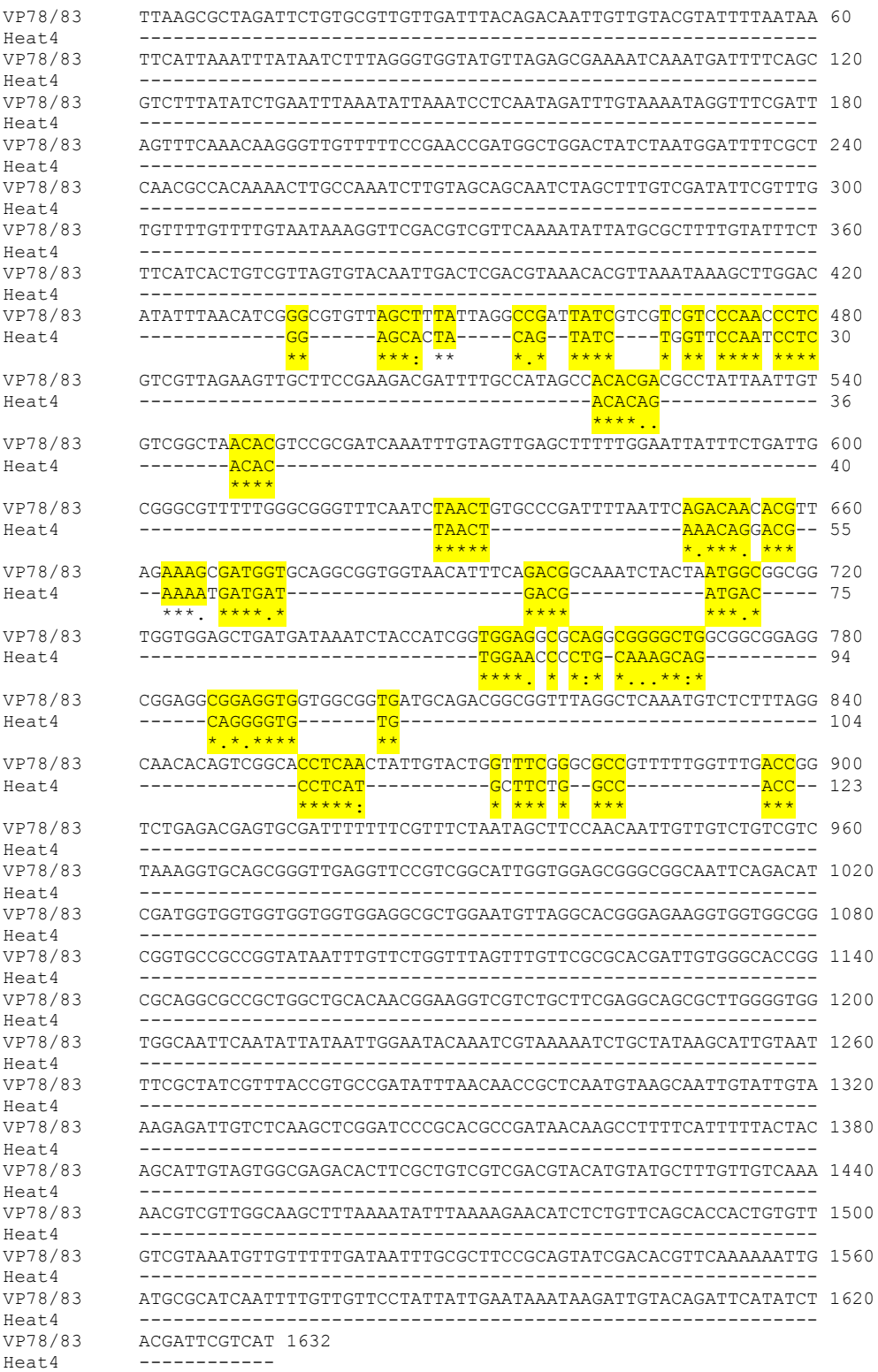
Sequence alignment of VP78/83 and HEAT2 domain



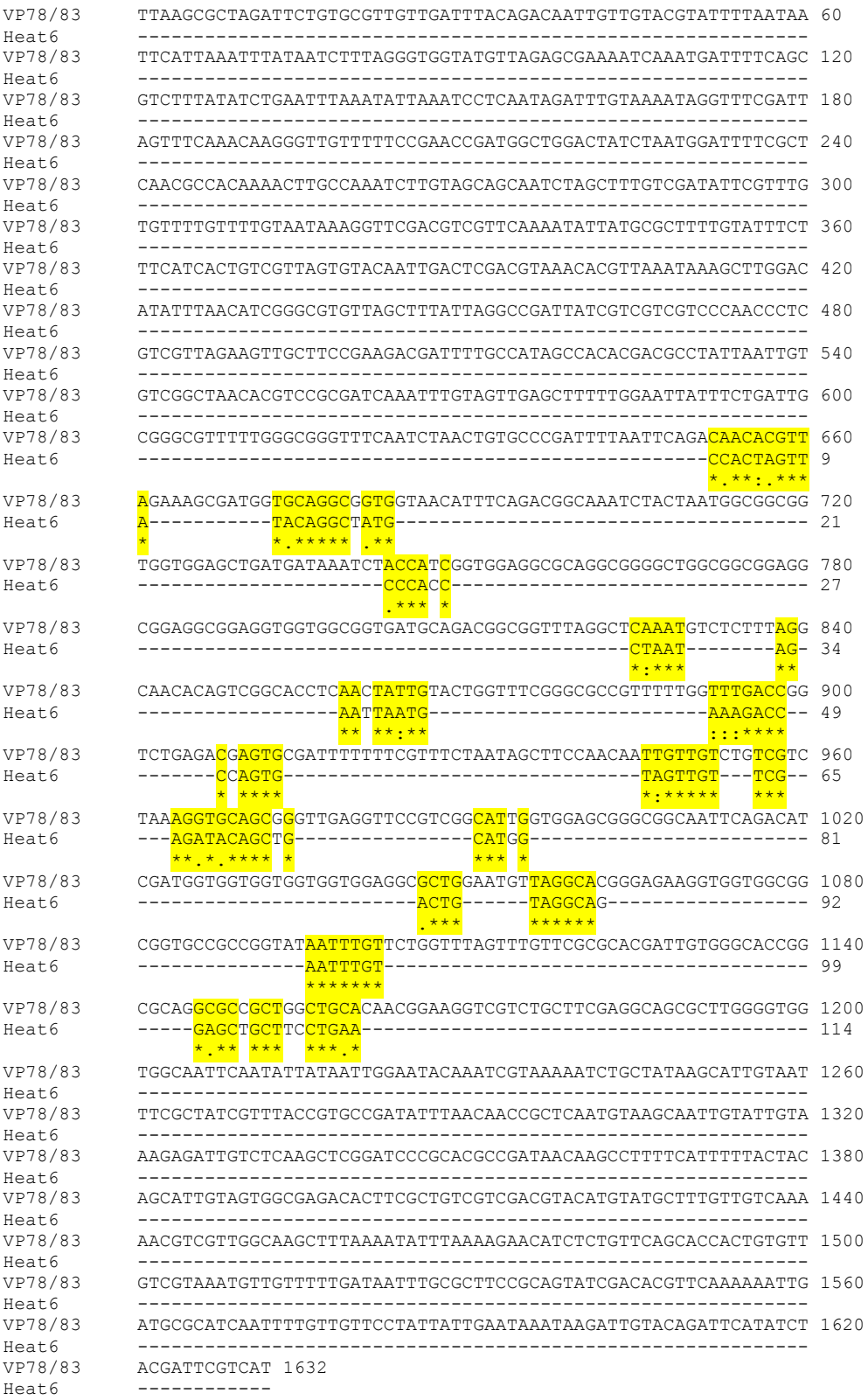
Sequence alignment of VP78/83 and HEAT3 domain



Sequence alignment of VP78/83 and HEAT4 domain



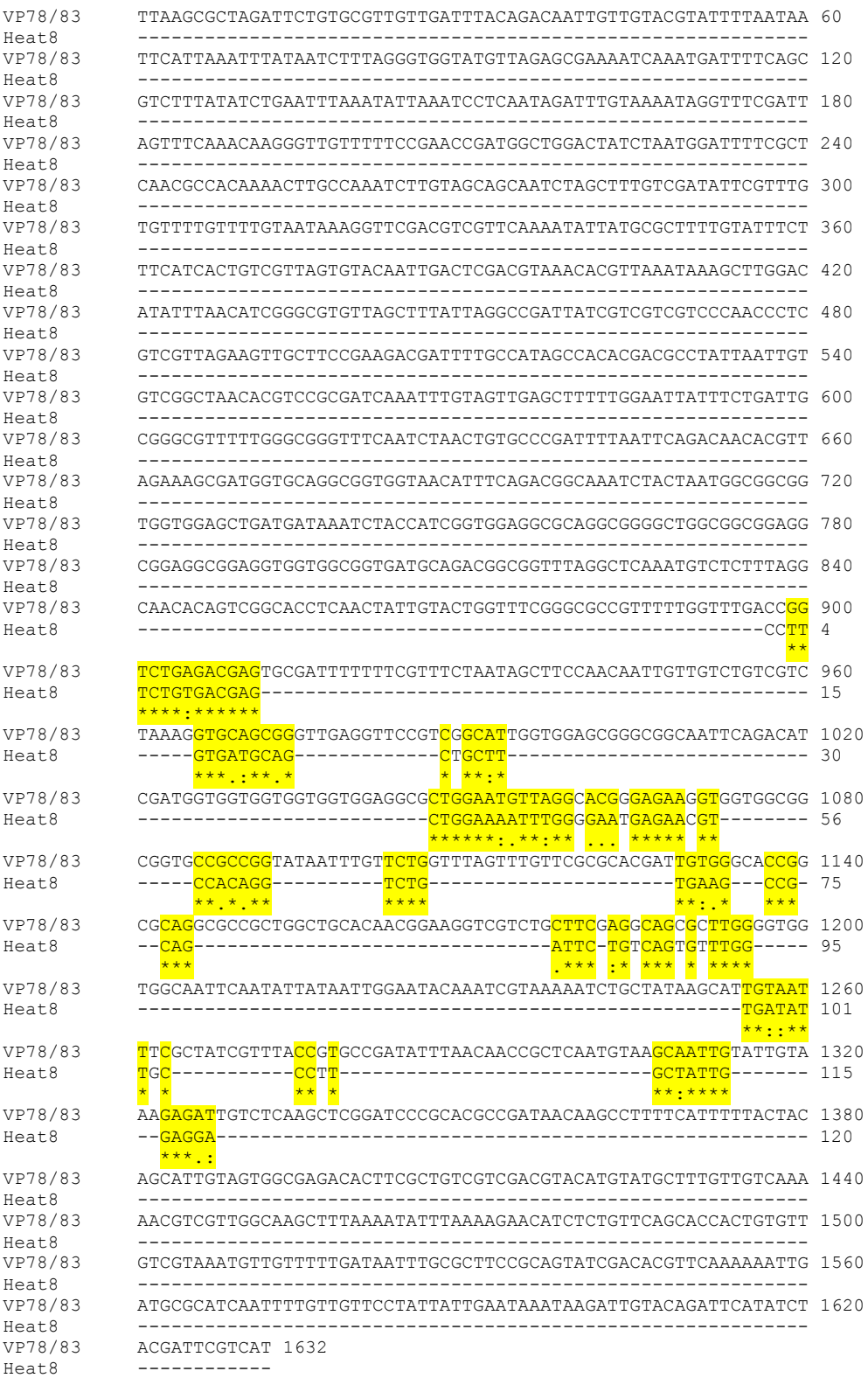
Sequence alignment of VP78/83 and HEAT6 domain



Sequence alignment of VP78/83 and HEAT7 domain

```
VP78/83     TTAAGCGCTAGATTCTGTGCGTTGTTGATTTACAGACAATTGTTGTACGTATTTTAATAA 60
Heat7       ------------------------------------------------------------
VP78/83     TTCATTAAATTTATAATCTTTAGGGTGGTATGTTAGAGCGAAAATCAAATGATTTTCAGC 120
Heat7       ------------------------------------------------------------
VP78/83     GTCTTTATATCTGAATTTAAATATTAAATCCTCAATAGATTTGTAAAATAGGTTTCGATT 180
Heat7       ------------------------------------------------------------
VP78/83     AGTTTCAAACAAGGGTTGTTTTTCCGAACCGATGGCTGGACTATCTAATGGATTTTCGCT 240
Heat7       ------------------------------------------------------------
VP78/83     CAACGCCACAAAACTTGCCAAATCTTGTAGCAGCAATCTAGCTTTGTCGATATTCGTTTG 300
Heat7       ------------------------------------------------------------
VP78/83     TGTTTTGTTTTGTAATAAAGGTTCGACGTCGTTCAAAATATTATGCGCTTTTGTATTTCT 360
Heat7       ------------------------------------------------------------
VP78/83     TTCATCACTGTCGTTAGTGTACAATTGACTCGACGTAAACACGTTAAATAAAGCTTGGAC 420
Heat7       ------------------------------------------------------------
VP78/83     ATATTTAACATCGGGCGTGTTAGCTTTATTAGGCCGATTATCGTCGTCGTCCCAACCCTC 480
Heat7       ------------------------------------------------------------
VP78/83     GTCGTTAGAAGTTGCTTCCGAAGACGATTTTGCCATAGCCACACGACGCCTATTAATTGT 540
Heat7       ------------------------------------------------------------
VP78/83     GTCGGCTAACACGTCCGCGATCAAATTTGTAGTTGAGCTTTTTGGAATTATTTCTGATTG 600
Heat7       ------------------------------------------------------------
VP78/83     CGGGCGTTTTTGGGCGGGTTTCAATCTAACTGTGCCCGATTTTAATTCAGACAACACGTT 660
Heat7       ------------------------------------------------------------
VP78/83     AGAAAGCGATGGTGCAGGCGGTGGTAACATTTCAGACGGCAAATCTACTAATGGCGGCGG 720
Heat7       ------------------------------------------------------------
VP78/83     TGGTGGAGCTGATGATAAATCTACCATCGGTGGAGGCGCAGGCGGGGCTGGCGGCGGAGG 780
Heat7       ------------------------------------------------------------
VP78/83     CGGAGGCGGAGGTGGTGGCGGTGATGCAGACGGCGGTTTAGGCTCAAATGTCTCTTTAGG 840
Heat7       ----------------------------CAGAT-----------CTCTGATG---------- 13
                                         ****           ***:.***
VP78/83     CAACACAGTCGGCACCTCAACTATTGTACTGGTTTCGGGCGCCGTTTTTGGTTTGACCGG 900
Heat7       ------------------------------TGGTTATGGCCTCC------------------ 27
                                           *****: ** * **
VP78/83     TCTGAGACGAGTGCGATTTTTTTCGTTTCTAATAGCTTCCAACAATTGTTGTCTGTCGTC 960
Heat7       -CTGTTAAGGATG-----------------------TTCCAAA------------------ 46
             ***: *.*..**                       ******.
VP78/83     TAAAGGTGCAGCGGGTTGAGGTTCCGTCGGCATTGGTGGAGCGGGCGGCAATTCAGACAT 1020
Heat7       -----GCACAGCTGG----------GTC-----TGGGGGAGT--------------ACA- 71
                 * .**** **           ***     ***  ****                ***
VP78/83     CGATGGTGGTGGTGGTGGTGGAGGCGCTGGAATGTTAGGCACGGGAGAAGGTGGTGGCGG 1080
Heat7       ----------------AGAGGATGCCCTG------------------------ATGGC-- 89
                            .*:*** **  ***                      .****
VP78/83     CGGTGCCGCCGGTATAATTTGTTCTGGTTTAGTTTGTTCGCGCACGATTGTGGGCACCGG 1140
Heat7       ------------------------------------AGTTAGCACAC-----TGG------- 103
                                                 :***.**.***     ***
VP78/83     CGCAGGCGCCGCTGGCTGCACAACGGAAGGTCGTCTGCTTCGAGGCAGCGCTTGGGGTGG 1200
Heat7       ------------------------TGGAAG------TGTT---------------GGGTGG 119
                                     *****      ** *               ******
VP78/83     TGGCAATTCAATATTATAATTGGAATACAAATCGTAAAAATCTGCTATAAGCATTGTAAT 1260
Heat7       T------------------------------------------------------------ 120
            *
VP78/83     TTCGCTATCGTTTACCGTGCCGATATTTAACAACCGCTCAATGTAAGCAATTGTATTGTA 1320
Heat7       ------------------------------------------------------------
VP78/83     AAGAGATTGTCTCAAGCTCGGATCCCGCACGCCGATAACAAGCCTTTTCATTTTTACTAC 1380
Heat7       ------------------------------------------------------------
VP78/83     AGCATTGTAGTGGCGAGACACTTCGCTGTCGTCGACGTACATGTATGCTTTGTTGTCAAA 1440
Heat7       ------------------------------------------------------------
VP78/83     AACGTCGTTGGCAAGCTTTAAAATATTTAAAAGAACATCTCTGTTCAGCACCACTGTGTT 1500
Heat7       ------------------------------------------------------------
VP78/83     GTCGTAAATGTTGTTTTTGATAATTTGCGCTTCCGCAGTATCGACACGTTCAAAAAATTG 1560
Heat7       ------------------------------------------------------------
VP78/83     ATGCGCATCAATTTTGTTGTTCCTATTATTGAATAAATAAGATTGTACAGATTCATATCT 1620
Heat7       ------------------------------------------------------------
VP78/83     ACGATTCGTCAT 1632
Heat7       ------------
```

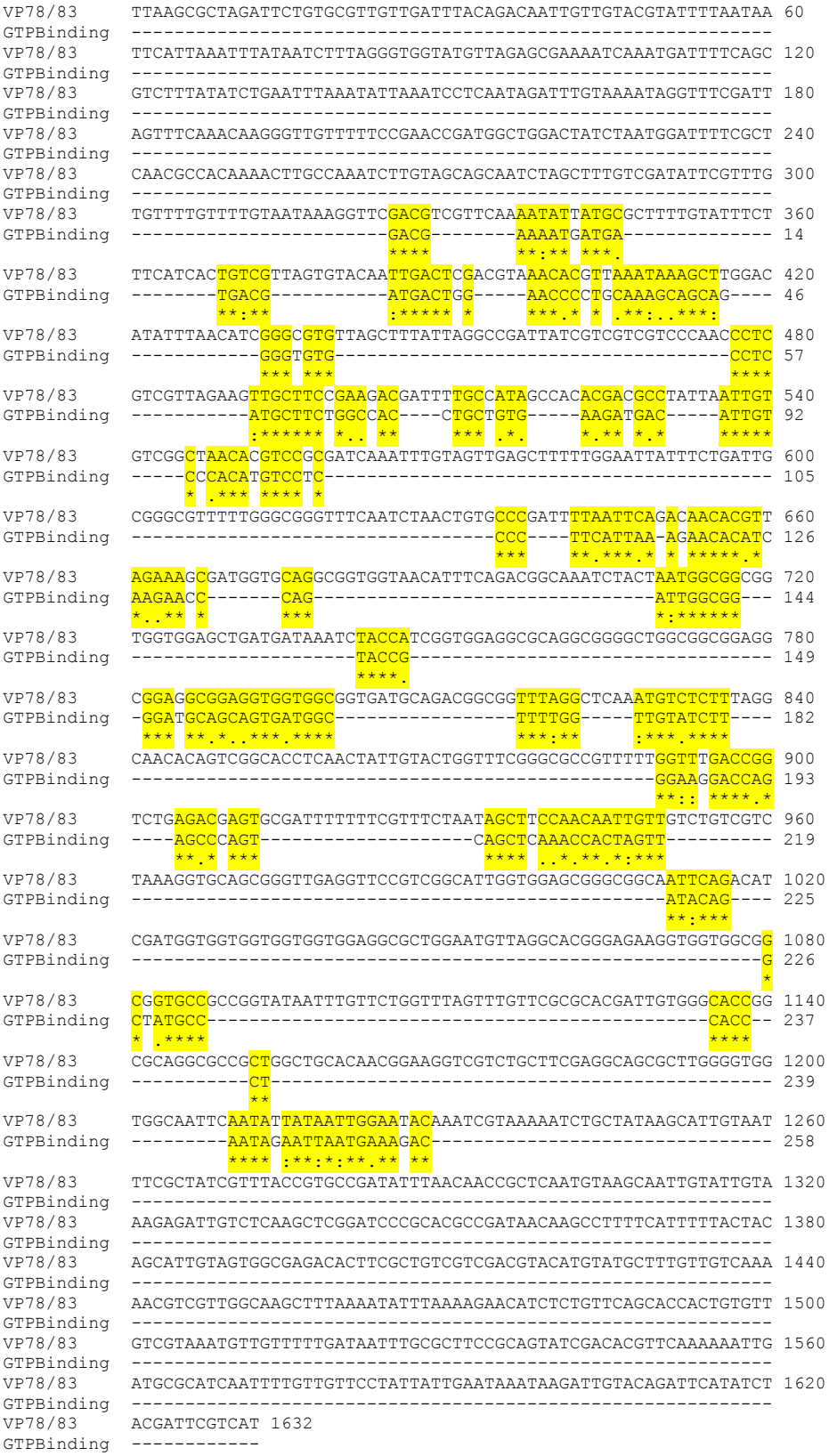
Sequence alignment of VP78/83 and HEAT8 domain



Sequence alignment of VP78/83 and importin-α binding domain

```
VP78/83     TTAAGCGCTAGATTCTGTGCGTTGTTGATTTACAGACAATTGTTGTACGTATTTTAATAA 60
Importin-α  ------------------------------------------------------------
VP78/83     TTCATTAAATTTATAATCTTTAGGGTGGTATGTTAGAGCGAAAATCAAATGATTTTCAGC 120
Importin-α  ------------------------------------------------------------
VP78/83     GTCTTTATATCTGAATTTAAATATTAAATCCTCAATAGATTTGTAAAATAGGTTTCGATT 180
Importin-α  ------------------------------------------------------------
VP78/83     AGTTTCAAACAAGGGTTGTTTTTCCGAACCGATGGCTGGACTATCTAATGGATTTTCGCT 240
Importin-α  ------------------------------------------------------------
VP78/83     CAACGCCACAAAACTTGCCAAATCTTGTAGCAGCAATCTAGCTTTGTCGATATTCGTTTG 300
Importin-α  ------------------------------------------------------------
VP78/83     TGTTTTGTTTTGTAATAAAGGTTCGACGTCGTTCAAAATATTATGCGCTTTTGTATTTCT 360
Importin-α  ------------------------------------------------------------
VP78/83     TTCATCACTGTCGTTAGTGTACAATTGACTCGACGTAAACACGTTAAATAAAGCTTGGAC 420
Importin-α  ------------------------------------------------------------
VP78/83     ATATTTAACATCGGGCGTGTTAGCTTTATTAGGCCGATTATCGTCGTCGTCCCAACCCTC 480
Importin-α  ------------------------------------------------------------
VP78/83     GTCGTTAGAAGTTGCTTCCGAAGACGATTTTGCCATAGCCACACGACGCCTATTAATTGT 540
Importin-α  ------------------------------------------------------------
VP78/83     GTCGGCTAACACGTCCGCGATCAAATTTGTAGTTGAGCTTTTTGGAATTATTTCTGATTG 600
Importin-α  ------------------------------------------------------------
VP78/83     CGGGCGTTTTTGGGCGGGTTTCAATCTAACTGTGCCCGATTTTAATTCAGACAACACGTT 660
Importin-α  ----------------------ACACTAACT-----------------AAACAGGACG-- 19
                                   *.:******                 *.***. ***
VP78/83     AGAAAGCGATGGTGCAGGCGGTGGTAACATTTCAGACGGCAAATCTACTAATGGCGGCGG 720
Importin-α  --AAAATGATGAT----------------------GACG------------ATGAC----- 39
              ***. ****.*                       ****            ***.*
VP78/83     TGGTGGAGCTGATGATAAATCTACCATCGGTGGAGGCGCAGGCGGGGCTGGCGGCGGAGG 780
Importin-α  TGG---------------------------------------------------------- 42
            ***
VP78/83     CGGAGGCGGAGGTGGTGGCGGTGATGCAGACGGCGGTTTAGGCTCAAATGTCTCTTTAGG 840
Importin-α  ------------------------------------------------------------
VP78/83     CAACACAGTCGGCACCTCAACTATTGTACTGGTTTCGGGCGCCGTTTTTGGTTTGACCGG 900
Importin-α  ------------------------------------------------------------
VP78/83     TCTGAGACGAGTGCGATTTTTTTCGTTTCTAATAGCTTCCAACAATTGTTGTCTGTCGTC 960
Importin-α  ------------------------------------------------------------
VP78/83     TAAAGGTGCAGCGGGTTGAGGTTCCGTCGGCATTGGTGGAGCGGGCGGCAATTCAGACAT 1020
Importin-α  ------------------------------------------------------------
VP78/83     CGATGGTGGTGGTGGTGGAGGCGCTGGAATGTTAGGCACGGGAGAAGGTGGTGGCGG 1080
Importin-α  ------------------------------------------------------------
VP78/83     CGGTGCCGCCGGTATAATTTGTTCTGGTTTAGTTTGTTCGCGCACGATTGTGGGCACCGG 1140
Importin-α  ------------------------------------------------------------
VP78/83     CGCAGGCGCCGCTGGCTGCACAACGGAAGGTCGTCTGCTTCGAGGCAGCGCTTGGGGTGG 1200
Importin-α  ------------------------------------------------------------
VP78/83     TGGCAATTCAATATTATAATTGGAATACAAATCGTAAAAATCTGCTATAAGCATTGTAAT 1260
Importin-α  ------------------------------------------------------------
VP78/83     TTCGCTATCGTTTACCGTGCCGATATTTAACAACCGCTCAATGTAAGCAATTGTATTGTA 1320
Importin-α  ------------------------------------------------------------
VP78/83     AAGAGATTGTCTCAAGCTCGGATCCCGCACGCCGATAACAAGCCTTTTCATTTTTACTAC 1380
Importin-α  ------------------------------------------------------------
VP78/83     AGCATTGTAGTGGCGAGACACTTCGCTGTCGTCGACGTACATGTATGCTTTGTTGTCAAA 1440
Importin-α  ------------------------------------------------------------
VP78/83     AACGTCGTTGGCAAGCTTTAAAATATTTAAAAGAACATCTCTGTTCAGCACCACTGTGTT 1500
Importin-α  ------------------------------------------------------------
VP78/83     GTCGTAAATGTTGTTTTTGATAATTTGCGCTTCCGCAGTATCGACACGTTCAAAAAATTG 1560
Importin-α  ------------------------------------------------------------
VP78/83     ATGCGCATCAATTTTGTTGTTCCTATTATTGAATAAATAAGATTGTACAGATTCATATCT 1620
Importin-α  ------------------------------------------------------------
VP78/83     ACGATTCGTCAT 1632
Importin-α  ------------
```

Sequence alignment of VP78/83 and RanGTP binding domain



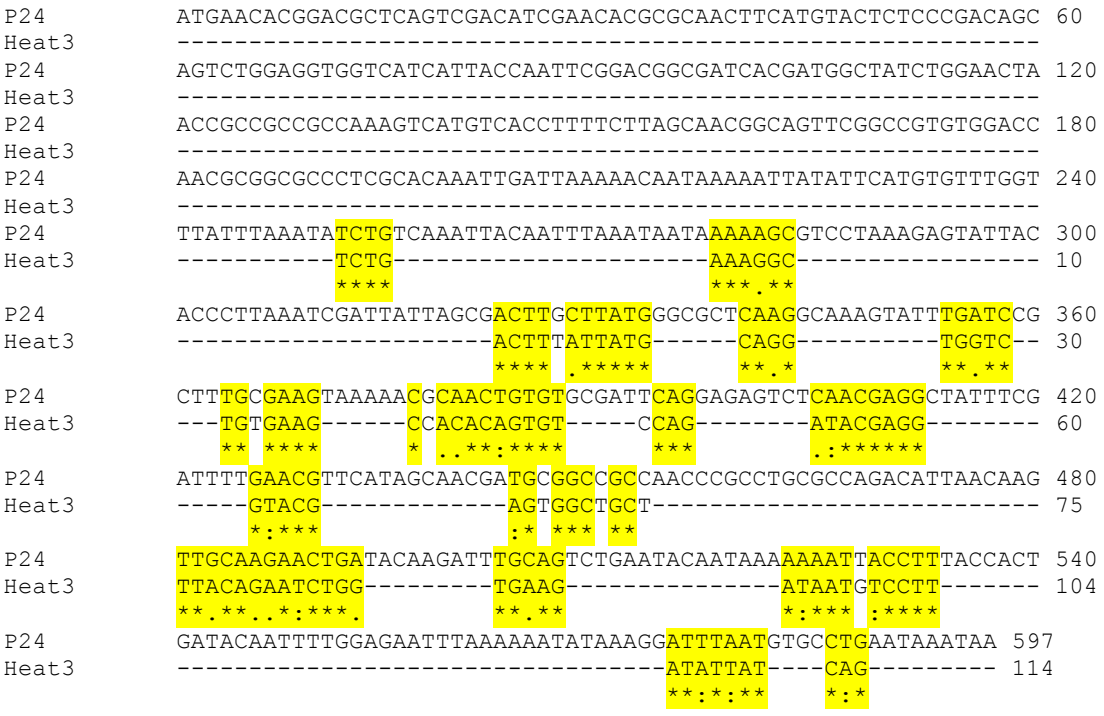
Sequence alignment of P24 and HEAT1 domain

```
P24          ATGAACACGGACGCTCAGTCGACATCGAACACGCGCAACTTCATGTACTCTCCCGACAGC 60
Heat1        ---------AAC---CAGTGGCCA--------------------GAACTCATTCCTCAGC 28
                      .**    **** *.**                    *:****:  * :****

P24          AGTCTGGAGGTGGTCATCATTACCAATTCGGACGGCGATCACGATGGCTATCTGGAACTA 120
Heat1        TG-------GTGG---------CCAAT---GTC----------------------ACAA 46
             :*       ****         *****   *:*                     **:*

P24          ACCGCCGCCGCCAAAGTCATGTCACCTTTTCTTAGCAACGGCAGTTCGGCCGTGTGGACC 180
Heat1        ACC-CCAACAGCACAG-----------------AGCACATGAAG-------GAGTCGACA 81
             *** **..*. **.**                 ****.. *.**       *:** ***.

P24          AACGCGGCGCCCTCGCACAAATTGATTAAAAACAATAAAAATTATATTCATGTGTTTGGT 240
Heat1        TTG--GAAGCCATCG-------------------------------------------GT 96
             ::   *..***.***                                           **

P24          TTATTTAAATATCTGTCAAATTACAATTTAAATAATAAAAAGCGTCCTAAAGAGTATTAC 300
Heat1        T-------ATATTTGCCAAGATATAG---------------------------------- 115
             *       **** ** ***.:** *.

P24          ACCCTTAAATCGATTATTAGCGACTTGCTTATGGGCGCTCAAGGCAAAGTATTTGATCCG 360
Heat1        ACCCA------------------------------------------------------- 120
             ****:

P24          CTTTGCGAAGTAAAAACGCAACTGTGTGCGATTCAGGAGAGTCTCAACGAGGCTATTTCG 420
Heat1        ------------------------------------------------------------

P24          ATTTTGAACGTTCATAGCAACGATGCGGCCGCCAACCCGCCTGCGCCAGACATTAACAAG 480
Heat1        ------------------------------------------------------------

P24          TTGCAAGAACTGATACAAGATTTGCAGTCTGAATACAATAAAAAAATTACCTTTACCACT 540
Heat1        ------------------------------------------------------------

P24          GATACAATTTTGGAGAATTTAAAAAATATAAAGGATTTAATGTGCCTGAATAAATAA 597
Heat1        ----------------------------------------------------------
```

