The Role of Terminal Protein in Adenovirus Nuclear Delivery

by

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BTech (hons)

Submitted in total fulfilment of the requirements for the degree of
Doctor of Philosophy

Drug Discovery Biology
Monash Institute of Pharmaceutical Sciences
Monash University

July 2013

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Summary

The future of gene therapy is dependent on the development of vectors that can selectively and efficiently deliver therapeutic DNA to target cells *in vivo* with minimal toxicity. Virus vectors are the most widely used transducing systems. However, there are many ethical and safety concerns associated with the use of viruses in humans. Non-viral vectors are more suitable because of the lack of a specific immune response; but these are currently inefficient due to the intracellular barriers between nucleus and extracellular milieu preventing sufficient therapeutic DNA delivery to the cell nucleus. Non-viral vectors must be tailored to overcome these barriers for sufficient delivery. One of the most substantial barriers for DNA delivery is the nuclear envelope which is a double membrane surrounded the nucleus. The vector must pass the nuclear envelope to enter the nucleus.

Research on viral tropism suggests that some viruses have evolved means of interaction with nuclear transport systems, and there is a good chance that active transport can be utilized for delivery of plasmid DNA. There is a widespread interest in the use of adenoviruses primarily because of their ability to efficiently deliver double-stranded DNA to the nucleus. In addition, their large genome allows for extensive modification and incorporation of therapeutic genes. The basic understanding of how adenovirus nuclear delivery systems work is fundamental to ensure further development and enhancement of the non-viral gene therapy.

This PhD project investigated into the mechanisms of adenovirus nuclear delivery by examining the role of the adenovirus terminal protein, a protein that is covalently coupled to the 5' ends of the adenoviral DNA.

To identify the encoded NLS on terminal protein (Chapter 4), PCR-based approaches were used to amplify several truncated terminal protein derivatives and to create site-directed terminal protein mutants. The products were inserted into mammalian expression plasmids pcDNA6.2/C-EmGFP (fused in frame to the C-terminus of the EmGFP) and

pcDNA6.2/N-YFP (fused in frame to the N-terminus of the YFP) using the Gateway recombination system. Transfection of mammalian cells (HeLa and COS-7 cells) allowed evaluation of the extent of nuclear delivery of each fusion protein by determining the nuclear/cytoplasmic fluorescence ratio F(n/c). Two NLSs were identified; the bipartite sequences (MRRRR^{370} and PVRRRRRRV^{390}) and monopartite sequence (PGARPRGRF^{671}). The arginine residues were found to be critical part of the bipartite NLS. The PGARPRGRF^{671} sequence was also shown to have a nucleolus delivery property when not masked by the flanking regions.

To identify the nuclear import pathway of terminal protein (Chapter 5), ALPHAScreen® assay was carried out for the terminal protein and terminal protein derivatives encoding NLSs. It was found that the nuclear delivery of terminal protein is independent of the functional microtubules and the two NLSs employed different pathways. This suggested that TP is not involved in the intracellular trafficking of Adv. The nuclear delivery of the bipartite NLS (MRRRR^{370} and PVRRRRRRV^{390}) is dependent on the IMPα/β mediated pathway, whereas the monopartite NLS (PGARPRGRF^{671}) employs alternative nuclear trafficking pathway, most likely IMPβ mediated pathway. It was identified that the negatively rich domain, proximal to the bipartite NLS, reduces the binding specificity between NLS and its IMP binding partner. It also influences the binding affinity of NLS with IMP receptors by either masking or enhancing NLS-IMP interaction.

Declaration

This thesis contains no material that has been accepted for the award of any other degree in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is given in the text.

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Vidyaporn Ann Chailertvanitkul

List of Publications

Publications

Chailertvanitkul V.A. and Pouton C.W., "Adenovirus: A Blue Print for Non-Viral Gene Therapy", Current Opinion in Biotechnology, 21 (5), 627-632, 2010

Abstracts for Conference

Chailertvanitkul V.A. and Pouton C.W., "Identification of Nuclear Localisation Signal Sequences in Adenoviral Terminal Protein", The 5th Australian Health and Medical Research Congress, 2010

Chailertvanitkul V.A., Mantamadiotis T. and Pouton C.W., "An Investigation of the Nuclear Localisation Signal in Adenovirus Terminal Protein", The 11th Annual Meeting of American Society of Gene Therapy, 2008

Acknowledgements

First, I would like to thank my supervisors Professor Colin Pouton and Dr. Theo Mantamadiotis. Thank you Colin for the opportunity to work on this interesting project, for your continuous advice and guidance, but most importantly for finding the time out of your busy schedule to read my thesis. Thank you Theo for your support and for reading part of my thesis even when you were in Greece.

I would also like to thank Fred, Helen and Paul for your invaluable suggestions; your expertise has certainly helps shaped this PhD. Thank you also to Adrian, Jian and Rebecca for making the ordering process a smooth one.

I am forever grateful for all the friends I have made throughout my candidature. Anupa, thank you for being a great friend and an awesome office buddy! To Michelle and Shannen, thank you for making MIPS a great place to work; I enjoyed our many discussions throughout the years and look forward to many more in the future. Christian, Nick, Ken and Yu Hua thank you for making lab 111 a fun place to be. It was definitely nice to not be the only one left in the lab at 10pm or to be alone in the weekend!

To Simer, thank you for being my gym and chocolate buddy. I miss our weekly BodyPump class and our frequent chocolate visits – I look forward to tasting all the best hot chocolate Melbourne has to offer together when you move back later this year. Thanks also for reading part of my thesis and for being an example that there is a better life at the end of the tunnel!

Angela, thank you for being such a great friend and for making my PhD life an enjoyable one. I miss our regular lunches and our walk home together. Thank you for always being there and for your support. Well done for completing your PhD and all the best with the job hunting.

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Abbreviations

Adv	Adenovirus
AD-293	Human embryonic kidney cell lines
ALPHAScreen	Amplified luminescent proximity homogeneous assay
ARM	Armadillo repeats
BTV	Blue tongue virus
BSA	Bovine serum albumin
Caco-2	Human epithelial colorectal adenocarcinoma cell lines
CAR	Coxsackie and adenovirus receptor
COS-7	African green monkey kidney cell lines
CRM1	Chromosomal region maintenance-1
DAB	Polypropylenimine dendrimer
DBP	DNA binding protein
DEL	Deletion
DEST	Destination
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DU145	Human prostate cancer cell lines
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
GDP	Guanosine-diphosphate

GFP	Green fluorescent protein
GTP	Guanosine-triphosphate
GST	Glutathione S-transferase
Hela	Human cervical carcinoma cell lines
HHV-6	Human herpes virus 6
HVR	Hyper variable region
IBB	Importin-β binding domain
IMP	Importin
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITR	Inverted terminal repeat
LB	Luria broth
LMB	Leptomycin-B
MAPKK	Mitogen-activated protein kinase kinase
MHSC	Major histone compability complex
MT	Microtubule
MTOC	Microtubule organizing centre
MUT	Mutation
NCZ	Nocodazole
NE	Nuclear envelope
NES	Nuclear export sequence
NF-κB	Nuclear factor kappa B
NLS	Nuclear localisation sequence
NPC	Nuclear pore complex
NTF-2	Nuclear transport factor-2
NuLS	Nucleolar localisation signal

Nup	Nucleoporin
OD	Optical density
ORF	Open reading frame
OTCD	Ornithine transcarbamylase deficiendy
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PC-3	Human prostate cancer cell lines
PEI	Polyethylenimine
PEG	Polyethylene glycol
Pol	Polymerase
pTP	Pre-terminal protein
p53	Tumour suppressor p53 protein
RanGAP	Ran GTP activating protein
RanGEF	Ran guanine nucleotide exchange factor
RGD	Arginine-glycine-aspatic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV40	Simian virus 40
T-ag	Large tumour antigen
TAE	Tris-acetate-EDTA
TGF	Transforming growth factor
TP	Terminal protein

T98G	Anchorage-independent human tumour cell lines
WT	Wild type
YFP	Yellow fluorescent protein

CHAPTER 1

THEORY

CHAPTER 1   THEORY

1.1 Introduction

The future of gene therapy is dependent on the development of vectors that can selectively and efficiently deliver therapeutic DNA to target cells with minimal toxicity. By 2012, according to the Clinical Trials Worldwide Database, over 400 protocols (Appendix A) had been approved for clinical trials of gene therapy using attenuated adenoviral vectors, 255 of which were open, but only 13 of which were Phase III trials (Appendix B).

Widespread interest in the use of Adenoviruses (Adv) developed primarily because of their ability to deliver double-stranded DNA to the nucleus efficiently. In addition, their large genome allows for extensive modification and incorporation of therapeutic genes. Although Adv vectors remain promising, clinical development of Adv constructs has been disappointing. Development into Phase III has been limited due to: 1) the incidence of an unexpected severe inflammatory reaction (Lehrman, 1999; Marshall, 1999; Tomanin *et al.*, 2004); 2) the fact that, once recognised by the immune system, Adv vectors are cleared rapidly which prevents repeat dosing of the same product; 3) the safety concerns common to all viral vectors (i.e. rare but finite incidences of DNA recombination that take place during biosynthesis in packaging cells or even *in vivo* after administration (Somia *et al.*, 2000; Tomanin *et al.*, 2004)) due to the chance that their manufacture or use could generate replication-competent viruses. During the past 20 years research on the molecular mechanisms of cell entry of Adv has continued. The basic understanding of how Adv delivery systems work, how they overcome each biological barrier, is fundamental to ensure further development and enhancement of the non-viral gene therapy. In short, the next-generation non-viral systems will need attributes which mimic Adenoviral DNA delivery.

1.2 Gene Therapy

Gene therapy is a technique which uses genes as medicine to treat or prevent diseases. It involves transferring a therapeutic gene into specific cells of an individual for genetic repair, thus gene therapy may replace a defect gene or introduced a new gene whose function is to correct or modify the clinical course of a condition. Gene therapy holds great promises for the future as means for effective treatment of genetic diseases. The success of gene therapy is primarily based upon the sufficient gene expression which is directly proportional to the efficiency of therapeutic gene nuclear delivery. There are two approaches in delivering DNA and these are through the use of viral and non-viral vectors. Both of the approaches have their advantages and disadvantages which are summarised in Table 1-1.

Table 1-1: Comparison between viral and non-viral vectors (Wang *et al.*, 2012)**.**

Viral Vectors	Advantages
	1. Transduction efficiency is high
	2. Natural tropism confers the capability for infection of many cell types
	3. Virus has intrinsic mechanism for endosomal escape.
	4. Virus evolved natural mechanism for nuclear import of genes.

Viral Vectors	Disadvantages
	1. Immune response is strong and multiple-injections are limited.
	2. Can cause chromosomal insertion and proto-oncogene activation.
	3. Complication in its construction and production.
	4. Can only carry limited sized genes.
	5. Can cause toxicity and may be contaminated with live virus.

Non-Viral Vectors	Advantages
	1. Immunogenicity is relatively low.
	2. No risk of chromosomal insertion.
	3. Ease of synthesis, and quality control in mass production.
	4. Can carry large-sized DNA.
	5. Can be functionalised for targeting, endosomal escape and nuclear import.

Non-Viral Vectors	Disadvantages
	1. Transfection efficiency is low.
	2. At high dose, current vectors show toxicity.
	3. Lack of intrinsic tropism.
	4. Lack of intrinsic mechanism for endosomal escape.
	5. Lack of intrinsic mechanism for nuclear import of genes.

Viral vectors can offer sufficient nuclear DNA delivery, but they can also initiate immunological responses and may introduce ethical issues and safety risks (Ioannou *et al.*, 2005). Due to these obstacles, there has been an increased interest in non-viral gene delivery system as the vectors carry no infectious or mutagenic capabilities and as a result no safety complications to overcome. Currently there is no non-viral gene delivery system which could delivery DNA as efficiently as virus vectors, thus much of the non-viral delivery research has been focusing on the development of vectors capable of achieving sufficiently high levels of target cell-specific transgene expression (Lehn *et al.*, 1998; Wang *et al.*, 2012).

1.3 **Vectors for Gene Therapy**

1.3.1 Viral system

Viruses are extremely efficient at introducing nucleic acids into the nucleus of the cells as part of their replication cycles. However, virus vectors also pose several obstacles including insertional mutagenesis and toxicity problems.

The viral gene therapy clinical trial started as early as 1970 but without much success and hit major drawback in 1999 when a patient died while admitting in the Adenovirus clinical trials for partial ornithine transcarbamylase deficiency (OTCD) which lead to an immediate termination of clinical trials employing similar route and set back several other Adenovirus-based trials (Ioannou *et al.*, 2005; Kaffman *et al.*, 1998; Marshall, 1999; Somia *et al.*, 2000)..In 2000, viral gene therapy had its first break through when Alain Fischer and colleagues reported successful long term cure using the retroviral gene therapy for twenty children with lethal severe combined immunodeficiency known as SCID-X1 (Ioannou *et al.*, 2005; Kaffman *et al.*, 1998). However, five patients subsequently developed leukaemia-like disorders, resulting in one death, following the insertional mutagenesis caused by the retrovirus vectors (Ioannou *et al.*, 2005; Kaffman *et al.*, 1998; Somia *et al.*, 2000). These fatalities raised many ethical and safety concerns, resulting in difficulties in continuation of viral gene therapy research and many researchers chose to change direction and concentrate on the alternative delivery system (Glover *et al.*, 2005; Ioannou *et al.*, 2005; Kaffman *et al.*, 1998; Li *et al.*, 2000; Somia *et al.*, 2000).

1.3.2 Non-viral system

Non-viral gene delivery attracts many interests as it does not involve in ethical barriers or safety concerns faced by viral gene delivery. The developments of non-viral delivery systems have been following two major approaches, physical and chemical approaches.

The physical approaches do not rely on the carrier to deliver therapeutic DNA but instead employ physical force to permeate the cell membranes to facilitate intracellular gene

transfer. These included needle injection of naked DNA, electroporation, gene gun, ultrasound and hydrodynamic delivery (Campbell *et al.*, 2005; Wells, 2004). Although the physical methods are simple and have met with success *in vitro* and in small animals, the same high level of delivery efficiency could not be achieved *in vivo* and surgical procedure would be needed for localised gene transfer in human. Due to these limitations, the chemical approaches are the more frequently studied strategy for the non-viral delivery system.

The chemical approach is based on the formulation of condensed DNA particles using cationic lipids, cationic polymers or lipid/polymer hybrids, for subsequent delivery by the cells intracellular trafficking mechanism (Chan *et al.*, 2012; Kiselev *et al.*, 2012; Niidome *et al.*, 2002; Plank *et al.*, 1996). Similarly with the physical methods, the chemical methods have been found to efficiently delivered DNA *in vitro* but the same success have not been met in the clinical trials. Understanding the barriers that limited the gene delivery *in vivo* would be essential to further enhance effective delivery system.

1.4 Barriers in Gene Delivery

A method of achieving systemic gene delivery would be clinically valuable. Although viral vectors mediate high levels of transgene expression, clinical protocols usually require site-specific injection or *ex vivo* techniques due to toxicity and efficient blood clearance. The inefficiency in nuclear delivery of non-viral vectors is due to series of barriers existing in the route from the site of injection to the cell nucleus which consequently reduces the amount of therapeutic (usually a DNA plasmid) reaching its destination. These barriers can be considered as extracellular and intracellular barriers. The significance of each barrier will depend on the route of administration of the therapeutic DNA and on the location of the target cells. Barriers and the current measures being taken to overcome them are described in this section.

1.4.1 Extracellular barriers

1.4.1.1   Packaging of therapeutic drug (nucleic acids)

Primary challenge of systemic delivery of therapeutic drugs targeted at the nuclear compartment is the hostile extracellular environment which presents a number of barriers: extreme pH (in the gastrointestinal tract), enzymes (proteases and nucleases) and immune defence; which can cause the nucleic acid degradations. For effective transportation of therapeutic drugs, nucleic acids are required to be protected from such environmental factors. One way of achieving this is through the complexation with polymers, such as the cationic polyethylenimine (PEI) (Wang *et al.*, 2012). DNA can be condensed with PEI via the electrostatic interaction between the negatively charged nucleic acids and positively charged PEI (Biri *et al.*, 2010). PEI/DNA complexes become positively charged which promote interaction with negatively charged cell surface for cell binding, resulting in higher levels of gene expression (Wang *et al.*, 2012).

Although these DNA/polymer complexes showed great promise *in vitro,* they were shown to accumulate in organs with very fine capillary structures, such as skin, lung, and the intestine *in vivo* (Pouton, 2001; Pouton et al., 2001). It is thought that this is due to aggregation between serum proteins and the macromolecule (Pouton *et al.*, 2001). Currently research is being conducted to overcome this dilemma, including the use of poly(ethylene glycol) (PEG) as a protective shield on the particle surface.

1.4.1.2   Circulation

During systemic administration, it was shown that both naked DNA and lipoplexes had rapid hepatic clearance due to phagocytosis by Kupffer cells (Pouton, 2001; Pouton *et al.*, 2001; Zhang *et al.*, 2005). Plasma proteins interact with hydrophobic particles causing their removal from circulation. One of the ways to prevent this systemic removal is the addition of hydrophilic groups on the surface of the particles. Modification of non-viral vectors such as lipoplexes with hydrophilic molecules like PEG, creates a hydrophilic atmosphere around the particle surface, causing steric hindrance between the plasma proteins and the delivery

vectors (Muzykantov *et al.*, 2011). PEG plays an important role in preventing interference from blood components by reducing the particle-plasma protein interactions and also by preventing particle aggregation (Wang *et al.*, 2012).

However, the complication involved in using PEG is achieving binding and uptake into target cells, or achieving its removal from the particle surface once the delivery vector reaches the intended destination. Some of the strategies employed include: pH-responsive PEG cleavage, enzymatic PEG cleavage and the use of diffusible PEG conjugates, however to date there is no single method which can be used to remove PEG successfully.

1.4.1.3   *Targeting the correct cell type*

Most non-viral gene delivery system interacts non-specifically with the cell surface. To increase the interaction, hence the delivery efficiency, a ligand on the cell surface needs to be targeted. There are a number of considerations when choosing a ligand with which to target a gene delivery vector. The ligand receptor should either be unique to the target cell or be upregulated. In diseased conditions, such as in cancer, there is an increased expression of receptors and/or antigens that can be targeted with specific ligands such as antibodies, peptides or proteins that recognise and bind to the cells of interest resulting in high transfection efficiency (Ogris *et al.*, 2002).

One of the main potential targets for gene delivery is the integrin $\alpha_v\beta_3$ due to its high expression level in cancer cells and tumour vasculatures (Wang *et al.*, 2012). Arginine-glycine-aspatic acid (RGD) peptide is a classic targeting ligand for integrin $\alpha_v\beta_3$ (Wang *et al.*, 2012). When introduced to PEI/pDNA polyplexes, a cluster of RGD ligands were shown to increase transfection efficiency in HeLa cells in comparison to unmodified PEI/pDNA polyplexes (Ng *et al.*, 2009). Transferrin receptors, which are found to overexpress in cancer cells, also showed a great promise as a potential ligand. Koppu and colleagues had successfully designed a conjugation of transferrin with polypropylenimine dendrimer (DAB) which improved the targeted delivery of galactosidase-encoding plasmid in both A431 and T98G cell lines (Mah *et al.*, 2002). A dual targeting gene vector composing of transferrin

with RGD peptide and PEG/PEI polyplexes were also shown to increase reporter gene expression in both DU145 and PC3 cells, 60 and 20-fold respectively (Cho *et al.*, 2003).

Unfortunately, increased delivery efficiency does not translate to increased expression efficiency which is an ultimate goal for non-viral delivery system. Once the delivery system enters the target cells, it has to face multiple intracellular barriers before arriving at the final destination of the cell nucleus. These intracellular barriers are discussed below in Section 1.4.2.