Sequence alignment of P24 and HEAT2 domain

```
P24          ATGAACACGGACGCTCAGTCGACATCGAACACGCGCAACTTCATGTACTCTCCCGACAGC 60
Heat2        ------------------------------------------------------------
P24          AGTCTGGAGGTGGTCATCATTACCAATTCGGACGGCGATCACGATGGCTATCTGGAACTA 120
Heat2        --------GAT-------AAATCCAATGAG--------------AT-----TCTG------ 21
                     *.*       *:::*****  .*                **     ****
P24          ACCGCCGCCGCCAAAGTCATGTCACCTTTTCTTAGCAACGGCAGTTCGGCCGTGTGGACC 180
Heat2        ------ACTGCCATAATCCAG------------------GGGA---------TGAGGAAA 48
                   .*  ****:*.**.:*                  **  *         **:***..
P24          AACGCGGCGCCCTCGCACAAATTGATTAAAAACAATAAAAATTATATTCATGTGTTTGGT 240
Heat2        GAAGAG----CCTAG------------------------------TAATAATGTG------ 69
             .*.*.*    ***.*                              **:*.*****
P24          TTATTTAAATATCTGTCAAATTACAATTTAAATAATAAAAAGCGTCCTAAAGAGTATTAC 300
Heat2        ----------------------------------------AAGC------------------ 73
                                                       ****
P24          ACCCTTAAATCGATTATTAGCGACTTGCTTATGGGCGCTCAAGGCAAAGTATTTGATCCG 360
Heat2        -----------------TAGCTGCTA-CGAATG--CACTCCTG------------------ 96
                              ****  .**:  *  :***   *.***.:*
P24          CTTTGCGAAGTAAAAACGCAACTGTGTGCGATTCAGGAGAGTCTCAACGAGGCTATTTCG 420
Heat2        --------------AACTCATTGGAGT-----------------TCACCAAAGCA------ 120
                           ***  **:   *:**                ***.*.*.**:
P24          ATTTTGAACGTTCATAGCAACGATGCGGCCGCCAACCCGCCTGCGCCAGACATTAACAAG 480
Heat2        ------------------------------------------------------------
P24          TTGCAAGAACTGATACAAGATTTGCAGTCTGAATACAATAAAAAAATTACCTTTACCACT 540
Heat2        ------------------------------------------------------------
P24          GATACAATTTTGGAGAATTTAAAAAATATAAAGGATTTAATGTGCCTGAATAAATAA 597
Heat2        ------------------------------------------------------------
```

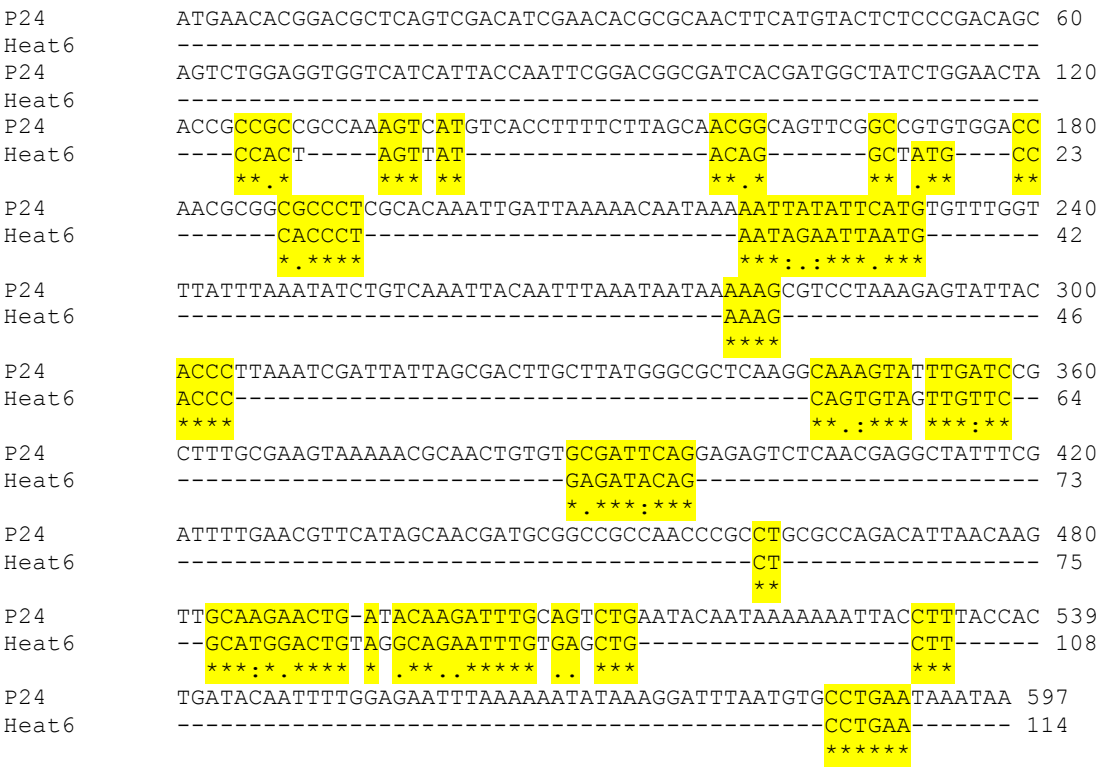
Sequence alignment of P24 and HEAT3 domain



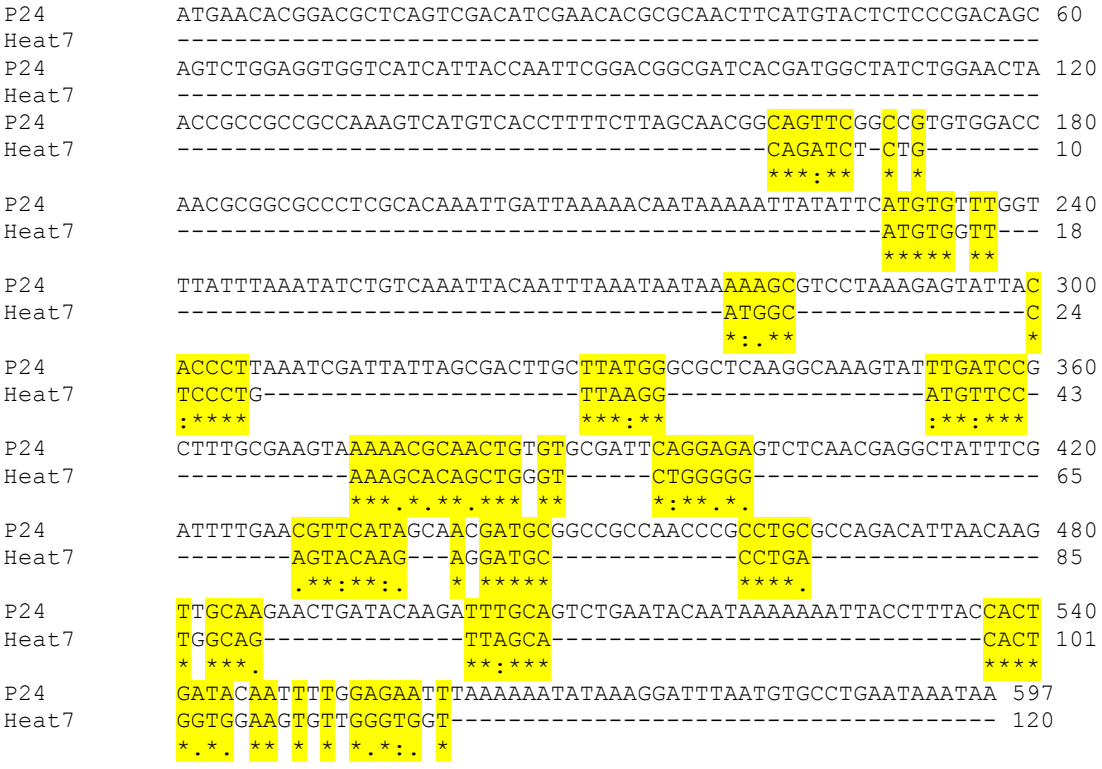
Sequence alignment of P24 and HEAT4 domain

```
P24          ATGAACACGGACGCTCAGTCGACATCGAACACGCGCAACTTCATGTACTCTCCCGACAGC 60
Heat4        ------------------------------------------------------------
P24          AGTCTGGAGGTGGTCATCATTACCAATTCGGACGGCGATCACGATGGCTATCTGGAACTA 120
Heat4        -----GGAG--------------CACTACAG-----------------TATCTG------ 18
                  ****              **.*:*.*                  ******
P24          ACCGCCGCCGCCAAAGTCATGTCACCTTTTCTTAGCAACGGCAGTTCGGCCGTGTGGACC 180
Heat4        ----------------------------------------GTTC------------C 23
                                                    ****            *
P24          AACGCGGCGCCCTCGCACAAATTGATTAAAAACAATAAAAATTATATTCATGTGTTTGGT 240
Heat4        AAT-------CCTCACACAG-----------ACACTAA---------------------- 43
             **        ****.****.           ***.***
P24          TTATTTAAATATCTGTCAAATTACAATTTAAATAATAAAAAGCGTCCTAAAGAGTATTAC 300
Heat4        ----------------------------------------CTAAACAG------ 51
                                                     *****  **
P24          ACCCTTAAATCGATTATTAGCGACTTGCTTATGGGCGCTCAAGGCAAAGTATTTGATCCG 360
Heat4        --------GACGAAAAT------------------GATGATGACGATG-ACTGGAACCC 83
                     .:***::**                 *.* *:*.*.*:* * * **:**
P24          CTTTGCGAAGTAAAAACGCAACTGTGTGCGATTCAGGAGAGTCTCAACGAGGCTATTTCG 420
Heat4        CT--GCAAAG--------CAGCAGG----------GGTGTGCCTCAT----GCTTCT--- 116
             **  **.***         **.*:*          **:*:* ****:    ***: *
P24          ATTTTGAACGTTCATAGCAACGATGCGGCCGCCAACCCGCCTGCGCCAGACATTAACAAG 480
Heat4        --------------------------GGCCACC---------------------------- 123
                                        ****.**
P24          TTGCAAGAACTGATACAAGATTTGCAGTCTGAATACAATAAAAAAATTACCTTTACCACT 540
Heat4        ------------------------------------------------------------
P24          GATACAATTTTGGAGAATTTAAAAAATATAAAGGATTTAATGTGCCTGAATAAATAA 597
Heat4        ------------------------------------------------------------
```

Sequence alignment of P24 and HEAT6 domain



Sequence alignment of P24 and HEAT7 domain



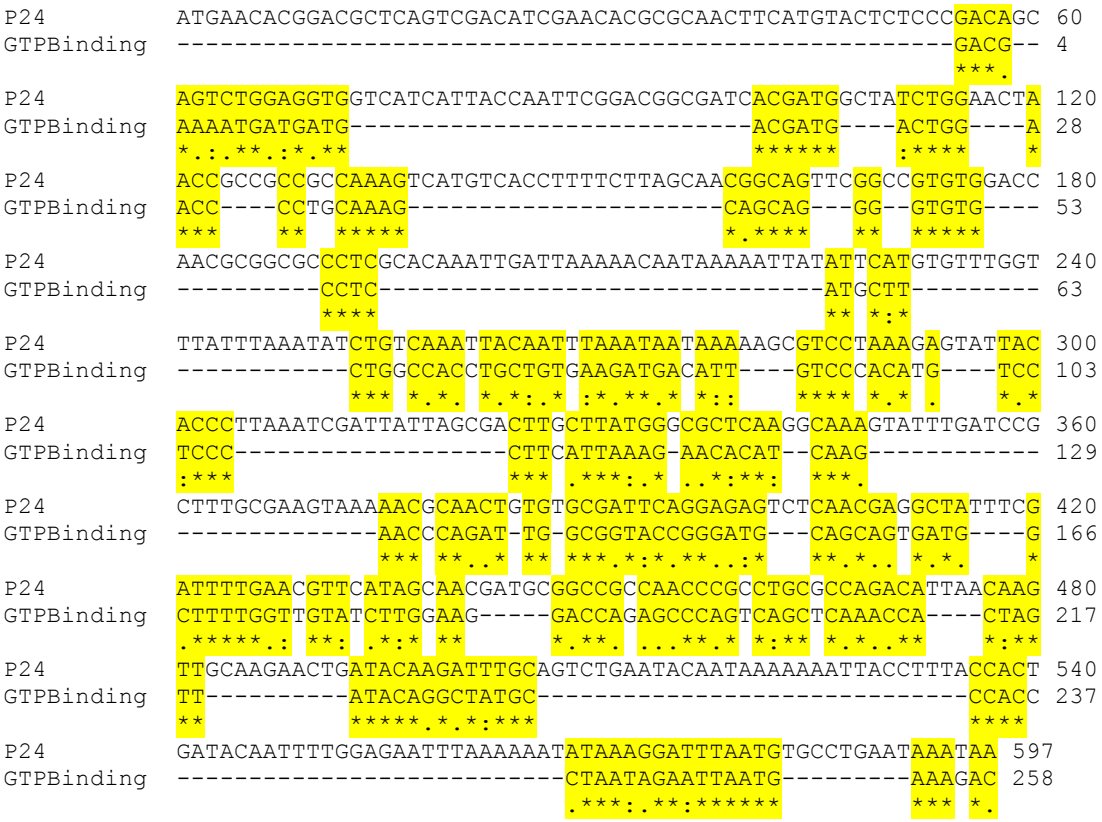
Sequence alignment of P24 and HEAT8 domain

```
P24         ATGAACACGGACGCTCAGTCGACATCGAACACGCGCAACTTCATGTACTCTCCCGACAGC 60
Heat8       ------------------------------------------------------------
P24         AGTCTGGAGGTGGTCATCATTACCAATTCGGACGGCGATCACGATGGCTATCTGGAACTA 120
Heat8       ------------------------------------------------------------
P24         ACCGCCGCCGCCAAAGTCATGTCACCTTTTCTTAGCAACGGCAGTTCGGCCGTGTGGACC 180
Heat8       ------------------------CCTTT--------------------CTGTG------ 10
                                     *****                    *  ***
P24         AACGCGGCGCCCTCGCACAAATTGATTAAAAACAATAAAAATTATATTCATGTGTTTGGT 240
Heat8       -ACGAG-------------------------------------------GTG------ 18
             ***.*                                             ***
P24         TTATTTAAATATCTGTCAAATTACAATTTAAATAATAAAAAGCGTCCTAAAGAGTATTAC 300
Heat8       ----------------------------------------ATGC----------------- 22
                                                     *:**
P24         ACCCTTAAATCGATTATTAGCGACTTGCTTATGGGCGCTCAAGGCAAAGTATTTGATCCG 360
Heat8       -----------------AGC----TGCTTCTGG----------AAAA------------ 38
                             ***    *****.***          .***
P24         CTTTGCGAAGTAAAAACGCAACTGTGTGCGATTCAGGAGAGTCTCAACGAGGCTATTTCG 420
Heat8       -TTTGGGGAATGAGAACG-------------------------TCCACAGGTCTG----- 67
             **** *.*.*.*.****                         **.**..* **.
P24         ATTTTGAACGTTCATAGCAACGATGCGGCCGCCAACCCGCCTGCGCCAGACATTAACAAG 480
Heat8       ----------------------TGAAGCCGC---------------------------- 76
                                   **..*****
P24         TTGCAAGAACTGATACAAGATTTGCAGTCTGAATACAATAAAAAAATTACCTTTACCACT 540
Heat8       -----------------AGATT--CTGTCAGTGTTTGGTG---ATATTGCCCTTGC---- 110
                             *****  *:***:*:.*: ..*.   *:***.** **.*
P24         GATACAATTTTTGGAGAATTTAAAAAATATAAAGGATTTAATGTGCCTGAATAAATAA 597
Heat8       -------TATTGGAGGA---------------------------------------- 120
                   *:******.*
```

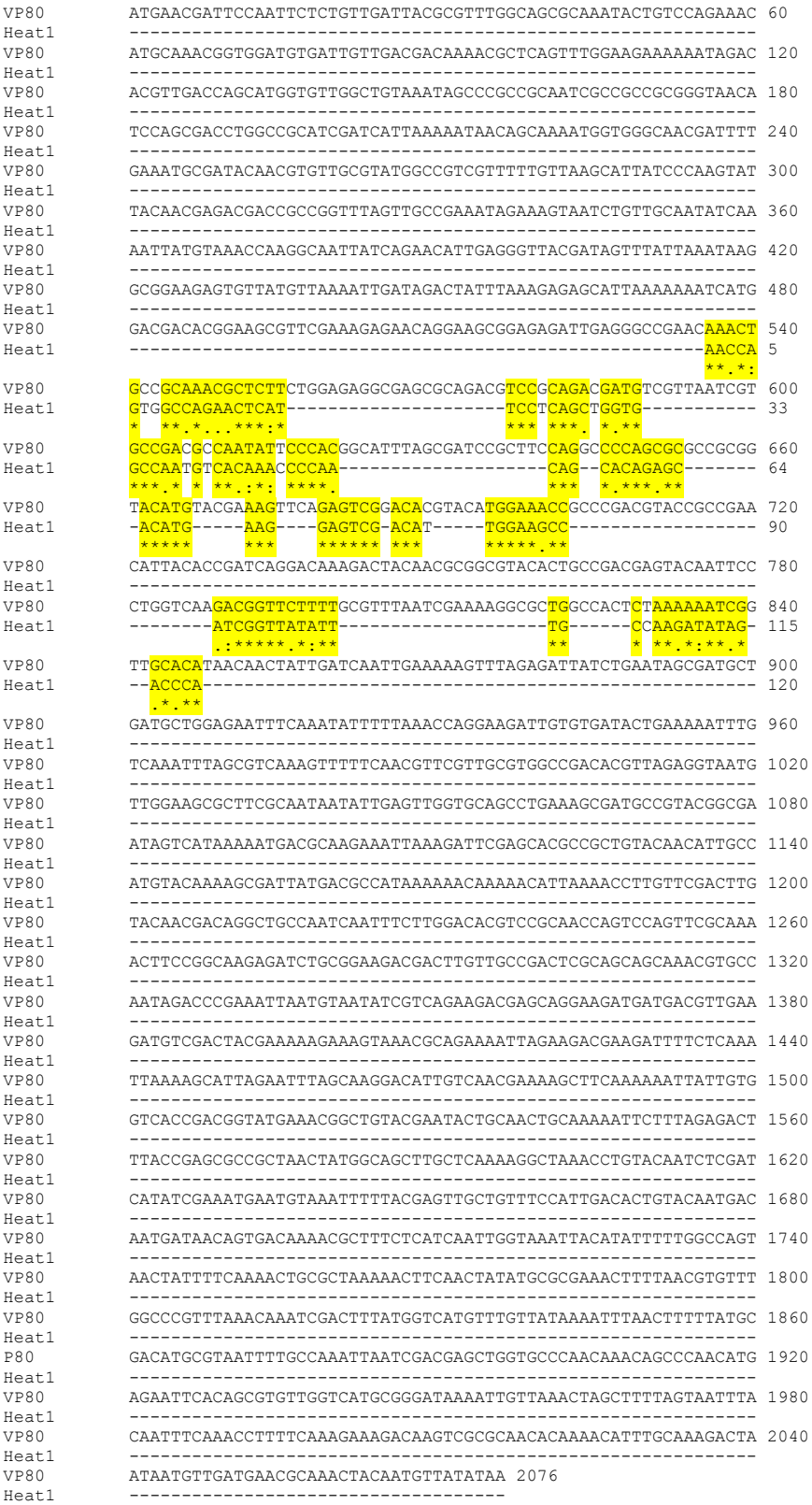
Sequence alignment of P24 and importin-α binding domain

```
P24          ATGAACACGGACGCTCAGTCGACATCGAACACGCGCAACTTCATGTACTCTCCCGACAGC 60
Importin-α   ----------------------------ACAC---TAACTAAA--------CAGGACG-- 19
                                         ****    ****:.*        *. ***.

P24          AGTCTGGAGGTGGTCATCATTACCAATTCGGACGGCGATCACGATGGCTATCTGGAACTA 120
Importin-α   AAAATGATGATG----------------------------ACGATG----ACTGG----- 42
             *.:.**.:*.**                            ******    :****

P24          ACCGCCGCCGCCAAAGTCATGTCACCTTTTCTTAGCAACGGCAGTTCGGCCGTGTGGACC 180
Importin-α   ------------------------------------------------------------

P24          AACGCGGCGCCCTCGCACAAATTGATTAAAAACAATAAAAATTATATTCATGTGTTTGGT 240
Importin-α   ------------------------------------------------------------

P24          TTATTTAAATATCTGTCAAATTACAATTTAAATAATAAAAAGCGTCCTAAAGAGTATTAC 300
Importin-α   ------------------------------------------------------------

P24          ACCCTTAAATCGATTATTAGCGACTTGCTTATGGGCGCTCAAGGCAAAGTATTTGATCCG 360
Importin-α   ------------------------------------------------------------

P24          CTTTGCGAAGTAAAAACGCAACTGTGTGCGATTCAGGAGAGTCTCAACGAGGCTATTTCG 420
Importin-α   ------------------------------------------------------------

P24          ATTTTGAACGTTCATAGCAACGATGCGGCCGCCAACCCGCCTGCGCCAGACATTAACAAG 480
Importin-α   ------------------------------------------------------------

P24          TTGCAAGAACTGATACAAGATTTGCAGTCTGAATACAATAAAAAAATTACCTTTACCACT 540
Importin-α   ------------------------------------------------------------

P24          GATACAATTTTGGAGAATTTAAAAAATATAAAGGATTTAATGTGCCTGAATAAATAA 597
Importin-α   ----------------------------------------------------------
```

Sequence alignment of P24 and RanGTP binding domain



Sequence alignment of VP80 and HEAT1 domain



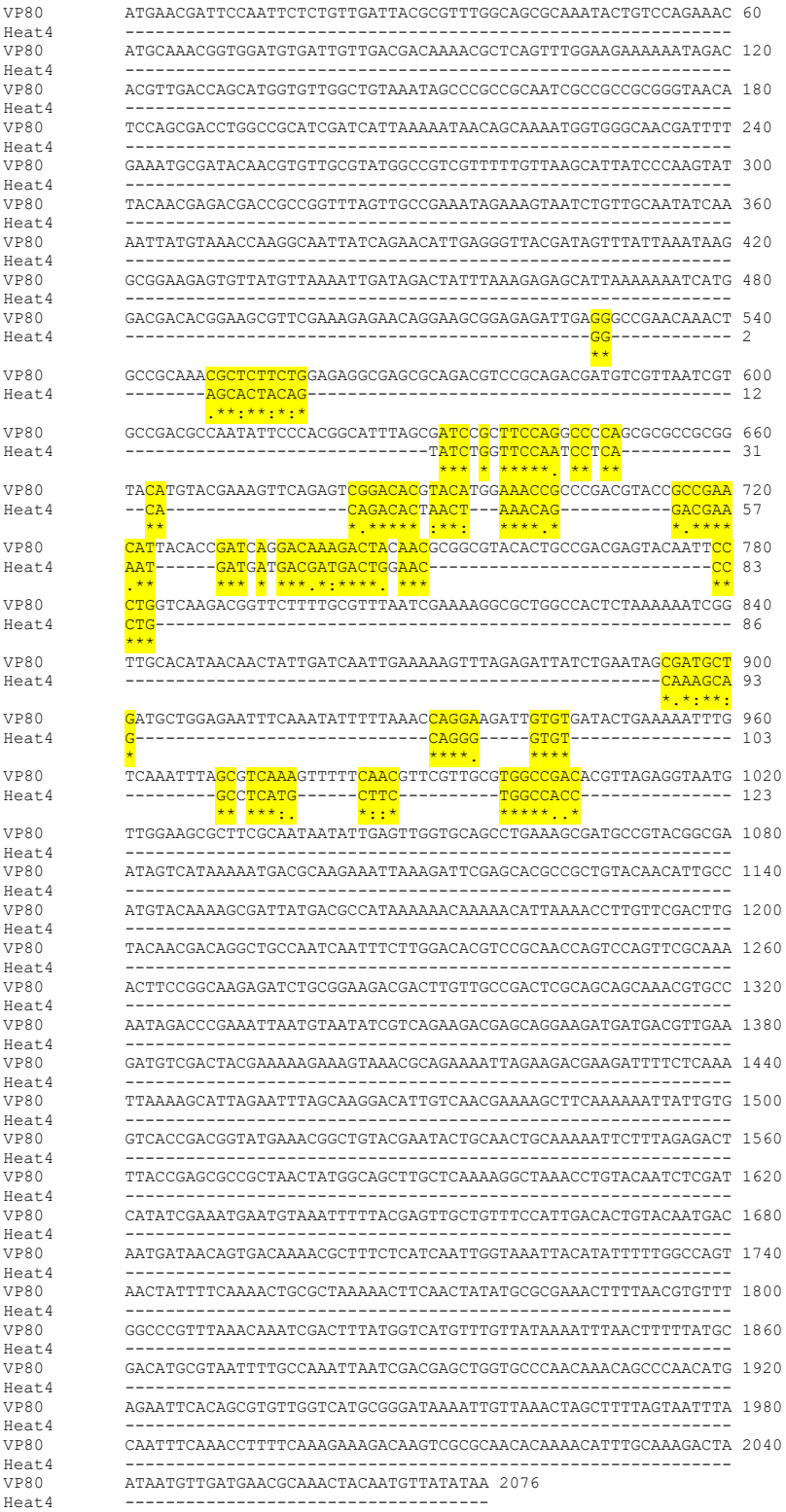
Sequence alignment of VP80 and HEAT2 domain

```
VP80        ATGAACGATTCCAATTCTCTGTTGATTACGCGTTTGGCAGCGCAAATACTGTCCAGAAAC 60
Heat2       ------------------------------------------------------------
VP80        ATGCAAACGGTGGATGTGATTGTTGACGACAAAACGCTCAGTTTGGAAGAAAAAATAGAC 120
Heat2       ------------------------------------------------------------
VP80        ACGTTGACCAGCATGGTGTTGGCTGTAAATAGCCCGCCGCAATCGCCGCCGCGGGTAACA 180
Heat2       ------------------------------------------------------------
VP80        TCCAGCGACCTGGCCGCATCGATCATTAAAAATAACAGCAAAATGGTGGGCAACGATTTT 240
Heat2       ------------------------------------------------------------
VP80        GAAATGCGATACAACGTGTTGCGTATGGCCGTCGTTTTTGTTAAGCATTATCCCAAGTAT 300
Heat2       ----------------------------------------GATAA-----ATCCAATGAGA 16
                                                     *:***     ****.*:*:.:

VP80        TACAACGAGACGACCGCCGGTTTAGTTGCCGAAATAGAAAGTAATCTGTTGCAATATCAA 360
Heat2       TTCT-------GACTGCCA---------------------------------------- 28
            *:*:       *** ***.

VP80        AATTATGTAAACCAAGGCAATTATCAGAACATTGAGGGTTACGATAGTTTATTAAATAAG 420
Heat2       -------TAATCCAGG------------------------------------------- 37
                  ***:***.*

VP80        GCGGAAGAGTGTTATGTTAAAATTGATAGACTATTTAAAGAGAGCATTAAAAAATCATG 480
Heat2       --GGATGAG---------------GAAAGA----------AGAGCCT---AGTAATAATG 67
              ***:***               **:***          *****.*   *.:***.***

VP80        GACGACACGGAAGCGTTCGAAAGAGAACAGGAAGCGGAGAGATTGAGGGCCGAACAAACT 540
Heat2       ----------------------------------------TGAAG------CTAGCT 78
                                                     ***.*      *:*.**

VP80        GCCGCAAACGCTCTTCTGGAGAGGCGAGCGCAGACGTCCGCAGACGATGTCGTTAATCGT 600
Heat2       GCTACGAATGCACTCCTG--------AACTCA---------------------------- 102
            ** .*.** **:** ***        *.* **

VP80        GCCGACGCCAATATTCCCACGGCATTTAGCGATCCGCTTCCAGGCCCCAGCGCGCCGCGG 660
Heat2       -------------------------TTGGAGTTCA----------CCAAAGCA-------- 120
                                      **.*.*:**.          **..***.

VP80        TACATGTACGAAAGTTCAGAGTCGGACACGTACATGGAAACCGCCCGACGTACCGCCGAA 720
Heat2       ------------------------------------------------------------
VP80        CATTACACCGATCAGGACAAAGACTACAACGCGGCGTACACTGCCGACGAGTACAATTCC 780
Heat2       ------------------------------------------------------------
VP80        CTGGTCAAGACGGTTCTTTTGCGTTTAATCGAAAAGGCGCTGGCCACTCTAAAAAATCGG 840
Heat2       ------------------------------------------------------------
VP80        TTGCACATAACAACTATTGATCAATTGAAAAAGTTTAGAGATTATCTGAATAGCGATGCT 900
Heat2       ------------------------------------------------------------
VP80        GATGCTGGAGAATTTCAAATATTTTTAAACCAGGAAGATTGTGTGATACTGAAAAATTTG 960
Heat2       ------------------------------------------------------------
VP80        TCAAATTTAGCGTCAAAGTTTTTCAACGTTCGTTGCGTGGCCGACACGTTAGAGGTAATG 1020
Heat2       ------------------------------------------------------------
VP80        TTGGAAGCGCTTCGCAATAATATTGAGTTGGTGCAGCCTGAAAGCGATGCCGTACGGCGA 1080
Heat2       ------------------------------------------------------------
VP80        ATAGTCATAAAAATGACGCAAGAAATTAAAGATTCGAGCACGCCGCTGTACAACATTGCC 1140
Heat2       ------------------------------------------------------------
VP80        ATGTACAAAAGCGATTATGACGCCATAAAAAACAAAAACATTAAAACCTTGTTCGACTTG 1200
Heat2       ------------------------------------------------------------
VP80        TACAACGACAGGCTGCCAATCAATTTCTTGGACACGTCCGCAACCAGTCCAGTTCGCAAA 1260
Heat2       ------------------------------------------------------------
VP80        ACTTCCGGCAAGAGATCTGCGGAAGACGACTTGTTGCCGACTCGCAGCAGCAAACGTGCC 1320
Heat2       ------------------------------------------------------------
VP80        AATAGACCCGAAATTAATGTAATATCGTCAGAAGACGAGCAGGAAGATGATGACGTTGAA 1380
Heat2       ------------------------------------------------------------
VP80        GATGTCGACTACGAAAAAGAAAGTAAACGCAGAAAATTAGAAGACGAAGATTTTCTCAAA 1440
Heat2       ------------------------------------------------------------
VP80        TTAAAAGCATTAGAATTTAGCAAGGACATTGTCAACGAAAAGCTTCAAAAAATTATTGTG 1500
Heat2       ------------------------------------------------------------
VP80        GTCACCGACGGTATGAAACGGCTGTACGAATACTGCAACTGCAAAAATTCTTTAGAGACT 1560
Heat2       ------------------------------------------------------------
VP80        TTACCGAGCGCCGCTAACTATGGCAGCTTGCTCAAAAGGCTAAACCTGTACAATCTCGAT 1620
Heat2       ------------------------------------------------------------
VP80        CATATCGAAATGAATGTAAATTTTTACGAGTTGCTGTTTCCATTGACACTGTACAATGAC 1680
Heat2       ------------------------------------------------------------
VP80        AATGATAACAGTGACAAAACGCTTTCTCATCAATTGGTAAATTACATATTTTTGGCCAGT 1740
Heat2       ------------------------------------------------------------
VP80        AACTATTTTCAAAACTGCGCTAAAAACTTCAACTATATGCGCGAAACTTTTAACGTGTTT 1800
Heat2       ------------------------------------------------------------
VP80        GGCCCGTTTAAACAAATCGACTTTATGGTCATGTTTGTTATAAAATTTAACTTTTTATGC 1860
Heat2       ------------------------------------------------------------
VP80        GACATGCGTAATTTTGCCAAATTAATCGACGAGCTGGTGCCCAACAAACAGCCCAACATG 1920
Heat2       ------------------------------------------------------------
VP80        AGAATTCACAGCGTGTTGGTCATGCGGGATAAAATTGTTAAACTAGCTTTTAGTAATTTA 1980
Heat2       ------------------------------------------------------------
VP80        CAATTTCAAACCTTTTCAAAGAAAGACAAGTCGCGCAACACAAAACATTTGCAAAGACTA 2040
Heat2       ------------------------------------------------------------
VP80        ATAATGTTGATGAACGCAAACTACAATGTTATATAA 2076
Heat2       -----------------------------------
```

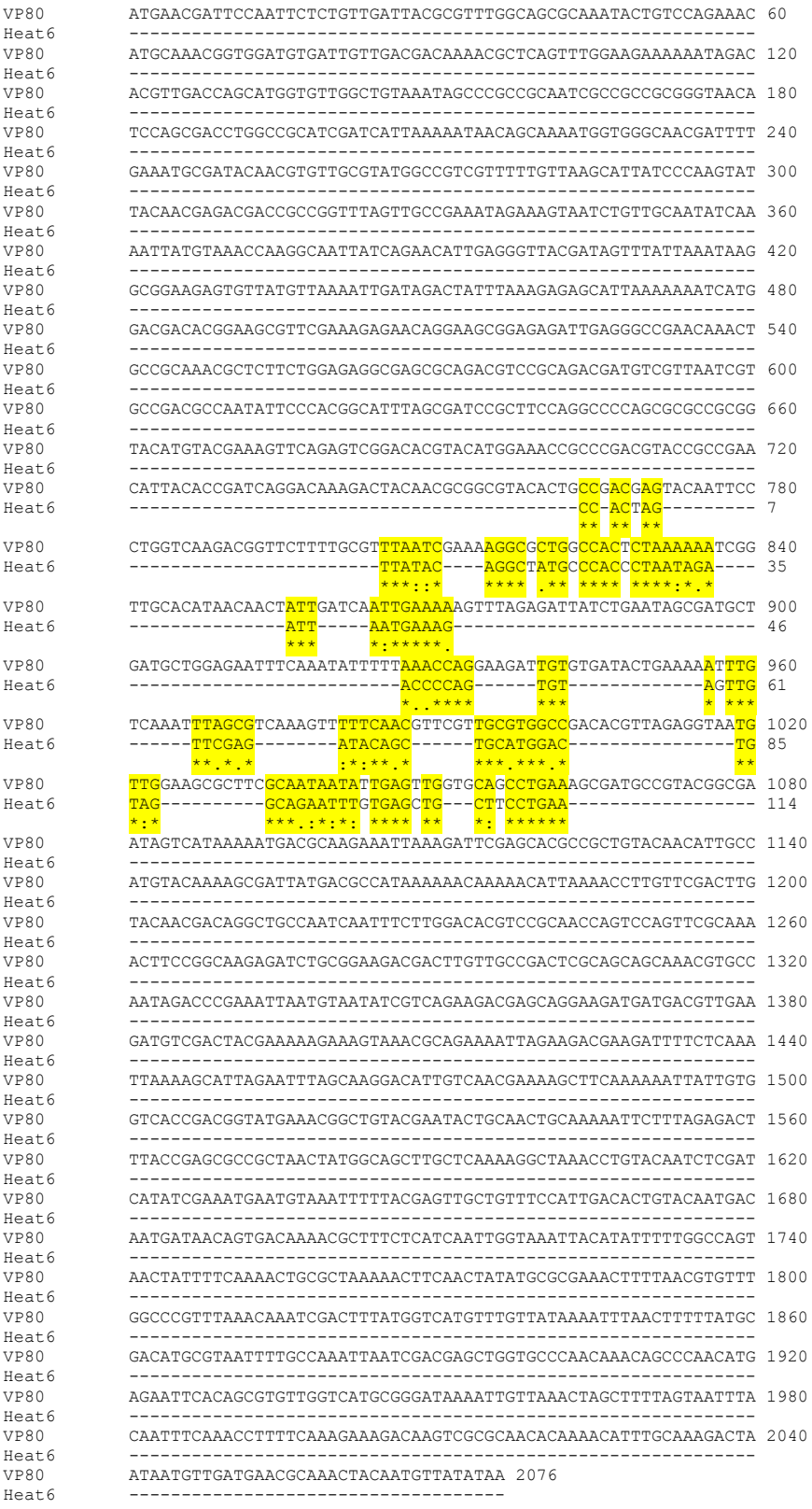
Sequence alignment of VP80 and HEAT3 domain

```
VP80        ATGAACGATTCCAATTCTCTGTTGATTACGCGTTTGGCAGCGCAAATACTGTCCAGAAAC 60
Heat3       ------------------------------------------------------------
VP80        ATGCAAACGGTGGATGTGATTGTTGACGACAAAACGCTCAGTTTGGAAGAAAAAATAGAC 120
Heat3       ----------------------------------------TCTG-------------AAAGGC 10
                                                    **:*             *:**.*
VP80        ACGTTGACCAGCATGGTGTTGGCTGTAAATAGCCCGCCGCAATCGCCGCCGCGGGTAACA 180
Heat3       ACTTTATTATGCA-GGTG--GTCTGTGAA------GCCACA-------------------- 42
            **  **.:  .:***  ****  *  ****.**      ***.**
VP80        TCCAGCGACCTGGCCGCATCGATCATTAAAAATAACAGCAAAATGGTGGGCAACGATTTT 240
Heat3       --CAGTGTCCAG---------------------------------------------------- 52
              ***  *:**:*
VP80        GAAATGCGATACAACGTGTTGCGTATGGCCGTCGTTTTTGTTAAGCATTATCCCAAGTAT 300
Heat3       --------ATACGAGGG--TACGAGTGGCTG--------------------------CTT 76
                    ****.*  *   *.**:.**** *                          :*
VP80        TACAACGAGACGACCGCCGGTTTAGTTGCCGAAATAGAAAGTAATCTGTTGCAATATCAA 360
Heat3       TACAG-----------------------------------AATCTGGTG-------AA 92
            ****.                                   ******  **       **
VP80        AATTATGTAAACCAAGGCAATTATCAGAACATTGAGGGTTACGATAGTTTATTAAATAAG 420
Heat3       GATAATGT---CCTT--ATATTATCAG---------------------------------- 114
            .**:****   **::  .:********
VP80        GCGGAAGAGTGTTATGTTAAAATTGATAGACTATTTAAAGAGAGCATTAAAAAAATCATG 480
Heat3       ------------------------------------------------------------
VP80        GACGACACGGAAGCGTTCGAAAGAGAACAGGAAGCGGAGAGATTGAGGGCCGAACAAACT 540
Heat3       ------------------------------------------------------------
VP80        GCCGCAAACGCTCTTCTGGAGAGGCGAGCGCAGACGTCCGCAGACGATGTCGTTAATCGT 600
Heat3       ------------------------------------------------------------
VP80        GCCGACGCCAATATTCCCACGGCATTTAGCGATCCGCTTCCAGGCCCCAGCGCGCCGCGG 660
Heat3       ------------------------------------------------------------
VP80        TACATGTACGAAAGTTCAGAGTCGGACACGTACATGGAAACCGCCCGACGTACCGCCGAA 720
Heat3       ------------------------------------------------------------
VP80        CATTACACCGATCAGGACAAAGACTACAACGCGGCGTACACTGCCGACGAGTACAATTCC 780
Heat3       ------------------------------------------------------------
VP80        CTGGTCAAGACGGTTCTTTTGCGTTTAATCGAAAAGGCGCTGGCCACTCTAAAAAATCGG 840
Heat3       ------------------------------------------------------------
VP80        TTGCACATAACAACTATTGATCAATTGAAAAAGTTTAGAGATTATCTGAATAGCGATGCT 900
Heat3       ------------------------------------------------------------
VP80        GATGCTGGAGAATTTCAAATATTTTTAAACCAGGAAGATTGTGTGATACTGAAAAATTTG 960
Heat3       ------------------------------------------------------------
VP80        TCAAATTTAGCGTCAAAGTTTTTCAACGTTCGTTGCGTGGCCGACACGTTAGAGGTAATG 1020
Heat3       ------------------------------------------------------------
VP80        TTGGAAGCGCTTCGCAATAATATTGAGTTGGTGCAGCCTGAAAGCGATGCCGTACGGCGA 1080
Heat3       ------------------------------------------------------------
VP80        ATAGTCATAAAAATGACGCAAGAAATTAAAGATTCGAGCACGCCGCTGTACAACATTGCC 1140
Heat3       ------------------------------------------------------------
VP80        ATGTACAAAAGCGATTATGACGCCATAAAAAACAAAAACATTAAAACCTTGTTCGACTTG 1200
Heat3       ------------------------------------------------------------
VP80        TACAACGACAGGCTGCCAATCAATTTCTTGGACACGTCCGCAACCAGTCCAGTTCGCAAA 1260
Heat3       ------------------------------------------------------------
VP80        ACTTCCGGCAAGAGATCTGCGGAAGACGACTTGTTGCCGACTCGCAGCAGCAAACGTGCC 1320
Heat3       ------------------------------------------------------------
VP80        AATAGACCCGAAATTAATGTAATATCGTCAGAAGACGAGCAGGAAGATGATGACGTTGAA 1380
Heat3       ------------------------------------------------------------
VP80        GATGTCGACTACGAAAAAGAAAGTAAACGCAGAAAATTAGAAGACGAAGATTTTCTCAAA 1440
Heat3       ------------------------------------------------------------
VP80        TTAAAAGCATTAGAATTTAGCAAGGACATTGTCAACGAAAAGCTTCAAAAAATTATTGTG 1500
Heat3       ------------------------------------------------------------
VP80        GTCACCGACGGTATGAAACGGCTGTACGAATACTGCAACTGCAAAAATTCTTTAGAGACT 1560
Heat3       ------------------------------------------------------------
VP80        TTACCGAGCGCCGCTAACTATGGCAGCTTGCTCAAAAGGCTAAACCTGTACAATCTCGAT 1620
Heat3       ------------------------------------------------------------
VP80        CATATCGAAATGAATGTAAATTTTTACGAGTTGCTGTTTCCATTGACACTGTACAATGAC 1680
Heat3       ------------------------------------------------------------
VP80        AATGATAACAGTGACAAAACGCTTTCTCATCAATTGGTAAATTACATATTTTTGGCCAGT 1740
Heat3       ------------------------------------------------------------
VP80        AACTATTTTCAAAACTGCGCTAAAAACTTCAACTATATGCGCGAAACTTTTAACGTGTTT 1800
Heat3       ------------------------------------------------------------
VP80        GGCCCGTTTAAACAAATCGACTTTATGGTCATGTTTGTTATAAAATTTAACTTTTTATGC 1860
Heat3       ------------------------------------------------------------
VP80        GACATGCGTAATTTTGCCAAATTAATCGACGAGCTGGTGCCCAACAAACAGCCCAACATG 1920
Heat3       ------------------------------------------------------------
VP80        AGAATTCACAGCGTGTTGGTCATGCGGGATAAAATTGTTAAACTAGCTTTTAGTAATTTA 1980
Heat3       ------------------------------------------------------------
VP80        CAATTTCAAACCTTTTCAAAGAAAGACAAGTCGCGCAACACAAAACATTTGCAAAGACTA 2040
Heat3       ------------------------------------------------------------
VP80        ATAATGTTGATGAACGCAAACTACAATGTTATATAA 2076
Heat3       -----------------------------------
```

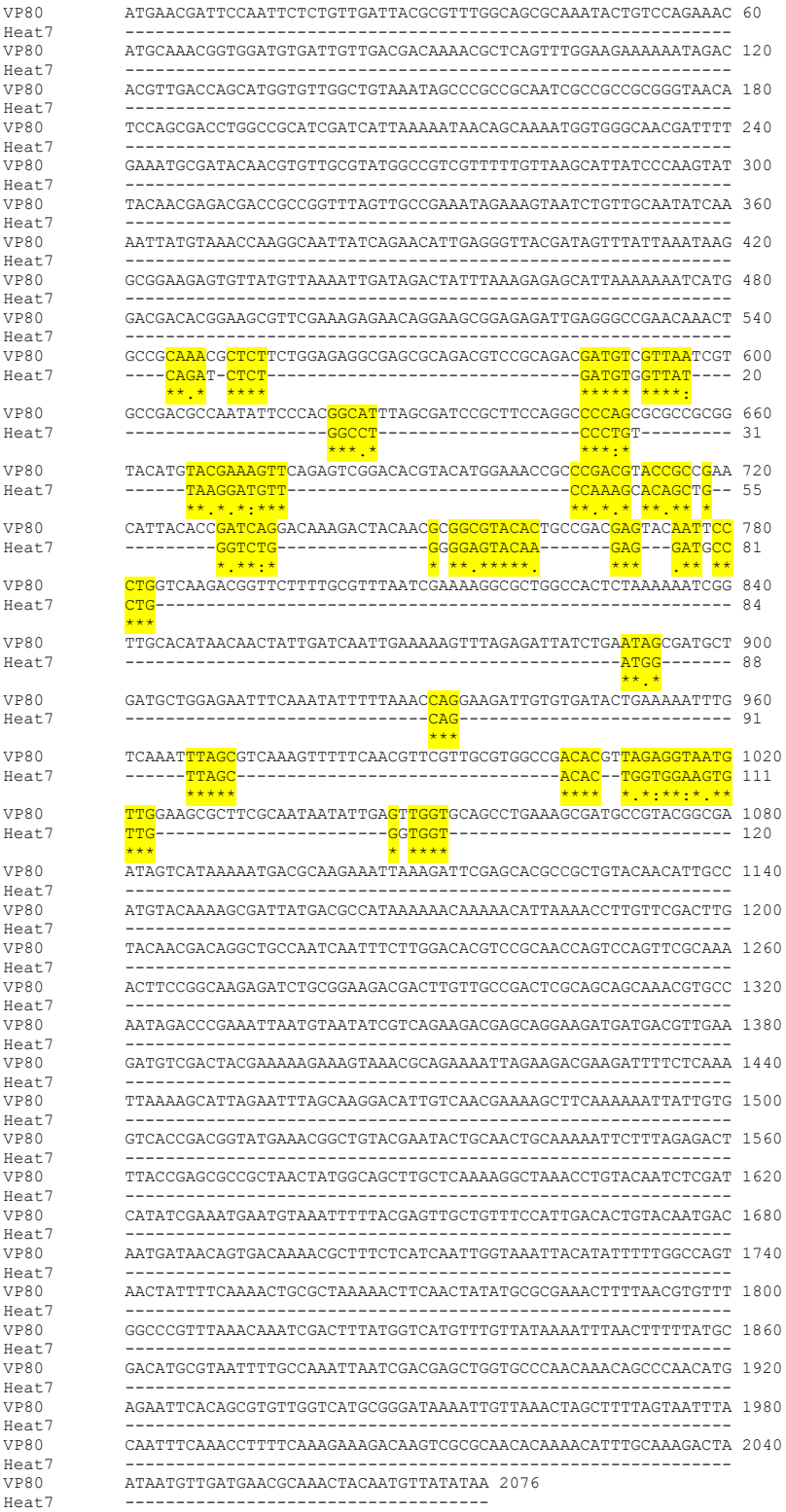
Sequence alignment of VP80 and HEAT4 domain



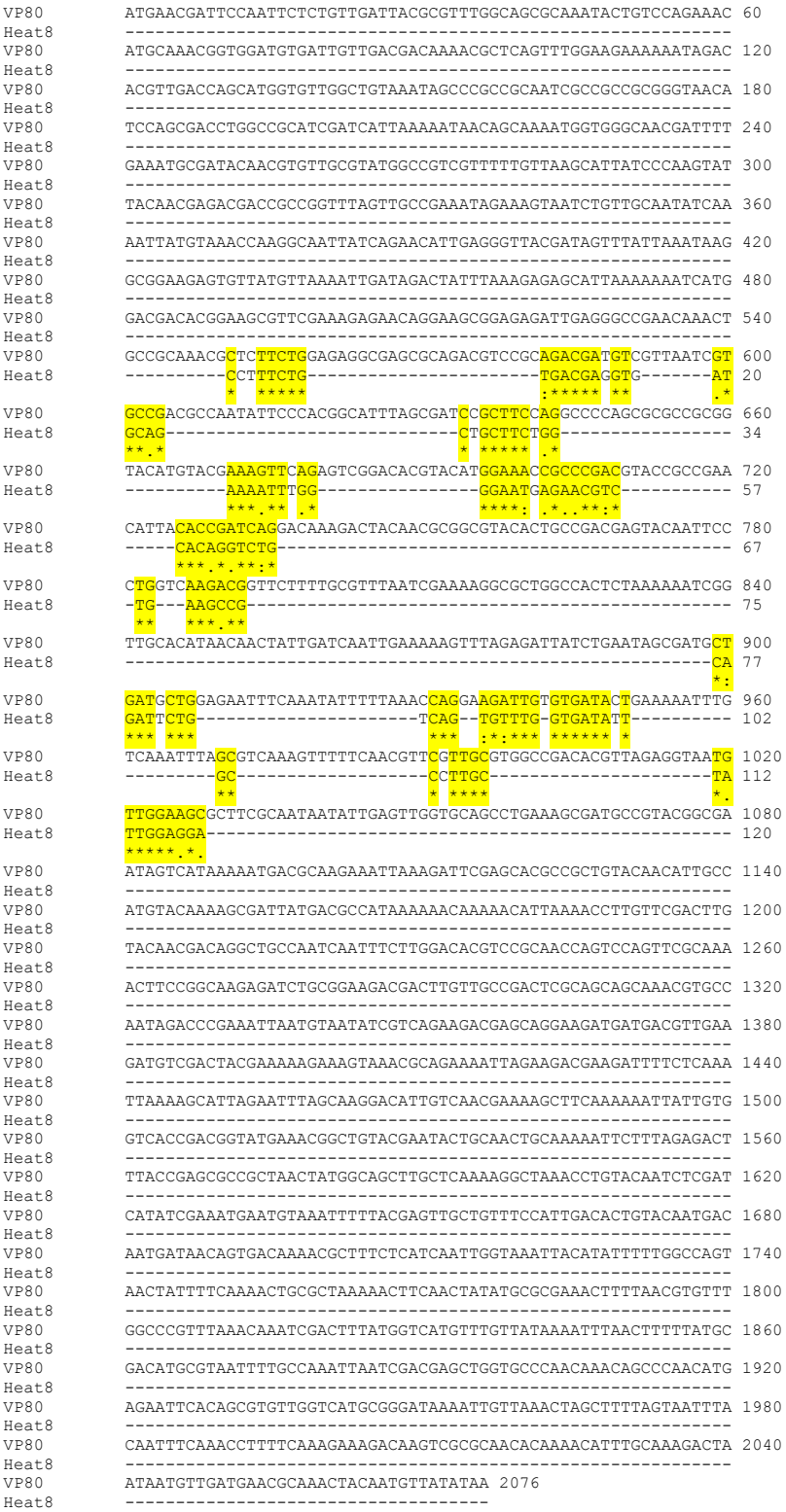
Sequence alignment of VP80 and HEAT6 domain



Sequence alignment of VP80 and HEAT7 domain



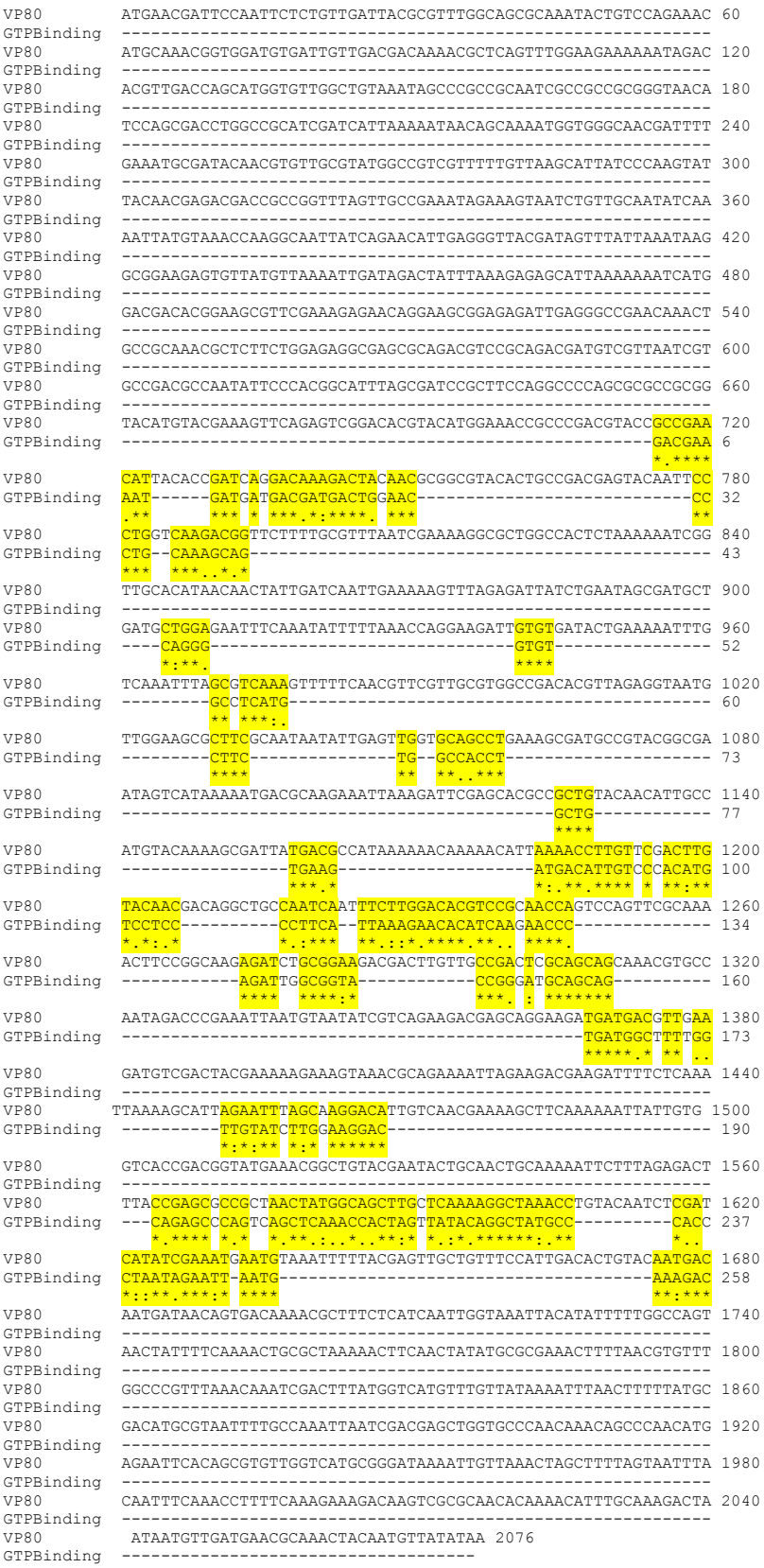
Sequence alignment of VP80 and HEAT8 domain



Sequence alignment of VP80 and importin-α binding domain

```
VP80        ATGAACGATTCCAATTCTCTGTTGATTACGCGTTTGGCAGCGCAAATACTGTCCAGAAAC 60
Importin-α  ------------------------------------------------------------
VP80        ATGCAAACGGTGGATGTGATTGTTGACGACAAAACGCTCAGTTTGGAAGAAAAAATAGAC 120
Importin-α  ------------------------------------------------------------
VP80        ACGTTGACCAGCATGGTGTTGGCTGTAAATAGCCCGCCGCAATCGCCGCCGCGGGTAACA 180
Importin-α  ------------------------------------------------------------
VP80        TCCAGCGACCTGGCCGCATCGATCATTAAAAATAACAGCAAAATGGTGGGCAACGATTTT 240
Importin-α  ------------------------------------------------------------
VP80        GAAATGCGATACAACGTGTTGCGTATGGCCGTCGTTTTTGTTAAGCATTATCCCAAGTAT 300
Importin-α  ------------------------------------------------------------
VP80        TACAACGAGACGACCGCCGGTTTAGTTGCCGAAATAGAAAGTAATCTGTTGCAATATCAA 360
Importin-α  ------------------------------------------------------------
VP80        AATTATGTAAACCAAGGCAATTATCAGAACATTGAGGGTTACGATAGTTTATTAAATAAG 420
Importin-α  ------------------------------------------------------------
VP80        GCGGAAGAGTGTTATGTTAAAATTGATAGACTATTTAAAGAGAGCATTAAAAAAATCATG 480
Importin-α  ------------------------------------------------------------
VP80        GACGACACGGAAGCGTTCGAAAGAGAACAGGAAGCGGAGAGATTGAGGGCCGAACAAACT 540
Importin-α  ------------------------------------------------------------
VP80        GCCGCAAACGCTCTTCTGGAGAGGCGAGCGCAGACGTCCGCAGACGATGTCGTTAATCGT 600
Importin-α  ------------------------------------------------------------
VP80        GCCGACGCCAATATTCCCACGGCATTTAGCGATCCGCTTCCAGGCCCCAGCGCGCCGCGG 660
Importin-α  ------------------------------------------------------------
VP80        TACATGTACGAAAGTTCAGAGTCGGACACGTACATGGAAACCGCCCGACGTACCGCCGAA 720
Importin-α  ------------------------------------------------------------
VP80        CATTACACCGATCAGGACAAAGACTACAACGCGGCGTACACTGCCGACGAGTACAATTCC 780
Importin-α  ------------------------------------------------------------
VP80        CTGGTCAAGACGGTTCTTTTGCGTTTAATCGAAAAGGCGCTGGCCACTCTAAAAAATCGG 840
Importin-α  ------------------------------------------------------------
VP80        TTGCACATAACAACTATTGATCAATTGAAAAAGTTTAGAGATTATCTGAATAGCGATGCT 900
Importin-α  ------------------------------------------------------------
VP80        GATGCTGGAGAATTTCAAATATTTTTAAACCAGGAAGATTGTGTGATACTGAAAAATTTG 960
Importin-α  ------------------------------------------------------------
VP80        TCAAATTTAGCGTCAAAGTTTTTCAACGTTCGTTGCGTGGCCGACACGTTAGAGGTAATG 1020
Importin-α  ------------------------------------------------------------
VP80        TTGGAAGCGCTTCGCAATAATATTGAGTTGGTGCAGCCTGAAAGCGATGCCGTACGGCGA 1080
Importin-α  ------------------------------------------------------------
VP80        ATAGTCATAAAAATGACGCAAGAAATTAAAGATTCGAGCACGCCGCTGTACAACATTGCC 1140
Importin-α  ------------------------------------------------------------
VP80        ATGTACAAAAGCGATTATGACGCCATAAAAAACAAAAACATTAAAACCTTGTTCGACTTG 1200
Importin-α  ------------------------------------------------------------
VP80        TACAACGACAGGCTGCCAATCAATTTCTTGGACACGTCCGCAACCAGTCCAGTTCGCAAA 1260
Importin-α  ------------------------------------------------------------
VP80        ACTTCCGGCAAGAGATCTGCGGAAGACGACTTGTTGCCGACTCGCAGCAGCAAACGTGCC 1320
Importin-α  ------------------------------------------------------------
VP80        AATAGACCCGAAATTAATGTAATATCGTCAGAAGACGAGCAGGAAGATGATGACGTTGAA 1380
Importin-α  -----ACACTAACTAAA-----------CAG--GACGAAAA---TGATGATGACGAT--- 36
                 **.*  **.*:**           ***  *****..*   :**********:*
VP80        GATGTCGACTACGAAAAAGAAAGTAAACGCAGAAAATTAGAAGACGAAGATTTTCTCAAA 1440
Importin-α  ------GACTG-G---------------------------------------------- 42
                   ****. *
VP80        TTAAAAGCATTAGAATTTAGCAAGGACATTGTCAACGAAAAGCTTCAAAAAATTATTGTG 1500
Importin-α  ------------------------------------------------------------
VP80        GTCACCGACGGTATGAAACGGCTGTACGAATACTGCAACTGCAAAAATTCTTTAGAGACT 1560
Importin-α  ------------------------------------------------------------
VP80        TTACCGAGCGCCGCTAACTATGGCAGCTTGCTCAAAAGGCTAAACCTGTACAATCTCGAT 1620
Importin-α  ------------------------------------------------------------
VP80        CATATCGAAATGAATGTAAATTTTTACGAGTTGCTGTTTCCATTGACACTGTACAATGAC 1680
Importin-α  ------------------------------------------------------------
VP80        AATGATAACAGTGACAAAACGCTTTCTCATCAATTGGTAAATTACATATTTTTGGCCAGT 1740
Importin-α  ------------------------------------------------------------
VP80        AACTATTTTCAAAACTGCGCTAAAAACTTCAACTATATGCGCGAAACTTTTAACGTGTTT 1800
Importin-α  ------------------------------------------------------------
VP80        GGCCCGTTTAAACAAATCGACTTTATGGTCATGTTTGTTATAAAATTTAACTTTTTATGC 1860
Importin-α  ------------------------------------------------------------
VP80        GACATGCGTAATTTTGCCAAATTAATCGACGAGCTGGTGCCCAACAAACAGCCCAACATG 1920
Importin-α  ------------------------------------------------------------
VP80        AGAATTCACAGCGTGTTGGTCATGCGGGATAAAATTGTTAAACTAGCTTTTAGTAATTTA 1980
Importin-α  ------------------------------------------------------------
VP80        CAATTTCAAACCTTTTCAAAGAAAGACAAGTCGCGCAACACAAAACATTTGCAAAGACTA 2040
Importin-α  ------------------------------------------------------------
VP80        ATAATGTTGATGAACGCAAACTACAATGTTATATAA 2076
Importin-α  -----------------------------------
```

Sequence alignment of VP80 and RanGTP binding domain



Sequence alignment of VP1054 and HEAT1 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat1       ---------AACCAGTGGCC---------AGAACTCATT-CCTCAG-------------- 27
                     *.*.**:..**         ***.***: *  ****.*

VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat1       ------CTGGTG-----GCCAATGTC---------------------------------- 42
                  * *.**      ***.*:* *

VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat1       ------------------------------ACAAACCCC---------------------- 51
                                           :**.*  ***

VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat1       ---------------AACAGC---------ACAGAGCACATGAAG---------------- 72
                           ******          *..******.  ***.

VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat1       ----------------------GAG----------------------TCGACA-------- 81
                                   ***                      *****

VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat1       ------------------------------------TTGGAAGC---------------- 89
                                                 **.*****

VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat1       ------------------------------CATCGGT------------------------ 96
                                           *****.*

VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat1       ---------------TATATTT---------------------------------------- 103
                           **  ****

VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat1       ---------GCCAAGATATAGACCCA---------------------------------- 120
                     ****:  **:*:*.*  **

VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat1       ------------------------------------------------------------

VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat1       ------------------------------------------------------------

VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat1       ------------------------------------------------------------

VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat1       ------------------------------------------------------------

VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat1       ------------------------------------------------------------

VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat1       ------------------------------------------------------------

VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat1       ------------------------------------------------------------

VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat1       ------------------------------------------------------------

VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat1       ------------------------------------------------------------

VP1054      CACGCACACAACGTGTAG 1098
Heat1       ------------------
```

Sequence alignment of VP1054 and HEAT2 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat2       ------------------------------------------------------------
VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat2       ------------------------------------------------------------
VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat2       ------------------------------------------------------------
VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat2       ------------------------------------------------------------
VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat2       ------------------------------------------------------------
VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat2       ------------------------------------------------------------
VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat2       ----------------------------------GAT---------AAATCCAATG----- 13
                                               ***         **.*.**:**

VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat2       -------------AG-----------------------------------------ATTCT 20
                         **                                         ****

VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat2       GACT-----GCCATAAT----CC--------------------AGGGGAT---GAGGAA 47
            **.*     *****:**    **                    ***.***    *..***

VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat2       AG------------------AAGAG---------------------------CCTAG-TAA 62
            **                 ***:*                           ***:* :**

VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat2       TAATGTGAAGCTAGCTG-----------CTACG---------------------------- 84
            *:*: *...** *.*:.           *****

VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat2       ----------------------AATGCACTCCTGAAC------------------------- 99
                                   ***.** ** ***.*

VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat2       ----------------------------------------TCA-----TTGG-------- 106
                                                   ***     ****

VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat2       ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat2       ----------------------AGTTCACCAAAGCA------------------------ 120
                                   **:********..*

VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat2       ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat2       ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat2       ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Heat2       ------------------
```

Sequence alignment of VP1054 and HEAT3 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat3       ----------------------------------TCTG--------------AAAGGCAC 12
                                               ****              :** **.*

VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat3       TTTATT--------------------------ATGCAG----------------GTGGTC 30
            **  *::                          ****:*                * *.:

VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat3       TGTGAAG--------CCAC---ACAGT-------GTCCAGATA------------------ 55
            **  .***        ****   ***.*       .***.****

VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat3       ------------------------------------------------------------

VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat3       --------------------CGAGG--------------------------------GTAC 64
                                *****                                ****

VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat3       GAGT----------------------------------------------------GGCTG-- 73
            ****                                                    *****

VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat3       ----------------CTTTACAG---------------------------------AAT 84
                            ****.**                                    **:

VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat3       CTGG---------------TGAAGATA-------------ATGTC--------------- 101
            ****               *  ::****            *****

VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat3       --------------------------------CTTATATTATCAG---------------- 114
                                             * ** *******.

VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat3       ------------------------------------------------------------

VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat3       ------------------------------------------------------------

VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat3       ------------------------------------------------------------

VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat3       ------------------------------------------------------------

VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat3       ------------------------------------------------------------

VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat3       ------------------------------------------------------------

VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat3       ------------------------------------------------------------

VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat3       ------------------------------------------------------------

VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat3       ------------------------------------------------------------

VP1054      CACGCACACAACGTGTAG 1098
Heat3       ------------------
```

Sequence alignment of VP1054 and HEAT4 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat4       ----------------------------GGAGCACT-----ACAGT------------- 13
                                        .**  *:**     :*.**
VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat4       ----ATCTGGT-------TCCAA---------------------TCCTC----------- 30
                *:*  *.*         ****                    *****
VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat4       ---------ACACAGACAC-TAAC---TAAACAGG--------ACGAAAATG--ATGATG 67
                     **.*. .***  ****   **::***.        *******  *  ***:**
VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat4       ACGATGACTG-------------------GAAC---------------------------- 81
            ..  *****  *                 ****
VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat4       ------------------CCCTG-----------------CAAAGCAG-CAGGG----- 99
                             **  :*                 ***.**.*  ***  *
VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat4       GTGTGCCT--------------------------CAT------------------GCT--- 113
            *:****.*                         ***                  ***
VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat4       ------------------------------------------------------------T 114
                                                                         :
VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat4       CTGGCC---------------------------ACC---------------------------- 123
            ******                           ***
VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat4       ------------------------------------------------------------
VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat4       ------------------------------------------------------------
VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat4       ------------------------------------------------------------
VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat4       ------------------------------------------------------------
VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat4       ------------------------------------------------------------
VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat4       ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat4       ------------------------------------------------------------
VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat4       ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat4       ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat4       ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Heat4       ------------------
```

Sequence alignment of VP1054 and HEAT6 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat6       ------------------CC--ACTAGTTATACA----------GG-------------- 16
                             **  :*:***** **.          **

VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat6       -------CTATGC--CCACCCTAATAGAATTAATG------------------------- 42
                  * **** **.***:*. .**:  ****

VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat6       ---AAAG----------------------------ACCCC---------------------- 51
               ****                            * ***

VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat6       --------------------AGTGTAGTTGTTCGAG------------------------- 67
                               .****: ***::****

VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat6       -------------------------------------AT-----------ACAGC------ 74
                                                  **           *****

VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat6       ---TGCATG---------------------------------------------GACTG-- 85
               ******                                              *.***

VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat6       ------------------------------------------------------TAGGCAGA 93
                                                                   *:****.*

VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat6       AT-----------------------TTGTGAGCTGCTT---------------------- 108
            .*                        *:* *..* **:*

VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat6       ----------------------CCTGAA---------------------------------- 114
                                   *** **

VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat6       ------------------------------------------------------------
VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat6       ------------------------------------------------------------
VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat6       ------------------------------------------------------------
VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat6       ------------------------------------------------------------
VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat6       ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat6       ------------------------------------------------------------
VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat6       ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat6       ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat6       ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Heat6       ------------------
```

Sequence alignment of VP1054 and HEAT7 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat7       ----------------------------CAGATCTCTG---------------------- 10
                                        *** *****
VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat7       ---------ATG------------------------TGGTTATGGCCTC----------- 26
                     ***                        ***:** . ****
VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat7       ------------------------------------CCTGTTAAGG--------ATGTTC 42
                                               ** *:*** *        *****
VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat7       CAA--------------------------------AGCAC---------------------- 50
            **.                                *****
VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat7       -----------------AGCTGGGTCTGG----------------------GGGAGTAC 70
                             *** *.* * .*                      * *:****
VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat7       AAGAGGATG---------CC-----------------------------------CTG-- 84
            .**:* ***         **                                   ***
VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat7       ---------ATG-------------------------GCAG------------TTAGCACA 99
                     ***                        **.*            **.***.*
VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat7       CTGG-------------------TGGAAGTG------------------------------ 111
            ****                   **.:** *
VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat7       -----TTGGG---------------------------------------------------- 116
                  *:***
VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat7       -----TGGT---------------------------------------------------- 120
                  *.**
VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat7       ------------------------------------------------------------
VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat7       ------------------------------------------------------------
VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat7       ------------------------------------------------------------
VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat7       ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat7       ------------------------------------------------------------
VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat7       ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat7       ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat7       ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Heat7       ------------------
```

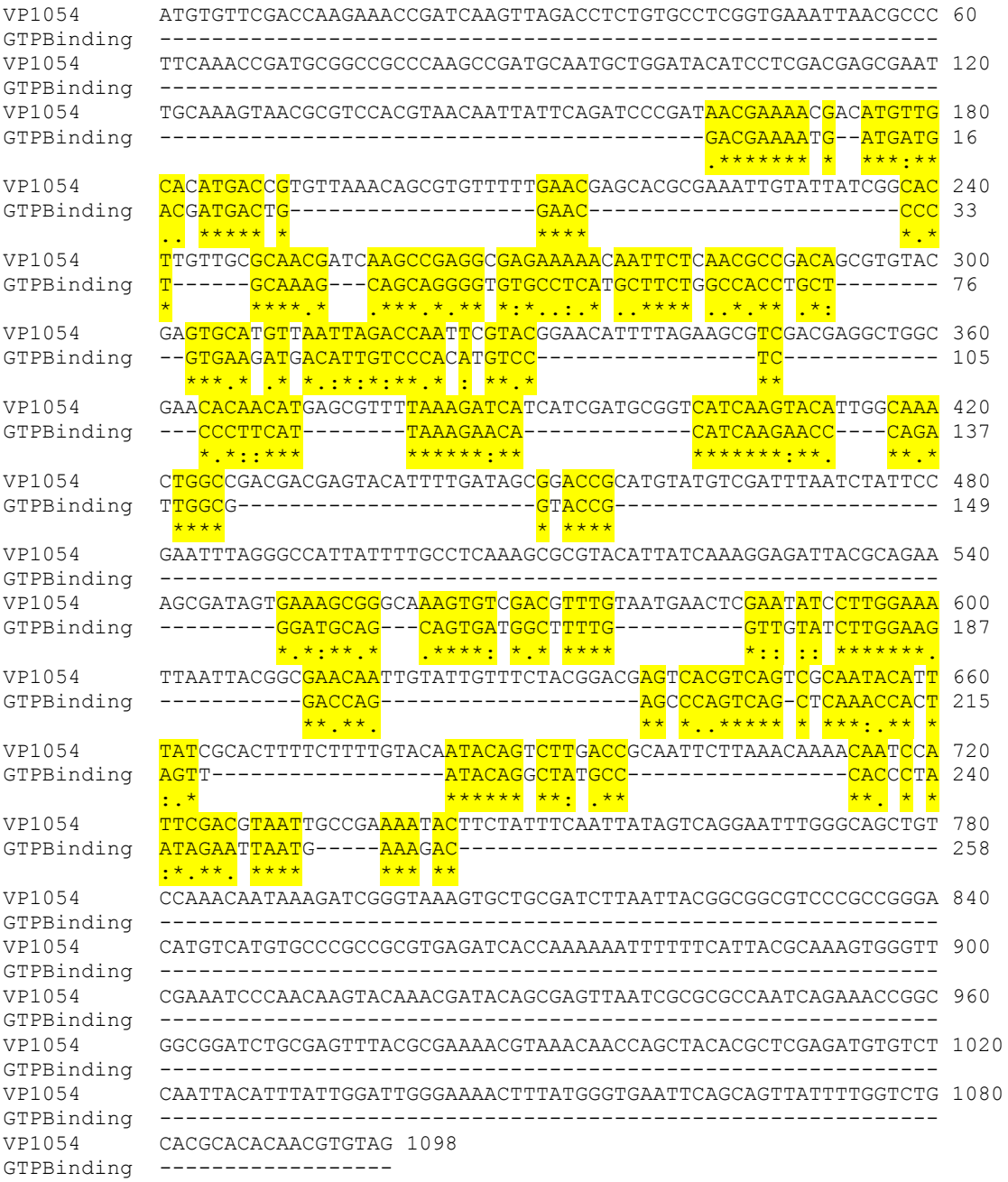
Sequence alignment of VP1054 and HEAT8 domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Heat8       ----------CCTT--------------------TCTGTGACGAGGT------------- 17
                      **::                    ******.* .***
VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Heat8       --------GATGCAGCTGC--------------TTCTGGAAA---------------ATT 40
                    *****.**  **              * *****:*               *:*
VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Heat8       TG-------------------------------------GGGAATGAGAACGTC------ 57
            **                                     *. ** **.****:*
VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Heat8       CACAGGTCTGTG---------------------------------------------- 69
            **** *:* ***
VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Heat8       ----------------AAGCCGCAG-----------ATTCT-------GTCAGTGT---- 91
                            ******..*           *****       *:***  **
VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Heat8       ------------------------------------------------------------
VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Heat8       ----------------------------------------------------TTGGTGAT 99
                                                               **** .*:
VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Heat8       ATTGCC-------------------------------CTTG--------------CTATT-- 114
            .* ***                               *:**              *****
VP1054      GAATTTAGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Heat8       --------GG-----------------------------------AGGA------------ 120
                    **                                   ****
VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Heat8       ------------------------------------------------------------
VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Heat8       ------------------------------------------------------------
VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Heat8       ------------------------------------------------------------
VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Heat8       ------------------------------------------------------------
VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Heat8       ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Heat8       ------------------------------------------------------------
VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Heat8       ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Heat8       ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Heat8       ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Heat8       ------------------
```

Sequence alignment of VP1054 and importin-α binding domain

```
VP1054      ATGTGTTCGACCAAGAAACCGATCAAGTTAGACCTCTGTGCCTCGGTGAAATTAACGCCC 60
Importin-α  ------------------------------------------------------------
VP1054      TTCAAACCGATGCGGCCGCCCAAGCCGATGCAATGCTGGATACATCCTCGACGAGCGAAT 120
Importin-α  ------------------------------------------------------------
VP1054      TGCAAAGTAACGCGTCCACGTAACAATTATTCAGATCCCGATAACGAAAACGACATGTTG 180
Importin-α  ---------------ACAC-TAAC----------------------------------- 8
                           .***  ****
VP1054      CACATGACCGTGTTAAACAGCGTGTTTTTGAACGAGCACGCGAAATTGTATTATCGGCAC 240
Importin-α  -------------TAAACAG-------------GA-----CGAAAATG------------ 25
                        *******              **     *****:**
VP1054      TTGTTGCGCAACGATCAAGCCGAGGCGAGAAAAACAATTCTCAACGCCGACAGCGTGTAC 300
Importin-α  ------------------------------------------------------------
VP1054      GAGTGCATGTTAATTAGACCAATTCGTACGGAACATTTTAGAAGCGTCGACGAGGCTGGC 360
Importin-α  ------ATG------------------------------------------------- 28
                  ***
VP1054      GAACACAACATGAGCGTTTTAAAGATCATCATCGATGCGGTCATCAAGTACATTGGCAAA 420
Importin-α  ---------ATGA-------------------CGATG----------------------A 38
                     ****                   *****                      *
VP1054      CTGGCCGACGACGAGTACATTTTGATAGCGGACCGCATGTATGTCGATTTAATCTATTCC 480
Importin-α  CTGG-------------------------------------------------------- 42
            ****
VP1054      GAATTTAGGGCCATTATTTTGCCTCAAAGCGCGTACATTATCAAAGGAGATTACGCAGAA 540
Importin-α  ------------------------------------------------------------
VP1054      AGCGATAGTGAAAGCGGGCAAAGTGTCGACGTTTGTAATGAACTCGAATATCCTTGGAAA 600
Importin-α  ------------------------------------------------------------
VP1054      TTAATTACGGCGAACAATTGTATTGTTTCTACGGACGAGTCACGTCAGTCGCAATACATT 660
Importin-α  ------------------------------------------------------------
VP1054      TATCGCACTTTTCTTTTGTACAATACAGTCTTGACCGCAATTCTTAAACAAAACAATCCA 720
Importin-α  ------------------------------------------------------------
VP1054      TTCGACGTAATTGCCGAAAATACTTCTATTTCAATTATAGTCAGGAATTTGGGCAGCTGT 780
Importin-α  ------------------------------------------------------------
VP1054      CCAAACAATAAAGATCGGGTAAAGTGCTGCGATCTTAATTACGGCGGCGTCCCGCCGGGA 840
Importin-α  ------------------------------------------------------------
VP1054      CATGTCATGTGCCCGCCGCGTGAGATCACCAAAAAATTTTTTCATTACGCAAAGTGGGTT 900
Importin-α  ------------------------------------------------------------
VP1054      CGAAATCCCAACAAGTACAAACGATACAGCGAGTTAATCGCGCGCCAATCAGAAACCGGC 960
Importin-α  ------------------------------------------------------------
VP1054      GGCGGATCTGCGAGTTTACGCGAAAACGTAAACAACCAGCTACACGCTCGAGATGTGTCT 1020
Importin-α  ------------------------------------------------------------
VP1054      CAATTACATTTATTGGATTGGGAAAACTTTATGGGTGAATTCAGCAGTTATTTTGGTCTG 1080
Importin-α  ------------------------------------------------------------
VP1054      CACGCACACAACGTGTAG 1098
Importin-α  ------------------
```

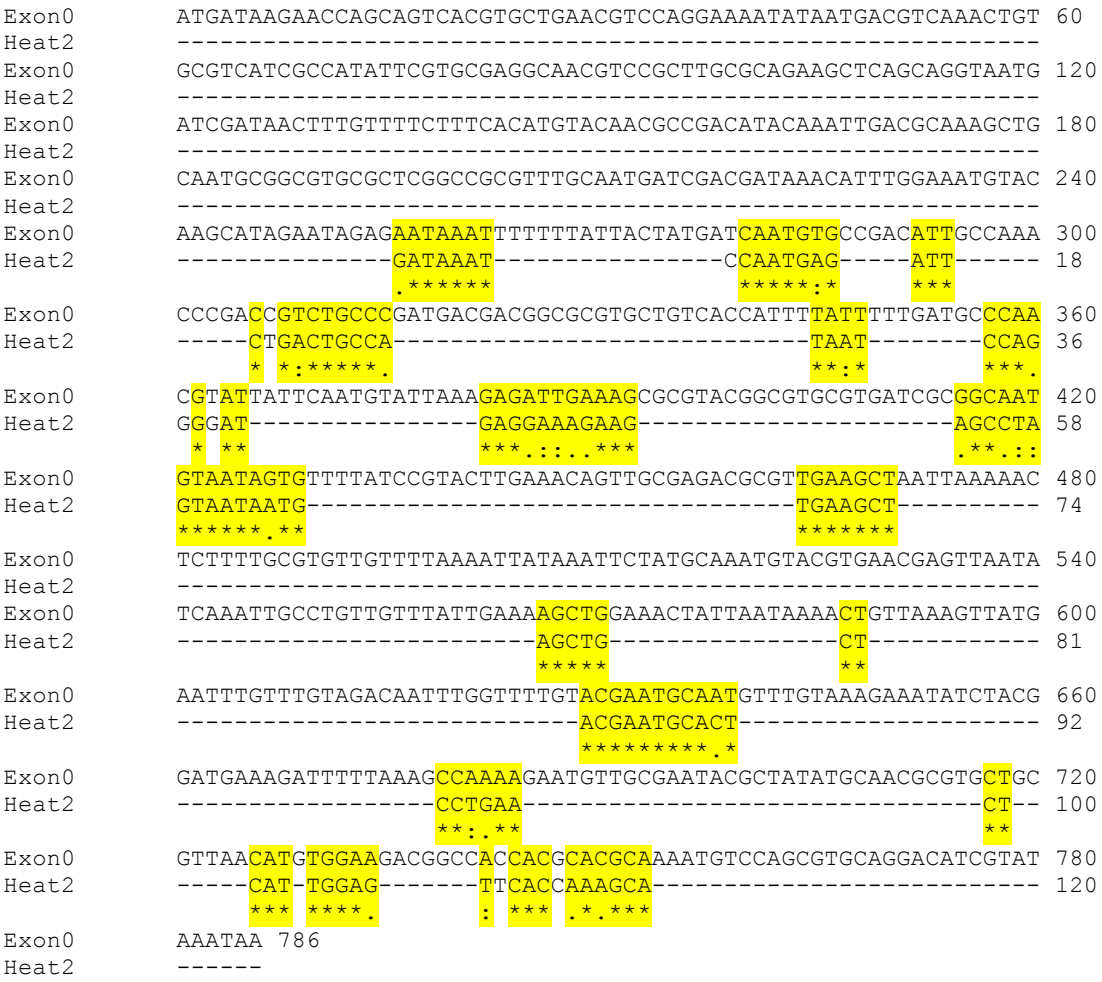
Sequence alignment of VP1054 and RanGTP binding domain



Sequence alignment of Exon0 and HEAT1 domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Heat1       --------AACCAGTGG---------------CCAG------------------AACT-- 17
                    ******.*                ****                  ****

Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Heat1       ------------CATTC-----------------------------CTCAGCTGG---TG 33
                        ****                             ******:**    **

Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Heat1       GCCAATG-------------TCACA-----AACCCCAACAG---------CACAGAGCA- 65
            . *.**.             *****     ***  **.***          *.**.***:

Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Heat1       ------------------------------------------------------------

Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Heat1       ---CATG-----------------------------------AAGGAGTCGACATTG----- 84
               ***.                                   **  *:*  ********

Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Heat1       ---------------GAAGCCATCGG-----------------TTATATTTG----CCAA 108
                           **:*.*.:***                 ****:****    ****

Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Heat1       GATATAGACCCA------------------------------------------------- 120
             .***:.: *.*

Exon0       GTAATAGTGTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Heat1       ------------------------------------------------------------

Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Heat1       ------------------------------------------------------------

Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Heat1       ------------------------------------------------------------

Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Heat1       ------------------------------------------------------------

Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Heat1       ------------------------------------------------------------

Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Heat1       ------------------------------------------------------------

Exon0       AAATAA 786
Heat1       ------
```

Sequence alignment of Exon0 and HEAT2 domain



Sequence alignment of Exon0 and HEAT3 domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Heat3       ------------------------------------------------------------
Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Heat3       ------------------------------------------------------------
Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Heat3       ----------------------------------------------------------TCTG 4
                                                                     ***
Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Heat3       AAAGGC--------------------------------------ACTTT----ATTATGC 22
            .**  **                                     **:**    *:*.*.*
Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Heat3       AGG---------------------------------TGGTCTGTG-------------AAG 37
            *.*                                 **.**:.**               **.
Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Heat3       CCACACAGTGT-CCAGAT-ACGAGG---------------------------------- 60
            **.  **.**  *  **.***  ****  *
Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Heat3       ------------------------------------GTACG--------------------- 65
                                                *****
Exon0       GTAATAGTGTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Heat3       -----AGTG---------------------GCTGC-----------------TTTACAGAA 83
                 ****                     * ***                  :***.*.*.
Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Heat3       TCTGGTG---------------------------AAGATAATGTCCTT-------ATATTA 110
            ***   **                          *:*.:*****.* *       :**:**
Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Heat3       TCAG--------------------------------------------------------- 114
            ***.
Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Heat3       ------------------------------------------------------------
Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Heat3       ------------------------------------------------------------
Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Heat3       ------------------------------------------------------------
Exon0       AAATAA 786
Heat3       ------
```

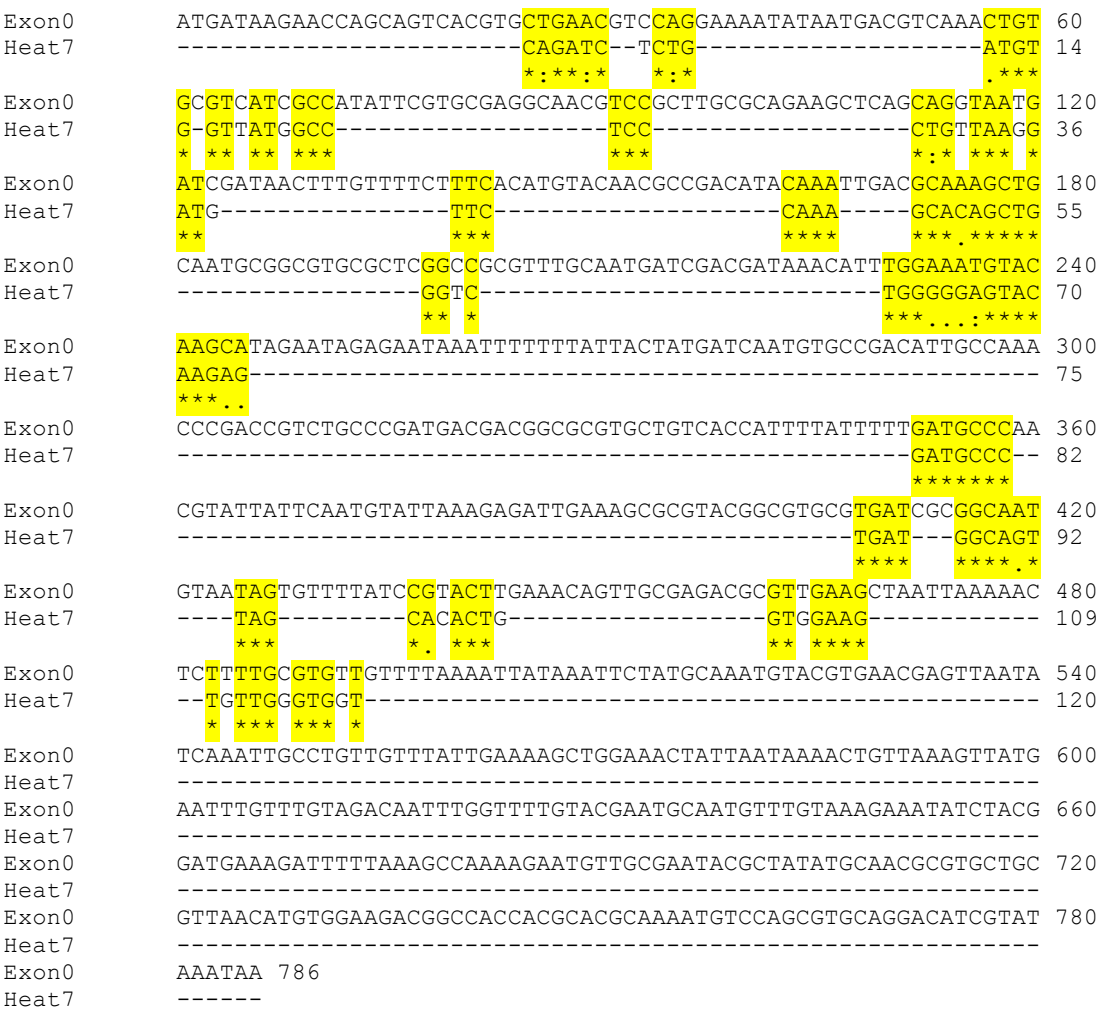
Sequence alignment of Exon0 and HEAT4 domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Heat4       ------------------------------------------------------------
Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Heat4       ----------------------------------------GGAGCACTACAG------ 12
                                                    *.***:*:.***
Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Heat4       --------------TATCTG------GTTCCAATCC-----TCACACAGACACTAA---- 43
                          *:***       **:*.*. **     :**.* :***.*:**
Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Heat4       CTAAACAG----------------------------GACGAAAATGAT------------ 63
            *:*:.*.*                           *****:**: **
Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Heat4       ------------------------------------------------------------
Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Heat4       ---------------GATGACGATG------ACTG----------------GAACCCCTG 86
                           ******** *      .***                **: ***:.
Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Heat4       C---------------------------AAAGC--------------------------- 92
            *                           *****
Exon0       GTAATAGTGTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Heat4       -------------------------AGCAG------------------------------ 97
                                     *.***
Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Heat4       ------GGGTG---------------------------------------------------- 102
                  * ***
Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Heat4       ------TGCCT---------------------------------------------------- 107
                  *****
Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Heat4       ------------------------------------------------------------
Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Heat4       ----------------------------------------------------CATGCTTC 115
                                                               *.**** *
Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Heat4       ----------------TGGCCACC------------------------------------ 123
                            *******
Exon0       AAATAA 786
Heat4       ------
```