1.4.2 Intracellular barriers

The success of gene delivery is dependent on the expression of therapeutic gene which could only be achieved when DNA reaches the nucleus of target cell. A diagram outlining these barriers is shown in Figure 1-1.
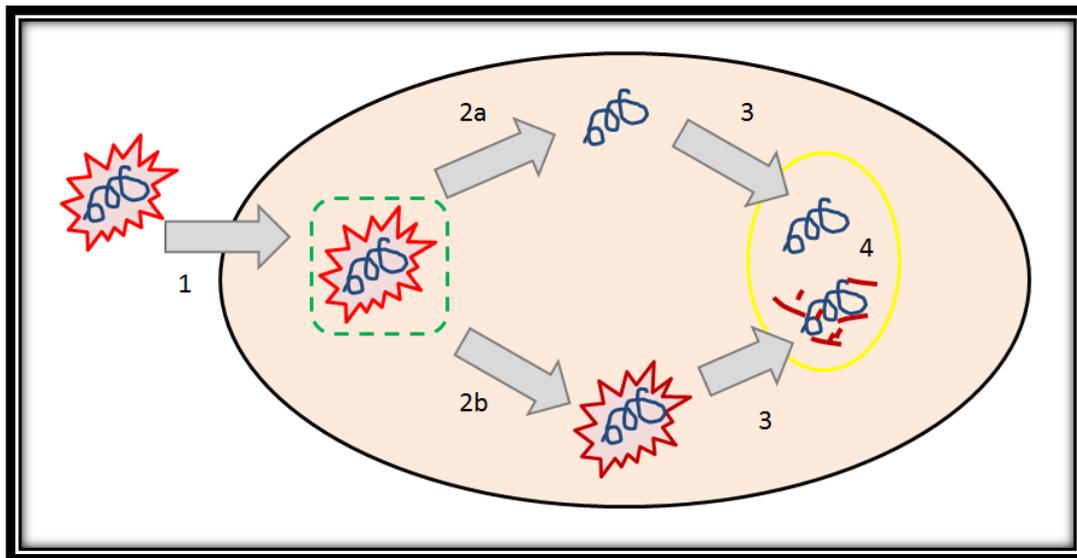
Figure 1-1: Intracellular barriers for gene delivery system. DNA is taken up into the cell by endocytosis (1) and may be released as either naked DNA (2a) or a complex (2b). Naked DNA is more vulnerable to the cytoplasmic nucleases which could lead to its degradation. DNA complexes or naked DNA are required to translocate through the cytoplasm (3) to the nucleus. The DNA then needs to cross the nuclear membrane to reach nucleus (4) and be available for transcription.

1.4.2.1   Cell membrane

Typically the cytoplasmic membrane of a cell is impermeable to nucleic acids due to their large size and hydrophilic nature. Strategies are required to effectively facilitate the transport of nucleic acids which may be through either physical approaches (the use of gene gun, microinjections and electroporation) or chemical approaches (i.e. development of vectors).

Although all of the physical approaches enable nucleic acid to enter cells, their clinical application is limited due to their invasive nature and potential damage to the overall structure of the cells. Chemical approaches have produced promising outcomes with the development of lipoplex/polyplex vectors. Lipoplexes and polyplexes are often formulated into particles with net positive charges, favouring endocytosis through electrostatic interaction with the negatively charged heparin sulphate proteoglycan found on the surface of the cell (Belting *et al.*, 2005; Ruponen *et al.*, 2003; Wang *et al.*, 2012). Although chemical vectors are able to cross the cell membrane with ease, there are complications with using polymers as discussed in Section 1.4.1.1 and furthermore, simple delivery systems based on complexes of plasmids with polycations are unable to efficiently overcome the next barrier faced.

1.4.2.2   Endosome escape

Once lipoplexes or polyplexes have entered the cells via the endocytotic pathway, endosomal entrapment and subsequent lysosomal degradation is a major barrier that limits the efficiency of gene delivery. Gene delivery vectors, therefore, need to be designed with endosomal-escape in mind.

Many strategies have been developed to mimic viral mechanism for endosome destabilisation in an attempt to facilitate endosome escape, mainly mimicking the fusogenic peptide which is responsible for endosomal escape in influenza virus (Kakudo *et al.*, 2004; Mah *et al.*, 2002). There have also been reports of the design of synthetic

peptides based on this fusogenic peptide. The GALA (glutamic-ananine-leucine-aninine) was synthesised with pH-sensitive fusogenic properties and showed to enhanced transfection efficiency of plasmid DNA and siRNA duplex *in vitro* and *in vivo* (Hatakeyama et al., 2009; Kakudo et al., 2004).

1.4.2.3   Nuclear Import

The last major barrier for gene delivery system, and a focus for this PhD project, is the DNA delivery from cytoplasm across the nuclear envelope (NE) into the nucleus. The NE is a nuclear import barrier for most macromolecules, unless they are below 45kDa in molecular weight, which is not likely to be the case for gene delivery systems or macromolecular therapeutic drugs (Talcott *et al.*, 1999; Wente, 2000).

Attempts to achieve nuclear delivery of DNA have been reported, making use of the native nuclear import machinery. The nuclear delivery mechanism is discussed in detail in Section 1.5. It is known that a nuclear localisation sequence (NLS) is required for large proteins to be transported through nuclear pore complexes (NPCs) into the nucleus (Poon *et al.*, 2005; Ribbeck *et al.*, 2001). Identification of NLSs has encouraged the design of non-viral gene vectors to improve nuclear targeting properties. NLSs have been attached to the DNA vectors via both covalent and non-covalent conjugation. The approach employed for non-covalent linkage is the electrostatic interaction between a positive NLS and negative DNA (Henkel *et al.*, 1992; Ludtke *et al.*, 1999; Pouton *et al.*, 2007). However there are three main issues with this approach: the positively charged NLS has been shown to interfere with the electrostatic interaction between cationic polymer and DNA complex; the electrostatic interactions may sterically hinder the NLS limiting its binding to importins; and due to the reversible nature of the interaction the NLS may dissociate from the DNA before the complex reaches the nucleus (Wang *et al.*, 2012). Due to these problems, a strong covalent-bond linkage is preferred although the chemistry involved may be complicated. A major issue for this approach, however, is that the chemical modifications may interfere and reduce DNA transcription activity. To overcome this hurdle, a peptide nucleic acid (PNA) was developed as a bi-functional linker between NLS and DNA. The SV40 NLS-PNA-DNA tertiary complex was successfully imported into the nucleus both *in vivo* and *in vitro*

without influencing the DNA transcription activity (Brandén *et al.*, 2001; Lars *et al.*, 1999; Sforza *et al.*, 2010).

1.5 **Nuclear Delivery**

1.5.1 Mechanisms of nuclear transport

The idea of selective nuclear entry was first introduced by Bonner in 1975 when an experiment showed that small proteins and larger proteins that were known to act in the nucleus, accumulated in the nucleus when microinjected into the cytoplasm of *Xenopus* oocyte, while very large or cytosolic proteins remained in the cytoplasm (Bonner, 1975a; Bonner, 1975b). It was not until 1980s when an electron micrographs showed proteins and gold-labelled RNA moving through NPCs that the mechanism by which proteins were translocated into the nucleus was discovered, although the concept of NPCs was first described back in 1962 (Dworetzky *et al.*, 1988; Feldherr, 1962; Feldherr *et al.*, 1984). An introduction to nuclear import is given here and this is developed further in Chapter 5.

The nucleus is separated from the cytoplasm by the nuclear envelope (Zhao *et al.*, 1988). Nuclear import and export of both proteins and nucleic acids are controlled by the NPC (Zhao *et al.*, 1988). The NPC is a large 125 MDa multi-protein structure, spanning across nuclear envelope, which extends into cytoplasm and nucleoplasm (Hoelz *et al.*, 2011; Stoffler *et al.*, 1999). The diameter of the pore is approximately 120 nm with a 9 nm aqueous channel in the centre where passive diffusion of proteins smaller than 45kDa can occur (Hoelz *et al.*, 2011; Jamali *et al.*, 2011; Paine *et al.*, 1975; Strambio-De-Castillia *et al.*, 2010). For a detailed structure of NPC refer to Section 5.2.1 of Chapter 5.

Nuclear import of large proteins is dependent on nuclear localisation signals. The process of nuclear delivery can be operationally separated into 2 steps; (1) the energy-independent attachment of the transport ligand to the cytoplasmic face of the NPC and (2) the energy-dependent translocation (Greber *et al.*, 1997).

1.5.2 Nuclear Localisation Signal

Nuclear localisation signals are short peptide motifs responsible for binding to their cytoplasmic receptors, referred to as importins (IMPs) or karyopherins, which facilitate the nuclear import of proteins (Chen *et al.*, 1996; Chowdhury, 2009; Christophe *et al.*, 2000; Dingwall *et al.*, 1991; Greber *et al.*, 2003; Kanno *et al.*, 2010; Ludtke *et al.*, 1999; Moroianu, 1999; Smith *et al.*, 1998; Strambio-De-Castillia *et al.*, 2010).

Most of the identified NLSs are referred to as classical NLSs and can be categorised into two classes; monopartite and bipartite NLS. The monopartite NLSs have a single cluster of basic amino acid residues and can be divided into two types, one with at least four consecutive basic amino acids (represented by SV40 large antigen NLS: PKKKRKV) and one with only three basic amino acids (putative consensus: K(K/R)X(K/R), exemplified by the c-Myc NLS: PAAKRVKLD) (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007). For the bipartite NLS, the putative consensus sequences is defined by (K/R)(K/R)$X_{10\text{-}12}$(K/R)$_{3/5}$, where (K/R)$_{3/5}$ represents at least three of either lysine or arginine of five consecutive amino acids (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007).

However, there are many experimentally identified NLS that do not match any of the classical putative consensus sequences. More details are discussed in Section 4.3 of Chapter 4.

1.5.3 Nuclear localisation signal receptors

IMPs or karyopherins, are molecules responsible for mediating the active nuclear import of NLS-containing proteins. The IMPα and IMPβ family of proteins serve as adaptor and receptor molecules used during the nuclear delivery process. Often the initiation of nuclear import occurs when a ternary complex between IMPα, IMPβ1 and a NLS-containing protein cargo has formed (Dingwall *et al.*, 1991; Jans *et al.*, 2000; Quimby *et al.*, 2001; Yoneda *et al.*, 1999). Importin β1 then docks the complex to the NPC and with the help of Ran-GTP, releases the protein cargo into the nucleus (Quimby *et al.*, 2001; Yoneda *et al.*, 1999). The nuclear import and export of proteins is regulated by an asymmetric distribution of Ran-

GTP and Ran-GDP between the nucleus and cytoplasm (Moroianu, 1999; Quimby *et al.*, 2001; Yoneda *et al.*, 1999). The Ran-GTP and Ran-GDP gradient is maintained by various Ran-associated regulatory factors (Moroianu, 1999; Quimby *et al.*, 2001; Yoneda *et al.*, 1999).

For more details of the receptors and pathways that are involved refer to Section 5.3 of Chapter 5.

1.6 **Adenovirus**

Adenoviruses (Adv) are usually transmitted by respiratory infection and cause a variety of respiratory complications from a common cold to bronchitis (Engelke *et al.*, 2011; Russell, 2009). Adenoviruses can also cause ocular, genitor-urinary and gastrointestinal diseases (Engelke *et al.*, 2011; Russell, 2009).

The *Adenoviridae* virus family consists of over 50 distinct serotypes and can be categorised into six subgroups (A-F) based on their DNA sequence similarity. The human Adv are non-enveloped, double stranded DNA viruses which contain a linear genome of approximately 36kb (Leopold *et al.*, 2007; McConnell *et al.*, 2004). The adenovirus genome encodes for over 70 gene products, but the mature virion contains only 11 structural proteins (Fields *et al.*, 2007). Although all of the adenovirus subgroups share a similar structure and genomic organisation, most of the molecular studies have focused on Adenovirus serotype 2 and 5 (both in subgroup C).

1.6.1 Adenovirus Structure

The Adv genome contains twelve transcription units; five early units (E1A, E1B, E2, E3 and E4), two early delayed or intermediate units (pIX and IVa2) and five late units (L1, L2, L3, L4 and L5) (Fields *et al.*, 2007). The late transcription units are mostly responsible for the encoding of the structural proteins for the capsid and the internal core (Fields *et al.*, 2007).

The Adv virion consists of an outer icosahedra capsid surrounded by an inner nucleoprotein core as illustrated in Figure 1-2 (Lehrman, 1999; Marshall, 1999; Reddy *et al.*, 2010). Adenoviruses are composed of 7 capsid proteins and 4 core proteins. Hexon, a structural protein, is the most prevalent protein of the viral capsid with other minor protein components being protein IIIa, protein VI, protein VIII and protein IX (Tandon *et al.*, 2012). At each of the twelve vertices found on the capsid are penton base proteins that are non-covalently attached to the fiber proteins (Bai *et al.*, 1993; Leopold *et al.*, 2007; McConnell *et al.*, 2004; Reddy *et al.*, 2010; Tandon *et al.*, 2012). The Adenovirus core consists of viral DNA, protein V, protein VII, Mu and 2 copies of terminal protein (TP). Terminal protein is initially synthesised as pre-terminal protein (pTP) and can be found covalently coupled to the inverted terminal repeat (ITR) at the 5' ends of each strand of the viral genome (Leopold *et al.*, 2007; McConnell *et al.*, 2004; Smart *et al.*, 1982; Webster *et al.*, 1997b).

The Adv proteins were named based on their position on the polyacrylamide gel, with complex components responsible for forming the largest band, hence no protein I was named (Fields *et al.*, 2007). This section will explore the current understandings on the structure and function of Adv proteins.
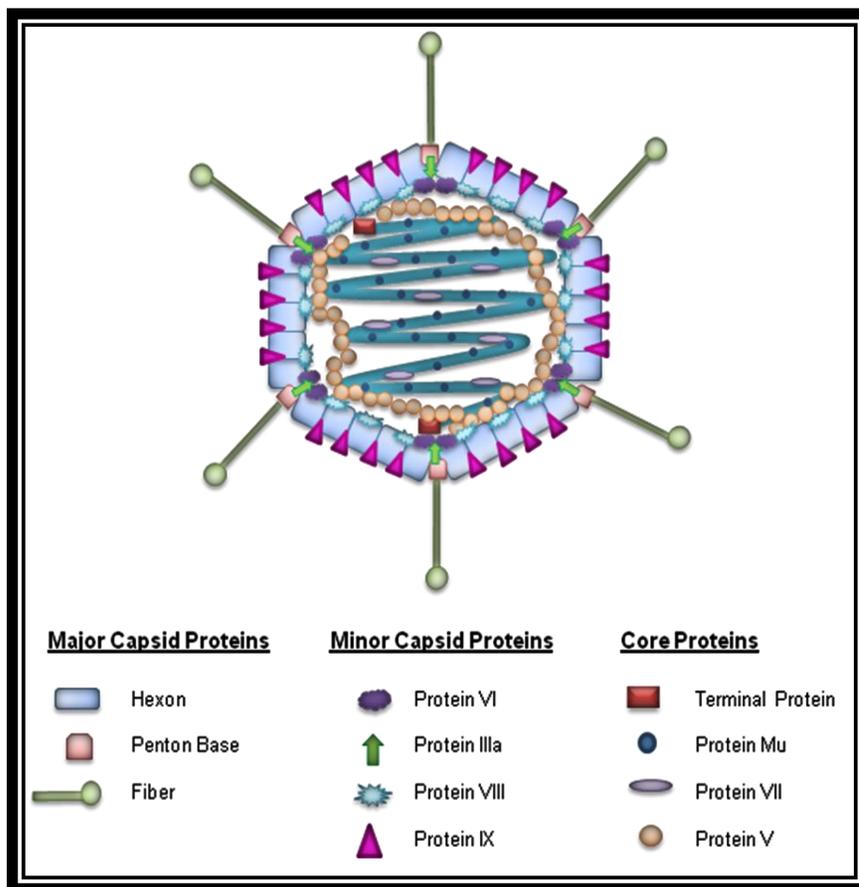
Figure 1-2: Schematic diagram of Adenovirus structure. Structure of Adenovirus based on cryo-electron microscopy and crystallography. Adenovirus particles include eleven polypeptides that condense Adenoviral DNA and assemble into the viral capsid .The viral capsid also contains a cysteine protease that is involved in post-translational modification of viral proteins after nuclear export of the viral DNA. [diagram as published in (Chailertvanitkul *et al.*, 2010)].

1.6.1.1 *Capsid Proteins*

Hexon (Protein II)

Hexon is the major structural protein which is synthesised during the late infection stage of the Adv life cycle (Russell, 2009; Rux *et al.*, 2004). Hexon is involved in the intracellular trafficking of Adv capsid towards the nucleus, by way of microtubules, with the help of host dyneins or dynein-associated proteins (Greber *et al.*, 2006; Russell, 2009). The Adv is thought to be delivered to the microtubular organising centre in the vicinity of the NE. Hexon is also responsible for triggering host immune responses (Molinier-Frenkel *et al.*, 2002; Russell, 2009).

There are 720 hexon subunits, arranged as 240 trimers, in the Adv capsid and the size of hexon molecule varies between Adv serotypes, the largest being found in Adv serotype 2 which is comprised of 967 amino acids (Burnett, 1985; Ebner *et al.*, 2005; Reddy *et al.*, 2010; Russell, 2009; Rux *et al.*, 2004). Hexon proteins are categorised into four kinds, according to their different surrounding environment; H1 (associated with the penton base), H2 (also known as 'groups of nine' or GONs), H3 (on the threefold axes) and H4 (Russell, 2009). The crystal structure of hexon reveals two eight-stranded ß-barrels and a long loop at the base of each hexon molecule (Athappily *et al.*, 1994; Reddy *et al.*, 2010; Russell, 2009).

The sequences of hexon proteins for Adenovirus serotype 2 and 5 are closely related with 86% similarities (Ebner *et al.*, 2005). The differences are due to the hyper variable region (HVR) which is conserved for a given serotype but varies between serotypes (Crawford-Miksza *et al.*, 1996). Hexon consists of seven HVR sites on the outer surfaces of the virion; HVR1-6 are located on loop 1 and HVR7 are located on loop 2 (Biere *et al.*, 2010; Ebner *et al.*, 2005).

Penton Base (Protein III)

Penton base is one of the major proteins involved in Adv cell entry. Penton base initiates clathrin-mediated internalisation through the binding with the host integrin heterodimer $\alpha_v\beta_5$ (Lyle *et al.*, 2010). Penton base can also mediate Adv attachment to the host cell in the absence of the primary attachment receptor; Coxsackie and Adenovirus Receptor (CAR) (Lyle *et al.*, 2010; Medina-Kauwe, 2003; Schoggins *et al.*, 2006; Shayakhmetov *et al.*, 2005).

Penton base sequences share high homology across several Adenovirus subgroups with exception of the highly variable region found at the centre which contains the conserved Arg-Gly-Asp sequence (RGD sequence) (Bai *et al.*, 1993). This RGD sequence, together with the fibre knob, is responsible for the binding of the penton base with the cellular integrins during viral cell entry (Russell, 2009; Shayakhmetov *et al.*, 2005). To date the function of penton base after the cell entry remains unclear although it has been shown to interact with a number of cellular components such as ubiquitin ligases (Chroboczek *et al.*, 2003).

Furthermore, the RGD loop may also be involved in facilitating the release of the virion from the endosome (Schoggins *et al.*, 2006; Shayakhmetov *et al.*, 2005).

Protein IIIa

Temperature-sensitive mutation of protein IIIa has suggested its role in the assembly of Adenovirus (Boudin *et al.*, 1980; Fields *et al.*, 2007). Protein IIIa is likely to also play a role in Adv cell entry due to its early released from the virion and being one of the most thermally labile capsid components (Boudin *et al.*, 1980; Lemay *et al.*, 1980).

There are 60 copies of protein IIIa per virion (Rux *et al.*, 2004). During virus maturation, protein IIIa is cleaved by the Adv proteases near the C-terminus to form a mature protein of 63.5 kDa (Boudin *et al.*, 1980). Protein IIIa composes of a highly helical structure with at least 14 helices at the N-terminal (Russell, 2009; Saban *et al.*, 2006). It is at this N-terminal where protein IIIa interacts with the penton base, hexons and protein VI (Russell, 2009; Rux *et al.*, 2004). Protein IIIa is highly phosphorylated at multiple sites and its phosphorylation occurs during early infection of Adv (Boudin *et al.*, 1980; Fields *et al.*, 2007).

Fibre (Protein IV)

The Adenovirus fibre protein is a major capsid protein responsible for attachment of the virus to a specific cell surface receptor (Philipson *et al.*, 1968; Wu *et al.*, 2003). Fibre swap experiments also suggested its role in modulating intercellular trafficking of Adv by directing the virus into a specific intracellular environment (Miyazawa *et al.*, 1999).

All of the human Adenovirus serotypes include 12 fibres, with each fibre projecting from each of the penton base at the vertices of the icosahedral capsid (Miyazawa *et al.*, 1999; Nemerow *et al.*, 2012; Schoggins *et al.*, 2006). Structurally, the fibre protein consists of three segments; a NH2-terminal *tail*, a central 15-residue motif forming a *shaft* and a COOH-terminal globular *knob* (Devaux *et al.*, 1990; Henry *et al.*, 1994; Russell, 2009). The

tail is responsible for interaction with penton base while the knob is a ligand for binding to the Adv receptor (Devaux *et al.*, 1990; Henry *et al.*, 1994; Nemerow *et al.*, 2012).