Sequence alignment of Exon0 and HEAT6 domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Heat6       ------------------------------------------------------------
Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Heat6       ------------------------------------------------------------
Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Heat6       ------------------------------------------------------------
Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Heat6       -------------------CCAC-------------------------TAG---TTATAC 13
                               **.*                         *:*   :*.***
Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Heat6       AG---------------------------------------------------------- 15
            *.
Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Heat6       --------------------------------GCT------------------ATGCCCAC 26
                                           ***                   *******.
Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Heat6       CCTAATA------GAATTAATGA-----AAGACCC---------------------CAGT 54
            *  **:**      *:*****:**      **..* *                     **.*
Exon0       GTAATAGTGTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Heat6       GTAGTTGT-----------TCGAGATACAGCTGC--------------------------- 77
            ***.*:**           :* :**:**** ***
Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Heat6       ------------------------------------------------------------
Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Heat6       ----ATGGACTGTAG-----------GCAG------------------------------ 92
                ** *.****:*           **:*
Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Heat6       ---------------AATTTG------------TGAGCTGCTT----------------- 108
                           ******            **...** **
Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Heat6       ------------------CCTGAA------------------------------------ 114
                             **:.**
Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Heat6       ------------------------------------------------------------
Exon0       AAATAA 786
Heat6       ------
```

Sequence alignment of Exon0 and HEAT7 domain



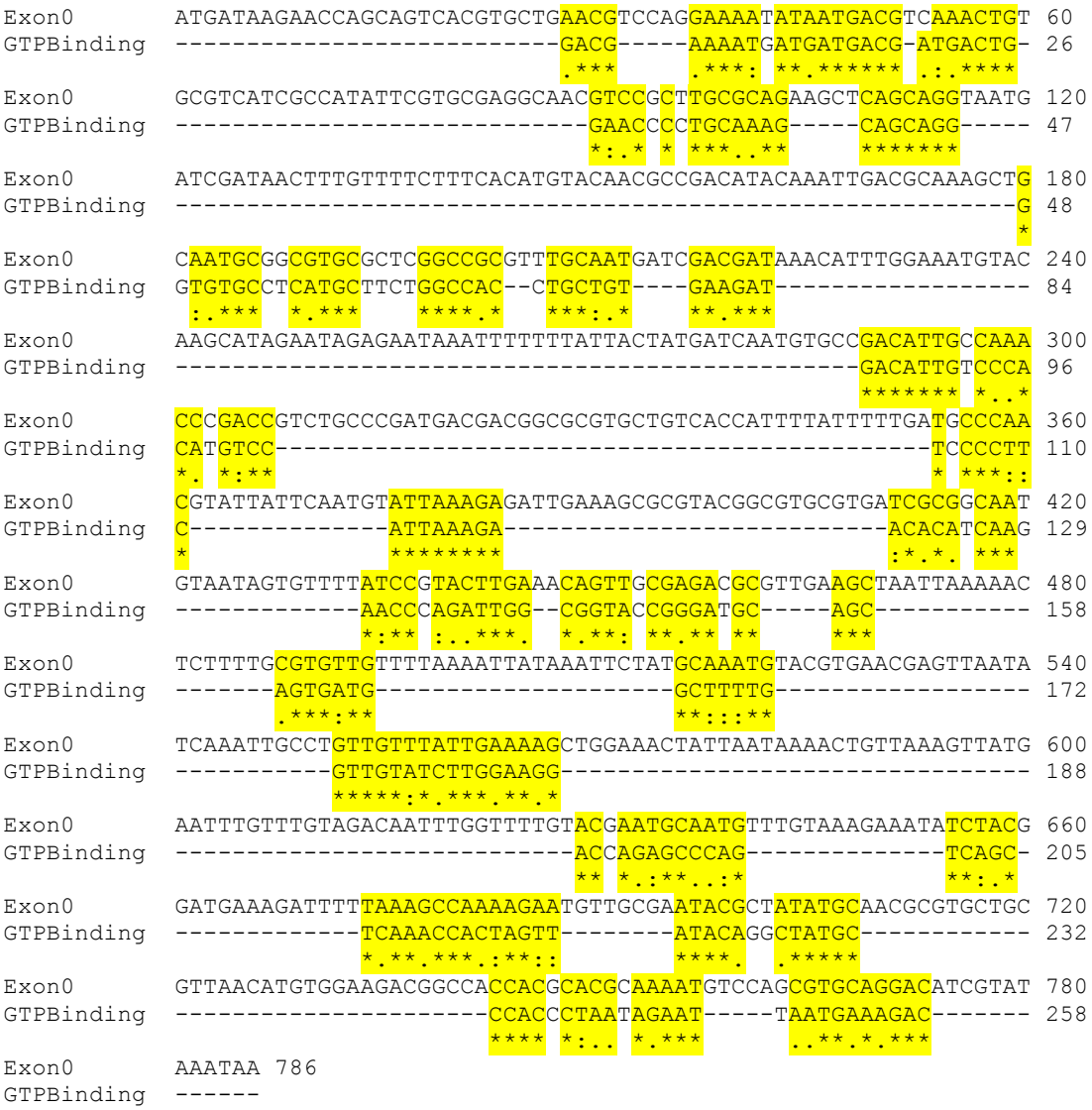
Sequence alignment of Exon0 and HEAT8 domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Heat8       ----------------------------------------------------CCTTTCTGT 9
                                                               *:::****
Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Heat8       GA------------------CGAGGTGATG------------------CAGCTG------ 27
            *.                  *****  .*  *                 ****:*
Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Heat8       --------CTT---------------------------CTGGAAAATT------------ 40
                    ***                           *:  ..*****
Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Heat8       ---TGGGGAATGAGAACGTCCAC------------------------------------- 60
               **  **..**.*.:**  **.*
Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Heat8       -AG---------------------------------------------------------- 62
             **
Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Heat8       -------GTCTG-----TGAAGCCG-CAGATTCTGTCA----------------------- 87
                   *****      ***.*.**  *. .*  ******
Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Heat8       ----------------------GTGTTTG---------------------------------- 94
                                  *:*:***
Exon0       GTAATAGTGTTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Heat8       ------GTGATATTGCC---CTTG---CTATTGGAGGA---------------------- 120
                  ***:*:*:  **    ****    *:.***  ..**
Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Heat8       ------------------------------------------------------------
Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Heat8       ------------------------------------------------------------
Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Heat8       ------------------------------------------------------------
Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Heat8       ------------------------------------------------------------
Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Heat8       ------------------------------------------------------------
Exon0       AAATAA 786
Heat8       ------
```

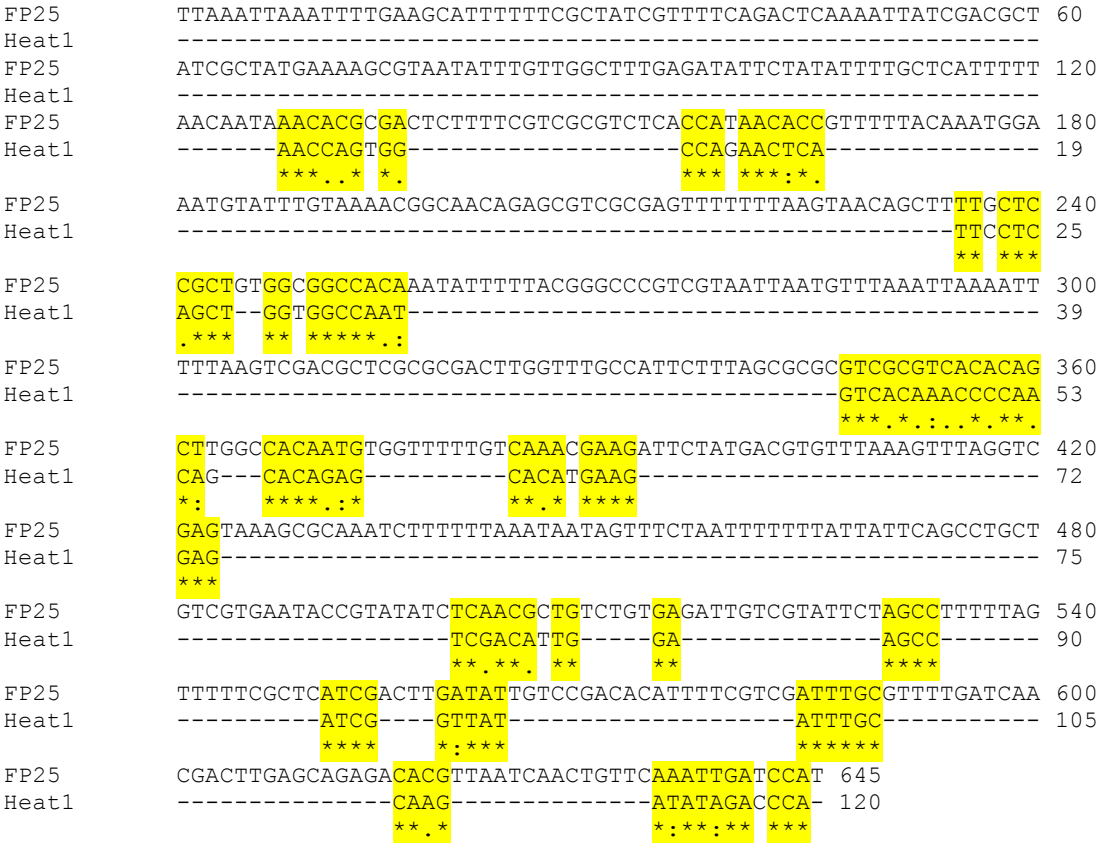
Sequence alignment of Exon0 and importin-α binding domain

```
Exon0       ATGATAAGAACCAGCAGTCACGTGCTGAACGTCCAGGAAAATATAATGACGTCAAACTGT 60
Importin-α  ------------------------------------------------------------
Exon0       GCGTCATCGCCATATTCGTGCGAGGCAACGTCCGCTTGCGCAGAAGCTCAGCAGGTAATG 120
Importin-α  --------------------------------ACACT--------AACTAAACAG------ 15
                                             .*.**        *.**.*.***
Exon0       ATCGATAACTTTGTTTTCTTTCACATGTACAACGCCGACATACAAATTGACGCAAAGCTG 180
Importin-α  ------------------------------------------------GACGAAAA---- 23
                                                             ****.***
Exon0       CAATGCGGCGTGCGCTCGGCCGCGTTTGCAATGATCGACGATAAACATTTGGAAATGTAC 240
Importin-α  ---TG--------------------------ATGAT-GACGATG-AC---TGG-------- 42
               **                           *****  ******. **    ***
Exon0       AAGCATAGAATAGAGAATAAATTTTTTTATTACTATGATCAATGTGCCGACATTGCCAAA 300
Importin-α  ------------------------------------------------------------
Exon0       CCCGACCGTCTGCCCGATGACGACGGCGCGTGCTGTCACCATTTTATTTTTGATGCCCAA 360
Importin-α  ------------------------------------------------------------
Exon0       CGTATTATTCAATGTATTAAAGAGATTGAAAGCGCGTACGGCGTGCGTGATCGCGGCAAT 420
Importin-α  ------------------------------------------------------------
Exon0       GTAATAGTGTTTTATCCGTACTTGAAACAGTTGCGAGACGCGTTGAAGCTAATTAAAAAC 480
Importin-α  ------------------------------------------------------------
Exon0       TCTTTTGCGTGTTGTTTTAAAATTATAAATTCTATGCAAATGTACGTGAACGAGTTAATA 540
Importin-α  ------------------------------------------------------------
Exon0       TCAAATTGCCTGTTGTTTATTGAAAAGCTGGAAACTATTAATAAAACTGTTAAAGTTATG 600
Importin-α  ------------------------------------------------------------
Exon0       AATTTGTTTGTAGACAATTTGGTTTTGTACGAATGCAATGTTTGTAAAGAAATATCTACG 660
Importin-α  ------------------------------------------------------------
Exon0       GATGAAAGATTTTTAAAGCCAAAAGAATGTTGCGAATACGCTATATGCAACGCGTGCTGC 720
Importin-α  ------------------------------------------------------------
Exon0       GTTAACATGTGGAAGACGGCCACCACGCACGCAAAATGTCCAGCGTGCAGGACATCGTAT 780
Importin-α  ------------------------------------------------------------
Exon0       AAATAA 786
Importin-α  ------
```

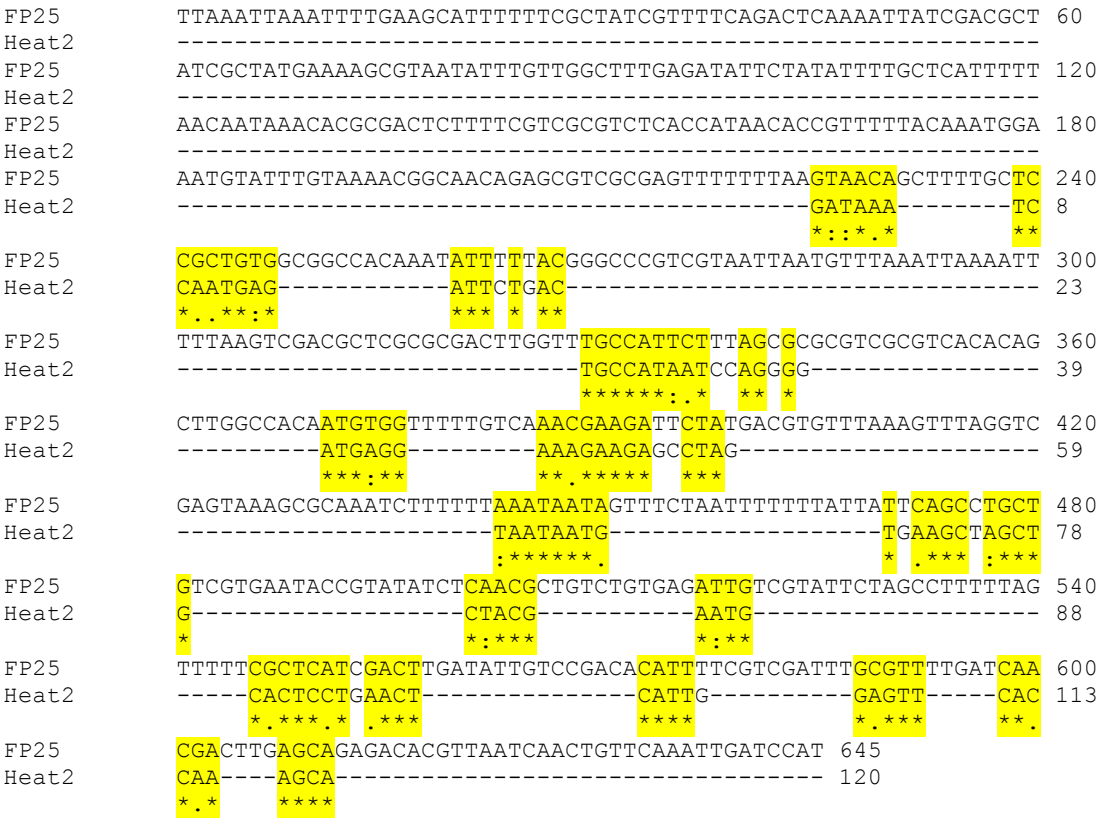
Sequence alignment of Exon0 and RanGTP binding domain



Sequence alignment of FP25 and HEAT1 domain



Sequence alignment of FP25 and HEAT2 domain



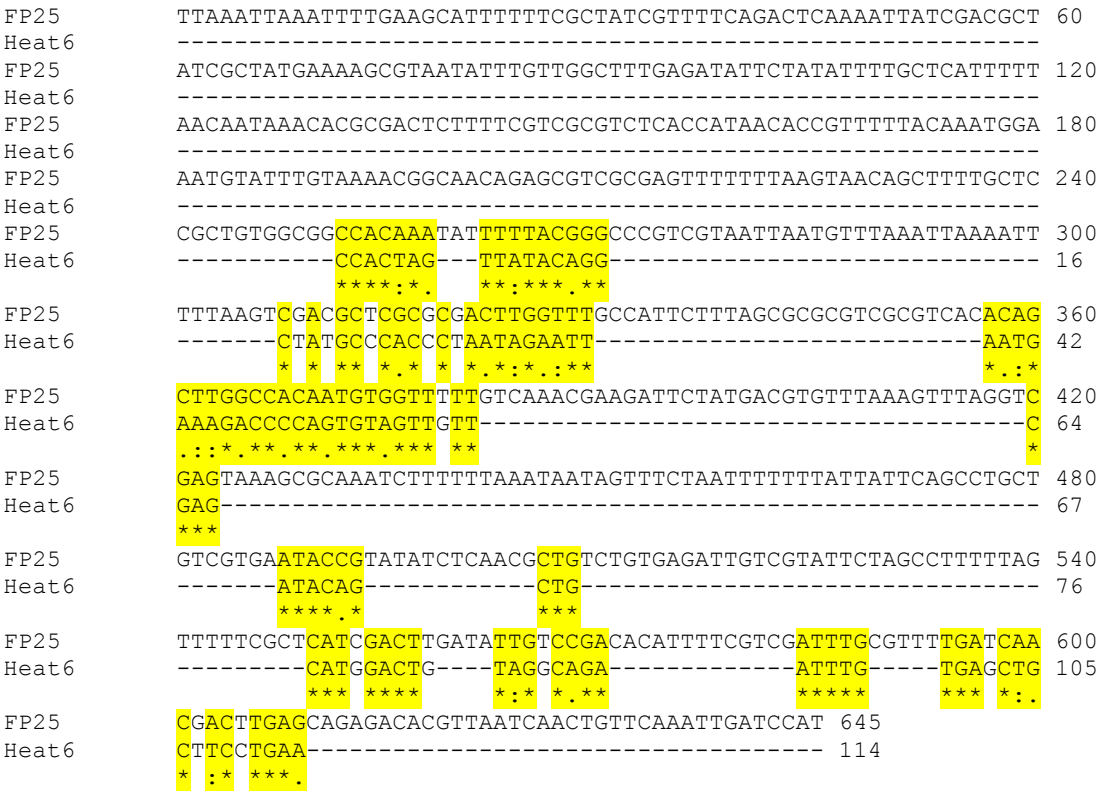
Sequence alignment of FP25 and HEAT3 domain

```
FP25        TTAAATTAAATTTTGAAGCATTTTTTCGCTATCGTTTTCAGACTCAAAATTATCGACGCT 60
Heat3       ------------------------------------------------------------
FP25        ATCGCTATGAAAAGCGTAATATTTGTTGGCTTTGAGATATTCTATATTTTGCTCATTTTT 120
Heat3       -----TCTGAAAGGC-------------------------ACTTTATTATG--------- 21
                 *.*****.**                         :**:****:**
FP25        AACAATAAACACGCGACTCTTTTCGTCGCGTCTCACCATAACACCGTTTTTACAAATGGA 180
Heat3       ------------------------------------------------CAGGTG-- 27
                                                             **..**
FP25        AATGTATTTGTAAAACGGCAACAGAGCGTCGCGAGTTTTTTTAAGTAACAGCTTTTGCTC 240
Heat3       ----------------------------GTC------------------------------ 30
                                        ***
FP25        CGCTGTGGCGGCCACAAATATTTTTACGGGCCCGTCGTAATTAATGTTTAAATTAAAATT 300
Heat3       ---TGTG------------------------------------------------------ 34
                ****
FP25        TTTAAGTCGACGCTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAG 360
Heat3       ------------------------------------AAGC-----------CACACAG 45
                                                :***           *******
FP25        CTTGGCCACAATGTGGTTTTTGTCAAACGAAGATTCTATGACGTGTTTAAAGTTTAGGTC 420
Heat3       --TGTCCAG---------------ATACGAGGGT------ACGAGT-------------- 68
              **  ***               *:****.*.*      ***:**
FP25        GAGTAAAGCGCAAATCTTTTTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCT 480
Heat3       ------------------------------------------------------GGCTGCT 75
                                                               *  *****
FP25        GTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG 540
Heat3       -------TTACAGAAT---------CTG---GTGAAG-------ATAATGTCCTT----- 104
                   :***.*:**         ***   ****..       **:.*.  ****
FP25        TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAA 600
Heat3       -------------------ATATTATCAG------------------------------ 114
                                *****.**.*
FP25        CGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT 645
Heat3       ---------------------------------------------
```

Sequence alignment of FP25 and HEAT4 domain

```
FP25        TTAAATTAAATTTTGAAGCATTTTTTCGCTATCGTTTTCAGACTCAAAATTATCGACGCT 60
Heat4       --------------GGAGCA--------CTACAG-TATCTGGTTC-------------CA 24
                          *.****        *** .* *:**:*. **             *:

FP25        ATCGCTATGAAAAGCGTAATATTTGTTGGCTTTGAGATATTCTATATTTTGCTCATTTTT 120
Heat4       ATC-------------------------------------------------CTCA----- 31
            ***                                                 ****

FP25        AACAATAAACACGCGACTCTTTTCGTCGCGTCTCACCATAACACCGTTTTTACAAATGGA 180
Heat4       ---------CAC-AGACACT---------AACTAAACAGGACG---------AAAATGAT 63
                     *** .***:**          .:**.*.** .**.         .*****.:

FP25        AATGTATTTGTAAAACGGCAACAGAGCGTCGCGAGTTTTTTTAAGTAACAGCTTTTGCTC 240
Heat4       GATG----------ACGATGACTGG---------------------AACCCCT---GCAA 89
            .***           ***. .**:*.                    ***. **    **:.

FP25        CGCTGTGGCGGCCACAAATATTTTTACGGGCCCGTCGTAATTAATGTTTAAATTAAAATT 300
Heat4       AGCAG---------------------CAGG------------------------------ 98
            .**:*                    *.**

FP25        TTTAAGTCGACGCTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAG 360
Heat4       ------------------------GGTGTGCCT--------CATGCTTC----------- 115
                                    *** ****:        *. ** **

FP25        CTTGGCCACAATGTGGTTTTTGTCAAACGAAGATTCTATGACGTGTTTAAAGTTTAGGTC 420
Heat4       --TGGCCACC---------------------------------------------------- 123
              *******.

FP25        GAGTAAAGCGCAAATCTTTTTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCT 480
Heat4       ------------------------------------------------------------

FP25        GTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG 540
Heat4       ------------------------------------------------------------

FP25        TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAA 600
Heat4       ------------------------------------------------------------

FP25        CGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT 645
Heat4       ---------------------------------------------
```

Sequence alignment of FP25 and HEAT6 domain



Sequence alignment of FP25 and HEAT7 domain

```
FP25        TTAAATTAAATTTTGAAGCATTTTTTCGCTATCGTTTTCAGACTCAAAATTATCGACGCT 60
Heat7       ------------------------------------------------------------
FP25        ATCGCTATGAAAAGCGTAATATTTGTTGGCTTTGAGATATTCTATATTTTGCTCATTTTT 120
Heat7       ------------------------------------------------------------
FP25        AACAATAAACACGCGACTCTTTTCGTCGCGTCTCACCATAACACCGTTTTTACAAATGGA 180
Heat7       ------------------------------------------------------------
FP25        AATGTATTTGTAAAACGGCAACAGAGCGTCGCGAGTTTTTTTAAGTAACAGCTTTTGCTC 240
Heat7       ----------------------------------------------CAGAT----CTC   8
                                                           ***.*    ***

FP25        CGCTGTGGCGGCCACAAATATTTTTACGGGCCCGTCGTAATTAATGTTTAAATTAAAATT 300
Heat7       TGATGTGG---------------TTATGG--CCTCCCTG---------------------  30
             *.*****               ***  **   **  * *.

FP25        TTTAAGTCGACGCTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAG 360
Heat7       -TTAAG--GATGTTC-----------------------------------CAAAGCACAG  52
             *****  **  * **                                  *.:..*****

FP25        CTTGGCCACAATGTGGTTTTTGTCAAACGAAGATTCTATGACGTGTTTAAAGTTTAGGTC 420
Heat7       CTGGGTC----TGGGG--------------------------GAGTACAAG----AGG-- 76
            **  **  *    **  **                      *:**:  **.     ***

FP25        GAGTAAAGCGCAAATCTTTTTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCT 480
Heat7       -----ATGC-------------------------------------------CCTGAT  86
                 *:**                                          ****.*

FP25        GTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG 540
Heat7       GGCAG--TTAGC----------ACACTG---GTGGAAGTGTTGGG------------TGG 119
            *  *.   :** *          **.***   ***..* *** * .            *.*

FP25        TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAA 600
Heat7       T------------------------------------------------------------ 120
            *

FP25        CGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT 645
Heat7       ---------------------------------------------
```

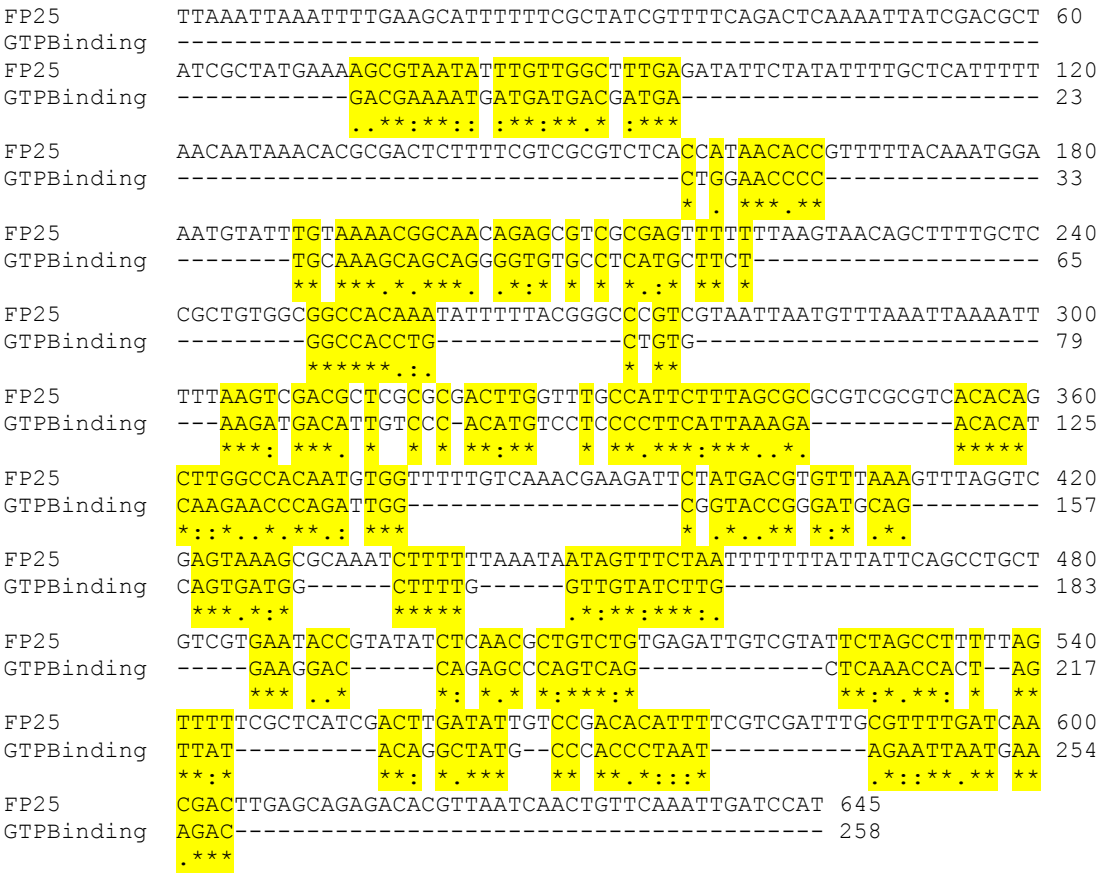
Sequence alignment of FP25 and HEAT8 domain

```
FP25        TTAAATTAAATTTTGAAGCATTTTTTCGCTATCGTTTTCAGACTCAAAATTATCGACGCT 60
Heat8       ------------------------------------------------------------
FP25        ATCGCTATGAAAAGCGTAATATTTGTTGGCTTTGAGATATTCTATATTTTGCTCATTTTT 120
Heat8       ------------------------------------------------------------
FP25        AACAATAAACACGCGACTCTTTTCGTCGCGTCTCACCATAACACCGTTTTTACAAATGGA 180
Heat8       ------------------------------------------------------------
FP25        AATGTATTTGTAAAACGGCAACAGAGCGTCGCGAGTTTTTTTAAGTAACAGCTTTTGCTC 240
Heat8       ------------------------------------------------------------
FP25        CGCTGTGGCGGCCACAAATATTTTTACGGGCCCGTCGTAATTAATGTTTAAATTAAAATT 300
Heat8       ------------------------------------------------------------
FP25        TTTAAGTCGACGCTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAG 360
Heat8       ------------------------------CCTTTCTGTGACGAG------GTGATGCAG 24
                                           **:**** *..**.*      ** * .***

FP25        CTTGGCCACAATGTGGTTTTTGTCAAACGAAGATTCTATGACGTGTTTAAAGTTTAGGTC 420
Heat8       CT------------------------------GCTTCTGG---------AAAATTTGG--- 43
            **                             *.****.          ***.***.*

FP25        GAGTAAAGCGCAAATCTTTTTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCT 480
Heat8       --GGAATGAG---------------------------------------------------- 51
              * **:*.*

FP25        GTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG 540
Heat8       ---------AACG------TCCACAG-GTCTGTGAAGCCG-CAGATTCTG------TCAG 88
                    *.**      **.**.  ********..  * *. *****.      * **

FP25        TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAA 600
Heat8       TGTTTGG----------TGATATTGCCC-----------------TTGC-TATTG----- 115
            * *** *          ******** **                 **** *:***

FP25        CGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT 645
Heat8       ------GAGGA----------------------------------- 120
                  *** *
```

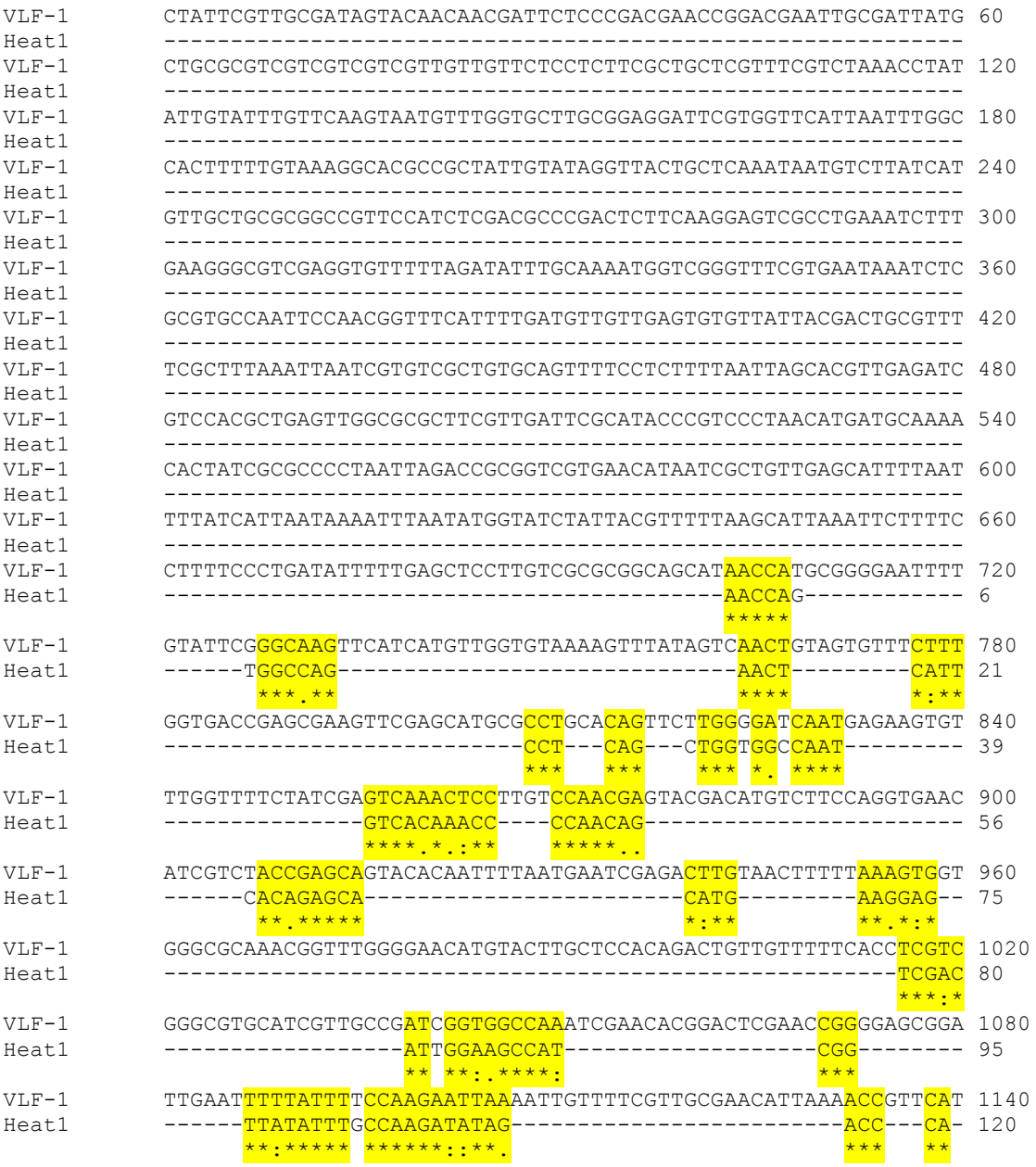
Sequence alignment of FP25 and importin-α binding domain

```
FP25        TTAAATTAAATTTTGAAGCATTTTTTCGCTATCGTTTTCAGACTCAAAATTATCGACGCT 60
Importin-α  ------------------------------------------------------------
FP25        ATCGCTATGAAAAGCGTAATATTTGTTGGCTTTGAGATATTCTATATTTTGCTCATTTTT 120
Importin-α  ------------------------------------------------------------
FP25        AACAATAAACACGCGACTCTTTTCGTCGCGTCTCACCATAACACCGTTTTTACAAATGGA 180
Importin-α  ------------------------------------------------------------
FP25        AATGTATTTGTAAAACGGCAACAGAGCGTCGCGAGTTTTTTTAAGTAACAGCTTTTGCTC 240
Importin-α  ------------------------------------------------------------
FP25        CGCTGTGGCGGCCACAAATATTTTTACGGGCCCGTCGTAATTAATGTTTAAATTAAAATT 300
Importin-α  ------------------------------------------------------------
FP25        TTTAAGTCGACGCTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAG 360
Importin-α  ----------------------------------------------------ACACT- 5
                                                                 ****:
FP25        CTTGGCCACAATGTGGTTTTTGTCAAACGAAGATTCTATGACGTGTTTAAAGTTTAGGTC 420
Importin-α  -------------------------AACTAA---------AC-------------AGGAC 18
                                       *** **         **             ***:*
FP25        GAGTAAAGCGCAAATCTTTTTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCT 480
Importin-α  GA--------------------AAATGAT----------------------------GAT 30
            **                     ****.**                            *.*
FP25        GTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG 540
Importin-α  G----------------------ACGATGACTG-G--------------------------- 42
            *                      ***.**:*** *
FP25        TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAA 600
Importin-α  ------------------------------------------------------------
FP25        CGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCAT 645
Importin-α  ---------------------------------------------
```

Sequence alignment of FP25 and RanGTP binding domain



Sequence alignment of VLF-1 and HEAT1 domain



Sequence alignment of VLF-1 and HEAT2 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat2       -----------GATAAATCCAATGAGATTCT---GAC-------------TGCCATAAT- 32
                       ****.::*.*.:..******   ***             ***  **:**
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat2       C------------------------------------------------------------ 33
            *
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat2       --------------------------------CAGGGGAT--GAGG-------------- 45
                                             *.*.****  *:**
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat2       ----------AAAGAAGAGCC----------TAG----------TAATAATG--------T 68
                      ****....***          ***           :*******        *
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat2       GAAGCT-AGCTGCT-----------ACGAATG-CACT-----------CCTGAACTCATT 104
            *::*** .** **             ***.. * *:**           ******.**:**
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat2       GGAG---------------------------------------TTC------------- 111
            *.**                                      ***
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat2       ----------ACCAAAG----CA-------------------------------------- 120
                      :****.*    **
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat2       ------------------------------------------------------------
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat2       ------------------------------------------------------------
VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat2       ------------------------------------------------------------
VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat2       ------------------------------------------------------------
VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat2       ------------------------------------------------------------
VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat2       ------------------------------------------------------------
VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Heat2       ------------------------------------------------------------
VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat2       ------------------------------------------------------------
VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Heat2       ------------------------------------------------------------
VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat2       ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat2       ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat2       ------------------------------------------------------------
```

Sequence alignment of VLF-1 and HEAT3 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat3       ------------------------------------------------------------
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat3       ------------------------------------------------------------
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat3       ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat3       ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat3       ------------------------------------------------------TCT 3
                                                                  * *
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat3       GAAAGGC------------------------------------------------------ 10
            ***.***
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat3       --------------AC---TTTATTATGCAGGTGGT------------------------- 29
                          **   **  ***:**.:* **  *
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat3       ----------------------CTGTGAAG-------------------------------- 37
                                  *****.**
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat3       --CCACACAGTGT---CCAGATACGAGGGTACGAGTG--------------GCTGCTTTA 78
              ****.*:*:**   *  .*.*:**:  *.*:**..*.              *.***:::*
VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat3       CAGAATC----------------------TGGTGAAGATAATG------------------ 99
            **  :***                     *  *****  *****
VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat3       ------------------------------------------------------------TC 101
                                                                        **
VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat3       CTT-------ATATTAT-------------------CAG---------------------- 114
            ***       *****:*                   ***
VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat3       ------------------------------------------------------------
VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Heat3       ------------------------------------------------------------
VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat3       ------------------------------------------------------------
VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Heat3       ------------------------------------------------------------
VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat3       ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat3       ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat3       ------------------------------------------------------------
```

Sequence alignment of VLF-1 and HEAT4 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat4       ------------------------------------------------------------
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat4       ------------------------------------------------------------
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat4       ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat4       ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat4       ------------------------------------------------------------
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat4       ------------------------------------------------------------
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat4       ------------------------------------------------------------
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat4       ------------------------------------------------------------
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat4       ----------------------------------------------------GGAGCACTA 9
                                                               *.:***.:*

VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat4       CAGTATC------------------------------------------------------ 16
            **  ****

VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat4       ----------------------TGG--------------------------TTCCAATC 27
                                   ***                        ***  ::**

VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat4       CT--------------------CACACAGACAC---------TAACTAAACAGG------- 52
            **                    *:*. :*:*.*         **** *:.*.**

VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat4       ----ACG---------AAAATGATGATGACGATG----------ACTGGA---------- 79
                :**           *:.***:**.**:..*:*          ****  *

VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Heat4       ----ACC--------------------CCTGCAAAGCAGCAGGGGT----------GTG- 104
                ***                    ******.** :  :****:           ***

VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat4       ----------------------CCT----------------CATG-CTTCTGG------ 118
                                   ***                ****  ****  .*

VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Heat4       -----CCACC---------------------------------------------------- 123
                  * ***

VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat4       ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat4       ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat4       ------------------------------------------------------------
```

Sequence alignment of VLF-1 and HEAT6 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat6       ------------------------------------------------------------
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat6       ------------------------------------------------------------
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat6       ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat6       ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat6       ------------------------------------------------------------
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat6       ------------------------------------------------------------
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat6       ------------------------------------------------------------
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat6       ----------------------------------CCACT---AGTTA------------ 10
                                               **:**   *.***

VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat6       -TACAGGCT-------------------------ATGCCC-------------------- 24
             *.**  ***                        **.***

VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat6       ----------ACCCTAATAG---------------------------------------- 34
                      .*******:.

VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat6       -----AATTAATGAAAG-----------------AC------------------------ 48
                  .******.***                 **

VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat6       -----CCCAG------------------------------------------------- 53
                  ***:*

VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat6       ------------------------------------------TGTAGT-------- 59
                                                      ******

VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Heat6       -------------TGTTCGAG-ATACAGCTGCATGG----------ACTGTAGGCAGAAT 95
                         :******* **.*. ***** .*          :*:.*..*.**:.*

VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat6       TTG---------TGAG--------CTG--------------------CTTCCTG---AA- 114
            ***          ***        **                    *****:*   **

VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Heat6       ------------------------------------------------------------
VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat6       ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat6       ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat6       ------------------------------------------------------------
```

Sequence alignment of VLF-1 and HEAT7 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat7       ------------------------------------------------------------
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat7       ------------------------------------------------------------
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat7       ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat7       ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat7       ------------------------------------------------------------
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat7       ------------------------------------------------------------
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat7       ------------------------------------------------------------
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat7       ------------------------------------------------------------
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat7       ------------------------------------------------------------
VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat7       -------------------------CAG-----------ATCTCTGATGTG--------- 15
                                     *.*           *** ***:**:*
VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat7       --------------------GTTATGG-----------------------------------C 23
                                .:*****                                    *
VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat7       CT---CCCTGTTAAGGATGT--TCCAA--AGCACAGCT------------GGG------- 57
            **    *****:**:  :**:  ***::  .**.*.**:            ***
VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat7       ---TCTGGGGGAGTACAAGAGGATG--------------------------------CCCT 83
               *  *** .***:**:  *  *:**                               *  *
VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Heat7       GATGGCAGT-------------------TAGCACA---CTGGTG---------GAAGTGT 112
            *.**.*.*:                   :*****   ** * *         *******
VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat7       TGGGTGGT-------------------------------------------------- 120
            * ***  *
VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Heat7       ------------------------------------------------------------
VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat7       ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat7       ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat7       ------------------------------------------------------------
```

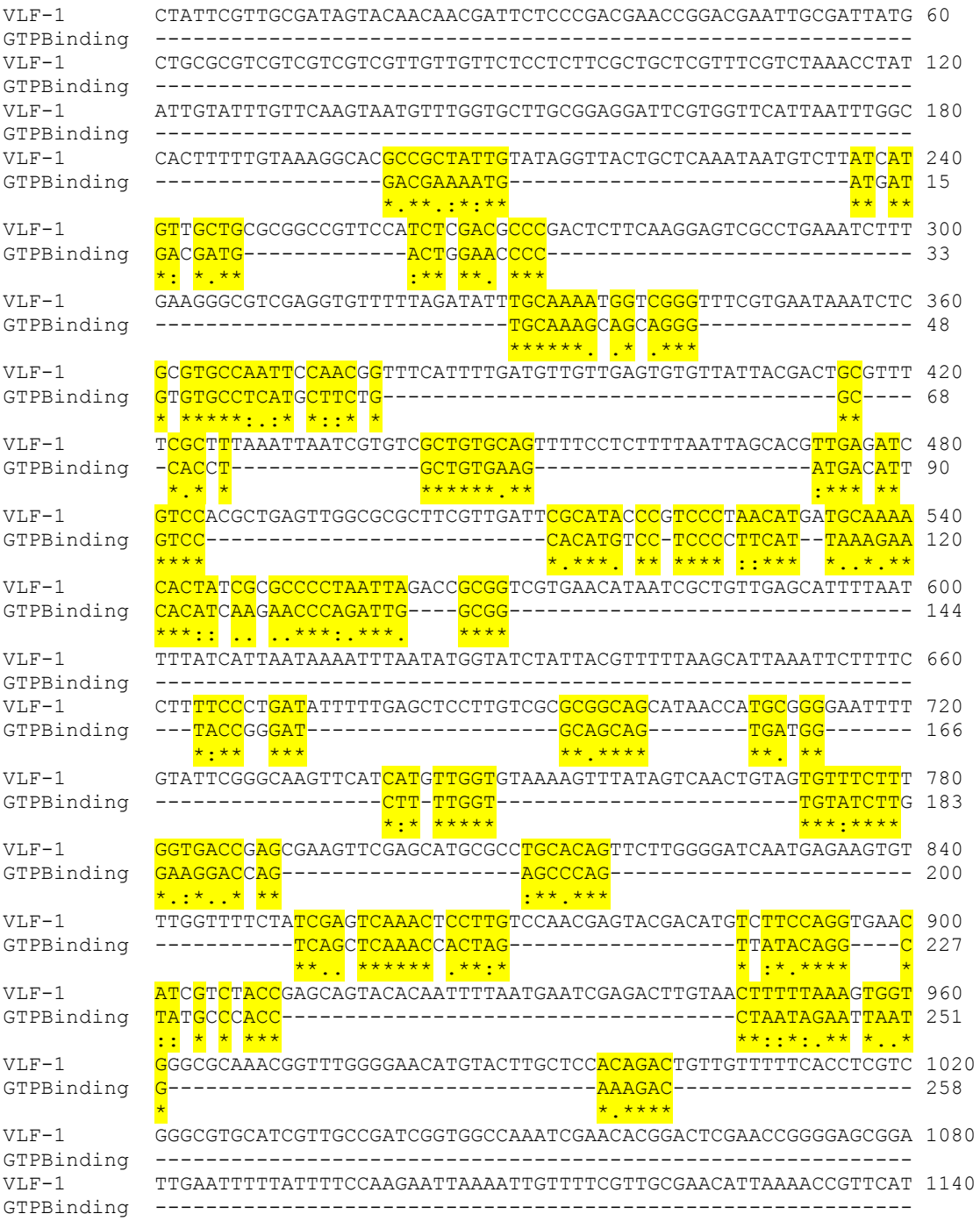
Sequence alignment of VLF-1 and HEAT8 domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Heat8       ------------------------------------------------------------
VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Heat8        -----------------------------------------------------------
VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Heat8       ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Heat8       ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Heat8       ------------------------------------------------------------
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Heat8       ------------------------------------------------------------
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Heat8       ------------------------------------------------------------
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Heat8       ------------------------------------------------------------
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Heat8       ------------------------------------------------------------
VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Heat8       ------------------------------------------------------------
VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Heat8       ------------------------------------------------------------C 1
                                                                         *

VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Heat8       CTTT---CTG-------TGA-----------CGAGG-------------------------- 16
            ****   ***       ***           **.**

VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Heat8       --------------------TGATG-----------------CAGCTG--------CTT- 30
                                **:**                 **.***         ***

VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGGATCAATGAGAAGTGT 840
Heat8       ----------------------------CTGGAAAAT--TTGGGG---AATGAGAA---- 53
                                         *** *.*.* ******   ********

VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Heat8       ------------CG--------TCC----------------ACAGGTCT-----GTGAAG 72
                        **        ***                ***  ****     *****

VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTTAAAGTGGT 960
Heat8       --------CC--GCAG-------------------------------ATTCTGTCAGTG-- 90
                  **  ****                                  .**  *  :.****

VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Heat8       ------------TTTGGTGATATTGCCCTTGCT---------------------------- 111
                        *****  **:.:**  .******

VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Heat8       ------------------ATTGGAGGA----------------------------------- 120
                             **  **:**.

VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Heat8       ------------------------------------------------------------
```

Sequence alignment of VLF-1 and importin-α binding domain

```
VLF-1       CTATTCGTTGCGATAGTACAACAACGATTCTCCCGACGAACCGGACGAATTGCGATTATG 60
Importin-α  -----------------ACACTAACTA-----------AACAGGACGAAA----ATGATG 28
                              ***. *** *           ***.*******:    **  ***

VLF-1       CTGCGCGTCGTCGTCGTCGTTGTTGTTCTCCTCTTCGCTGCTCGTTTCGTCTAAACCTAT 120
Importin-α  AT-------------GACGATG--------------ACTGG------------------- 42
            .*             *:**:**             .***

VLF-1       ATTGTATTTGTTCAAGTAATGTTTGGTGCTTGCGGAGGATTCGTGGTTCATTAATTTGGC 180
Importin-α  ------------------------------------------------------------
VLF-1       CACTTTTTGTAAAGGCACGCCGCTATTGTATAGGTTACTGCTCAAATAATGTCTTATCAT 240
Importin-α  ------------------------------------------------------------
VLF-1       GTTGCTGCGCGGCCGTTCCATCTCGACGCCCGACTCTTCAAGGAGTCGCCTGAAATCTTT 300
Importin-α  ------------------------------------------------------------
VLF-1       GAAGGGCGTCGAGGTGTTTTTAGATATTTGCAAAATGGTCGGGTTTCGTGAATAAATCTC 360
Importin-α  ------------------------------------------------------------
VLF-1       GCGTGCCAATTCCAACGGTTTCATTTTGATGTTGTTGAGTGTGTTATTACGACTGCGTTT 420
Importin-α  ------------------------------------------------------------
VLF-1       TCGCTTTAAATTAATCGTGTCGCTGTGCAGTTTTCCTCTTTTAATTAGCACGTTGAGATC 480
Importin-α  ------------------------------------------------------------
VLF-1       GTCCACGCTGAGTTGGCGCGCTTCGTTGATTCGCATACCCGTCCCTAACATGATGCAAAA 540
Importin-α  ------------------------------------------------------------
VLF-1       CACTATCGCGCCCCTAATTAGACCGCGGTCGTGAACATAATCGCTGTTGAGCATTTTAAT 600
Importin-α  ------------------------------------------------------------
VLF-1       TTTATCATTAATAAAATTTAATATGGTATCTATTACGTTTTTAAGCATTAAATTCTTTTC 660
Importin-α  ------------------------------------------------------------
VLF-1       CTTTTCCCTGATATTTTTGAGCTCCTTGTCGCGCGGCAGCATAACCATGCGGGGAATTTT 720
Importin-α  ------------------------------------------------------------
VLF-1       GTATTCGGGCAAGTTCATCATGTTGGTGTAAAAGTTTATAGTCAACTGTAGTGTTTCTTT 780
Importin-α  ------------------------------------------------------------
VLF-1       GGTGACCGAGCGAAGTTCGAGCATGCGCCTGCACAGTTCTTGGGGATCAATGAGAAGTGT 840
Importin-α  ------------------------------------------------------------
VLF-1       TTGGTTTTCTATCGAGTCAAACTCCTTGTCCAACGAGTACGACATGTCTTCCAGGTGAAC 900
Importin-α  ------------------------------------------------------------
VLF-1       ATCGTCTACCGAGCAGTACACAATTTTAATGAATCGAGACTTGTAACTTTTTAAAGTGGT 960
Importin-α  ------------------------------------------------------------
VLF-1       GGGCGCAAACGGTTTGGGGAACATGTACTTGCTCCACAGACTGTTGTTTTTCACCTCGTC 1020
Importin-α  ------------------------------------------------------------
VLF-1       GGGCGTGCATCGTTGCCGATCGGTGGCCAAATCGAACACGGACTCGAACCGGGGAGCGGA 1080
Importin-α  ------------------------------------------------------------
VLF-1       TTGAATTTTTATTTTCCAAGAATTAAAATTGTTTTCGTTGCGAACATTAAAACCGTTCAT 1140
Importin-α  ------------------------------------------------------------
```

Sequence alignment of VLF-1 and RanGTP binding domain



Sequence alignment of P49 and HEAT1 domain

```
P49         ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat1       ------------------------------------------------------------
P49         ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat1       ------------------------------------------------------------
P49         CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat1       ------------------------------------------------------------
P49         AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat1       ------------------------------------------------------------
P49         AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat1       ------------------------------------------------------------
P49         TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat1       ----------------------AACCAG---------TG---GCCAG--AACT------- 17
                                   ****.*          **   ****   *.**
P49         TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat1       --CATTCCTC---AGCTGGTGGC---------------------CAATGT---------- 41
              **:*** *   **.**.****                     ***  **
P49         AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat1       --------CACAAACCCCAACAGCACAGAGCACATGAAGGAG------------------- 75
                    *. ****.  *****.*:..*.  .*.**::**.*
P49         TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat1       -TCGAC-------------ATTGGAAG---------------------------------- 88
             *****             *******
P49         AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat1       ------------CCAT--CGGTTATATTTGC----------CAAG---------------- 109
                        **.*  **. *.*** *.*          **:*
P49         GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat1       -------ATATAG---------ACCCA---------------------------------- 120
                    *****          ****:
P49         ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat1       ------------------------------------------------------------
P49         GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat1       ------------------------------------------------------------
P49         GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat1       ------------------------------------------------------------
P49         CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat1       ------------------------------------------------------------
P49         AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat1       ------------------------------------------------------------
P49         GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat1       ------------------------------------------------------------
P49         ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat1       ------------------------------------------------------------
P49         GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat1       ------------------------------------------------------------
P49         AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat1       ------------------------------------------------------------
P49         CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat1       ------------------------------------------------------------
P49         GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat1       ------------------------------------------------------------
P49         CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat1       ------------------------------------------------------------
P49         CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat1       ----------------------------------------------------
```

Sequence alignment of P49 and HEAT2 domain

```
P49         ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat2       ------------------------------------------------------------
P49         ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat2       ------------------------------------------------------------
P49         CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat2       ------------------------------------------------------------
P49         AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat2       ------------------------------------------------------------
P49         AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat2       ------------------------------------------------------------
P49         TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat2       ------------------------------------------------------------
P49         TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat2       ------------------------------------------------------------
P49         AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat2       ------------------------------------------------------------
P49         TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat2       ------------------------------------------------------------
P49         AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat2       ------------------------------------------------------------
P49         GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat2       ------------------------------------------------------------
P49         ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat2       ------------------------------------------------------------
P49         GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat2       ----------------------------------------GATAAATCCAATGAGATT------ 18
                                                    ****:**  ********:

P49         GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat2       --------------CTGACTG---------------------------------------- 25
                          ***:* *

P49         CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat2       ----------------------------------------CCATAA------------ 31
                                                     ***:**

P49         AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat2       ---------TCCAGG-----GGATGAGGAAAG-------AAGAGC--------------- 55
                     :***.*     *.:***.****.       *:****

P49         GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat2       ---------CT------------AGTAATAATGTGAAGCTAGCTG---CTACGAATG--- 88
                     **            ****.:.*: *.:.**:*****   .**..****

P49         ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat2       ----------------CACTCCTGAAC--TCAT---------------------------- 103
                            *: **.**:**   ****

P49         GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat2       -------TGGAGTT-------------------------CACCAAAGCA---------- 120
                   ** *.**                         *.**.*****

P49         AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat2       ------------------------------------------------------------
P49         CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat2       ------------------------------------------------------------
P49         GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat2       ------------------------------------------------------------
P49         CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat2       ------------------------------------------------------------
P49         CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat2       ----------------------------------------------------
```

Sequence alignment of P49 and HEAT3 domain

```
P49          ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat3        ------------------------------------------------------------
P49          ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat3        -----------------------------------------------------------T 1
                                                                         *
P49          CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat3        C---------TGAAAGGCAC-TTTATTATGCAG--------------------------- 24
             *         ****:  **** ***.:*:***:.
P49          AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat3        ----------------------------------GTGG----------------------- 28
                                                ****
P49          AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat3        ---TCTGTGAAGCCACA-------------------------CAGTG------------ 47
                * ***.********                          *:***
P49          TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat3        ------------------------------------------------------------
P49          TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat3        --------------------------TCCAG------------------------------ 52
                                        ***:*
P49          AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat3        ------------------------------------------------------------
P49          TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat3        -----------ATACGAGG-----------GTACGAGTG--------GCTGC------TT 76
                         .****:**           **.***.**        **  **      :*
P49          AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat3        TACAGAAT----------------------CTGG----------------------TGAA 92
             :***..**                      ****                      **:.
P49          GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat3        GATAATGT----------------CCTTATATT---------------ATCAG------- 114
             ******.:                ****.**:               ****.
P49          ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat3        ------------------------------------------------------------
P49          GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat3        ------------------------------------------------------------
P49          GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat3        ------------------------------------------------------------
P49          CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat3        ------------------------------------------------------------
P49          AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat3        ------------------------------------------------------------
P49          GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat3        ------------------------------------------------------------
P49          ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat3        ------------------------------------------------------------
P49          GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat3        ------------------------------------------------------------
P49          AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat3        ------------------------------------------------------------
P49          CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat3        ------------------------------------------------------------
P49          GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat3        ------------------------------------------------------------
P49          CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat3        ------------------------------------------------------------
P49          CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat3        ----------------------------------------------------
```

Sequence alignment of P49 and HEAT4 domain

```
P49         ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat4       ------------------------------------------------------------
P49         ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat4       ------------------------------------------------------------
P49         CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat4       ------------------------------------------------------------
P49         AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat4       ----------------------------------GG--AGCAC-------TACAGTATC 16
                                               **  *****       :***  *:**
P49         AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat4       TGGTT-------CCAATCCT----------------CACACA------------------ 35
            :.***       ***.:. *                *.****
P49         TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat4       ---GACACTAACT---------AAACAGG----------------ACGAAAATG------ 61
               *****  ..*:         **.*.**                *****..**
P49         TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat4       ---------------ATGATG----------ACGATGACT-------GG----------- 78
                           ******          ***.***.*        **
P49         AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat4       ------------AACCCCTGCAAA----GCAGC-------AGGGGTGTGCC----TCATG 111
                        ***.  *:.***.    **:**        .***  :*****    **:**
P49         TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat4       ------------------------------------------------------------
P49         AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat4       ----------------------------CTTCTGG---------------------------- 118
                                         **:****
P49         GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat4       ---------------CCACC---------------------------------------- 123
                           **.**
P49         ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat4       ------------------------------------------------------------
P49         GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat4       ------------------------------------------------------------
P49         GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat4       ------------------------------------------------------------
P49         CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat4       ------------------------------------------------------------
P49         AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat4       ------------------------------------------------------------
P49         GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat4       ------------------------------------------------------------
P49         ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat4       ------------------------------------------------------------
P49         GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat4       ------------------------------------------------------------
P49         AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat4       ------------------------------------------------------------
P49         CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat4       ------------------------------------------------------------
P49         GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat4       ------------------------------------------------------------
P49         CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat4       ------------------------------------------------------------
P49         CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat4       ----------------------------------------------------
```

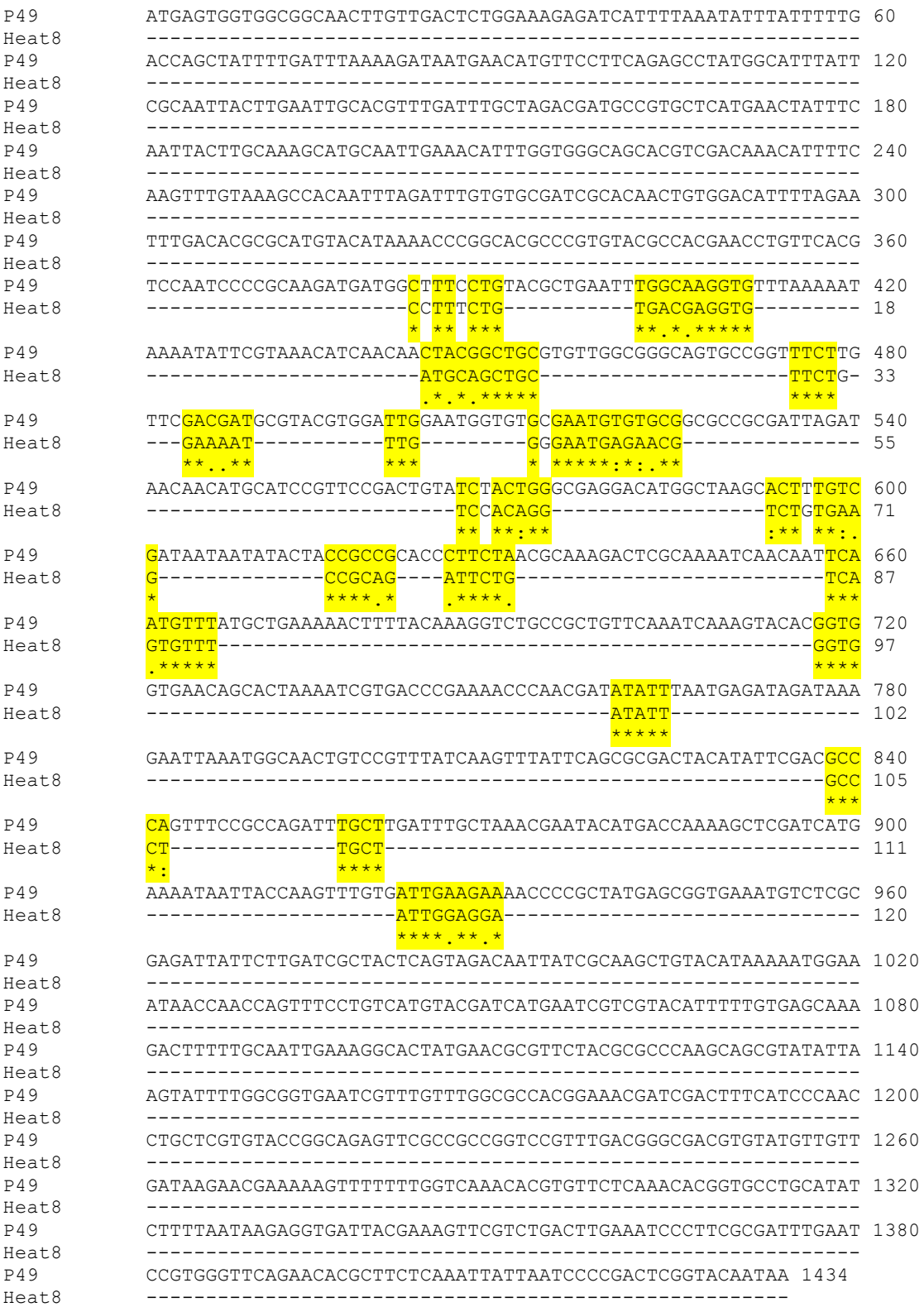
Sequence alignment of P49 and HEAT6 domain

```
P49         ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat6       ------------------------------------------------------------
P49         ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat6       ------------------------------------------------------------
P49         CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat6       ------------------------------------------------------------
P49         AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat6       ------------------------------------------------------------
P49         AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat6       ------------------------------------------------------------
P49         TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat6       ------------------------------------------------------------
P49         TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat6       ------------------------------------------------------------
P49         AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat6       ------------------------------------------------------------
P49         TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat6       ------------------------------------------------------------
P49         AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat6       ------------------------------------------------------------
P49         GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat6       ------------------------------------------------------------
P49         ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat6       ------------------------------------------------------------
P49         GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat6       ------------------------------------------------------------
P49         GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat6       -----------CCACT------------AG-TTATACAGG-------------CTATGCC 23
                       *.***            ** ****:***             * * ***
P49         CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat6       CA----CC-------------------CTAATAGAATTAAT------------------- 41
            **    **                   ****:.****:.**
P49         AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat6       ---------------------------GAAAGACCC--------CAGTGTAGT------- 59
                                       ****..***        *.***:*.*
P49         GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat6       -----------TGTTCGAGATACAG-----------------CTG--------------- 76
                       **:***. * :***                 ***
P49         ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat6       --------------------CATGGAC----------------------TGTAGGCAGA 93
                                ****  **                    ***..***.*
P49         GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat6       ----------ATTTGTGAG------------------------------------------ 102
                      *:***:.**
P49         AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat6       ------------------------------------------------------------
P49         CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat6       CTGCTT-----CCTGAA------------------------------------------- 114
            *****      ** *.*
P49         GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat6       ------------------------------------------------------------
P49         CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat6       ------------------------------------------------------------
P49         CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat6       ---------------------------------------------------
```