Protein VI

Current evidence indicates that virion releases protein VI when the acidic environment of endosome causes protein VI to undergo conformational change (Wiethoff *et al.*, 2005a). Once released, protein VI mediates disruption of the endosomal membrane allowing the virus to escape into cytosol and to translocate towards and into the nucleus (Moyer *et al.*, 2011; Wiethoff *et al.*, 2005a). The amino-terminal end of protein VI has an amphipathic helix which is responsible for inducing positive curvature in the inner leaflet of the lipid bilayer causing partial raptures of the endosomal membrane (Moyer *et al.*, 2011). Protein VI and its precursor are also essential for the maturation of Adv. Pre-protein VI facilitates the import of newly translated hexon into the nucleus while the mature protein VI is involved in virion assembly (Nemerow *et al.*, 2012; Wodrich *et al.*, 2003).

It is speculated that there are 360 copies of protein VI per virion (Russell, 2009). The full length protein VI (Pre-protein VI) is made up of 250 residues. Pre-protein VI undergoes two cleavage processes by protease via an intermediate, losing 33 residues from the N-terminus and 11 residues from the C-terminus, to form a mature 22 kDa protein VI (Matthews *et al.*, 1994; Matthews *et al.*, 1995). Protein VI has two long α-helices, however its structural association with other proteins and its precise location in the virion is still unclear (Matthews *et al.*, 1994; Matthews *et al.*, 1995; Nemerow *et al.*, 2012; Russell, 2009). It has been suggested protein VI interacts with the inner cavity of hexons, as well as protein IIIa, penton base and protein V (Nemerow *et al.*, 2012; Russell, 2009).

Protein VIII

Protein VIII is first synthesised as a larger precursor protein of approximately 227 residues, prior to proteolytic cleavage by adenaine (Stewart *et al.*, 1993; Vellinga *et al.*, 2005). Protein VIII contains relatively high content of serine (17-20%), proline (8%) and of basic residues arginine and lysine (11%) (Stewart *et al.*, 1993).

Protein VIII is the least studied capsid protein and very little is known about its function. It is located to the inner side of the Adv capsid with 5 copies in a ring around the hexon H1 connecting them to hexon H2, and 3 copies in further rings around hexon H3 (Vellinga *et al.*, 2005). It has been suggested that protein VIII is responsible for virion's structural stability by providing a bond between the peripentonal hexons and the rest of the capsid (Nemerow *et al.*, 2012; Russell, 2009; Stewart *et al.*, 1993; Vellinga *et al.*, 2005). This linkage is, however, relatively weak and may ruptured when the penton base is detached during the viral cell entry (Russell, 2009; Vellinga *et al.*, 2005).

Protein IX

Protein IX has been identified as a major protein involving in the stabilisation of the Adenovirus capsid although it appears to only be present in the family *Mastadenoviridae* (Boulanger *et al.*, 1979). Protein IX has been shown to have a great tendency to form coiled coils (Russell, 2009).

There are 240 protein IX molecules per virion (Marsh *et al.*, 2006). It is the only minor coat protein which is located on the outer part of Adv capsid. The images of hexon H2 reveal that there are 12 copies of protein IX arranged as four trimers within the capsid (Marsh *et al.*, 2006). The N-terminus is situated in the middle of each capsid facet with the C-terminus forming a four helix bundle, with one helix interacting with the hexon HVR4 loop (Marsh *et al.*, 2006; Russell, 2009). The N-terminus confers thermal stability of the virus and appears to be conserved between serotypes (Russell, 2009). The C-terminal coiled-coil regions, together with the central alanine-rich region, are more readily available to interact with other proteins (Russell, 2009).

1.6.1.2   Core Proteins

Protein V

Protein V, together with protein VII, is associated with Adv DNA. Protein V is not present in the family *Atadenoviridae*, hence it is speculated that protein V may be dispensable and

protein VII is more essential to Adv replication (Gorman *et al.*, 2005; Ugai *et al.*, 2007). Protein V contains multiple nuclear and nucleolar localisation signals and has been shown to be associated with the host nucleoli during the late phase of infection (Matthews, 2001). It has been suggested that protein V plays a role in the delivery of Adv DNA to the host cell during Adv infection (Matthews *et al.*, 1998a; Matthews *et al.*, 1998b).

There are approximately 160 copies of protein V per virion. There is little structural information available for protein V. It appears to interact with protein VI and also weakly interact with protein VII and the viral DNA (Harpst *et al.*, 1977; Matthews *et al.*, 1998b; Russell *et al.*, 1971). These interactions provide a bridge between the core and the capsid.

Protein VII

Protein VII is encoded by the late transcription unit L2. Protein VII is firstly synthesised as a precursor prior to undergoing protease-mediated cleavage, resulting in a removal of 24 amino acid N-terminal segment, to form a 174 residues mature protein VII (Johnson *et al.*, 2004; Lee *et al.*, 2003b; Wodrich *et al.*, 2006). There are over 800 copies of protein VII per virion. Protein VII is tightly associated with the Adenoviral DNA, but the interaction is not DNA sequence-specific (Russell *et al.*, 1982). The interaction appears to be charge-based between the basic arginine-lysine rich regions of protein VII and the phosphate backbone of 90-150 DNA bp (Vayda *et al.*, 1987). This interaction causes DNA to condense as a result of superfolding (Vayda *et al.*, 1987).

Protein VII is the major protein component of the core of Adenovirus. It is a nonspecific DNA-binding protein that condenses viral DNA inside the viral capsid (Chen *et al.*, 2007). Similar to protein V, protein VII also encodes for multiple nuclear and nucleolar localisation signals, and thus may be involved in directing the viral genome to the nucleus and nucleolus (Lee *et al.*, 2003b; Wodrich *et al.*, 2006). During Adenovirus infection, protein VII enters the nucleus, via microtubule network, through the NPC as a complex with Adv DNA (Greber *et al.*, 1997; Russell, 2009). It has been shown *in vitro* that protein VII inhibits synthesis and transcription of Adv DNA (Korn *et al.*, 1986; Nakanishi *et al.*, 1986; Russell, 2009). Indeed, protein VII reduces Adv early transcription but eventually transcription of

E1A triggers its release, leading to remodelling of the virus chromatin and late transcription (Chen *et al.*, 2007; Johnson *et al.*, 2004).

Mu (Protein X)

Mu protein is firstly synthesised as a precursor which undergoes protease cleavage, at both N- and C- terminal, to form mature 19 residues Mu protein (Lee *et al.*, 2004). Mu protein is tightly associated with Adv DNA. There also appears to be strong interactions between protein mu, protein V and protein VII to form a complex, although the precise topology of the complex is unknown (Chatterjee *et al.*, 1985).

The exact function of Mu protein remains unclear, however, it has been suggested to help condense Adv DNA as Mu protein has been shown to precipitate DNA *in vitro* (Anderson *et al.*, 1989; Lee *et al.*, 2004). It is thought that the condensation process is achieved through a charge-based interaction between the nine arginine residues on the protein Mu and the phosphate DNA backbone (Anderson *et al.*, 1989).

Terminal Protein

The Adenovirus terminal protein (TP) is first synthesised as an 80 kDa precursor, known as pre-terminal protein (pTP), which is later cleaved into a 55 kDa protein by Adv protease during late infection (Challberg *et al.*, 1981a; Enomoto *et al.*, 1981). For details of the cleavage process and site, refer to Section 4.6 of Chapter 4. The terminal protein is found covalently linked to the 5' end of both viral DNA strands via a phosphodiester bond between ß-OH of a serine residue to the 5'-OH of the terminal deoxycytidine residue (Challberg *et al.*, 1980; Desiderio *et al.*, 1981).

It was proposed that TP serves as a primer for Adv replication, however, it was later realised that this was due to its precursor; pre-terminal protein (Tamanoi *et al.*, 1982; Webster *et al.*, 1997b). This was supported when a complex was formed between pTP and an oligonucleotide corresponding to the 5' terminal of Adenovirus DNA (Lichy *et al.*, 1981). pTP, together with the virus-coded DNA polymerase and cellular protein NF1, bind to the origin of replication to initiate Adv replication process (Hay *et al.*, 1995; Liu *et al.*, 2003;

Webster *et al.*, 1997a). pTP is responsible for anchoring the replication complex to the nuclear matrix, through binding with the trifunctional enzyme CAD (carbamyl phosphate synthetise, aspartate transcarbamylase and dihydroorotase) (Angeletti *et al.*, 1998; Pronk *et al.*, 1993). The role of TP in Adv replication still remain unclear but it is speculated that TP may help aid the circularisation of the viral genome (Ruben *et al.*, 1983).

1.6.1.3   *Adenovirus Protease*

The Adenovirus protease plays an important role in Adenovirus cell entry as well as in the maturation of the virus, responsible for cleaving six virion precursors (protein IIIa, VII, VIII, IX, TP and Mu).

The protease of Adv is synthesised in an inactive form and is activated through a 2-stage process (Gupta *et al.*, 2004). First, it binds non-specifically to the viral DNA which partially activates the enzyme to cleave the precursor of protein VI (Gupta *et al.*, 2004). The product produced is a peptide derived from the C-terminus of the precursor (pVIc) which binds to the protease to induce its full activation (Gupta *et al.*, 2004). It has been suggested that once activated, the protease-peptide complex translocates along the viral DNA to cleave other virion precursors (protein IIIa, VII, VIII, IX, TP and Mu) (Mangel *et al.*, 2003). The Adenovirus protease is incorporated within the viral capsid and the peptide cleavage takes place either on the inner surface of the capsid or in the core of the virion (Fields *et al.*, 2007; Russell, 2009).

1.7 **Adenovirus Trafficking**

The majority of information on Adv trafficking is derived from studies on subgroup C (Miyazawa *et al.*, 1999). It has been observed that the route of Adv cell entry is consistent with a classical clathrin-mediated endocytic pathway (Leopold *et al.*, 2007; Nicola *et al.*, 2009). The Adv trafficking route can be divided into five different stages as illustrated in Figure 1-3. These include: cell receptor binding, cell entry, endosomal escape prior to degradation, microtubule translocation and nuclear import.
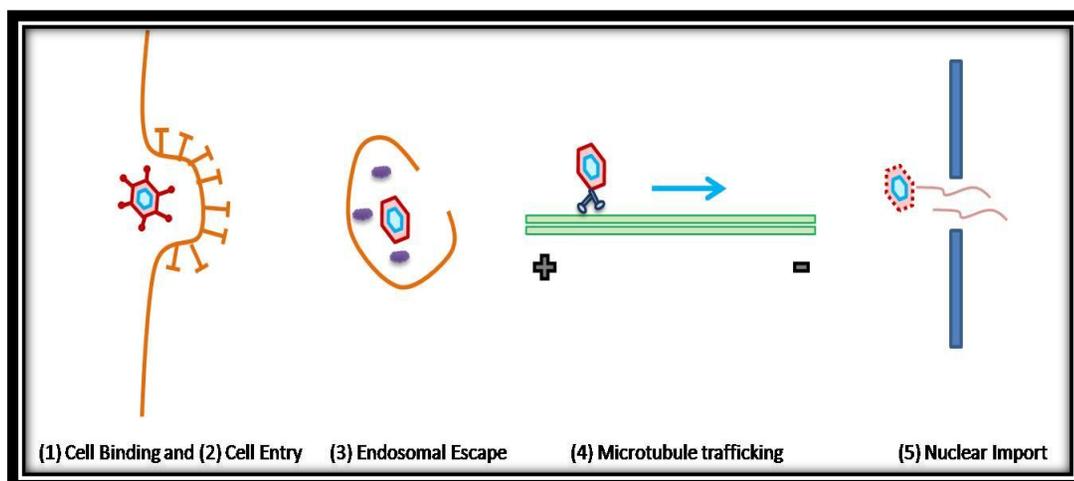
Figure 1-3: Adenovirus trafficking. There are five main stages involved in delivery of Adenovirus DNA to the nucleus. Binding is followed by internalisation into clathrin-coated vesicles (for the well studied Adv2 and Adv5 serotypes). Endosome escape involves loss of vertex proteins and subsequently the partially dissociated capsid binds to dynein and reaches the nucleus where it binds to Nup 214. The viral DNA is taken up by a process that is yet to be described in detail. [diagram as published in (Chailertvanitkul *et al.*, 2010)].

1.7.1 Binding to the Cell Receptor

Cell entry is initially mediated by high affinity binding between the virus fibre protein and the specific receptor present on the surface of the host cell (Miyazawa *et al.*, 1999). The receptor binding domain of the fibre protein is found in the C-terminal segment which folds into a globular knob (Bai *et al.*, 1993). All subgroups of the Adv, except subgroup B, use Coxsackie and Adenovirus Receptor (CAR) as their extracellular receptor (Johansson *et al.*, 2007; Nemerow *et al.*, 2009; Pache *et al.*, 2008a; Tomko *et al.*, 1997). The majority of Adv subgroup B particles bind to CD46 (Pache *et al.*, 2008a; Pache *et al.*, 2008b). Adv subgroup C initiates cell uptake through two specific receptor interactions. The first interaction is the high affinity interaction between the fiber knob and CAR (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). CAR is a 46-kDa single transmembrane protein in the immunoglobulin (Ig) superfamily that acts as a cell-to-cell adhesion molecule on the surface of epithelial cells (Farmer *et al.*, 2009a; Hidaka *et al.*, 1999; Honda *et al.*, 2000). The extracellular domain of CAR contains D1 and D2 regions. Although D1 alone is able to sufficiently bind to the fibre knob, both D1 and D2 interact with Adv 2 and Adv 12 fibre knobs (Farmer *et al.*, 2009b; Rux *et al.*, 2004). It is thought that the amino acid at position 140 on the fibre knob plays a role in CAR-binding by way of its presentation to a negatively

charged cluster on the receptor (Asp[56] and Glu[58]) (Durmort *et al.*, 2001; Hidaka *et al.*, 1999; McConnell *et al.*, 2004; Rux *et al.*, 2004; Tomko *et al.*, 1997). CAR-binding Advs contain either serine, threonine or proline, which are all neutral amino acids, at the key binding position (Bergelson *et al.*, 1997; Hidaka *et al.*, 1999; McConnell *et al.*, 2004; Rux *et al.*, 2004; Tomko *et al.*, 1997). However, Adv serotype 3 which is a non-CAR binding Adv, instead contains a glutamate residue which is negatively charged (Rux *et al.*, 2004). Thus charge repulsion may prevent high-affinity binding of Adv type 3 to CAR. The structural model of CAR-fibre binding from X-ray diffraction analysis shows that each fibre knob can bind to a maximum of three CAR molecules (Nemerow *et al.*, 2009). The initial fibre-CAR interaction is unable to promote efficient virus uptake and a lower affinity secondary interaction between the cell surface integrins ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) and a conserved viral arginine-glycine-aspartate (RGD) motif is an important requirement (Bai *et al.*, 1993; Farmer *et al.*, 2009b; Hidaka *et al.*, 1999; Nemerow *et al.*, 2009). The RGD motif is located in the variable region of the exterior surface of the penton base, at the end of a flexible loop. This interaction is thought to be responsible for virus internalisation (Rux *et al.*, 2004; Schoggins *et al.*, 2006; Wilson, 1996; Wodrich *et al.*, 2006). The internalisation of Adv requires the presence of free cholesterol in the plasma membrane, which is a feature of clathrin-mediated endocytosis (Imelli *et al.*, 2004). In the absence of cholesterol, Adv is internalised by way of a slow and less efficient pathway with limited virus particle delivery to the cytosol (Imelli *et al.*, 2004). The fibre protein may also play a role in directing the virus into a specific intracellular environment, as evidenced by a fibre swap experiment; a modified Ad5f7 vector, which is an Adv serotype 5 vector carrying Adv serotype 7 fibres, emulates the trafficking of Adv serotype 7 (Leopold *et al.*, 2007; Miyazawa *et al.*, 1999). This implies that the fibre influences a post-internalisation aspect of Adv trafficking. A recent reports suggests that when Adv serotype 5 infects cells that lack CAR, it can use lactoferrin as a bridge to enhance binding with the target cells (Johansson *et al.*, 2007). This observation was contradictory to the antiviral effects of lactoferrin, which is known to inhibit the virus entry into cells (Johansson *et al.*, 2007). It has been suggested that the concentration of lactoferrin may influence its role in either inhibiting or promoting Adv infection (Johansson *et al.*, 2007). Adv entry can also take place in the absence of CAR binding, when the key integrins may serve as both the primary cell receptor and activator of endocytosis (Schoggins *et al.*, 2006). This supports the idea that RGD-integrin binding may be a

convenient mechanism for facilitating endocytosis of non-viral delivery systems, given that RGD motifs could be grafted relatively easily to the surface of a DNA delivery particle. Indeed this approach has been used previously to facilitate cellular uptake of non-viral gene delivery systems (Jäkel *et al.*, 1998; Ribbeck *et al.*, 1998).

1.7.2 Endocytosis and Endosome escape

Once bound at the cell surface, Adv is taken up into clathrin-coated pits by receptor-mediated endocytosis. The interactions of the penton base with the cell surface promote integrins to aggregate, enhancing the downstream intracellular signaling pathway, which in turn induces endocytosis (Farmer *et al.*, 2009b; Johansson *et al.*, 2007; Nemerow *et al.*, 1999). An NPXY motif, which is an endocytotic sorting consensus sequence, can be found in the cytoplasmic tails of β3 and β5 integrin subunits (de Deyne *et al.*, 1998; Heilker *et al.*, 1999; Kirchhausen, 1999). This motif is responsible for mediating the localisation of receptors to coated pits (Medina-Kauwe, 2003). Adv internalisation is facilitated by activation of phosphatidylinositol 3-OH kinase (PI3K) which in turn activates Rac and CDC42 GTPases (Leopold *et al.*, 2007; Medina-Kauwe, 2003; Meier *et al.*, 2004). This process promotes polymerisation of actin filaments and subsequent endocytosis of Adv by way of clathrin-coated vesicles (Imelli *et al.*, 2004; Leopold *et al.*, 2007; Medina-Kauwe, 2003; Meier *et al.*, 2004). For the Adv to gain access to the cytoplasm, it needs to escape the endosome prior to the degradation by lysosomal proteases; the latter being the default path for the contents of late endosomal vesicles, after fusion with lysosomes. The acidic pH in the lumen of the endosome is maintained by the proton pump enzyme H^+ATPase. As the endosome matures, the pH falls to approximately pH 5 (Medina-Kauwe, 2003; Meier *et al.*, 2004). This acidification induces conformational changes in the Adv capsid causing the endosomal vesicle membrane to be disrupted, releasing partially disassembled Adv into the cytoplasm (Medina-Kauwe, 2003; Meier *et al.*, 2002; Meier *et al.*, 2004; Prchla *et al.*, 1995; Rux *et al.*, 2004; Wilson, 1996). The mechanism by which the escape occurs is of considerable interest because synthetic gene delivery systems also need to be provided with an endosomal escape system. The vertex regions of Adv, including protein VI, appear to be released during the first stage of viral disassembly (Wiethoff *et al.*, 2005b; Wodrich *et al.*, 2006). It is thought that exposure of protein VI helps mediate endosomal membrane

disruption by inducing curvature stress, which releases the remains of the Adv capsid (Johansson *et al.*, 2007; Maier *et al.*; Moyer *et al.*, 2011; Wiethoff *et al.*, 2005b). Other factors also contribute to the endosomal escape (Blumenthal *et al.*, 1986; Perez *et al.*, 1994; Prchla *et al.*, 1995; Rodriguez *et al.*, 1996). Integrins, particularly αvβ3, αvβ5, α5β1 and αvβ1, promote Adv permeabilization of the plasma membrane by way of attachment with the Adv penton base protein (Farmer *et al.*, 2009a; Wang *et al.*, 2000; Wickham *et al.*, 1994). The Adv fiber protein is thought to be involved in the timing of endosomal escape by acting as a pH sensor during the progress of endocytosis (Medina-Kauwe, 2003; Miyazawa *et al.*, 1999). After endosomal escape approximately 80% of the capsid is still intact, and retains its hexon and penton proteins (Saphire *et al.*, 2000). It appears that sometime during endocytosis or endosomal escape, the fiber protein and other capsid-stabilising proteins are lost (Saphire *et al.*, 2000). Protein VI remains behind in endosomes in a role which may mediate Adv endosomal escape (Puntener *et al.*, 2009; Wiethoff *et al.*, 2005b; Wodrich *et al.*, 2006). Since hexon and penton remain associated with the Adv particle at this stage, it has long been assumed that one or both proteins may facilitate translocation toward the nucleus (Saphire *et al.*, 2000). The molecular mechanisms of endosomal escape are partially understood but are worthy of further investigation because the escape systems used in synthetic gene delivery systems, making use of weakly basic polymers such as polyethyleneimine or cationic lipids, are known to cause endosomolysis (Pouton *et al.*, 2001). From a practical viewpoint viral escape systems may be too complex to replicate or simulate as part of a synthetic gene delivery system, but it would be useful to know if they are less damaging to the host cell.

1.7.3 Microtubule Trafficking

The crowded nature of the cytoplasm prevents the Adv capsid from freely diffusing towards the nucleus. It is thought that the Adv capsid reaches the nucleus by intracellular translocation along the microtubules by binding to the cytoplasmic dynein complex (Bailey *et al.*, 2003; Greber *et al.*, 2006; Kelkar *et al.*, 2006; Samir A *et al.*, 2004). Dynein is a microtubule-dependent molecular motor which aids the movement of protein cargoes towards the microtubule organisation centre (MTOC) and the nucleus (Bailey *et al.*, 2003). The strength by which the Adv interacts with MTOC is similar to that between the Adv and

nuclear envelope (Bailey *et al.*, 2003). The attachment between dynein and Adv is through interactions between the dynein IC and LIC_1 subunits and the capsid protein hexon (Bremner *et al.*, 2009a; Bremner *et al.*, 2009b). The L3/p23 protease of Adv is another candidate for dynein binding since it contains a light chain dynein consensus binding site similar to that identified in the other viral proteins (Martinez-Moreno *et al.*, 2003; Samir A *et al.*, 2004). Although the capsid still contains the penton base during trafficking towards the nucleus, it has been reported that this protein does not show any evidence of microtubular interaction and is therefore not likely to be involved in the translocation process (Samir A *et al.*, 2004).

Better understanding on Adv microtubular trafficking is required. It is possible that other Adv proteins are also involved in binding to dynein motor either directly, similar to that by hexon, or indirectly through help of other accessory proteins from existing transport pathways (Wodrich *et al.*, 2010). A dynactin protein complex, which interacts with dynein, is thought to be one of the potential candidates, since an overexpression of this complex results in disruption of Adv translocation (Samir A *et al.*, 2004). Further study is also required to identify the mechanism by which the Adv capsid is uncoupled from the dynein to form a more favourable interaction with the nuclear envelope.

1.7.4 Nuclear binding and Uptake of Adv DNA

The molecular details of the nuclear delivery of Adv DNA are yet to be elucidated (Hindley *et al.*, 2007b; Saphire *et al.*, 2000). However, it is clear that Adv DNA is delivered by an active process through the nuclear pore complex (NPC) (Hindley *et al.*, 2007b). Several studies suggest that the import factors; importin α, importin β and importin 7 may be responsible for the Adv DNA delivery to the nucleus (Hindley *et al.*, 2007b). Upon reaching the nucleus, Adv capsid protein hexon forms a stable association with the NPC via a nucleoporin, CAN/Nup214, which is located in the region of the cytoplasmic filaments (Greber *et al.*, 2003; Greber *et al.*, 1997; Strunze *et al.*, 2005; Trotman *et al.*, 2001). The Adv capsid then disassembles by an unknown mechanism, leaving the majority of capsid proteins at the perinuclear envelope, including most or all the copies of hexon. It has been estimated that only about 5% of hexon enters the nucleus (Greber *et al.*, 1996b; Puntener

et al., 2009). The dissociation of the capsid enables the Adv DNA, possibly with its associated DNA binding proteins, to reach the nucleus where it can employ the host cell nuclear enzymes for transcription (Bailey *et al.*, 2003; Greber *et al.*, 2003; Greber *et al.*, 1997; Wodrich *et al.*, 2003). Histone H1 is thought to be an important factor in the disassembly of Adv2 and Adv5 capsids. Once the Adv capsid is docked at the NPC, the capsid disassembly process is thought to be triggered by H1 and/or H1 import factors with the help of the additional cytosolic factors, including Hsp/Hsc70 (Hindley *et al.*, 2007b; Puntener *et al.*, 2009; Saphire *et al.*, 2000). Hsp/Hsc70 is a classical nuclear protein import factor (Hindley *et al.*, 2007b; Puntener *et al.*, 2009; Saphire *et al.*, 2000). Its direct function in the Adv capsid disassembly is unclear but it may have a role prior to Adv docking at the NPC (Greber *et al.*, 1996a; Greber *et al.*, 1997; Greber *et al.*, 1996b; Saphire *et al.*, 2000). The L3/p23 protease which is included in Adv particles may be a requirement to degrade internal protein VI for a full capsid dissociation (Greber *et al.*, 1996b). The nuclear export factor CRM1 is also involved in Adv nuclear delivery by acting as a positional indicator of the nucleus (Strunze *et al.*, 2005). Adv detachment from the microtubules and the binding between the Ad capsid and the nuclear envelope also requires the presence of CRM1 (Puntener *et al.*, 2009; Strunze *et al.*, 2005). Another factor which influences Adv nuclear targeting is the concentration of intracellular calcium. It has been shown that depletion of intracellular calcium inhibits nuclear import of Adv DNA (Greber *et al.*, 1997; Medina-Kauwe, 2003). It is possible that calcium induces conformational changes in the NPC to allow passage of macromolecular complexes. Although the contents of Adv core are well studied, it is yet to be established which of the core proteins enter the nucleus with the DNA. Due to the covalent attachment of TP to the 5' ends of the Adv DNA, TP is assumed to be imported into the nucleus coupled to the Adv DNA (Cardoso *et al.*, 2008; Puntener *et al.*, 2009; Zhao *et al.*, 1988). The degree to which DNA is condensed by core proteins during nuclear uptake, and the role these proteins might play in uptake is not known. pVII is thought to enter the nucleus as it is tightly associated with the Adv DNA and a basic nuclear localisation signal (NLS) sequence has been identified in its precursor (Hindley *et al.*, 2007b; Lee *et al.*, 2003b; Wodrich *et al.*, 2006). However, more research needs to be conducted to identify the complete list of Adv nuclear localized proteins and their potential functions in Adv nuclear delivery. The cationic proteins are indeed localized in the nucleus after host cell expression but accumulation would be expected to occur simply as a result

of their polycationic nature. Their uptake may also be needed to facilitate export of the replicated Adv genome, but as yet the degree of condensation required for export of Adv DNA has not been reported.

1.8 **Research Objectives**

The main aim of this project was to determine how Adenovirus terminal protein achieves its nuclear delivery. The current theory of Adenovirus trafficking was discussed in this Chapter and it is apparent that there is knowledge lacking in the nuclear delivery stage of the trafficking. As such, this research focussed on the nuclear delivery of terminal protein, which is known to associate with Adv DNA. By understanding the mechanism of terminal protein nuclear import, it will provide insights into how Adenovirus achieves its nuclear delivery.

This PhD project aimed to create new knowledge by examining:

1. The nuclear localisation signals encoded on terminal protein

2. The role of NLS flanking negatively rich domain regions in terminal protein nuclear delivery

3. The nuclear import pathway of terminal protein

4. The interaction between NLS of terminal protein with IMPα, IMPβ and IMPα/β

CHAPTER 2

Materials

CHAPTER 2   Materials

2.1 Materials

2.1.1 Cell Lines and Bacterial Strains

Cell Lines	Catalogue Number	Supplier
AD-293 Human Embryonic Kidney	240085	Stratagene, USA
HeLa Human Cervical Carcinoma	CCL-2	ATCC, USA
Cos-7 African Green Monkey Kidney Fibroblast	CRL-1651	ATCC, USA
Caco-2 Human epithelial colorectal adenocacinoma	HTB-37	ATCC, USA
PC-3 Human prostate cancer	CRL-1435	ATCC, USA

Bacterial Strains	Catalogue Number	Supplier
DH5α-T1 E. coli F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44thi-1 gyrA96 relA1 tonA (confers resistance to phage T1)	12297-016	Invitrogen, USA
BL21-AI E. coli *F- ompT hsdSB (rB-mB-) gal dcm araB::T7RNAP-tetA*	C6070-03	Invitrogen, USA

2.1.2 Cell Culture

Solutions	Catalogue Number	Supplier
F-12K Nutrient Mixture	21127-022	Invitrogen, USA
Dulbecco's Modified Eagle Medium (DMEM)	11995-065	Invitrogen, USA
DMEM (no phenol red)	21063-029	Invitrogen, USA
MEM Sodium Pyruvate Solution	11360-070	Invitrogen, USA
Opti-MEM® I Reduced-Serum Medium	11058-021	Invitrogen, USA
Fetal Bovine Serum (FBS)	10100-147	Invitrogen, USA
Trypsin-EDTA	25200-056	Invitrogen, USA
Lipofectamine™ 2000 Transfection Reagent	11668-019	Invitrogen, USA
Penicillin-Streptomycin (penstrep)	15140-148	Invitrogen, USA

Cell Culture Media	Components
For AD-293, Cos-7, and Caco-2	10% FBS 90% DMEM (optional: 1% penstrep)
For PC-3 cells	10% FBS 90% F-12K (optional: 1% penstrep)
For HeLa cells	5% FBS 95% DMEM (optional: 1% penstrep)

2.1.3 Bacterial Culture

Bacterial Culture Media	Components
Luria Bertani (LB)	10 g tryptone 5 g yeast extract 10 g NaCl 1 ml NaOH Made up to 1 L with MilliQ water
LB Agar	1 L LB media 15 g agar
LB Agar - ampicillin	LB Agar Ampicillin to final concentration of 100 μg/mL
LB Agar - kanamycin	LB Agar Kanamycin to final concentration of 50 μg/mL
SOC	20 g tryptone 5 g yeast extract 0.5 g NaCl 2.5 mM KCl 10 mM $MgCl_2$ Made up to 1 L with MilliQ water Glucose to final concentration of 20 mM

The Luria Bertani (LB) media and agar were made up according to the composition listed and autoclaved. For antibiotic selective plates, the LB agar was cooled to 55°C prior to addition of antibiotic. SOC media was also autoclaved prior to adding of filter-sterile glucose.

Chemicals/Solutions	Catalogue Number	Supplier
Tryptone	LP0042	Oxoid, Australia
Yeast Extract	92144	Sigma-Aldrich, Australia
NaCl	73575	Sigma-Aldrich, Australia
NaOH	S8045	Sigma-Aldrich, Australia
Agar	05038	Sigma-Aldrich, Australia
Ampicillin Powder	A0166	Sigma-Aldrich, Australia
Kanamycin Solution	K0254	Sigma-Aldrich, Australia

2.1.4 Vectors

Vectors	Catalogue Number	Suppliers
pAdEasy-1	240010	Stratagene, USA
pDonR221	12536-017	Invitrogen, USA
pcDNA 6.2/C-EmGFP-DEST	V355-20	Invitrogen, USA
pcDNA-6.2/N-YFP-DEST	V358-20	Invitrogen, USA
pDEST 15	11802-014	Invitrogen, USA
pDEST 24	11802-016	Invitrogen, USA

2.1.5 Buffers

All the buffers were autoclaved and kept at 4°C, otherwise stated.

Buffers	Components
50x TAE	242 g Tris base 57.1 mL glacial acetic acid 100 mL 0.5 m EDTA (pH 8.0) Made up to 1 L with MilliQ water
Te Buffer	10mM Tris-HCl 1 mM EDTA 2 µg/mL DNase-inactivated RNase
10x PBS	80 g NaCl 2 g KCl 11.5 g Na_2HPO_4 2 g KH_2PO_4 Made up to 1 L with Distilled water Adjust to pH 7.4
PBST	1x PBS 0.3% (v/v) Triton X100
10x Tris Buffer	60.57 g Tris base 87 g NaCl 2.03 g $MgCl_2$ 1.11 g $CaCl_2$ Made up to 1 L with MilliQ water Adjust to pH 7.6
5x SDS Running Buffer	15 g Tris base 72 g glycine 5 g SDS Made up to 1 L with Distilled water

SDS Loading Buffer	50% (w/v) glycerol
	10% (w/v) SDS
	14.96% 1.5 mM Tris-HCl (pH 6.8)
	0.04% (w/v) bromophenol blue
	25% β-mercaptoethanol
Transfer Buffer	3.03 g Tris
	14.41 g glycine
	100 mL methanol
	Made up to 1 L with Distilled water
Binding Buffer	0.5 mM Tris-HCl pH 8.0
	0.5 mM NaCl
Intracellular Buffer	110 mM KCl
	5 mM $NaHCO_3$
	5 mM $MgCl_2$
	1 mM EGTA
	mM $CaCl_2$
	pH to 7.4 with NaOH
Staining Solution	0.05% Comassie blue
	50% Methanol
	10% Acetic acid
	40% Water
Destain Solution I	50% Methanol
	10% Acetic acid
	40% Water
Destain Solution II	5% Methanol
	7% Acetic acid
	88% Water

Chemicals/Solutions	Catalogue Number	Supplier
Tris base	0497	Astral Scientific, Australia
Bromophenol blue	B0126-25G	Sigma-Aldrich, Australia
β-mercaptoethanol	M3148	Sigma-Aldrich, Australia
Tween-20	P5927	Sigma-Aldrich, Australia
Glacial acetic acid	1000562500	MERCK Chemicals, Australia
EDTA	ED	Sigma-Aldrich, Australia
EGTA	E3889	Sigma-Aldrich, Australia
HCl	H1758	Sigma-Aldrich, Australia
NaCl	71376	Sigma-Aldrich, Australia
Glucose	G8270	Sigma-Aldrich, Australia
KCl	A383	Univar, USA
Na_2HPO_4	255793	Sigma-Aldrich, Australia
KH_2PO_4	P0662	Sigma-Aldrich, Australia
Glycine	G8898	Sigma-Aldrich, Australia
SDS	28365	Thermo Scientific, Australia

PBS	P5493	Sigma-Aldrich, Australia
DTT	D9779	Sigma-Aldrich, Australia
Triton X100	28314	Thermo Scientific, Australia
$MgCl_2$	63020	Sigma-Aldrich, Australia
$CaCl_2$	449709	Sigma-Aldrich, Australia
Glycerol	17904	Thermo Scientific, Australia
Methanol	4102322500	MERCK Chemicals, Australia
Ethanol	4100502500	MERCK Chemicals, Australia
6x Agarose Gel Loading Dye	R0611	Fermentas, USA
PBS Lysis Buffer	9803S	Gene Search, Australia
Odyssey Blocking Buffer	927-40000	Licor, USA

2.1.6 Enzymes

Enzymes	Catalogue Number	Supplier
HotStar HiFidelity Polymerase	202605	QIAGEN, Germany
Proteinase K	AM2546	Invitrogen, USA
BP Clonase II	11789-020	Invitrogen, USA

LR Clonase II	11791-020	Invitrogen, USA
DpnI endonuclease	R0176S	New England BioLabs, USA
Pfu DNA Polymerase	M7741	Promega, Australia
Thrombin Protease	27084601	GE Healthcare, Australia
Lysozyme	10837059001	Roche applied science, Australia
Protease Inhibitor	05892791001	Roche applied science, Australia

2.1.7 Antibodies

Antibody	Catalogue Number	Supplier
Mouse Anti-V5	R960-25	Invitrogen, USA
Mouse Anti-GST	13-6700	Invitrogen, USA
IRDye 680LT Goat anti-Mouse IgG	926-68020	Licor, USA

2.1.8 Other Materials, Chemicals and Kits

Materials/Chemicals/Kits	Catalogue Number	Supplier
Bovine serum albumin (BSA)	23210	Thermo Scientific, Australia
Comassie blue	20279	Thermo Scientific, Australia
Ethidium Bromide	E1510-10ML	Sigma-Aldrich, Australia

Agarose Powder	161-3102EDU	BioRad, Australia
PageRuler prestained protein ladder	SM0671	Thermo Scientific, Australia
Odyssey two-colour protein molecular weight marker	928-40001	Licor, USA
Immun-blot PVDF membrane	162-0175	BioRad, Australia
GeneRuler 1 kb DNA ladder	SM0313	Fermentas, USA
QIAprep Spin Miniprep Kit	27106	QIAGEN, Australia
QIAGEN Plasmid Midi Kit	12143	QIAGEN, Australia
QIAGEN Plasmid Maxi Kit	12163	QIAGEN, Australia
PureLink Quick Plasmid Miniprep Kit	K2100-10	Invitrogen, USA
QIAquick Gel Extraction Kit	28704	QIAGEN, Australia
QIAquick PCR Purification Kit	28104	QIAGEN, Australia
Qproteome Nuclear Protein Kit	37582	QIAGEN, Australia
Glutathione Agarose	16101	Thermo Scientific, Australia
Benzamidine Sepharose 6B beads	17-0568-01	GE Healthcare, Australia
Glutathione Sepharose 4B beads	17-0756-01	GE Healthcare, Australia
Glutathione Acceptor beads	AL109R	Perkin Elmer, Australia
Streptavidin Donor beads	6760002B	Perkin Elmer, Australia
PD-10 Desalting Columns	17-0851-01	GE Healthcare, Australia
Amicon Ultra-15 (50kDa)	UFC905008	Millipore, Australia
Amicon Ultra-15 (100kDa)	UFC910008	Millipore, Australia

384-well opti-plate	6007290	Perkin Elmer, Australia
25 cm2 flask	430639	Corning Life Sciences, Australia
75 cm2 flask	430641	Corning Life Sciences, Australia
150 cm2 flask	430825	Corning Life Sciences, Australia
6-well microplate	3506	Corning Life Sciences, Australia
12-well microplate	3512	Corning Life Sciences, Australia
24-well microplate	3524	Corning Life Sciences, Australia
96-well microplate	3599	Corning Life Sciences, Australia

CHAPTER 3

Method

CHAPTER 3   Method

3.1 **Introduction**

This thesis examines the function of adenovirus Terminal Protein (TP) in nuclear delivery. The experimental procedure has been divided into two main parts.

To identify peptide sequences in the adenovirus TP which encode for nuclear localisation signal (NLS).

To identify the binding affinity between adenovirus TP and classical importin receptors (IMPα, IMPβ and IMPα/β heterodimer).

3.2 **Polymerase Chain Reaction (PCR) Amplification**

3.2.1 Overview

To identify the peptide sequences in TP that relate to the NLSs, several truncated TP derivative fragments were designed and produced. The truncations were designed after possible sequences had been identified by sequence homology to known NLSs. PCR was used to amplify full-length pTP, TP and truncated TP derivative fragments from the pAdEasy-1 vector. The pAdEasy-1 vector is a plasmid containing most of the human adenovirus serotype 5 genome, minus of the E1 and E3 genes, as illustrated in Figure 3-1. The virus is unable to replicate in the absence of these genes.
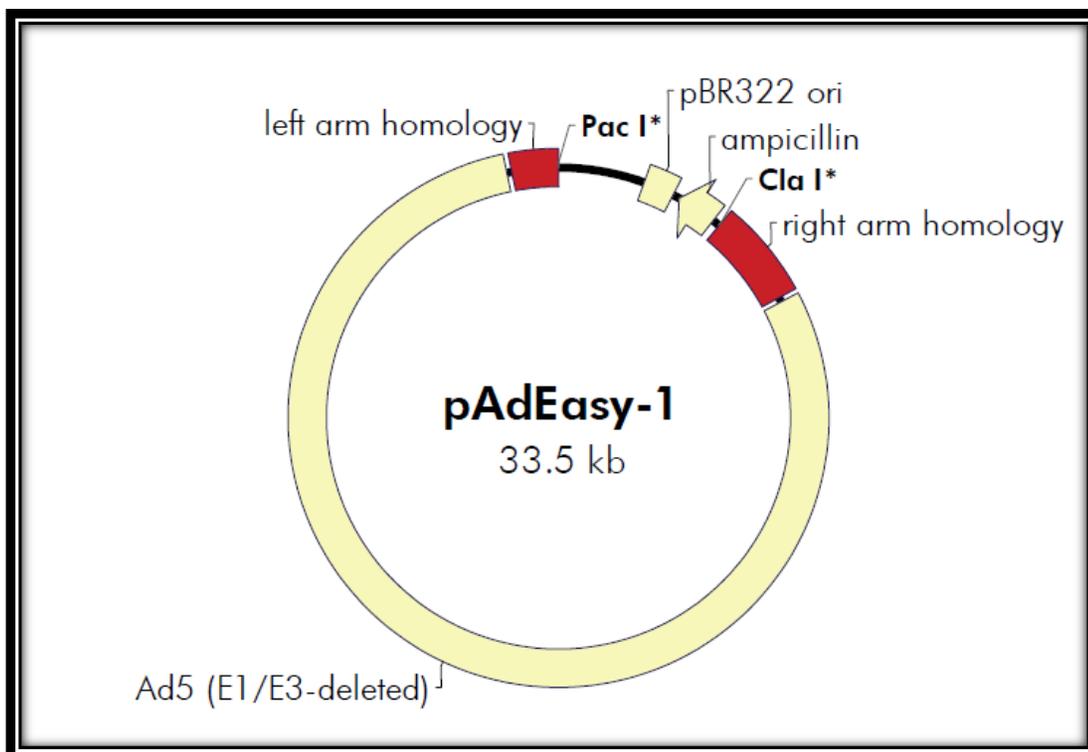
Figure 3-1: pAdEasy-1 vector map (taken from AdEasy^TM Adenoviral vector system manual, Agilent Technologies)

The procedure of PCR can be divided into two parts: primer design and PCR reactions.