Sequence alignment of P49 and HEAT7 domain

```
P49         ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Heat7       ------------------------------------------------------------
P49         ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Heat7       ------------------------------------------------------------
P49         CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Heat7       ------------------------------------------------------------
P49         AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Heat7       ------------------------------------------------------------
P49         AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Heat7       --------------------CAGAT-------------CTCTGATGTGGTTATG------ 21
                                ****             *:*:..*****: **
P49         TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Heat7       ----------------------------------------GCCTC---CCTGTTAAG- 35
                                                     ***:*   ******.*
P49         TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Heat7       -----------------GATG---TTCCAAAGC---------------------------- 48
                             ****   ****:.:.*
P49         AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Heat7       --------------------ACAGCTGGGTCTG---------GGGGAGTACAAG------- 73
                               ***.**. * ***         *** ***.*..*
P49         TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Heat7       ----AGGATGC---CCTG-------ATG----------------------GCAGTTAG-- 95
                * *****   *  **       ***                    **..****
P49         AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Heat7       -----------------CACACTG------------GTGGAAGTG----------TTG-- 114
                             *. ****            *:***..**          ***
P49         GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Heat7       ------------------------------------------------------------
P49         ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Heat7       ----------------------------------------------------------GGTG 118
                                                                       ****
P49         GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Heat7       GT---------------------------------------------------------- 120
            **
P49         GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Heat7       ------------------------------------------------------------
P49         CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Heat7       ------------------------------------------------------------
P49         AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Heat7       ------------------------------------------------------------
P49         GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Heat7       ------------------------------------------------------------
P49         ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Heat7       ------------------------------------------------------------
P49         GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Heat7       ------------------------------------------------------------
P49         AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Heat7       ------------------------------------------------------------
P49         CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Heat7       ------------------------------------------------------------
P49         GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Heat7       ------------------------------------------------------------
P49         CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Heat7       ------------------------------------------------------------
P49         CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Heat7       ----------------------------------------------------
```

Sequence alignment of P49 and HEAT8 domain



Sequence alignment of P49 and importin-α binding domain

```
P49          ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
Importin-α   ------------------------------------------------------------
P49          ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
Importin-α   ------------------------------------------------------------
P49          CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
Importin-α   ------------------------------------------------------------
P49          AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
Importin-α   ------------------------------------------------------------
P49          AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
Importin-α   ------------------------------------------------------------
P49          TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
Importin-α   ----ACAC-------TAACTAAA--CAGG----------------ACGAAAATG------ 25
                 ****        **.****  *.**                *****..**
P49          TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
Importin-α   ---------------ATGATG----------ACGATGACT--GG---------------- 42
                           ******          ***.***.*  **
P49          AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
Importin-α   ------------------------------------------------------------
P49          TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
Importin-α   ------------------------------------------------------------
P49          AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
Importin-α   ------------------------------------------------------------
P49          GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
Importin-α   ------------------------------------------------------------
P49          ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
Importin-α   ------------------------------------------------------------
P49          GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
Importin-α   ------------------------------------------------------------
P49          GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
Importin-α   ------------------------------------------------------------
P49          CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
Importin-α   ------------------------------------------------------------
P49          AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
Importin-α   ------------------------------------------------------------
P49          GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
Importin-α   ------------------------------------------------------------
P49          ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
Importin-α   ------------------------------------------------------------
P49          GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
Importin-α   ------------------------------------------------------------
P49          AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
Importin-α   ------------------------------------------------------------
P49          CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
Importin-α   ------------------------------------------------------------
P49          GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
Importin-α   ------------------------------------------------------------
P49          CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
Importin-α   ------------------------------------------------------------
P49          CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
Importin-α   ----------------------------------------------------
```

Sequence alignment of P49 and RanGTP binding domain

```
P49          ATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAGAGATCATTTTAAATATTTATTTTTG 60
GTPBinding   ------------------------------------------------------------
P49          ACCAGCTATTTTGATTTAAAAGATAATGAACATGTTCCTTCAGAGCCTATGGCATTTATT 120
GTPBinding   ------------------GACGAAAATGATGATG--------------------------- 16
                               .*.**:*****: ***
P49          CGCAATTACTTGAATTGCACGTTTGATTTGCTAGACGATGCCGTGCTCATGAACTATTTC 180
GTPBinding   ----------------------------------ACGATGAC-TG-----GAAC------ 30
                                               ******.* **     ****
P49          AATTACTTGCAAAGCATGCAATTGAAACATTTGGTGGGCAGCACGTCGACAAACATTTTC 240
GTPBinding   ----CCCTGCAAAGCA-GCAGG----------GGTGTGCCTCATG-----------CTTC 64
                 .* ********* ***.           **** **. ** *           ***
P49          AAGTTTGTAAAGCCACAATTTAGATTTGTGTGCGATCGCACAACTGTGGACATTTTAGAA 300
GTPBinding   TG---------GCCAC------------------------CTGCTGTG----------AA 81
             :.         *****                       *:.*****          **
P49          TTTGACACGCGCATGTACATAAAACCCGGCACGCCCGTGTACGCCACGAACCTGTTCACG 360
GTPBinding   GATGACAT-----TG--------TCCC-----------------------------ACATG 100
              :*****       **       :***                           :** *
P49          TCCAATCCCCGCAAGATGATGGCTTTCCTGTACGCTGAATTTGGCAAGGTGTTTAAAAAT 420
GTPBinding   TCC--TCCCC--------------TTCAT-------------------------TAAAG-- 118
             ***  *****             ***.*                         ****.
P49          AAAATATTCGTAAACATCAACAACTACGGCTGCGTGTTGGCGGGCAGTGCCGGTTTCTTG 480
GTPBinding   ----------AACACATCAAGAACCCAG-------ATTGGCGG----TACCGG------- 150
                     :*.*******  ***  ..*       .*******    *.****
P49          TTCGACGATGCGTACGTGGATTGGAATGGTGTGCGAATGTGTGCGGCGCCGCGATTAGAT 540
GTPBinding   ------GATGCAGCAGTG-------ATG----------------------GCTTTTGGT- 174
                   *****. ..***        ***                     ** :**.*:
P49          AACAACATGCATCCGTTCCGACTGTATCTACTGGGCGAGGACATGGCTAAGCACTTTGTC 600
GTPBinding   ----------------------TGTATCT--TGG--AAGGACCAG--------------- 193
                                   *******  ***  .*****.:*
P49          GATAATAATATACTACCGCCGCACCCTTCTAACGCAAAGACTCGCAAAATCAACAATTCA 660
GTPBinding   ------------------------------------------------------------
P49          ATGTTTATGCTGAAAAACTTTTACAAAGGTCTGCCGCTGTTCAAATCAAAGTACACGGTG 720
GTPBinding   -------------------------------AGCC-CAG-TCAGCTCAAACCACTAG--- 217
                                           :*** *:* ***..***** **:.*
P49          GTGAACAGCACTAAAATCGTGACCCGAAAACCCAACGATATATTTAATGAGATAGATAAA 780
GTPBinding   ------------------------------------------------------------
P49          GAATTAAATGGCAACTGTCCGTTTATCAAGTTTATTCAGCGCGACTACATATTCGACGCC 840
GTPBinding   -------------------------------TTATACAGG-------------CTATGCC 233
                                           ****:***                * * ***
P49          CAGTTTCCGCCAGATTTGCTTGATTTGCTAAACGAATACATGACCAAAAGCTCGATCATG 900
GTPBinding   CA----CC-------------------CTAATAGAATTAATG---AAAGAC---------- 258
             **    **                  ****:.****:.***   ***..*
P49          AAAATAATTACCAAGTTTGTGATTGAAGAAAACCCCGCTATGAGCGGTGAAATGTCTCGC 960
GTPBinding   ------------------------------------------------------------
P49          GAGATTATTCTTGATCGCTACTCAGTAGACAATTATCGCAAGCTGTACATAAAAATGGAA 1020
GTPBinding   ------------------------------------------------------------
P49          ATAACCAACCAGTTTCCTGTCATGTACGATCATGAATCGTCGTACATTTTTGTGAGCAAA 1080
GTPBinding   ------------------------------------------------------------
P49          GACTTTTTGCAATTGAAAGGCACTATGAACGCGTTCTACGCGCCCAAGCAGCGTATATTA 1140
GTPBinding   ------------------------------------------------------------
P49          AGTATTTTGGCGGTGAATCGTTTGTTTGGCGCCACGGAAACGATCGACTTTCATCCCAAC 1200
GTPBinding   ------------------------------------------------------------
P49          CTGCTCGTGTACCGGCAGAGTTCGCCGCCGGTCCGTTTGACGGGCGACGTGTATGTTGTT 1260
GTPBinding   ------------------------------------------------------------
P49          GATAAGAACGAAAAAGTTTTTTTGGTCAAACACGTGTTCTCAAACACGGTGCCTGCATAT 1320
GTPBinding   ------------------------------------------------------------
P49          CTTTTAATAAGAGGTGATTACGAAAGTTCGTCTGACTTGAAATCCCTTCGCGATTTGAAT 1380
GTPBinding   ------------------------------------------------------------
P49          CCGTGGGTTCAGAACACGCTTCTCAAATTATTAATCCCCGACTCGGTACAATAA 1434
GTPBinding   ----------------------------------------------------
```

Sequence alignment of E27 and HEAT1 domain

```
E27          ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat1        ------------------------------------------------------------
E27          AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat1        ----------------------------------------------AACCAG------- 6
                                                           **  ***
E27          AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat1        -------------------------TGGC---CAG------------------------- 13
                                       ****   ***
E27          CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat1        --------AACTCATTC--------------------------------CTCAGCTGGTG 33
                     .*****: *                                ***.**:*.*
E27          GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat1        GCC--------------------------------------------------------- 36
             ***
E27          AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat1        --------------AATG----------TCACAAACCCCAACAGCACAG----------- 61
                           ****          ****:.*..*****..****.
E27          GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat1        -AGCACAT-----------------GAAGGAGT------------------------CGACA 81
              *.*.***                 *****:**                        **:*:
E27          ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat1        TTGG----------AAGCC-----------ATCG--------GTTATATTT--------- 103
             :* *          *****           * **        **:*:.***
E27          AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat1        ------------------GCCAAGATATAG------------------------------ 115
                               *******  .*:
E27          AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat1        --------------------------ACCCA------------------------------ 120
                                       *****
E27          ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat1        ------------------------------------------------------------
E27          TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat1        ------------------------------------------------------------
E27          CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat1        ------------------------------------------------------------
E27          TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat1        ------------------------------------------------------------
E27          TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat1        ---------------------------------
```

Sequence alignment of E27 and HEAT2 domain

```
E27         ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat2       ------------------------------------------------------GAT--- 3
                                                                   ***

E27         AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat2       -AAATCCAATG-----------------------------AGATTCTGA----------- 22
             ********:.                              *.*:***.*

E27         AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat2       ----------------------------------CTGC-----------------CATAATC 33
                                             *:**                ** .* *

E27         CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat2       CAGGGGATGA-------------GG--------------AAAGAAGAGCCTAG------- 59
            *:**..****              **              ****.*** .**.*

E27         GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat2       ------------------------------------------------------------

E27         AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat2       --------TAATAATGTGAAGCTAG-------------CTGCTACGAATGCACTCCTG-- 96
                    *** **:.**.** *:*              *:.* **..*:*** *** *

E27         GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat2       -AACTCATTG------------------------------------------------- 105
             *** ****

E27         ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat2       ----------------------GAGTT---CACCAAAGCA---------------------- 120
                                   *****    ****.*.**:

E27         AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat2       ------------------------------------------------------------

E27         AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat2       ------------------------------------------------------------

E27         ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat2       ------------------------------------------------------------

E27         TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat2       ------------------------------------------------------------

E27         CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat2       ------------------------------------------------------------

E27         TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat2       ------------------------------------------------------------

E27         TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat2       --------------------------------
```

Sequence alignment of E27 and HEAT3 domain

```
E27          ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat3        ------------------------------------------------------------
E27          AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat3        ------------------------------------------------------------
E27          AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat3        ----------------------------------------------------TCTG---- 4
                                                                **:*
E27          CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat3        ----------------------------------------AAAG---------------- 8
                                                       ****
E27          GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat3        ----------------GCACTTTA----TTATGCAG------------------------ 24
                             ****:***    ****.**
E27          AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat3        ------------------------GTGGTCTGTGAAGCCA----CACAG--TGTCCAG-- 52
                                     ******: ****.***    ****.  : ***.*
E27          GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat3        --------------------ATACGAGGGT------------------------------ 62
                                 *:****.***
E27          ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat3        --------------------ACGAGT----------GGCTG-------CTTT----ACAGA 82
                                 .*****          ****        ****    **...
E27          AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat3        ATCTG----GTGAAGATA---ATGTCCTT-------------------------------- 104
             *:***    ***..****    *:*:* **
E27          AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat3        -----------------ATATTATCAG---------------------------------- 114
                               *:**:*:**.
E27          ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat3        ------------------------------------------------------------
E27          TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat3        ------------------------------------------------------------
E27          CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat3        ------------------------------------------------------------
E27          TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat3        ------------------------------------------------------------
E27          TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat3        --------------------------------
```

Sequence alignment of E27 and HEAT4 domain

```
E27          ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat4        ------------------------------------------------------------
E27          AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat4        ------------------------------------------------------------
E27          AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat4        --------------------------GGAGCACTACAG---------TAT---------- 15
                                        **.**:*:.**.         ***

E27          CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat4        CTG--------------------------------------------------------- 18
             ***

E27          GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat4        ----------------------------------------GTTCCAATCCTCA------- 31
                                                     *:* *.**** *:

E27          AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat4        ----------------------------CACAGACACTAACTAAACAGG---------- 52
                                         ***:**.** *** *.***.*

E27          GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat4        ---------------ACG-AAAATGATG-------------------------------- 64
                             *** **** **:*

E27          ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat4        ---------ATG-------------------------------------------ACGAT 72
                      ***                                           ****

E27          AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat4        GACTGGAAC----------CCCTG-------------CAAAGC--------------AGC 95
             .**** *** **.:* *:**** .**

E27          AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat4        AGGGGTG----------------------------------------------------- 102
             ** *.**

E27          ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat4        --------------TGCCTC-------------ATGCTTCTGGCCACC------------ 123
                           :***:*             .** * ***.**. *

E27          TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat4        ------------------------------------------------------------
E27          CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat4        ------------------------------------------------------------
E27          TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat4        ------------------------------------------------------------
E27          TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat4        ---------------------------------
```

Sequence alignment of E27 and HEAT6 domain

```
E27          ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat6        ------------------------------------------------------------
E27          AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat6        --------------CCACTAGTT------------------------------------ 9
                           *  **  *.**
E27          AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat6        ----------------------------------------ATACA-GGCTATGCCCA---CC 27
                                                     *****  ********  **    **
E27          CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat6        CT---------------------------------------------------------- 29
             **
E27          GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat6        ------------------------------------------------------------
E27          AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat6        --------------AATAGAATTAATG------------AAAGAC---------CCCAGT 54
                           ***.**.**:.**              **.***          ***.*:
E27          GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat6        GTAG-----TTGTT---------CGAG---ATACAGCTG-----CATGGACTG------- 85
             *:*      *****         ***.   .*.*:****     *.*****:*
E27          ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat6        -------------TAGGCAG--AATTTG----TGAG------------------------ 102
                          **.**.*   *.****     ***
E27          AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat6        --CTGCTTCCTGAA---------------------------------------------- 114
               ****::*  **..
E27          AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat6        ------------------------------------------------------------
E27          ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat6        ------------------------------------------------------------
E27          TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat6        ------------------------------------------------------------
E27          CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat6        ------------------------------------------------------------
E27          TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat6        ------------------------------------------------------------
E27          TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat6        --------------------------------
```

Sequence alignment of E27 and HEAT7 domain

```
E27         ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat7       ------------------------------------------------------------
E27         AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat7       ----------------------------------------------------CAG------- 3
                                                                   ***
E27         AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat7       --------ATCTCTGATG------------------------TGGTTATGG-------C 23
                    *:****.*:.                       ***  ****        *
E27         CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat7       CT-------------CC-CTGTTAAGGATGTTCCAAAGCACAGCTGGGTCTGGG------ 63
            **             **  *****....**.** ..**.**.***:*. :**  **
E27         GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat7       --------GGAGT---------------------ACAAGAGG----ATGCCCTG------ 84
                    **:**                     ****  **.    **  ****
E27         AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat7       ---------------ATG------------------------------GCA-------- 90
                           ***                              ***
E27         GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat7       -----------GTTAGC-----ACACTGGTG----GAAGTGTTGGGTGGT---------- 120
                       ***:.*     **..:****    *.:*****  ****:
E27         ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat7       ------------------------------------------------------------
E27         AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat7       ------------------------------------------------------------
E27         AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat7       ------------------------------------------------------------
E27         ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat7       ------------------------------------------------------------
E27         TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat7       ------------------------------------------------------------
E27         CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat7       ------------------------------------------------------------
E27         TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat7       ------------------------------------------------------------
E27         TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat7       --------------------------------
```

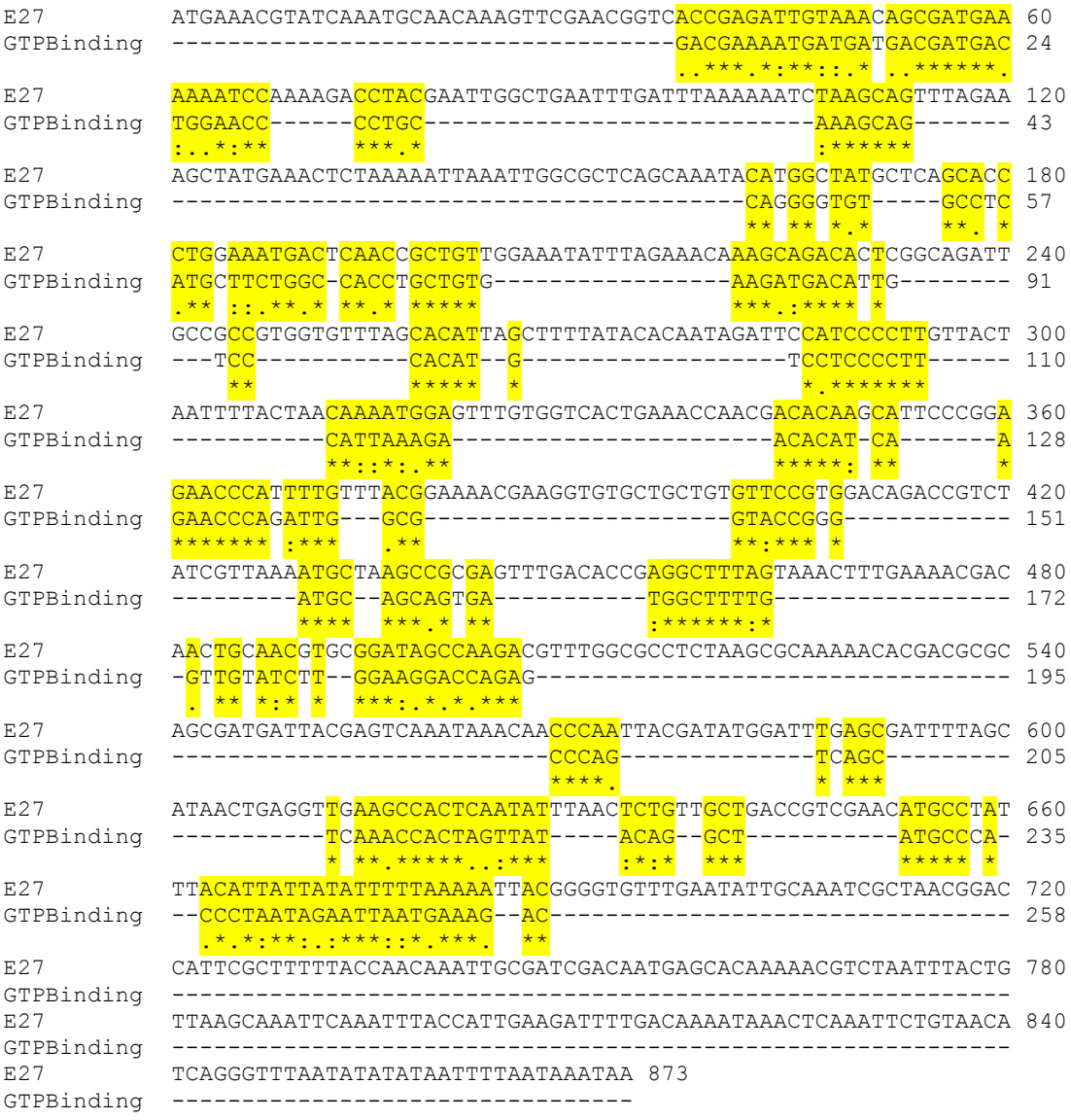
Sequence alignment of E27 and HEAT8 domain

```
E27         ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Heat8       ------------------------------------------------------------
E27         AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Heat8       ------------------------------------------------------------
E27         AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Heat8       ------------------------------------------------------------
E27         CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Heat8       ------------------------------------------------------------
E27         GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Heat8       ------------------------------------------------------------
E27         AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Heat8       ------------------------------------------------------------
E27         GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Heat8       ----CCTTTCTGTG--------ACGAGGTGATGCAGCTG----------------CTTCT 32
                **:** ***          ****.*   .***:****                * ***
E27         ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Heat8       G------------------GAAAATTT------GGGG-----------AATGAGAACGTC 57
            .                  *..*.***      *.**           ::***.****:*
E27         AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Heat8       CACAG----------------------GTCTG------------------------------ 67
            .**:*                      ** **
E27         AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Heat8       ------------------------------------------------------------
E27         ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Heat8       -----------TGAAGCCG--------------CAGATTCTGTCAG-------------- 88
                       *******.              *:*:* ***:*.*
E27         TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Heat8       -----------TGTTT-------------GGTG-----ATATTGCCCTT-GCTATTGGAG 118
                       *.***            ****     *******..:* ****: ***
E27         CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Heat8       GA------------------------------------------------------------ 120
             *
E27         TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Heat8       ------------------------------------------------------------
E27         TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Heat8       --------------------------------
```

Sequence alignment of E27 and importin-α binding domain

```
E27          ATGAAACGTATCAAATGCAACAAAGTTCGAACGGTCACCGAGATTGTAAACAGCGATGAA 60
Importin-α   ------------------------------------------------------------
E27          AAAATCCAAAAGACCTACGAATTGGCTGAATTTGATTTAAAAAATCTAAGCAGTTTAGAA 120
Importin-α   ------------------------------------------------------------
E27          AGCTATGAAACTCTAAAAATTAAATTGGCGCTCAGCAAATACATGGCTATGCTCAGCACC 180
Importin-α   ------------------------------------------------------------
E27          CTGGAAATGACTCAACCGCTGTTGGAAATATTTAGAAACAAAGCAGACACTCGGCAGATT 240
Importin-α   ------------------------------------------------------------
E27          GCCGCCGTGGTGTTTAGCACATTAGCTTTTATACACAATAGATTCCATCCCCTTGTTACT 300
Importin-α   ------------------------------------------------------------
E27          AATTTTACTAACAAAATGGAGTTTGTGGTCACTGAAACCAACGACACAAGCATTCCCGGA 360
Importin-α   ----------------------------ACACT------AACTAAACAGG---------- 16
                                         :****      *** *.***.*
E27          GAACCCATTTTGTTTACGGAAAACGAAGGTGTGCTGCTGTGTTCCGTGGACAGACCGTCT 420
Importin-α   ---------------ACG-AAAATGATGATG-----------ACGATGACTGG------ 42
                           *** **** **:*.**            .**: ***:*.
E27          ATCGTTAAAATGCTAAGCCGCGAGTTTGACACCGAGGCTTTAGTAAACTTTGAAAACGAC 480
Importin-α   ------------------------------------------------------------
E27          AACTGCAACGTGCGGATAGCCAAGACGTTTGGCGCCTCTAAGCGCAAAAACACGACGCGC 540
Importin-α   ------------------------------------------------------------
E27          AGCGATGATTACGAGTCAAATAAACAACCCAATTACGATATGGATTTGAGCGATTTTAGC 600
Importin-α   ------------------------------------------------------------
E27          ATAACTGAGGTTGAAGCCACTCAATATTTAACTCTGTTGCTGACCGTCGAACATGCCTAT 660
Importin-α   ------------------------------------------------------------
E27          TTACATTATTATATTTTTAAAAATTACGGGGTGTTTGAATATTGCAAATCGCTAACGGAC 720
Importin-α   ------------------------------------------------------------
E27          CATTCGCTTTTTACCAACAAATTGCGATCGACAATGAGCACAAAAACGTCTAATTTACTG 780
Importin-α   ------------------------------------------------------------
E27          TTAAGCAAATTCAAATTTACCATTGAAGATTTTGACAAAATAAACTCAAATTCTGTAACA 840
Importin-α   ------------------------------------------------------------
E27          TCAGGGTTTAATATATATAATTTTAATAAATAA 873
Importin-α   --------------------------------
```

Sequence alignment of E27 and RanGTP binding domain



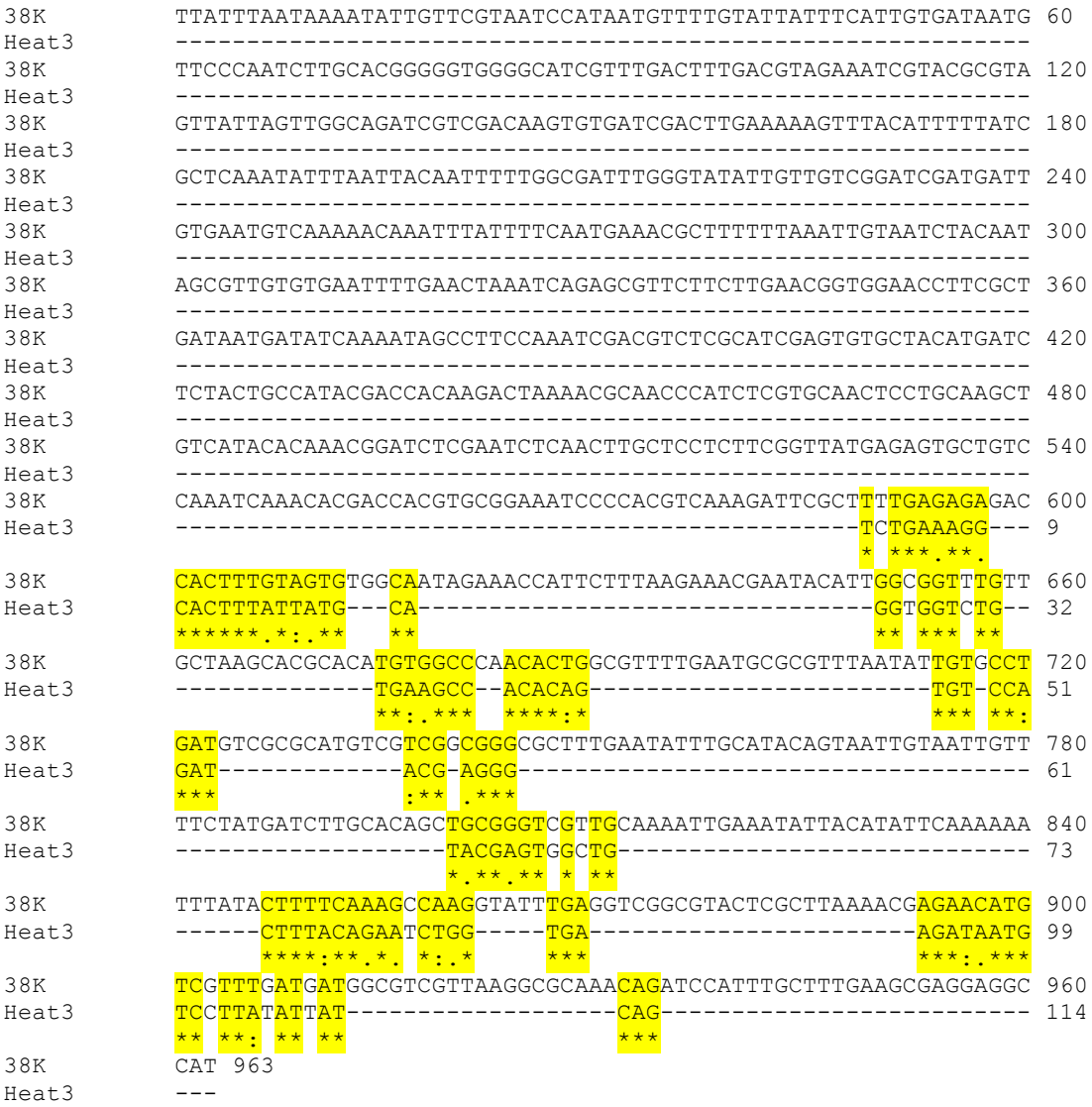
Sequence alignment of 38K and HEAT1 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat1       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat1       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat1       ------------------------------------------------------------
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat1       ------------------------------------------------------------
38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat1       ------------------------------------------------------------
38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat1       ------------------------------------------------------------
38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat1       -------------------------------------------AACCAGTG------------ 8
                                                       *:* ****
38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat1       ------GCCAG-----------------------------------AACTCAT------- 20
                  ****                                    *****.*
38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat1       ------------------------------------TCCTCAGC-----------TGGTGGC 35
                                                *****: *           **  **  *
38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat1       CAAT---GTCAC------------AAACCCCAACAGCACAGA---GCACATGAAGGAGTC 77
            ***:   .:***             ***  ***.**.  **.***    **:  :***..***:*
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat1       GACATTG-------------GAAGCCAT-----------CGGTTATATT----------T 103
             **:***             ***.****           **.:**  ***          *
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat1       GCCAAG-------ATATAGACCCA------------------------------------ 120
            **  ***        **.*.*.**.*
38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat1       ------------------------------------------------------------
38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat1       ------------------------------------------------------------
38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat1       ------------------------------------------------------------
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat1       ------------------------------------------------------------
38K         CAT 963
Heat1       ---
```

Sequence alignment of 38K and HEAT2 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat2       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat2       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat2       ----------------------------------------GATAAA---------------  6
                                                    **:***
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat2       --TCCAATG-------------------------------------------------AGATT 18
              **.***.                                                 :****
38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat2       CTGACTGCCATAATCCAG----------------------------------------- 36
              ***.**  **:**:*.*.
38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat2       -GGGATGAGG------------AAAGAAGAGC--------------------------CT 57
             *  *:**:*              ***  .*****                        **
38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat2       AGTAATAATGTGAAGCTAGCT-----------------GCTACGAATG------------ 88
            ..****.**.*  **..****                 **::***.**
38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat2       ----------------------------------------------CACTCCTGAA---- 98
                                                          .*******.*
38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat2       ------------------------------------CTCATTG-------GAGTTCA--C 113
                                                 ***:* *       ****  *:   *
38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat2       CAAAGCA---------------------------------------------------- 120
            ****  **
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat2       ------------------------------------------------------------
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat2       ------------------------------------------------------------
38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat2       ------------------------------------------------------------
38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat2       ------------------------------------------------------------
38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat2       ------------------------------------------------------------
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat2       ------------------------------------------------------------
38K         CAT 963
Heat2       ---
```