3.2.2 Selection of Truncated TP Derivative Fragments

Three distinct sequences within TP were proposed to be the potential NLS. In addition it was thought that a domain rich in negatively charged residues located directly behind the positively rich regions might play a role in nuclear delivery. Nine different TP truncated derivative fragments were designed surrounding these regions of interest.

The amino acid sequence of full length pTP, TP and 9 truncated derivative fragments can be found below. In each case the full sequence represents the sequence of pTP, the sequence in green represents the TP sequence found in the mature virion, the sequence in blue represents the truncated fragment that was used to make fusion proteins, the underlined sequence represents the negatively rich domain and the bold sequences represents the proposed NLS sequences.

pTP (1-671) and TP Sequence (350-671)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA

PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG

AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD

AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST

SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPPEEEEGEAL

MEEEIEEEEEAPVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW

VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL

MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP

VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F1: First and Second Proposed NLS (1-430)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA

PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG

AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD

AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST

SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPPEEEEGEAL

MEEEIEEEEEAPVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW

VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL

MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP

VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F2: Without Negatively Charged Domain (1-390)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA

PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG

AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD

AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST

SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRLP**VRRRRRRV**PPPPPPPEEEEGEAL

MEEEIEEEEEAPVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW
VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL
MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP
VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F3: First Proposed NLS (1-380)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA
PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG
AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD
AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST
SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP
PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPPEEEEGEAL
MEEEIEEEEEAPVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW
VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL
MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP
VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F4: Before NLS (1-356)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA
PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG
AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD
AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST
SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP
PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPPEEEEGEAL
MEEEIEEEEEAPVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW
VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL
MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP
VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F5: Second and Third Proposed NLS (374-671)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA
PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG
AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD
AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST
SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP
PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPP<u>EEEEGEAL</u>
<u>MEEEIEEEEEA</u>PVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW
VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL
MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP
VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F6: Third NLS with Negatively Charged Domain (391-671)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA
PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG
AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD
AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST
SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP
PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPP<u>EEEEGEAL</u>
<u>MEEEIEEEEEAPVA</u>FEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW
VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL
MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP
VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F7: Third Proposed NLS (541-671)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA
PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG
AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD
AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST
SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPP<u>EEEEGEAL</u>

<u>MEEEIEEEEEA</u>PVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW

VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL

MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP

VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F8: No Third NLS (391-654)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA

PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG

AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD

AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST

SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRL**PVRRRRRRV**PPPPPPP<u>EEEEGEAL</u>

<u>MEEEIEEEEEA</u>PVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW

VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL

MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP

VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

F9: Second NLS (371-480)

MALSVNDCARLTGQSVPTMEHFLPLRNIWNRVRDFPRASTTAAGITWMSRYIYGYHRLMLEDLAPGA

PATLRWPLYRQPPPHFLVGYQYLVRTCNDYVFDSRAYSRLRYTELSQPGHQTVNWSVMANCTYTINTG

AYHRFVDMDDFQSTLTQVQQAILAERVVADLALLQPMRGFGVTRMGGRGRHLRPNSAAAAAIDARD

AGQEEGEEEVPVERLMQDYYKDLRRCQNEAWGMADRLRIQQAGPKDMVLLSTIRRLKTAYFNYIISST

SARNNPDRRPLPPATVLSLPCDCDWLDAFLERFSDPVDADSLRSLGGGVPTQQLLRCIVSAVSLPHGSP

PPTHNRDMTGGVFQLRPRENGRAVTET**MRRRR**GEMIERFVDRLP**VRRRRRRV**PPPPPPP<u>EEEEGEAL</u>

<u>MEEEIEEEEEA</u>PVAFEREVRDTVAELIRLLEEELTVSARNSQFFNFAVDFYEAMERLEALGDINESTLRRW

VMYFFVAEHTATTLNYLFQRLRNYAVFARHVELNLAQVVMRARDAEGGVVYSRVWNEGGLNAFSQL

MARISNDLAATVERAGRGDLQEEEIEQFMAEIAYQDNSGDVQEILRQAAVNDTEIDSVELSFRLKLTGP

VVFTQRRQIQEINRRVVAFASNLRAQHQLLPARGADVPLPPLPAGPEPPLPPG**ARPRHR**F

The programme OligoPerfect[TM] Designer, available from Invitrogen, was used to design oligonucleotide primers to amplify cDNAs for each target sequence and allow creation of expression vectors in the subsequent Gateway® recombination process. Primers were designed with the Gateway specification sequence to produce 11 different cDNAs encoding; the full length pTP, mature TP and 9 truncated TP derivatives as specified above (F1 to F9 respectively). Primers were selected to ensure a GC content of 50-60% and were terminated with either G or C. These general rules are known to increase primer efficiency.

Two expression vectors were selected for use in the project; vivid colours pcDNA6.2/C-EmGFP-DEST and pcDNA6.2/N-YFP-DEST Gateway® vector so that fusion proteins could be expressed with the fluorescent protein at either the N-terminus or C-terminus. This strategy was used to compensate for any influence that the fluorescent part of the fusion protein might have on nuclear import. Due to the specific requirements of recombination with two different vectors, two separate sets of forward primers were designed. For use with the pcDNA[TM] 6.2/N-YFP-DEST, the forward primers were designed to encode the Gateway® sequence follow by the start codon for amplification of the open reading frame of the products. For the vector pcDNA[TM] 6.2/C-EmGFP-DEST, the forward primers were designed to contain both the Gateway® sequence and the kozak consensus sequence follow by the start codon. The kozak consensus sequence is required for proper translational initiation of the products. However, only one set of the reverse primers was designed. This is because the requirement for the reverse primers was identical between the two vectors. The reverse primers were created without the stop codon to allow expression of the V5 epitope in pcDNA[TM] 6.2/N-YFP-DEST and for expression of fluorescent protein in pcDNA[TM] 6.2/C-EmGFP-DEST. The V5 epitope was used as the target for an antibody in subsequent Western blot experiments to confirm the presence of the fusion proteins of interest. The Gateway® specific sequence and primer sequences can be found in Table 3-1, Table 3-2 and Table 3-3.

Table 3-1: Gateway specific sequence.

Primer	Sequence (from 5' to 3')	Length (bp)
Gateway FWD (GWF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTC	30
Gateway REV (GWR)	GGGGACCACTTTGTACAAGAAAGCTGGGTC	30

Table 3-2: Primer sequences used to amplify gene of interest for recombination with pcDNA^{TM} 6.2/N-YFP-DEST.

Gene/ Fragments	Sequence (from 5' to 3')	Product Size (bp)
pTP	(GWF)- ATGGCCCTGAGCGTCAAC (GWR)- AAAGCGGTGACGCGG	2076
TP	(GWF)- ATGGTCTTCCAACTGCGCCC (GWR)- AAAGCGGTGACGCG	1026
F1	(GWF)- ATGATCGAGCGCTTTG (GWR)- AAAGCGGTGACGCGGG	903
F2	(GWF)- ATGGCCCTGAGCGTC (GWR)- CTCGGCGACAGTGTCG	1350
F3	(GWF)- ATGATCGAGCGCTTTGTCG (GWR)- AAAGCGGTGACGCG	954
F4	(GWF)- ATGGCCCGCATTTC (GWR)- AAAGCGGTGACGCGGG	453
F5	(GWF)- ATGGCCCTGAGCGTCAACG (GWR)- GCGGCGACGGCGC	1230
F6	(GWF)- ATGGCCCTGAGCGTCAAC (GWR)- GCGGGGGCGCAGTTGG	1128
F7	(GWF)- ATGATCGAGCGCTTTG (GWR)- GAAGTACATAACCCAGCGTCG	390
F8	(GWF)- ATGGCCCTGAGCGTC (GWR)- GCGGTCGACAAAGCG	1200
F9	(GWF)- ATGGAAGAGGAGATTGAAGAAG (GWR)- CGGAGGTAGGGGGGGCTC	810

Table 3-3: Primer sequences used to amplify gene of interest for recombination with pcDNA^{TM} 6.2/C-GFP-DEST.

Gene/ Fragments	Sequence (from 5' to 3')	Product Size (bp)
pTP	(GWF)- ACCATGGATGGCCCTGAGCGTCAAC (GWR)- AAAGCGGTGACGCGG	2076
TP	(GWF)- ACCATGGGTCTTCCAACTGCGCCC (GWR)- AAAGCGGTGACGCG	1026
F1	(GWF)- ACCATGGGGGGGAGATGATCGAGCGC (GWR)- AAAGCGGTGACGCGGG	903
F2	(GWF)- ACCATGGATGGCCCTGAGCGTC (GWR)- CTCGGCGACAGTGTCG	1350
F3	(GWF)- ACCATGGATCGAGCGCTTTGTCG (GWR)- AAAGCGGTGACGCG	954
F4	(GWF)- ACCATGGCAGCTCATGGCCCGC (GWR)- AAAGCGGTGACGCGGG	453
F5	(GWF)- ACCATGGATGGCCCTGAGCGTCAACG (GWR)- GCGGCGACGGCGC	1230
F6	(GWF)- ACCATGGATGGCCCTGAGCGTCAAC (GWR)- GCGGGGGCGCAGTTGG	1128
F7	(GWF)- ACCATGGGGGGAGATGATCGAGCG (GWR)- GAAGTACATAACCCAGCGTCG	390
F8	(GWF)- ACCATGGATGGCCCTGAGCGTC (GWR)- GCGGTCGACAAAGCG	1200
F9	(GWF) – ACCATGGAAGAGGAGATTGAAGAAG (GWR) – CGGAGGTAGGGGGGGCTC	810

3.2.3 PCR Reactions

PCR was performed using the HotStar HiFidelity PCR Kit (Invitrogen) on a My Cycler thermal cycler (Bio-Rad, USA). PCR reaction conditions were optimised for each set of primers. The optimisation process involved investigating the effect of magnesium concentration, annealing temperature, and DNA template (pAdEasy-1) concentrations on the yield. Magnesium concentrations of 1.5 mM, 2 mM, 2.5 mM and 3 mM, and annealing temperature of T_m ± 5°C were assessed. Specifications for the general PCR reaction environment are given in Table 3-4 and Table 3-5 respectively.

Table 3-4: General PCR reaction mix used in optimising PCR conditions.

Component	Volume per reaction (µl)	Final Concentration
5x HotStar HiFidelity PCR Buffer (contains dNTPs)	10	1x
25mM $MgSO_4$	Variable	1.5 mM, 2.0 mM, 2.5 mM or 3.0 mM
Forward Primer	Variable	1 µM
Reverse Primer	Variable	1 µM
HotStar HiFidelity DNA Polymerase	1	2.5 units
RNase-free water	Variable	-
DNA Template	Variable	1-200ng
TOTAL VOLUME	50	-

Table 3-5: General PCR conditions.

Step	Time	Temperature (˚C)	
Initial Activation	5 minutes	95	
Denaturation	15 seconds	94	
Annealing	1 minute	60-70	35 cycles
Extension	1 minute/kb	72	
Final Extension	10 minutes	72	
End of PCR Cycling	∞	4	

3.3 **Agarose Gel Electrophoresis**

Following PCR amplification, agarose gel electrophoresis was used to verify that the expected PCR products were present. 10 µl of each PCR product was mixed with 2 µl of 6x DNA loading dye and run using a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/mL) in the electrophoresis gel tank (Bio-Rad Mini Sub® Cell GT) filled with 1x TAE buffer. Samples were run against GeneRuler™ 1kb plus DNA ladder at 100V for 60 minutes. The resultant bands were visualised and photographed under ultra violet (UV) light using a Gene Flash Syngene Bio Imaging and Video Graphic Printer UP-895MD system. Figure 3-2 illustrates a typical PCR image.
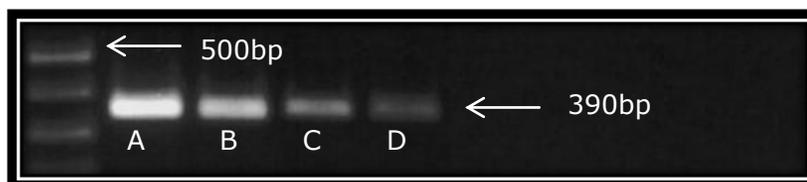
Figure 3-2: A typical PCR image with 1kb plus DNA ladder. A PCR image of F7 fragments at different magnesium concentration against 1kb plus ladder. (a) 1.5mM MgSO$_4$, (b) 2.0 MgSO$_4$, (c) 2.5 MgSO$_4$ and (d) 3.0 MgSO$_4$.

3.4 PCR Product Purification

After confirmation of the correct PCR products, the remainder of the products were purified using QIAquick PCR Purification Kit. This purification procedure removes primers, nucleotides, enzymes, salts and other impurities from DNA samples using a size exclusion procedure enabled by centrifugation. The purified PCR products were used later in the Gateway recombination experiments.

As per the manufacturer's instructions, 5 volumes of Buffer PBI were added to 1 volume of the PCR sample and agitated using a vortex mixer. The mixed sample was then loaded on to the QIAquick column and centrifuged at maximum speed (19000 rcf) for 1 minute. The flow-through was discarded. To wash, 0.75 ml of Buffer PE was added to the QIAquick column and centrifuged for 1 minute at maximum speed. The flow-through was discarded and the QIAquick column was centrifuged further at maximum speed for 1 minute to remove any residual ethanol. QIAquick column was then placed in a new 1.5 ml microcentrifuge tube. For elution, 45 µl of MilliQ-water was added to the centre of the QIAquick column, incubated at room temperature for 1 minute and then centrifuged at maximum speed for 1 minute. The purified PCR products were stored at -20˚C.

3.5 DNA Quantification

The NanoDrop® ND-1000 spectrophotometer (ThermoScientific, USA) was used to quantify DNA concentration (PCR products and plasmids). The NanoDrop® ND-1000 is a full 220-750 nm spectrum spectrophotometer that can measure 1 µl samples with high accuracy and reproducibility. The NanoDrop® ND-1000 is able to measure samples in a concentration range of 2-3700 ng/µl.

To measure DNA concentrations, the "nucleic acids concentration" application and the sample type of "DNA-50" was selected. The machine was then equilibrated using 1 µl distilled water. Following this, a blank reference measurement was made using 1 µl of MilliQ water. DNA sample (1 µl)was then loaded and quantified.

3.6 Gateway® Technology with Clonase™ II

Gateway® technology available from Invitrogen is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda with improved recombination specificity and efficiency (Landy, 1989). Gateway® technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences between vectors. There are two reactions which constitute the basis of Gateway® technology; BP reaction to create entry vectors and LR reaction to create expression vectors.

3.6.1 BP Reactions: Production of Entry Vectors

The BP reaction is a facilitated recombination between the PCR product (*att*B substrate) and a donor vector (*att*P substrate), catalysed by the BP Clonase™ II enzyme. For this research, pDONR221 was used as a donor vector. The vector map is given in Figure 3-3. A successful BP reaction will create an *att*L-containing entry vector. This entry vector is used in the subsequent LR reactions for creation of an expression vector.

The reaction was conducted according to the manufacturer's instructions. 150 ng of PCR product and 150 ng of donor vectors were combined in 5 µl TE buffer (pH 8.0) with 2 µl BP clonase II enzymes. The sample was mixed and incubated for 16 hours at 25˚C. To terminate the clonase enzyme activity, 1 µl of proteinase K was added to the plasmid sample and incubated for 10 minutes at 37˚C. 2 µl of the plasmid sample was then transformed into competent DH5α bacteria using a heat shock process.
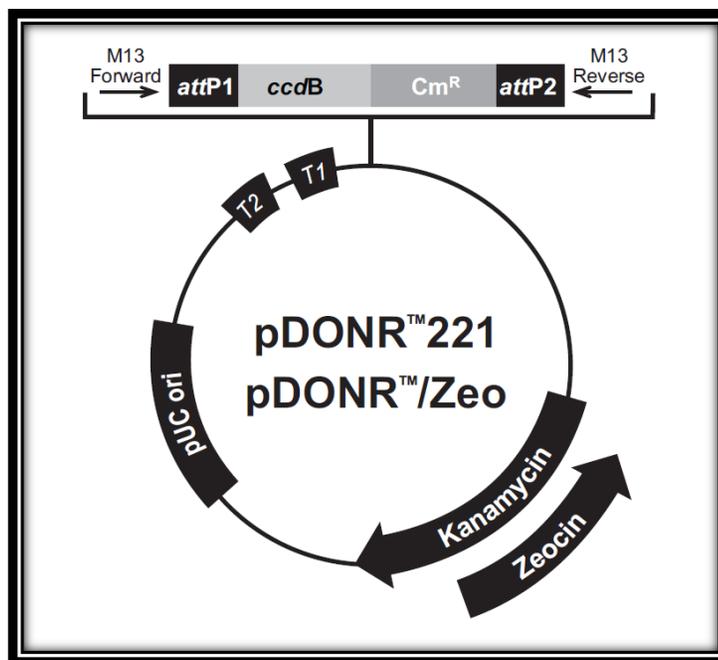
Figure 3-3: Vector map for pDONR221 (taken from the Gateway® Technology manual, Invitrogen).

3.6.2 Bacterial Transformation

Heat shock process is used to transform foreign plasmids into competent bacteria cells. For this project, heat shock was used to transform competent DH5α bacteria for production of entry vectors and expression vectors.

To start the process, competent DH5α bacteria cells were thawed on ice. Once thawed, 2 μl of the plasmid sample were added to 25 μl of competent cells. To ensure that the heat shock process was successful, pUC19 plasmid was used as a positive control. 2 μl of the pUC19 plasmid were added to 25 μl of competent cells. Both the sample and control mixtures were then incubated on ice for 30 minutes, heated in water bath at 42°C for 90 seconds, and placed back on ice for further 2 minutes. 200 μl SOC media were added to each vial of mixture and incubated in a shaking incubator for 1 hour at 37˚C and 250rpm. 50 μl of each mixture was then plated onto appropriate selective LB (Luria Broth) agar plates (kanamycin or ampicillin). The plates were incubated for 16 hours at 37°C to allow colony formation.

To extract plasmids from the bacteria, positive colonies were selected and grown in 10 mL LB with appropriate selective antibiotics. The bacteria were incubated in a 37°C bacterial shaker at 275 rpm for 16-20 hours. The plasmid DNA was then extracted using QIAprep miniprep kit as detailed in Section 3.7. The plasmid sample was quantified using Nanodrop® ND-1000 Spectrophotometer as described in section 3.5 and sequenced at Micromon (Monash University, Australia) to confirm the integrity of construct plasmids.

3.6.3 LR Reactions: Production of Expression Vectors

The LR reaction is a facilitated recombination step between the entry vector (*att*L substrate) and destination vector (*att*R substrate) catalysed by the LR Clonase™ II enzyme. A successful LR reaction will create an *att*B-containing expression clone. As mentioned previously in Section 3.2.2, two different destination vectors were used in this research, pcDNA™ 6.2/N-YFP-DEST and pcDNA™ 6.2/C-EmGFP-DEST. The vector map of these two vectors can be found in Figure 3-4 and Figure 3-5 respectively.
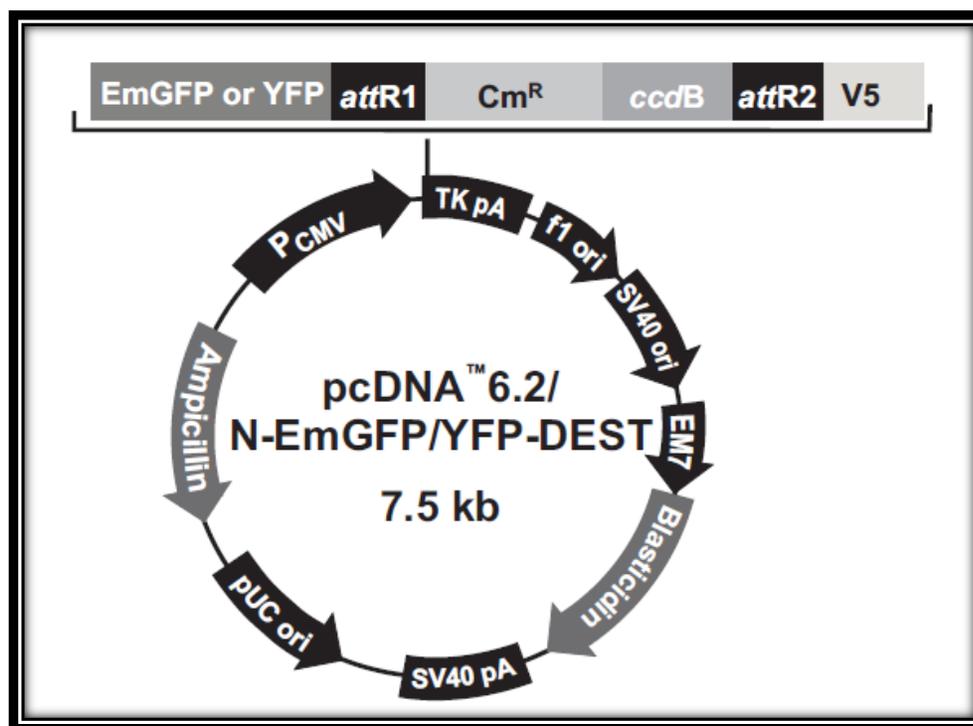
Figure 3-4: Vector Map for pcDNATM 6.2/N-YFP-DEST (taken from the Gateway® Technology manual, Invitrogen).
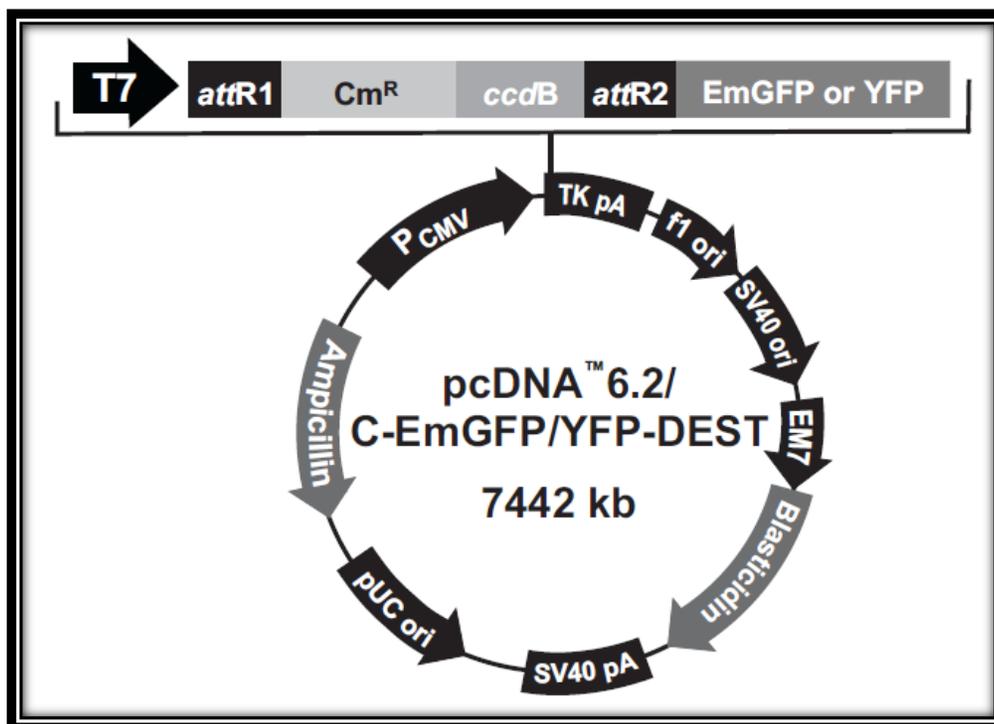
Figure 3-5: Vector Map for pcDNATM 6.2/C-EmGFP-DEST (taken from the Gateway® Technology manual, Invitrogen).

The reaction was conducted according to the manufacturer's instructions. 150 ng of entry clone and 150 ng of destination vectors were combined in 5 µl TE buffer (pH 8.0) with 2 µl LR clonase II enzymes. The sample was mixed and incubated overnight at 25°C. Proteinase K (1 µl) was then added to the sample, mixed and incubated for 10 minutes at 37°C. Sample (2 µl) was then transformed into competent DH5α bacteria by heat shock as described in section 0. 50 µl of each sample mixture was then plated onto ampicillin selective LB agar plates and incubated overnight at 37°C. Positive colonies were selected and grown up in 10 mL LB with 100 µg/mL ampicillin. The bacteria were incubated in a 37°C bacterial shaker at 275 rpm for 16-20 hours. Plasmid DNA was then extracted using the QIAprep miniprep kit as detailed in Section 3.7. The plasmid sample was quantified using a Nanodrop® ND-1000 Spectrophotometer as described in section 3.5 and sequenced by the Micromon service to confirm the integrity of constructs.

3.7 DNA Extraction

The QIAprep Miniprep kit was used to extract bacterial DNA plasmids. The kit has been designed to purify up to 20 µg of high-copy plasmid DNA from 5 ml overnight cultures of *Escherichia coli* in LB medium. All steps in the protocol were carried out according to the manufacturer's guidelines at room temperature unless otherwise stated.

Bacteria were grown for 16-20 hours in 10 ml LB medium with selective antibiotic (50 µg/mL kanamycin or 100 µg/mL ampicillin) in a shaking incubator at 37°C and 250 rpm. The bacterial cells were harvested by centrifugation at 19000 rcf at 4°C for 15 minutes. Pelleted bacterial cells were then resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. 250 µl of buffer P2 was then added and mixed by inverting the tube 6 times. Subsequently 350 µl of buffer N3 was added and mixed immediately by inverting the tube 6 times. The mixture solution then underwent centrifugation for 10 minutes at 19000 rcf. The supernatants were loaded onto QIAprep spin columns and centrifuged for 1 minute at 19000 rcf. The flow-through was discarded and 500 µl buffer PB was added to QIAprep spin column prior to centrifugation at 19000 rcf for 1 minute. The flow-through was discarded and 750 µl PE buffer was added and centrifuged for 1 minute at 19000 rcf. The flow-through was discarded and the QIAprep spin column underwent centrifugation for a further 1 minute to completely remove residual buffer. Afterwards the QIAprep spin column was placed in a new 1.5 ml microcentrifuge tube and the plasmid DNA was eluted from the column by adding 45 µl Milli-Q water to the centre of the column, incubated for 1 minute at room temperature and centrifuged at 19000 rcf for 1 minute. The plasmid DNA was then quantified and stored at -20°C.

3.8 Tissue Culture

For this research, five different mammalian cell lines (HeLa, Cos-7 cells, PC-3, Caco-2 and AD293) were used. Most transfection experiments were conducted on HeLa and COS-7 cells because of their distinct nuclear and cytoplasmic morphology allowing the localisation study to be analysed more effectively. HeLa cells were also used to express proteins for the purpose of western blotting since HeLa cells appeared to express the proteins at the highest yield in comparison with the other cell lines studied.

Unless otherwise stated, all the steps involved in tissue culture were carried out in a sterile environment. The tissue culture hood was cleaned before and after experiments with 70% ethanol. All materials were also cleaned with 70% ethanol prior to placing them in the hood. All the media and solutions were also warmed to 37°C prior to use.

3.8.1 Maintenance and Passage of Mammalian Cells

The specification of cell lines used in this study can be found in Chapter 2. All cells were maintained in a 37°C humidified incubator with 5% CO_2 in 75 cm^2 flasks containing 15 ml Dulbecco's Modified Eagle's Medium (DMEM) or F-12K supplemented with either 10% or 5% fetal bovine serum (FBS).

Routine passaging was performed every 48 – 72 hours as cells approached confluency. The media was removed and cells were washed in phosphate buffered saline (PBS) prior to incubation with 3 mL trypsin solution at 37°C with 5% CO_2 for approximately 5-10 minutes until all cells detached from the culture vessel. Cells were resuspended in 5 ml of supplemented DMEM by pipetting several times against the flask wall to ensure complete suspension. The cell suspension was transferred to a sterile Falcon conical tube. Cells were seeded onto 75cm^2 tissue culture flasks in $^1/_3$ dilution. If a transfection experiment was to be performed the next day, the cells were also seeded on 6 well plates in $^1/_{20}$ dilution.

3.8.2 Cryopreservation of Cells

For long term storage, trypsinised cells were resuspended in storage media. 1 ml of cells was aliquot to each cryostat vial, placed in Mr. Frosty® and transferred to a -80˚C freezer for at least 2 hours. Mr. Frosty® allows the cells to be frozen down slowly at the approximate rate of 1°C/min. The cells were then transferred to a liquid nitrogen dewar.

To start growing cells from the liquid nitrogen stock, cells were taken out of the dewar and rapidly warmed to room temperature. Immediately after thawing, the cells were

resuspended in 5 ml fresh medium and plated in 25 cm^2 flasks. The cells were then maintained in a 37°C humidified incubator with 5% CO_2. When approaching confluency the cells were trypsinised, resuspended in 15 ml fresh media and transferred to 75 cm^2 flask. The cells then underwent routine maintenance passage as described in section 3.8.1.

3.9 Lipofectamine^{2000TM} Transfection

The expression vectors needed to be transfected into mammalian cells for the localisation study and lipofectamine 2000^{TM} was selected as the transfection reagent for this research. Figure 3-6 is a schematic diagram of lipofectamine transfection. Transfection experiments were repeated in triplicate for each expression vector. With each set of experiments, destination vectors fused with the opening reading frame (ORF) of ovalbumin cDNA (OVA) were used as a control to produce a protein that should not enter the nucleus.

In preparation for the transfection experiment, mammalian cells were grown in 6 well plates to approximately 70% confluency. 30 minutes prior to transfection, 2 mL of Opti-MEM was added into each well in place of the DMEM media. The cells were kept in a 37°C incubator with 5% CO_2 until required for transfection.

Table 3-6: Dilution volume for DNA and Lipofectamine per 1 well of 6-well plate.

Volume of Plating Medium	DNA and Dilution Volume (µl)	Lipofectamine^{2000TM} and Dilution Volume (µl)
2 ml	12 µl in 38 µl	4 µl in 96 µl

DNA plasmid and lipofectamine were diluted with Opti-MEM as outlined in Table 3-6 and incubated at room temperature for 5 minutes. The DNA plasmid and lipofectamine were then mixed together and incubated for 20 minutes at room temperature. 200 µl DNA plasmid-lipofectamine complexes were used to transfect cells in each well. The cells were

incubated at 37°C with 5% CO_2 with the complexes for 25 minutes. Subsequently, the DNA plasmid-lipofectamine complexes were removed and 2 mL fresh DMEM media was added to each well. The cells were incubated at 37°C with 5% CO_2. Images of transfected cells were taken 48 hours post-transfection with confocal microscope as outlined in Section 3.16 for the localisation study.
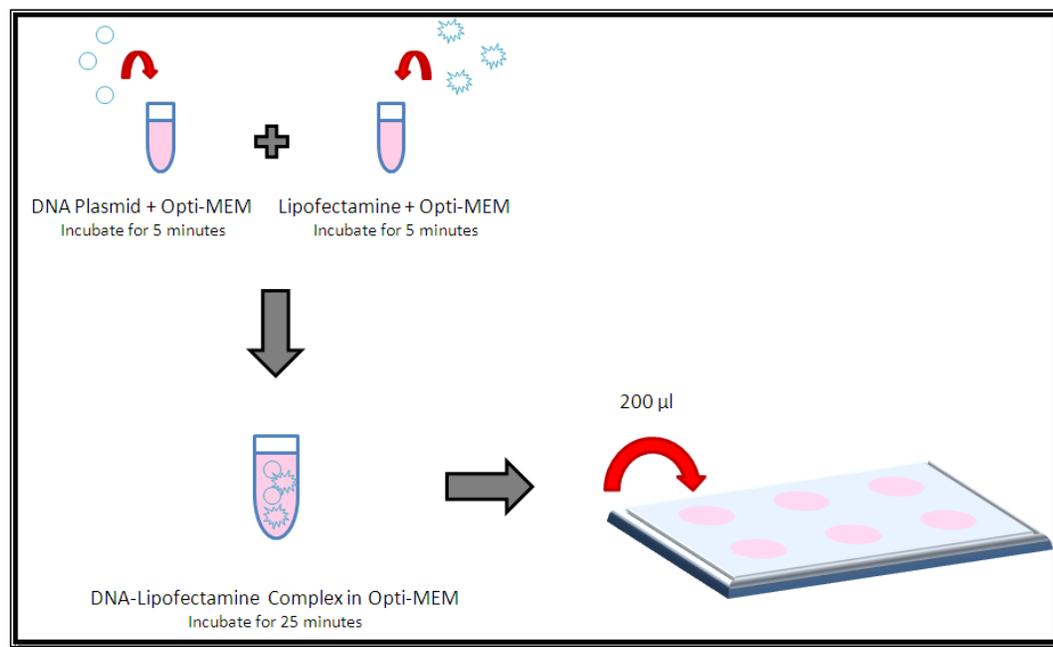
Figure 3-6: Scheme diagram of Lipofectamine transfection. First, DNA and lipofectamine were diluted in Opti-MEM media separately. DNA and lipofectamine were then incubated together in Opti-MEM at room temperature for 25 minutes to form DNA-lipofectamine complex. 200µl of the DNA-lipofectamine complexes were used to transfect one well of the 6-well microplate.

3.10 Mammalian Protein Extraction

To confirm that the mammalian cells are expressing the proteins of interest as fusion proteins, the proteins were extracted from HeLa cells after the completion of lipofectamine transfection and the localisation study.

The Qproteome^{TM} Nuclear Protein kit purchased from QIAGEN was used to extract the proteins since the proteins of interest are predominantly expressed in the nucleus. The

protocols were carried out as per the manufacturer's guidelines and at room temperature unless otherwise stated.

Table 3-7: Buffers for the Qproteome^{TM} Nuclear Protein extraction.

Buffer	Volume (µl)/sample	Protease Inhibitor Solution (µl)	M DTT (µl)	Benzonase Nuclease (µl)
Lysis Buffer NL	1000	10	5	-
Extraction Buffer NX1	50	0.5	-	-
Extraction Buffer NX2	100	1	1	1

Buffers were prepared as outlined in Table 3-7 prior to starting extraction. Once the transfected cells had been imaged under confocal microscope, the cells were washed twice with 2 ml ice-cold PBS to remove any trace of DMEM media. 5 ml ice-cold PBS was then added to each well. The cells were carefully removed with a cell-scraper and transferred to pre-chilled 15 ml conical tubes. The cell suspension then underwent centrifugation for 5 minutes at 450 g and 4°C. Supernatant was discarded. The cell pellet was then gently resuspended in 500 µl lysis buffer NL by pipetting up and down several times and then incubated on ice for 15 minutes. The resuspended cell suspension was then transferred to a pre-chilled microcentrifuge tube. 25 µl of detergent solutions NP was then added and agitated using a vortex mixer at maximum speed for 10 seconds. The cells then underwent centrifugation at 10,000 g for 5 minutes at 4°C. The supernatant (cytosolic fraction) was transferred to a new sterile microcentrifuge tube and stored at -80°C. The pellet (containing cell nuclei) was resuspended by agitation with vortex mixer at maximum speed for 5 minutes in 500 µl nuclear protein lysis buffer NL. The resuspended cells then underwent centrifugation at 10,000 g for 5 minutes at 4°C. The supernatant was discarded.

The pellet was resuspended in 50 μl extraction buffer NX1 by pipetting up and down several times and incubated at 4°C for 30 minutes at 750 rpm in a thermomixer. The cell suspension then underwent centrifugation at 4°C for 10 minutes at 12,000 g. The supernatant (nucleic-acid binding proteins) was transferred to a new sterile microcentrifuge tube and stored at -80°C. The pellet was resuspended in 100 μl extraction buffer NX2 by pipetting up and down several times and was then incubated at 4°C for 1 hour at 750 rpm in a thermomixer. The cell resuspension then underwent centrifugation at 4°C for 10 minutes at 12,000 g. The supernatant (containing insoluble nuclear proteins) was transferred to a new sterile microcentrifuge tube and stored at -80°C.

Protein was quantified using the Bradford method (described in Section 0). 50 μg of protein was mixed with 2 μl SDS loading dye and heated to 95°C for 5 minutes. Protein sample was then loaded onto a 12% SDS-PAGE gel.

3.11 **Bacterial Protein Extraction**

A bacterial expression system was used to express proteins for AlphaScreen® assay experiments as the bacterial system is capable of expressing recombinant protein at a high yield in comparison to mammalian expression systems. pDEST15 and pDEST24 bacterial expression vectors, available from Invitrogen, were selected for the purpose of this research. pDEST15 is designed for N-terminal fusion vectors while pDEST24 is a C-terminal fusion vector. However, both of the vectors contain a GST tag which allows purification of recombinant fusion protein using affinity chromatography on glutathione agarose. The map for these expression vectors can be found in Figure 3-7 and Figure 3-8.
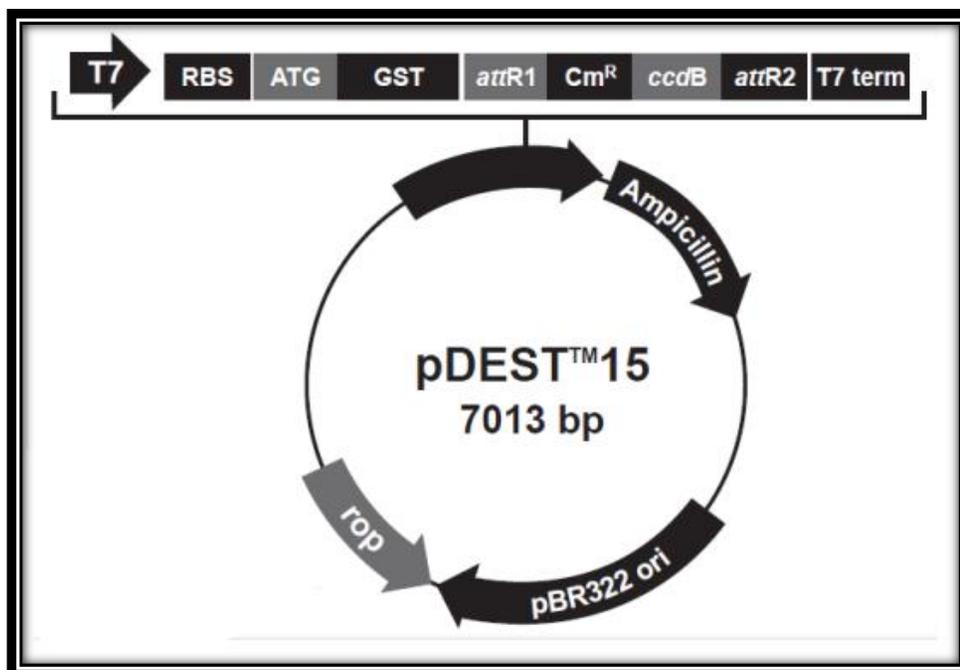
Figure 3-7: Vector map for pDEST15 (taken from the Gateway® Technology manual, Invitrogen).
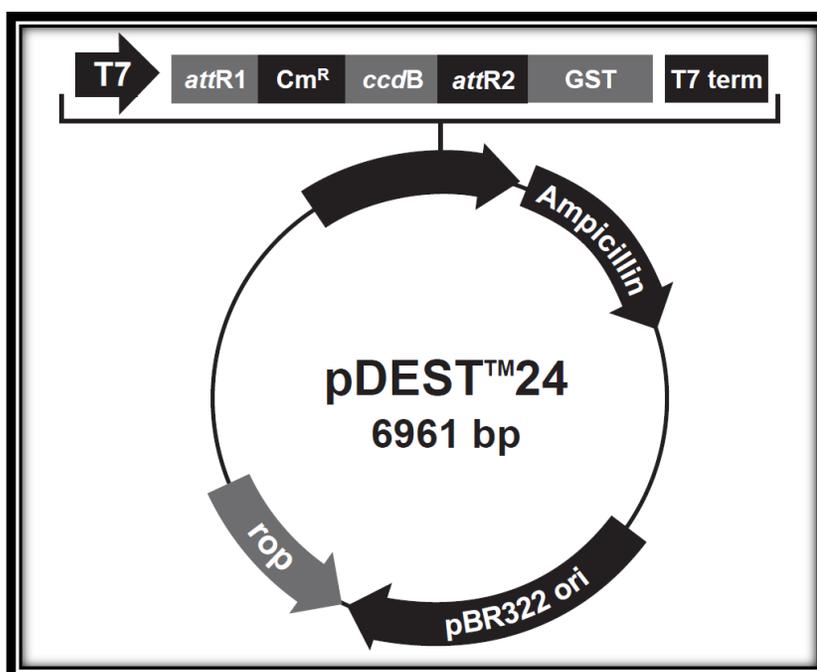
Figure 3-8: Vector map for pDEST24 (taken from the Gateway® Technology manual, Invitrogen).