Sequence alignment of 38K and HEAT3 domain



Sequence alignment of 38K and HEAT4 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat4       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat4       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat4       ------------------------------------------------------------
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat4       ------------------------------------------------------------
38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat4       ------------------------------------------------------------
38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat4       ------------------------------------------------------------
38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat4       ----------------------------------------------------------GGAGC 5
                                                                      **  *
38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat4       ACTACAG------------------------------TATCTGGTTCCAATCCT-CACACA 35
            :****:*                                **** ** *.*.**** **..*:
38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat4       G----ACACTAACT---------------------------------------------- 45
            *    ****:***
38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat4       ------AAACAGG--------------------ACGAAAATGAT--------------GAT 66
                  *****  *                  ***:.**:***                **
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat4       GACGATG---------ACTGGAACCCCT---------------------------------- 85
             **  :**         *.*.***.**.*
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat4       GCAAAGCAG------------------CAGGGG----------------------TGTGCCT 107
            **:*****                   *:** *                    *******
38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat4       ---------CATG----CTTCTGGC------------------------------------ 119
                     ****    *  *  ***
38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat4       -------------CACC--------------------------------------------- 123
                         ***.
38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat4       ------------------------------------------------------------
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat4       ------------------------------------------------------------
38K         CAT 963
Heat4       ---
```

Sequence alignment of 38K and HEAT6 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat6       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat6       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat6       ------------------------------------------------------------
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat6       ------------------------------------------------------------
38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat6       ------------------------------------------------------------
38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat6       ------------------------------------------------------------
38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat6       ------------------------------------------------------------
38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat6       ---------------CCACTAG---------------------------TTATACAGGCT 18
                           ****:**                           * .*.**.***
38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat6       ------------------------------------------------------------
38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat6       --------------------------ATGCCCAC-CCTAA---TAGAATTAATGAAAGAC 48
                                     ** ***** *:**   *.*.:**:.:**.****
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat6       CCCAGTGTAGT-TG----------------TTCGAGATACAGCTGCATGG---------- 81
            *.*: ****** **                ** .***:**...*.*** *
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat6       -------------------------ACTG------------------------------ 85
                                     ****
38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat6       ----------------TAGGCAG--------AATTTGTG---------------------- 100
                            *.***.*        ***:* **
38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat6       ----------------AGCTGC----------------------------TTCCTGAA- 114
                            ******                           ***.:.**
38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat6       ------------------------------------------------------------
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat6       ------------------------------------------------------------
38K         CAT 963
Heat6       ---
```

Sequence alignment of 38K and HEAT7 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat7       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat7       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat7       ------------------------------------------------------------
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat7       ----------------------------------------------CAGATCTCTGAT- 12
                                                           *.****  .****

38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat7       GTG--------------------------------------------------------- 15
            ***

38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat7       ---GTTATGG-----------------------------------------CCTCC-CT 29
               ***.**                                          ***  *  **

38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat7       GTTAAGGATG-----------TTCCAAAGC-AC---------------------------- 50
            *:***  ***.           *******  *  **

38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat7       ------------------------------------------------------AGCT 54
                                                                   ****

38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat7       G-------------GGTCTGG----------------------------GGGAGT------ 68
            *             *.***  *                          *.****

38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat7       ------------------------------------------------------------
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat7       ------------------------------------------------------------
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat7       -----------ACAAGAGG-------------------ATGC---------------CCT 83
                        ***:*:**                   ****               ***

38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat7       GATG---------------GCAG------------TTAGCACACTG---GTGGAAGTGTT 113
            ****               **.*            **:*** **:*    **  **  ****

38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat7       ----------------------GGGTGGT--------------------------------- 120
                                   ****  **

38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat7       ------------------------------------------------------------
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat7       ------------------------------------------------------------
38K         CAT 963
Heat7       ---
```

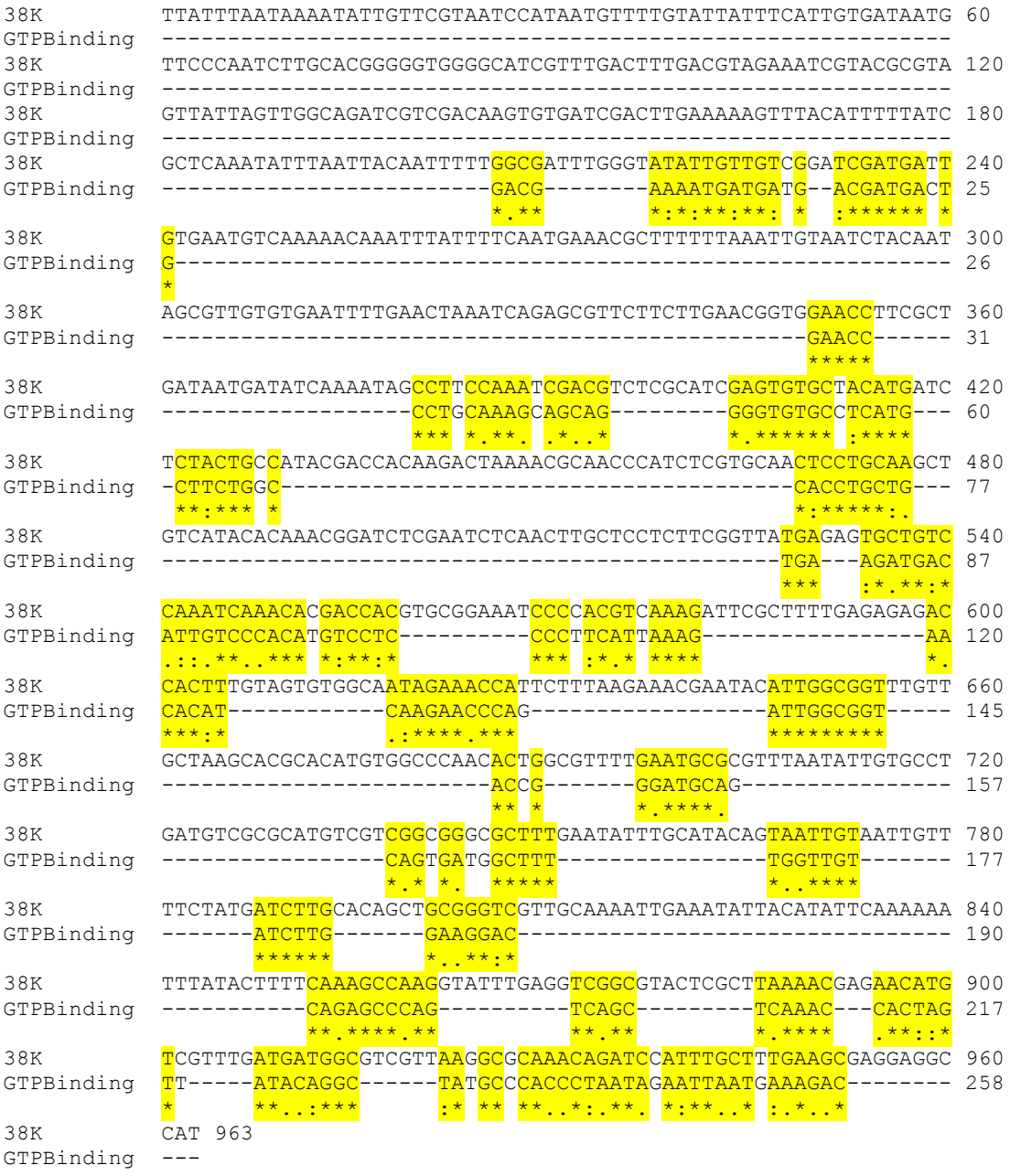
Sequence alignment of 38K and HEAT8 domain

```
38K         TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Heat8       ------------------------------------------------------------
38K         TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Heat8       ------------------------------------------------------------
38K         GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Heat8       ------------------------------------------------------------
38K         GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Heat8       ------------------------------------------------------------
38K         GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Heat8       ------------------------------------------------------------
38K         AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Heat8       ------------------------------------------------------------
38K         GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Heat8       --------------------CCTTTCT------------GTGACGAG-GTGATGCA----- 23
                                ****  *:            * .:****  ***.*.**
38K         TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Heat8       ------------------------------------------GCTGCTTCTGGAAAAT 39
                                                       **:.**  ***  **..*
38K         GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Heat8       ----------------------------------------TTGGGGAATGAGA-------- 52
                                                     **  **  :******
38K         CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Heat8       ----------------ACGT--------CCACAGGTC----------------------- 65
                             ****        **.**  ***
38K         CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Heat8       ----------TGTG------------------------------------------------ 69
                      ****
38K         GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Heat8       ---AAGC-CGCAGAT----------TCTG-------------------------------- 84
               ****  ****  **          :***
38K         GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Heat8       ------------------------------------------TCAGTGTTTG-------- 94
                                                       :****.:***
38K         TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Heat8       ----GTGATATTGCCCT--TGC---------------------------------------- 110
                .****.****.*:  ***
38K         TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Heat8       ----------------------TATTGGAGGA---------------------------- 120
                                  ****  ****:
38K         TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Heat8       ------------------------------------------------------------
38K         CAT 963
Heat8       ---
```

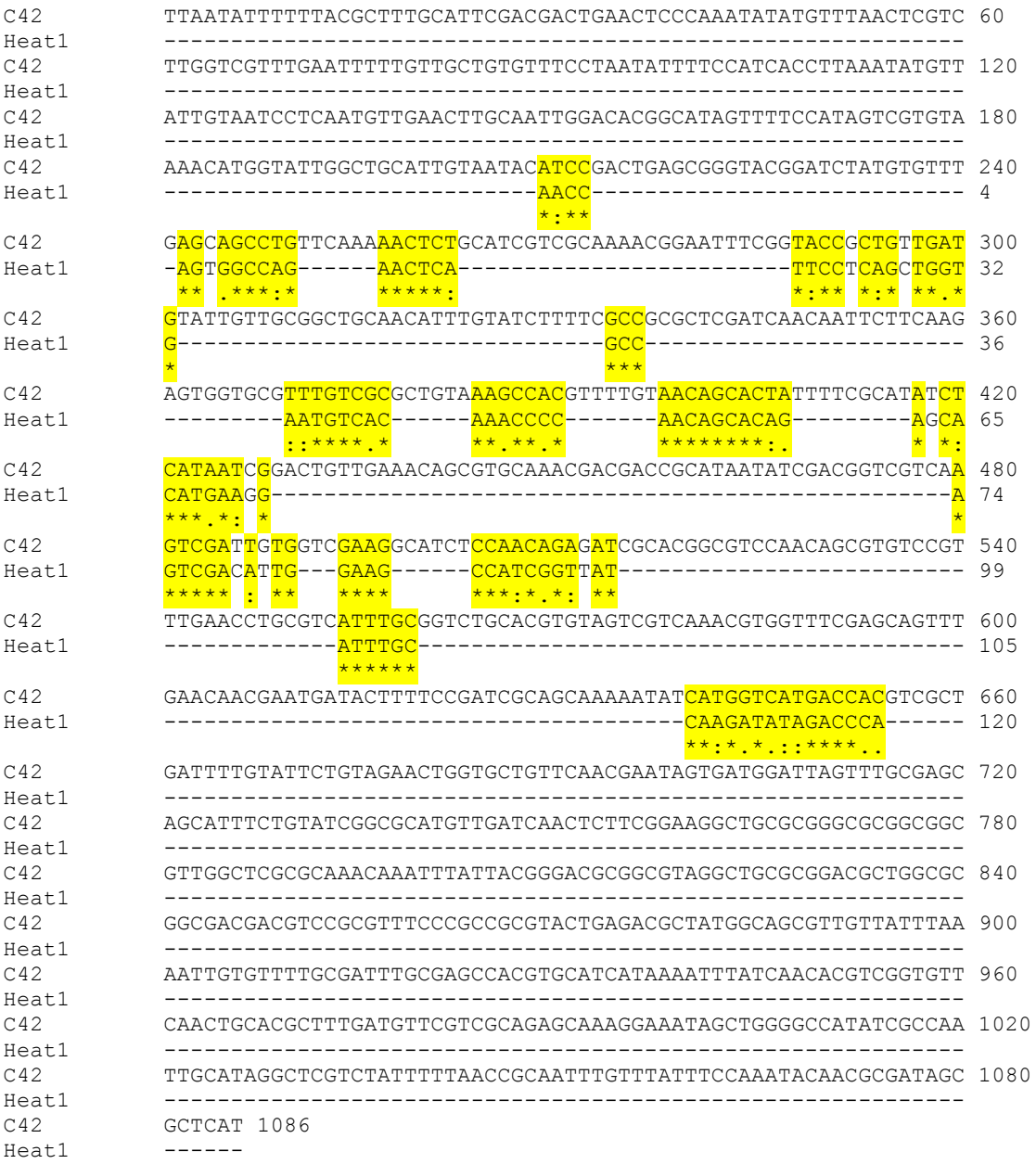
Sequence alignment of 38K and importin-α binding domain

```
38K          TTATTTAATAAAATATTGTTCGTAATCCATAATGTTTTGTATTATTTCATTGTGATAATG 60
Importin-α   ------------------------------------------------------------
38K          TTCCCAATCTTGCACGGGGGTGGGGCATCGTTTGACTTTGACGTAGAAATCGTACGCGTA 120
Importin-α   ------------------------------------------------------------
38K          GTTATTAGTTGGCAGATCGTCGACAAGTGTGATCGACTTGAAAAAGTTTACATTTTTATC 180
Importin-α   ------------------------------------------------------------
38K          GCTCAAATATTTAATTACAATTTTTGGCGATTTGGGTATATTGTTGTCGGATCGATGATT 240
Importin-α   ------------------------------------------------------------
38K          GTGAATGTCAAAAACAAATTTATTTTCAATGAAACGCTTTTTTAAATTGTAATCTACAAT 300
Importin-α   ----------------------------------ACACT---------------------- 5
                                                **.**
38K          AGCGTTGTGTGAATTTTGAACTAAATCAGAGCGTTCTTCTTGAACGGTGGAACCTTCGCT 360
Importin-α   ------------------AACTAAA-CAGGACG---------------------------- 19
                               ******* ***..**
38K          GATAATGATATCAAAATAGCCTTCCAAATCGACGTCTCGCATCGAGTGTGCTACATGATC 420
Importin-α   -AAAATGATG----------------ATGACGATGACTGG---------------------- 42
              *:******.                *:.:*** *:** *
38K          TCTACTGCCATACGACCACAAGACTAAAACGCAACCCATCTCGTGCAACTCCTGCAAGCT 480
Importin-α   ------------------------------------------------------------
38K          GTCATACACAAACGGATCTCGAATCTCAACTTGCTCCTCTTCGGTTATGAGAGTGCTGTC 540
Importin-α   ------------------------------------------------------------
38K          CAAATCAAACACGACCACGTGCGGAAATCCCCACGTCAAAGATTCGCTTTTGAGAGAGAC 600
Importin-α   ------------------------------------------------------------
38K          CACTTTGTAGTGTGGCAATAGAAACCATTCTTTAAGAAACGAATACATTGGCGGTTTGTT 660
Importin-α   ------------------------------------------------------------
38K          GCTAAGCACGCACATGTGGCCCAACACTGGCGTTTTGAATGCGCGTTTAATATTGTGCCT 720
Importin-α   ------------------------------------------------------------
38K          GATGTCGCGCATGTCGTCGGCGGGCGCTTTGAATATTTGCATACAGTAATTGTAATTGTT 780
Importin-α   ------------------------------------------------------------
38K          TTCTATGATCTTGCACAGCTGCGGGTCGTTGCAAAATTGAAATATTACATATTCAAAAAA 840
Importin-α   ------------------------------------------------------------
38K          TTTATACTTTTCAAAGCCAAGGTATTTGAGGTCGGCGTACTCGCTTAAAACGAGAACATG 900
Importin-α   ------------------------------------------------------------
38K          TCGTTTGATGATGGCGTCGTTAAGGCGCAAACAGATCCATTTGCTTTGAAGCGAGGAGGC 960
Importin-α   ------------------------------------------------------------
38K          CAT 963
Importin-α   ---
```

Sequence alignment of 38K and RanGTP binding domain



Sequence alignment of C42 and HEAT1 domain



Sequence alignment of C42 and HEAT2 domain

```
C42         TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat2       ------------------------------------------------------------
C42         TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat2       ------------------------------------------------------------
C42         ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat2       ------------------------------------------------------------
C42         AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat2       ------------------------------------------------------------
C42         GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat2       ------------------------------------------------------------
C42         GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat2       ------------------------------------------------------------
C42         AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat2       ------------------------------------------------------------
C42         CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat2       ------------------------------------------------------------
C42         GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat2       ----------------GATAAATCCAATG-AGAT---------TCTGACTGC-------- 26
                            *.  *:.*****  .  ****         **  .**:**
C42         TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat2       ------------CAT-------------------------AATCCAGG---------GGAT 41
                        ***                         **:*  :**         *  :*
C42         GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat2       GAGGAAAGAAG-----------------AGCCTAGTAATAATG----------TGAAGCT 74
            **.  **.***                 ***.:*.::**.***           *:.***
C42         GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat2       -----------------AGCTG--------CTACGAATG---------------------- 88
                             *.***         *:******.
C42         AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat2       --CACTCCTG------------------AACTCATTGGAG--------------------- 108
              **  *  ***                *****:*  ***.
C42         GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat2       -----TTCACCAAAGCA------------------------------------------- 120
                  **.*  .**.**
C42         GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat2       ------------------------------------------------------------
C42         AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat2       ------------------------------------------------------------
C42         CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat2       ------------------------------------------------------------
C42         TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat2       ------------------------------------------------------------
C42         GCTCAT 1086
Heat2       ------
```

Sequence alignment of C42 and HEAT3 domain

```
C42         TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat3       ------------------------------------------------------------
C42         TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat3       ------------------------------------------------------------
C42         ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat3       ------------------------------------------------------------
C42         AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat3       ------------------------------------------------------------
C42         GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat3       ------------------TCTG-----------AAAG---------------------- 8
                              ****           **.*
C42         GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat3       ------------------------------------GCACTT-------TATTATGCAG- 24
                                               **.**        :***.* **.
C42         AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat3       -GTGGT------------CTGTGAAGCCAC------------------------------ 41
             *****             ****.*******
C42         CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat3       ------------------------------------------------------------
C42         GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat3       ------------------------------------ACAGTGTCCAG------------ 52
                                                 **.* *****.
C42         TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat3       ----------------------------------------ATACGAGGGTACGAGTG---- 69
                                                     *:***:** *:****.
C42         GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat3       -----------GCTGCTTTACAGA-------------ATC-TGGTGAAGATAATGTC-CT 103
                       *.*.****:*.**             *** **** *:** .* *** **
C42         GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat3       TATATT---ATCAG------------------------------------------------ 114
             **:**   :**:*
C42         AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat3       ------------------------------------------------------------
C42         GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat3       ------------------------------------------------------------
C42         GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat3       ------------------------------------------------------------
C42         AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat3       ------------------------------------------------------------
C42         CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat3       ------------------------------------------------------------
C42         TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat3       ------------------------------------------------------------
C42         GCTCAT 1086
Heat3       ------
```

Sequence alignment of C42 and HEAT4 domain

```
C42         TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat4       ------------------------------------------------------------
C42         TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat4       ------------------------------------------------------------
C42         ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat4       ------------------------------------------------------------
C42         AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat4       ------------------------------------------------------------
C42         GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat4       ------------------------------------------------------------
C42         GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat4       ------------------------------------------------------------
C42         AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat4       ------------------------------------------------------------
C42         CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat4       ------------------------------------------------------------
C42         GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat4       ------------------------------------------------------------
C42         TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat4       ------------------------------------------------------------
C42         GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat4       ------------------------------------------------------------
C42         GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat4       ----------------------------------------------------------GG 2
                                                                       *

C42         AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat4       AGCACTACAGTATCTG--------GTTCCAATCCTCA--------CACAG---------- 36
            **** *:*:***** *        *:**.*.** **.        *.*.*

C42         GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat4       --------ACACTAACTAA------ACAGGACG------AAAAT-----GATGATG---- 67
                    .*.*:***:**      **.*****      *...*     **  *.**

C42         GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat4       ----ACGATGACTGG--AACCC--------CTGCAAAGC---AGCAGGGGTGT------- 103
                ****  *:*  *   ::***        ***..*.**   .****  *  ***

C42         AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat4       ----------------------GCCTCATGCTTCT------------------------- 116
                                  ***:*.***:**:

C42         CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat4       ----------------------------------------------GGCCA-----CC-- 123
                                                           *****     **

C42         TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat4       ------------------------------------------------------------
C42         GCTCAT 1086
Heat4       ------
```

Sequence alignment of C42 and HEAT6 domain

```
C42          TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat6        ------------------------------------------------------------
C42          TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat6        ------------------------------------------------------------
C42          ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat6        ------------------------------------------------------------
C42          AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat6        ------------------------------------------------------------
C42          GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat6        ----------------------------------------------CCACT------ 5
                                                           **.**
C42          GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat6        ----------------------------------------------AGTTATACAG- 15
                                                           *.**.*:**.
C42          AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat6        ----------------GCT---ATGCC--------------CAC--------------- 26
                             ***   *:***             ***
C42          CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat6        CCTAATAGAATTAATGAAA---------------GACC---------------------- 49
             *.****.*.* *.:*****               ****
C42          GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat6        ------------------------------------------------------------
C42          TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat6        -------------------------CCAGTGTAGTTG-----------TTCGA------- 66
                                      *..*******  *           *****
C42          GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat6        -----------GATACA---------GCTG----------CATGG---------------- 81
                        *****:         **:*          *****
C42          GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat6        ----------ACTGTAG-------GCAG----------------------AATTTGTGAGC 103
                       :******       **:*                      *.****  ****
C42          AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat6        TGC-TTCCTGAA-------------------------------------------------- 114
             :**  **  ***:*
C42          GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat6        ------------------------------------------------------------
C42          GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat6        ------------------------------------------------------------
C42          AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat6        ------------------------------------------------------------
C42          CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat6        ------------------------------------------------------------
C42          TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat6        ------------------------------------------------------------
C42          GCTCAT 1086
Heat6        ------
```

Sequence alignment of C42 and HEAT7 domain

```
C42          TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat7        ------------------------------CAGATCTC---------------------- 8
                                           *:**:***

C42          TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat7        --------------------TGATGTG------------------------------GTT 18
                                 **.****                              ***

C42          ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat7        ATGG----CCTCCCTGTT------------------------------------------ 32
             **  *    ****..****

C42          AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat7        ----------------------------------------AAGGAT----------- 38
                                                     *.****

C42          GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat7        ---------GTTCCAAAGC----------------------------ACAGCTG----- 55
                      ****.***.*                            **.****

C42          GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat7        ------------------------------------------------------------

C42          AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat7        ------------------------------------------------------------

C42          CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat7        -------GGTCTG----------------------------------------GG------- 63
                    **:***                                        **

C42          GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat7        ----------------------------------------GGAGTACAAGAGGATGCCC-- 82
                                                     **.**.***  **  .**  **

C42          TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat7        ------------------------------TGATG--------------------GCAGTTA 94
                                           **::*                    ******:

C42          GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat7        G-----------------------------------------------------CAC------ 98
             *                                                    ***

C42          GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat7        ------------------ACTGGTG-----------GAAGTGTTGGG-TGGT-------- 120
                                *******            .:****:***. *.**

C42          AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat7        ------------------------------------------------------------

C42          GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat7        ------------------------------------------------------------

C42          GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat7        ------------------------------------------------------------

C42          AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat7        ------------------------------------------------------------

C42          CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat7        ------------------------------------------------------------

C42          TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat7        ------------------------------------------------------------

C42          GCTCAT 1086
Heat7        ------
```

Sequence alignment of C42 and HEAT8 domain

```
C42         TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Heat8       ------------------------------------------------------------
C42         TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Heat8       ------------------------------------------------------------
C42         ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Heat8       ------------------------------------------------------------
C42         AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Heat8       ------------------------------------------------------------
C42         GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Heat8       ------CCT---------------------------------TTCTGTGACGAGG----- 16
                  ***                                 ***  **..**. *
C42         GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Heat8       ----TGATGCAGCTG-------------------------------------CTTCTGG 34
                **:***.****                                     ****:.*
C42         AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Heat8       A----------------------------AAATTTGG------------------------ 43
            *                           *..***  *
C42         CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Heat8       -------GGAATG----------------AGAACGTCCACA-------------------- 61
                   ***.**                 *  .***:**.**
C42         GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Heat8       ------------------------------------------------------------
C42         TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Heat8       ------------------GGTCT----GTGAAGCCG-----------------CAG--- 78
                              *****    ***:**  **                  ***
C42         GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Heat8       ------------------------------------------------------------
C42         GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Heat8       --------ATTCTG---------------TCAGTGTTTGGTGAT-------ATTGC---- 104
                     ******               ***.  *:::*.*****       :****
C42         AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Heat8       --CCTTGCT--ATTGGAGGA---------------------------------------- 120
              *.**  **   **  **.* *
C42         GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Heat8       ------------------------------------------------------------
C42         GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Heat8       ------------------------------------------------------------
C42         AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Heat8       ------------------------------------------------------------
C42         CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Heat8       ------------------------------------------------------------
C42         TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Heat8       ------------------------------------------------------------
C42         GCTCAT 1086
Heat8       ------
```

Sequence alignment of C42 and importin-α binding domain

```
C42          TTAATATTTTTTACGCTTTGCATTCGACGACTGAACTCCCAAATATATGTTTAACTCGTC 60
Importin-α   ------------------------------------------------------------
C42          TTGGTCGTTTGAATTTTTGTTGCTGTGTTTCCTAATATTTTCCATCACCTTAAATATGTT 120
Importin-α   ------------------------------------------------------------
C42          ATTGTAATCCTCAATGTTGAACTTGCAATTGGACACGGCATAGTTTTCCATAGTCGTGTA 180
Importin-α   ------------------------------------------------------------
C42          AAACATGGTATTGGCTGCATTGTAATACATCCGACTGAGCGGGTACGGATCTATGTGTTT 240
Importin-α   ------------------------------------------------------------
C42          GAGCAGCCTGTTCAAAAACTCTGCATCGTCGCAAAACGGAATTTCGGTACCGCTGTTGAT 300
Importin-α   ------------------------------------------------------------
C42          GTATTGTTGCGGCTGCAACATTTGTATCTTTTCGCCGCGCTCGATCAACAATTCTTCAAG 360
Importin-α   ------------------------------------------------------------
C42          AGTGGTGCGTTTGTCGCGCTGTAAAGCCACGTTTTGTAACAGCACTATTTTCGCATATCT 420
Importin-α   ------------------------------------------ACACTA------------- 6
                                                        .*****
C42          CATAATCGGACTGTTGAAACAGCGTGCAAACGACGACCGCATAATATCGACGGTCGTCAA 480
Importin-α   ---------ACT----AAACAG---------GACGAAA--ATGATGATGACG------AT 36
                      ***    ******         *****..  **.**.: ****      *:
C42          GTCGATTGTGGTCGAAGGCATCTCCAACAGAGATCGCACGGCGTCCAACAGCGTGTCCGT 540
Importin-α   GAC-----TGG---------------------------------------------------- 42
             *:*     ***
C42          TTGAACCTGCGTCATTTGCGGTCTGCACGTGTAGTCGTCAAACGTGGTTTCGAGCAGTTT 600
Importin-α   ------------------------------------------------------------
C42          GAACAACGAATGATACTTTTCCGATCGCAGCAAAAATATCATGGTCATGACCACGTCGCT 660
Importin-α   ------------------------------------------------------------
C42          GATTTTGTATTCTGTAGAACTGGTGCTGTTCAACGAATAGTGATGGATTAGTTTGCGAGC 720
Importin-α   ------------------------------------------------------------
C42          AGCATTTCTGTATCGGCGCATGTTGATCAACTCTTCGGAAGGCTGCGCGGGCGCGGCGGC 780
Importin-α   ------------------------------------------------------------
C42          GTTGGCTCGCGCAAACAAATTTATTACGGGACGCGGCGTAGGCTGCGCGGACGCTGGCGC 840
Importin-α   ------------------------------------------------------------
C42          GGCGACGACGTCCGCGTTTCCCGCCGCGTACTGAGACGCTATGGCAGCGTTGTTATTTAA 900
Importin-α   ------------------------------------------------------------
C42          AATTGTGTTTTGCGATTTGCGAGCCACGTGCATCATAAAATTTATCAACACGTCGGTGTT 960
Importin-α   ------------------------------------------------------------
C42          CAACTGCACGCTTTGATGTTCGTCGCAGAGCAAAGGAAATAGCTGGGGCCATATCGCCAA 1020
Importin-α   ------------------------------------------------------------
C42          TTGCATAGGCTCGTCTATTTTTAACCGCAATTTGTTTATTTCCAAATACAACGCGATAGC 1080
Importin-α   ------------------------------------------------------------
C42          GCTCAT 1086
Importin-α   ------
```

Sequence alignment of C42 and RanGTP binding domain