The Gateway® system was used to introduce proteins of interest into these expression vectors as described in Section 3.6. For high protein expression levels, the expression vectors were transformed into BL21-AI One Shot cells using heat shock as described previously in 3.6.1. The BL21-AI has been designed specifically to express recombinant proteins and therefore would express proteins of interest at a much higher yield than other bacteria cell lines. Once all the proteins had been transformed, regular bacterial protein extraction protocols were used.

A pilot expression study was carried out to identify the best time point for maximum protein expression. 3 positive colonies were selected and grown in 5 ml of LB containing 100 µg/ml ampicillin at 37°C with 250 rpm until the OD_{600} was between 0.6-1.0 (approximately 4-6 hours). The value of OD_{600} was measured using a spectrophotometer with LB media as blank. Afterwards, 1 ml of culture was transferred to 20 ml of LB media containing 100 µg/ml ampicillin. This dilution allows the cells to rapidly return to logarithmic growth phase. The cells were incubated at 37°C with 250 rpm for 3 hours until OD_{600} measured 0.4. Each culture was then divided into two separate tubes (containing 10ml each) and 0.2% L-arabinose was added to one of the tubes. At this stage each culture was processed in 2 ways; induced (contains L-arabinose) and un-induced. 500 µl aliquots from each culture were removed and centrifuged at maximum speed for 30 seconds. Supernatant was discarded and pellets kept at -20°C. These are the zero time point samples. The cells were kept incubating at 37°C with 250 rpm, and samples (induced and un-induced) were taken every hour for 4 hours. All the collected pellets were resuspended in 80 µl 1x SDS-PAGE sample buffer and heated to 95°C for 5 minutes. 10 µl of each sample was then loaded onto a 12% SDS-PAGE gel.

3.12 Protein Purification: Affinity Chromatography

3.12.1 Proteins of Interest

Affinity chromatography on glutathione agarose was used to purify GST-fusion proteins. The protocols were carried out as per the manufacturer's guidelines and at room

temperature unless otherwise stated. In brief, the cell sample was centrifuged to remove undissolved membranes and cellular debris. The affinity column was washed with 10 bed volumes of PBS to remove azide and equilibrated with 5 bed volumes of PBS containing 1% Triton X-100. The sample was added to the column and the eluate was collected. 10 bed volumes of PBS were used to wash the column each of 5 times until no proteins were detected in the eluate. The bound GST-fusion proteins were eluted with 5 bed volumes of 50 nM Tris-HCl buffer, pH 8, containing 5 mM glutathione. Each sample was concentrated using the appropriate Amicon MW cut-off membrane and quantified using the Bradford prior to SDS-PAGE.

3.12.2   Importin

The IMP proteins were purified from bacteria as GST-fusion proteins under native conditions. The bacteria were inoculated and grown with ampicillin until OD_{600} measured 1.2. The protein expression was then induced with 1 mM IPTG for 6 hours at 28°C and harvested by centrifugation at 2000 rcf at 4°C for 30 minutes. The pellet was resuspended in 25 ml of cold PBSD with 0.5 mM PMSF and frozen overnight at -80°C. Next day, the pellet was thawed prior to incubation with 1 mg/ml of lysozyme and Complete™ EDTA-free protease inhibitors for 15 minutes at 4°C. 1 mg/mL of sodium deoxycholate and 10U/mL of DNase I were added to the reaction and incubated for 15 minutes at 4°C. The insoluble material was pelleted at 22000 rcf for 1 hour at 4°C. 5 mL of GSH bead slurry was equilibrated in 40 mL PBSD, centrifuged at 1000 rpm for 5 minutes and the supernatant was discarded. This equilibration process was repeated a total of 3 times. The clear lysate mixture was added to the GSH beads and incubated for 2 hours at 4°C. The beads were centrifuged at 1000 rpm at 4°C for 5 minutes and supernatant was discarded. The beads were then washed with 40 mL PBSD 3 times, prior to incubation with 10 mL elution buffer for 5 minutes at room temperature. The beads underwent centrifugation at 120 rcf for 5 minutes at 4°C, the supernatant was collected, and the elution process was repeated a further 2 times. Each protein supernatant underwent dialysis at 4°C overnight using an Amicon 50 MW cut-off membrane. On the following day, the protein was concentrated using Amicon 50 MW cut-off for GST-IMPα (approx 86 kDa) and Amicon 100 MW cut-off for GST-IMPβ (approx. 123 kDa).

3.13 **Protein Quantification: Bradford Method**

The Bradford method was used to quantify proteins of interest. The Bovine serum albumin (BSA) standards were made fresh each time according to the Table 3-8;

Table 3-8: Set up of BSA Standards for protein quantification.

Standard Concentration (µg/ml)	Volume of 5mg/ml BSA stock solution (µl)	Volume of sample buffer (µl)
0	0	500
50	10	490
100	20	480
200	40	160
500	100	400
1000	200	300
1500	300	200
2000	400	100

245 µl of Bradford reagent was added to U-bottom 96 well plates with either 5 µl of each BSA standard or 5 µl of buffer for the blanks and mixed well without creating bubbles. Each standard was repeated in triplicate. For samples, 5 µl samples were added to 245 µl of Bradford reagent. Once set, the plate was wrapped in aluminium foil and incubated at room temperature for 5 minutes. The absorbance was read using the Perkin Elmer EnVision® Multilabel reader (USA) at 595nm excitation. Average values for the triplicates were calculated and a BSA standard curve was plotted using the Sigma Plot graphing

program. The sample concentration was calculated against the BSA standard concentrations.

3.14 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate and confirm the presence of proteins of interest. The proteins are coated with negatively charged SDS and move towards the positively charged electrode through the acrylamide gel. Smaller proteins migrate faster and thus proteins are separated according to its molecular size. SDS-PAGE gel is made up of two gel components: separation and stacking gel. The resolution of the gel is determined by the concentration of acrylamide; the lower the acrylamide concentration, the better the resolution for the higher molecular weight proteins. For this project, a 12% gel was shown to provide the best visual results. The gel recipe can be found in Table 3-9.

SDS-PAGE gel preparation is a two process step. First the separation gel was prepared, loaded on to the gel plate and left to polymerise at room temperature for at least 30 minutes. Once the separation gel was set, the stacking gel was prepared and added onto the top of the separating gel and left to set for at least 30 minutes at room temperature. Once the SDS-PAGE gel was ready, the protein sample was mixed with SDS loading dye and heated for 5 minutes at 95°C to denature the protein. The sample was then loaded and ran against PageRuler prestained protein ladder or Odyssey two-colour protein molecular weight markers if a Western blot was to be carried out. The electrophoresis was conducted in SDS running buffer for 15 minutes at 100V followed by at least 60 minutes at 200V until the dye reached the bottom of the gel.

Table 3-9: Recipes for separation and stacking gels.

12% Separation Gel	12% Stacking Gel
4.5 mL distilled water	6.4 mL distilled water
2.5 mL 1.5M Tris-HCl (pH 8.8)	2.5 mL 0.5M Tris-HCL (pH 6.8)
3.0 mL acrylamide/bis (40% stock),	1 mL acrylamide/bis (40% stock)
50 µL 10% ammonium persulfate	50 µL 10% APS
100 µL 10% (w/v) SDS	100 µL 10% (w/v) SDS
5 µL N,N,N′,N′-Tetramethylethylenediamine (TEMED).	10 µL TEMED

3.14.1   Coomassie Blue Staining

Coomassie brilliant blue was used to visualise proteins on SDS-PAGE gels. Once the SDS-PAGE was completed, the gel was transferred to a plastic box. Coomassie blue staining solution was poured into the box to cover the gel, which was then left to incubate at room temperature with gentle agitation for at least 2 hours to overnight. The gel was then destained in Destain I solution for 1 hour and in Destain II solution until the background of the gel was fully destained. The gel was visualised and photographed under ultra violet (UV) light using a Gene Flash Syngene Bio Imaging and Video Graphic Printer UP-895MD system.

3.15 **Western Blot**

Western blot is an analytical technique used to detect specific proteins with an antibody. For this research, Western blot was used to detect the presence of EmGFP- and YFP- fusion

proteins from the mammalian expression system and GST fusion protein from the bacterial expression system. The process of Western blotting can be divided into two steps. The first step consists of protein separation based on the molecular weight using gel electrophoresis as discussed in Section 0. The second step consists of specific binding of an antibody with a target protein.

3.15.1   Antibody Binding

Three primary antibodies were used for this research; mouse anti-GFP for detection of EmGFP-fusion proteins, mouse anti-V5 for detection of nYFP-fusion proteins and mouse anti-GST for detection of GST-fusion proteins. Once the SDS-PAGE is completed, the gel was blotted to a PVDF nitrocellulose membrane for 30 minutes at 15V using a Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell (Biorad, Australia). Membranes were blocked for 60 minutes at room temperature with Odyssey blocking buffer. Membranes were then incubated with 1:5000 dilutions of primary antibodies (anti-GFP, anti-V5 or anti-GST) for 16 hours at 4°C while shaking in Odyssey blocking buffer. The next day, membranes were washed 3 times (5 minutes each) with PBST. Afterwards the membranes were incubated for 60 minutes at room temperature on a shaker in 1:2000 diluted secondary antibodies (goat anti-mouse conjugated to 700nm infra red dyes (1:15000 dilution) for visualisation. During incubation, the membranes were protected from light with aluminium foil. After incubation, membranes were washed 4 times (5 minutes each) with PBST. The membranes were then viewed directly with the Odyssey Infrared Imaging system.

3.16 **Odyssey Infrared Imaging system**

The protein gel was placed face down and inverted onto the Odyssey machine (Licor, USA). On the computer screen, area where the gel is place was selected. The software then scanned the gel to produce a digitised image. The size of the protein was measured against the protein ladder.

3.17 **Confocal Imaging**

A Nikon A1R confocal microscope (USA) was used to image transfected cells. Live cell imaging was performed directly on 6 well plates in DMEM (no phenol red) media under 20 x objectives and saved as digitalised files using NIS-Elements AR 3.2 software. A minimum number of 50 transfected cell images were taken for each trial of expression vectors and for each cell lines. The 488nm filter was used to visualise both the EmGFP and YFP fluorescent proteins. The exact excitation and emission wavelengths of these proteins can be found in Table 3-10.

Table 3-10: Excitation and Emission wavelengths for EmGFP and YFP fluorescent proteins.

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509
YFP	514	527

3.18 **Statistical Analysis**

3.18.1 Image Analysis of transfected cells

The analysis of images was carried out using the digitalised confocal files using Image J 1.42q public domain software (National Institute of Health, MA, USA) to estimate the relative concentrations of fusion proteins. Once the image is opened with the Image J programme, an area of nuclear (Fn), cytoplasmic (Fc) and background was selected and fluorescence intensity was measured. In some cases, when there was clear homing of protein to nucleoli, fluorescence intensity of the nucleolus (Fnu) was also measured. This enables the Fn/Fc and Fnu/Fc ratios to be calculated. The Fn/Fc and Fnu/Fc were calculated based on the relative nuclear, nucleolus and cytoplasmic fluorescence intensity above background fluorescence. Quantitative Fn/Fc and Fnu/Fc analysis were performed on 150 cells and analysed by one way ANOVA.

3.18.2   One Way Analysis of Variance (ANOVA)

ANOVA was used to statistically analyse whether the specific fusion proteins had any influence on nuclear localisation. This was done by comparing the average of Fn/Fc or Fnu/Fc from the fusion proteins of interest with the Fn/Fc or Fnu/Fc of the control. In all cases, the difference between the sample and control is considered to be * significant when $P < 0.05$, ** significant when $P < 0.01$ and *** significant when $P < 0.001$.

3.18.3   Student T-test

Student's T-test was used to statistically analyse the difference in binding affinity between the protein studied and the three IMP family members tested. The InStat statistical analysis program was used to perform the test. In all cases, the binding affinity is considered to be * significant when $P < 0.05$, ** significant when $P < 0.01$ and *** significant when $P < 0.001$.

CHAPTER 4

Identification of Nuclear Localisation Signals in Adenovirus Terminal Protein

CHAPTER 4   Identification of nuclear localisation signals in Adenovirus terminal protein

4.1 Overview

The success of gene therapy is dependent on the development of vectors that can selectively and efficiently deliver therapeutic genes to target cells *in vivo* with minimal toxicity. Viral vectors are the most widely used transducing systems. However, there are many ethical and safety concerns associated with the use of viruses in humans. Non-viral vectors are more suitable because of the lack of a specific immune response; but these are currently inefficient due to poor gene transport.

For non-viral gene therapy to achieve its clinical potential, the transfection efficiency of the vector needs to approach that of a virus such as the Adenovirus. There are several extracellular and intracellular barriers which influence the amount of therapeutic gene (usually in a form of DNA plasmid) that reaches its intended destination, the nucleus. These barriers include the plasma membrane, endosomal or lysosomal degradation, trafficking within the cytoplasm, and ultimately the nuclear envelope. There have been developments in the design of lipid-based nanoparticles which allow them to successfully enter cells through endocytotic mechanisms and escape endosomes into cytoplasm. However, the rate-limiting step, and hence the most significant challenge, is the nuclear delivery stage, when the DNA needs to cross the nuclear membrane to reach the nucleus (Pouton *et al.*, 2001; Pouton *et al.*, 2007). One possible way of overcoming this barrier is to develop vectors that mimic the highly efficient processes that viruses use to achieve nuclear delivery.

There has been widespread interest in the use of Adv primarily because of its ability to efficiently deliver double-stranded DNA to the nucleus. In addition, its large genome allows for extensive modification and incorporation of therapeutic genes. Adv is a non-enveloped virus containing a 36-kb genome that consists of early genes which encode for regulatory proteins and late genes which encode for structural proteins (Rux *et al.*, 2004). The basic

understanding of how Adv nuclear delivery systems work is fundamentally important science and has the potential to result in further development and enhancement of the non-viral gene therapy. This chapter concentrates on the nuclear import of Adv with a focus on the Adv terminal protein which has been shown to localise in the nucleus. It is not known at this stage whether TP plays a vital role in the primary infection of cells by Adv, and as yet the sequences responsible for nuclear uptake of TP itself require to be studied further in detail. The aim of the chapter is to identify the NLS encoding sequence(s) within the TP of Adv serotype 5.

4.2 Nuclear Transport

The nucleus controls the activities of the cell by regulating gene expression. Access to the nucleus is strictly controlled by the nuclear envelope and nuclear pore complex. The NE is a double lipid bilayer membrane surrounding the nucleus to separate molecules between thecellular cytoplasm and nucleus (Kobe, 1999). Molecules, perhaps with the exception of small lipophilic molecules, are transported between the cytoplasm and nucleus compartment through an exchange channel known as the NPC. The NPC is a large 125 MDa multiprotein complex with a pore diameter of 25nm (Gasiorowski *et al.*, 2003). Small molecules with a diameter of up to 9nm are able to diffuse passively through the NPC, however, larger molecules require a specific signal sequence, known as nuclear localisation sequence, that facilitates active transport (Gasiorowski *et al.*, 2003; Kobe, 1999). Proteins require association with karyopherins known as importins to enter the nucleus and exportins (EXP) to escape the nucleus into the cytoplasm (Gasiorowski *et al.*, 2003; Macara, 2001; Whittaker *et al.*, 1998).

4.3 Nuclear Localisation Signals

The idea that NLSs may exist was first suggested by De Robertis *et al*. in 1978 but their discovery remained unresolved until 1982 (De Robertis *et al.*, 1978; Silver, 1991). The first NLS was discovered in the oocyte of *Xenopus*, when Dingwall *et al.* identified that an absence of C-terminal peptide in a major nuclear protein nucleoplasmin, prevented the protein from entering the nucleus, however, the C-terminal peptide segment alone was

able to transport into the nucleus efficiently. This finding suggested that a subset of amino acids were responsible for nuclear transport (Silver, 1991).

Current knowledge of NLSs suggests that they can be divided into several classes. The classical NLSs consist of one or two clusters of basic residues and can be referred to as monopartite NLSs and bipartite NLSs. The monopartite NLSs comprise a single cluster of basic amino acids (aa) and can be classified into 2 types, one with at least 4 consecutive basic aa, exemplified by SV40 large T antigen NLS: PKKKRKV, and one with only 3 basic aa (putative consensus sequence is defined as K-K/R-X-K/R), exemplified by the c-Myc NLS: PAAKRVKLD (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007). The bipartite NLS is typically composed of two stretches of basic aa separated by a linker of 10-12 residues (the putative consensus sequence is defined as $(K/R)(K/R)X_{10-12}(K/R)_{3/5}$ (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007)). Other NLS types include those that contain charged/polar residues with non-polar residues, resembling that of the yeast homeodomain-containing protein Matα2 NLS (NKIPIKDLLNPQ), or those composed of a basic cluster of aa surrounded by proline and aspartic acid residues, such as the proto-oncogene c-myc NLS (PAAKRVKLD[328]) (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007). Another class of non-classical NLSs include those lacking stretches of basic residues such as the largely hydrophobic M9 NLS (NQSSNFGPMKGGRSSGPYGGGGQYFAKPRNQGGY[305]) of the human mRNA-binding protein hnRNP A1 (Kosugi *et al.*, 2009).

Although classical NLSs are typically recognised by the IMPα/β heterodimer, there are many basic and non-classical NLSs that are recognised by different receptors, for example IMPβ1 alone, IMPβ2 or transportin. Selection of NLS types and their distinct IMP receptors can be found in Table 4-1.

Table 4-1: Selected examples of NLS consensus recognised by distinct IMP receptors.

IMP	Protein	NLS Sequence	Reference
IMPα/β	T-ag	PKKKRKV[132]	1
	NF-κB p50	QRKRQK[372]	2
	NF-κB p65	HRIEEKRKRTYETFKSI[345]	2
	UL44	PNTKKQK[433]	3
	BRCA1	KRKRRP[508], PKKNRLRRK[615]	4, 5
	LEF-1	KKKKRKREK[382]	6, 7
	Rb	KRSAEGSNPPKPLKKLR[877]	8
	DNA Helicase Q1	KKAANMLQQSGSKNTGAKKRK[645]	9
	Nucleoplasmin	KRPAATKKAGQAKKKK[170]	10
	P53	KRALPNNTSSSPQPKKKP[322]	11
	c-myc	PAAKRVKLD[328]	12
	Matα2	NKIPIKDLLNPQ[13]	13, 14
IMPβ1	PTHrP	RYLTQETNKVETYKEQPLKTPGKKKKGKP[94]	15
	CREB	RRKKKEYVK[309]	16
	SRY	RPRRKAK[136]	17
	TRF-1	KKKKESRR[356]	18
	REV	RQARRNRRRRWRE[47]	19

	GAL4	RLKKLKCSKEKPKCAKCLKNNWECRYSPKTK R[46]	20
IMPβ2 (transportin)	hnRNP A1 M9	NQSSNFGPMKGGRSSGPYGGGGQYFAKP RNQGGY[305]	21
IMPβ3 (RanBP5)	Transcription factor Pho4	SANKVTKNKSNSSPYLNKRKGKPGPDS[166]	22, 23
IMPβ3/ IMPβ4 (Yrb4p)	Ribosomal protein L25	MAPSAKATAAKKAVVKGTNGKKALKVRTS ATFRLPKTLKLAR[41]	24, 25

References 1 (Kalderon *et al.*, 1984); 2 (Nadler *et al.*, 1997); 3 (Alvisi *et al.*, 2005); 4 (Chen *et al.*, 1996); 5 (Li *et al.*, 1998); 6 (Prieve *et al.*, 1996); 7 (Herold *et al.*, 1998); 8 (Efthymiadis *et al.*, 1997); 9 (Miyamoto *et al.*, 1997); 10 (Robbins *et al.*, 1991); 11 (Liang *et al.*, 1999); 12 (Dang *et al.*, 1988); 13 (Hall *et al.*, 1984); 14 (Lanford *et al.*, 1990); 15 (Lam *et al.*, 1999); 16 (Forwood *et al.*, 2001b); 17 (Forwood *et al.*, 2001a); 18 (Forwood *et al.*, 2002); 19 (Henderson *et al.*, 1997); 20 (Chan *et al.*, 1999); 21 (Pollard *et al.*, 1996); 22 (Kaffman *et al.*, 1998); 23 (Nakielny *et al.*, 1999); 24 (Schlenstedt *et al.*, 1997); 25 (Rout *et al.*, 1997)

4.3.1 Importin Pathway

The best understood nuclear pathway is the classical importin pathway, which is dependent on the recognition of NLS found on proteins by IMPα/β heterodimer (Christophe *et al.*, 2000; Jans *et al.*, 2000; Kaffman *et al.*, 1998; Kosugi *et al.*, 2009; Schlenstedt, 1996; Strambio-De-Castillia *et al.*, 2010). The classical NLS is recognised by IMPα in cytoplasm and forms a complex with IMPβ1. IMPβ1 initiates interaction of the cargo complex with the NPC and nuclear transportation (Christophe *et al.*, 2000; Jans *et al.*, 2000; Kosugi *et al.*, 2009; Schlenstedt, 1996; Strambio-De-Castillia *et al.*, 2010). Once in the nucleus, RanGTP binds to IMPβ1 causing conformational change that reduces its affinity to IMP resulting in the release of cargo protein complex into the nucleus (Christophe *et al.*, 2000; Jans *et al.*, 2000; Kosugi *et al.*, 2009; Schlenstedt, 1996; Strambio-De-Castillia *et al.*, 2010).

4.3.2 Exportin Pathway

Exportins are involved in the nuclear export of proteins from nucleus into the cytoplasm (Cook *et al.*, 2007; Jamali *et al.*, 2011). Similarly to the importin pathway, nuclear export is an active process, obtaining its energy from the Ran protein, and is dependent on the nuclear export signal (NES) (Cook *et al.*, 2007; Jamali *et al.*, 2011). The export receptors, commonly CRM1 (chromosomal region maintenance-1), form a complex with the cargo protein in presence of RanGTP in the nucleus and release cargo protein in the cytoplasm when Ran undergoes hydrolysis to RanGDP (Cook *et al.*, 2007; Jamali *et al.*, 2011).

There have been many NES discovered, but the best characterised is the hydrophobic NES, which is a short amino acid sequence containing three to four hydrophobic residues (often leucine), exemplified by Rev NES (LPPLERLTL) (Niidome *et al.*, 2002; Pemberton *et al.*, 2005).

4.4 **Nucleolus Transport**

The nucleolus is the sub-domain within the nucleus with multiple cellular activities including synthesis of ribosomal RNA (rRNA), ribosomal biogenesis and viral infections (Olson, 2010; Olson *et al.*, 2002; Raška *et al.*, 2004; Scheer *et al.*, 1999). The nucleolus is a membrane free structure and is highly variable dependent on the type and synthetic activity of the cell in which the nucleolus is contained (Olson, 2010; Scheer *et al.*, 1999). Nucleoli may be divided into three main classes; reticulated nucleoli, compact nucleoli and ring-shaped nucleoli (Olson, 2010; Scheer *et al.*, 1999). The nucleolus is composed of many different proteins but three of the most abundant proteins are nucleolin, fibrillarin and B23 (Hiscox, 2002; Olson, 2010; Raška *et al.*, 2004).

Similar to nuclear transport, protein delivery to the nucleolus is dependent on a specific signal known as the nucleolar localisation signal (NuLS) (Birbach *et al.*, 2004; Hiscox, 2002). The NuLS can range from a few amino acids to over a hundred, and although some similarities have been observed within the identified NuLS no common consensus

sequence has emerged to date (Birbach *et al.*, 2004; Liu *et al.*, 2006). Table 4-2 lists examples of identified NuLS in virus proteins.

Table 4-2: Selected examples of NuLS in virus proteins.

Protein	NuLS Sequence	Reference
Adv Protein V	KKKKK[42], RRRRRR[337] and KR[160]-KR[164]-KR[179]-R[181]	1
Borna disease virus Capsid Protein	PPESRKKL[83]	2
Infectious bronchitis virus Nucleoprotein	PKKEKKLK[367]	3
Marek's disease virus MEQ Protein	RRRKR[66]-RRRRRK[77]	4
Semliki Forest virus Capsid Protein	KKKK[78] and KKKKK[104]	5
Transmissible gastroenteritis virus Nucleoprotein	RKRK[343]	6

References 1 (Matthews, 2001), 2 (Hiscox, 2002), 3 (Hiscox *et al.*, 2001), 4 (Liu *et al.*, 1997), 5 (Favre *et al.*, 1994) and 6 (Wurm *et al.*, 2001).

4.5 Adenovirus Trafficking

The majority of mechanistic information about Adv has been derived from studies on Adv subgroup C (Miyazawa *et al.*, 1999). Trafficking can be divided into five different stages. These include; cell receptor binding, cell entry, endosomal escape before degradation, microtubule translocation and nuclear import. The route of Adv cell entry is consistent with

a classical clathrin-mediated endocytotic pathway (Kobe, 1999; Leopold *et al.*, 2007; Nicola *et al.*, 2009).

Adenovirus enters the cells through a stepwise uncoating program (Greber *et al.*, 1994; Greber *et al.*, 1997). The fibers are released and bind to the specific cell binding receptor (Greber *et al.*, 1994; Greber *et al.*, 1997; Kobe, 1999). All subgroups of the Adv, except subgroup B, use Coxsackie and Adenovirus Receptor (CAR) as their extracellular receptor (Johansson *et al.*, 2007; Nemerow *et al.*, 2009; Pache *et al.*, 2008a; Tomko *et al.*, 1997). The majority of Adv subgroup B particles bind to CD46 (Pache *et al.*, 2008a; Pache *et al.*, 2008b). Interactions between the viral penton base with the secondary receptor, $\alpha v \beta 5$, promote Adv uptake into endosomes (Farmer *et al.*, 2009a; Greber *et al.*, 1997; Kobe, 1999; Wang *et al.*, 2000; Wickham *et al.*, 1994). By lysing the endosome, using an only partially understood mechanism, Adv is thought to reach the nucleus by intracellular translocation along the microtubules with the aid of the cytoplasmic dynein complex (Bailey *et al.*, 2003; Greber *et al.*, 2006; Kelkar *et al.*, 2006; Kobe, 1999; Samir A *et al.*, 2004). Dynein is a microtubule-dependent molecular motor that assists the movement of protein cargo toward the microtubule organisation center (MTOC) and the nucleus (Bailey *et al.*, 2003; Kobe, 1999). The molecular details of the nuclear delivery of Adv DNA are yet to be elucidated, however, it is clear that Adv DNA is delivered by an active process through the NPC (Hindley *et al.*, 2007b; Saphire *et al.*, 2000). Several studies suggest that import factors; IMPα, IMPβ, and IMP7 may be responsible for the Adv DNA delivery to the nucleus (Hindley *et al.*, 2007b).

Although the contents of Adv core structure are well studied, it is yet to be established which of the core proteins enter the nucleus with the DNA. Due to the covalent attachment of TP to the 5' ends of the Adv DNA, TP is assumed to be imported into the nucleus coupled to the Adv DNA (Cardoso *et al.*, 2008; Puntener *et al.*, 2009; Zhao *et al.*, 1988).

4.6 **Adenovirus Terminal Protein**

TP is one of the Adv core proteins and can be found covalently coupled to the inverted terminal repeat (ITR) at the 5' ends of each strand of the Adv genome (Leopold *et al.*, 2007; McConnell *et al.*, 2004; Smart *et al.*, 1982; Tamanoi *et al.*, 1982; Webster *et al.*, 1997b). The attachment between the Adv DNA and the TP is through a phosphodiester bond between the β-hydroxyl group of a 232 serine residue of TP and the 5'-terminal deoxycytidine residue of the Adv DNA (Bayliss *et al.*, 2000; Challberg *et al.*, 1981b; Ribbeck *et al.*, 2001; Smart *et al.*, 1982).

Initially TP is synthesised as an 80kDa precursor protein known as pre-terminal protein (Challberg *et al.*, 1981b; Freimuth *et al.*, 1986; Stillman *et al.*, 1981). pTP can be found covalently coupled to the replicating DNA. The cleavage process from pTP to TP occurs during late infection as new virions mature. First, pTP is cleaved at MRGF^{175}-G^{176} and MGGR^{183}-G^{184} by Adv protease to form an intermediate-TP (iTP) (Challberg *et al.*, 1981b; Freimuth *et al.*, 1986; Stillman *et al.*, 1981). iTP is then cleaved at the MTGG^{349}-V^{350} site to form a 55kDa COOH-terminal fragment (Challberg *et al.*, 1981b; Freimuth *et al.*, 1986; Stillman *et al.*, 1981). The sites giving rise to both iTPs have a glycine in the P1' position, while the TP site has the branched amino acid valine at P1'. pTP is encoded by the leftward-transcribed strand of the Adv genome and comprises part of a transcription unit that also encodes the single-strand DNA binding protein (Stillman *et al.*, 1981; Tamanoi *et al.*, 1982). The processing of pTP to TP is not required for the Adv DNA replication (Challberg *et al.*, 1981b). pTP function as a primer for DNA synthesis by forming a complex with dCMP with the aid of Adv DNA polymerase (Lichy *et al.*, 1982; Tamanoi *et al.*, 1982). However, the presence of TP increases the efficiency of the Adv replication process. This is due to its ability to stabilise origin binding by the pTP-Adv polymerase complex and induces changes in the origin structure. Furthermore, TP is involved in attachment of the DNA to the nuclear matrix which is significant for efficient replication and transcription of Adv DNA.

4.7 **Aim**

The aim of this chapter is to investigate the significance of three different moieties within TP of the Adv type 5, each of which has the characteristic features of an NLS.

4.8 **Method**

Otherwise stated, the detailed experimental procedures used in this chapter are described in Chapter 3.

4.8.1 Construction of Expression Plasmids

The coding sequence of full length pTP (aa 1-671), TP (aa 350-671) and 9 of its truncated derivatives: 1-430 (F2), 1-390 (F5), 1-380 (F8), 1-356 (F6), 374-671 (F3), 391-671 (F1), 541-671 (F4), 405-654 (F9), 371-480 (F7), were inserted into mammalian expression plasmids, pcDNA6.2/C-EmGFP (fused in frame to the C-terminus of the GFP) and pcDNA6.2/N-YFP (fused in frame to the N-terminus of the YFP) using the Gateway recombination system. For use as control expression vectors, the coding sequence of ovalbumin ORF (OVA: aa 1-385) was inserted into both the pcDNA6.2/C-EmGFP and pcDNA6.2/N-YFP expression vectors using the Gateway® recombination system. Schematic diagrams of the constructs are shown in Figure 4-3 and 4-4.

4.8.2 Site-Directed Mutagenesis

4.8.2.1   QuikChange® Site-Directed Mutagenesis

The QuikChange® Site-Directed Mutagenesis kit available from Stratagene can be used to introduce point mutations, switch amino acids and to delete/insert single or multiple amino acids. For this research, the mutagenesis kit was used to introduce mutations to the full length TP to identify the essential amino acids on the NLS-encoding regions. In total, 8 point mutants and 5 deletion mutants were generated using YFP-TP as the template: MUT-1 ($MRRRR^{370} \rightarrow MYYRR^{370}$), MUT-2 ($MRRRR^{370} \rightarrow MRRYY^{370}$), MUT-3 ($MRRRR^{370} \rightarrow$

MYYYY^{370}), MUT-4 (PVRRRRRRV^{390} → PVYYRRRRV^{390}), MUT-5 (PVRRRRRRV^{390} → PVRRYYRRV^{390}), MUT-6 (PVRRRRRRV^{390} → PVRRRRYYV^{390}), MUT-7 (PVRRRRRRV^{390} → PVYYYYRRV^{390}), MUT-8 (PVRRRRRRV^{390} → PVYYYYYYV^{390}), DEL-1 (MRRRR^{370} → M----^{370}), DEL-2 (PVRRRRRRV^{390} → PV------V^{390}), DEL-3 (MRRRR^{370} → M----^{370} and PVRRRRRRV^{390} → PV------V^{390}), DEL-4 (PGARPRGRF → PG------F^{671}) and DEL-5 (MRRRR^{370} → MYYYY^{370}, PVRRRRRRV^{390} → PVYYYYYYV^{390} and PGARPRGRF → PG------F^{671}).

Figure 4-1 provides an overview of the QuikChange$^{®}$ Site-Directed Mutagenesis method. Primers were designed to contain desired mutations. During each temperature cycle, a template vector (YFP-TP) was denatured and primers annealing start. The *Pfu* polymerase extends each complimentary strand to create a mutated plasmid. Once several cycles were completed, *Dpn* I endonuclease was used to digest the parental DNA template, leaving only the newly synthesised mutated DNA plasmid.

Step 1: Plasmid Preparation
Gene in plasmid with target mutation site

Step 2: Temperature Cycling
Denature of plasmids and annealing of primers

○ Target site for mutation

▣ Mutagenic primer

○ Parental DNA Plasmid

○ Mutated DNA Plasmid

Step 3: Digestion
Digestion of methylated, non-mutated parental DNA template

Step 4: Transformation
Transform the circular, nicked dsDNA into competent cells

Figure 4-1: Overview of the QuikChange® site-directed mutagenesis method (adapted from QuikChange® site-directed mutagenesis kit – instruction manual).

4.8.2.2   Designing Primers

8 point mutants (MUT-1 to MUT-8) and 5 deletion mutants (DEL-1 and DEL-5) were created for this project. Detailed of each mutation can be found in Table 4-3. The main focus when creating mutants was on the arginine residues as arginine is presented in majority of the known NLS putative sequences. Tyrosine was selected to replace arginine as they share the same side chain polarity (polar) and tyrosine side chain is predominantly unionised at physiological pH. This is to minimise any disruption of protein folding which may be caused by mutations.

The QuikChange® Primer design program available from Stratagene was used to create primers for site directed mutagenesis experiments as per the guideline.

Table 4-4 shows the lists and sequences of primers designed.

Table 4-3: List of mutants created from YFP-TP.

Mutants	
MUT-1	MRRRR^{370} → MYYRR^{370}
MUT-2	MRRRR^{370} → MRRYY^{370}
MUT-3	MRRRR^{370} → MYYYY^{370}
MUT-4	PVRRRRRRV^{390} → PVYYRRRRV^{390}
MUT-5	PVRRRRRRV^{390} → PVRRYYRRV^{390}
MUT-6	PVRRRRRRV^{390} → PVRRRRYYV^{390}
MUT-7	PVRRRRRRV^{390} → PVYYYYRRV^{390}
MUT-8	PVRRRRRRV^{390} → PVYYYYYYV^{390}
DEL-1	MRRRR^{370} → M----^{370}
DEL-2	PVRRRRRRV^{390} → PV------V^{390}
DEL-3	MRRRR^{370} → M----^{370} and PVRRRRRRV^{390} → PV------V^{390}
DEL-4	PGARPRHRF^{671} → PG------F^{671}
DEL-5	MRRRR^{370} → MYYYY^{370}, PVRRRRRRV^{390} → PVYYYYYYV^{390} and PGARPRHRF^{671} → PG------F^{671}

Table 4-4: A table showing the sequence of primers used in QuikChange® Site-Directed Mutagenesis.

Mutant Genes	Primer Sequence (from 5' to 3')
MUT-1	GTCACCGAGACCATGTACTATCGCCGCGGGGAGATGATC GATCATCTCCCCGCGGCGATAGTACATGGTCTCGGTGAC
MUT-2	GTCACCGAGACCATGCGCCGTTACTACGGGGAGATGATC GATCATCTCCCCGTAGTAACGGCGCATGGTCTCGGTGAC
MUT-3	GTCACCGAGACCATGTACTATTACTACGGGGAGATGATC GATCATCTCCCCGTAGTAATAGTACATGGTCTCGGTGAC
MUT-4	CGCCTCCCGGTGTACTATCGTCGCCGCCGTGTC GACACGGCGGCGACGATAGTACACCGGGAGGCG
MUT-5	CGCCTCCCGGTGCGCCGTTATTACCGCCGTGTC GACACGGCGGTAATAACGGCGCACCGGGAGGCG
MUT-6	CGCCTCCCGGTGCGCCGTCGTCGCTACTATGTC GACATAGTAGCGACGACGGCGCACCGGGAGGCG
DEL-1	GTCACCGAGACCATGGGGGGAGATGATCGAG CTCGATCATCTCCCCCATGGTCTCGGTGAC
DEL-2	GCCTCCCGGTGGTCCCCCCTCC GGAGGGGGGACCACCGGGAGGC
DEL-4	GCGGGTCCGGAGCCCCCCCCTACCTCCGGGGTTT AAACCCCGGAGGTAGGGGGGCTCCGGACCCGC

4.8.2.3   Mutagenesis Reactions

The PCR reaction for QuikChange® Site-Directed mutagenesis was optimised in the lab. For the reaction mixture, 2 units of *Pfu* polymerase, 5 µl of supplied 10x reaction buffer, 1 µl 10mM dNTPs, 2.5 µl DMSO, 20 ng template plasmid and 0.2 pmol of each primer were mixed together and made up to 50 µl with MilliQ water. Due to the sensitivity of the enzyme, the reactions were prepared on ice.   Reactions were then transferred to the MyCycler thermal cycler for temperature cycles. Table 4-5 shows the temperature profile used.

Table 4-5: Temperature Cycle profile for QuikChange Site-Directed Mutagenesis.

Temperature (˚C)	Time (seconds)
94	60
94	30
55	60
68	30 seconds/kb
10	∞

18 cycles (brace spanning the 94/30, 55/60, 68/30 seconds/kb rows)

Once the temperature cycle was completed, 5 µl of sample was run on 1% agarose to confirm the presence of product. If the correct size product was observed, the remainder of the sample was digested with 1 µl *DpnI* and incubated at 37˚C for 1 hour to remove methylated DNA template. Afterwards, 2 µl was transformed into DH5α competent cells using heat shock process as described previously in section 3.6.1 of Chapter 3. The plasmid DNA was extracted (detailed protocol in Section 3.7 of Chapter 3), quantified and sequenced for integrity confirmation.

4.8.3 Transfection

HeLa and COS-7 cells were maintained in DMEM, supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C. Cells were transfected at 70-80% confluency using Lipofectamine[2000] and imaged 48 hours post-transfection using confocal microscopy. Image analysis was carried out on the digitalised confocal files using the Image J 1.42q public domain software, to estimate the relative Fn, Fnu and Fc above background fluorescence.

Statistical analysis was performed on raw data using analysis tool pack on Microsoft Excel. Data was analysed by one way analysis of variance (ANOVA) with post-hoc Dunnett's test. All results are presented as mean ± standard error of the mean (SEM) of 3 separate experiments (n ≥ 50 from each experiment; total n ≥ 150). In all cases, the differences between the mean is considered to be * significant when $p < 0.05$, ** significant when $p < 0.01$ and *** significant when $p < 0.001$.

4.8.4 Protein Expression and Detection

The EmGFP- and YFP- fusion proteins (pTP, TP and 9 of its truncated derivatives) were expressed in HeLa cells and analysed 48 hours after transfection. The proteins were extracted of the lysate of the transfected cells and Western blot was performed on the fusion proteins on SDS-PAGE against mouse anti-GFP and mouse anti-V5 respectively.

4.9 **Results**

4.9.1 Adenovirus Terminal Protein encodes for multiple NLSs

One previous study has suggested that Ad TP has an NLS but in the present study it was considered that three regions of TP may have NLS activity, so in this study no assumptions were made based on the earlier study (Zhao *et al.*, 1988). The project set out to identify the most significant Ad TP region for nuclear accumulation from first principles. EmGFP-

and YFP- OVA fusion protein were used as controls and their constructs are shown in Figure 4-2. A range of truncated EmGFP- and YFP- fusion protein derivatives of TP were created as illustrated in Figure 4-3 andFigure 4-4 respectively.
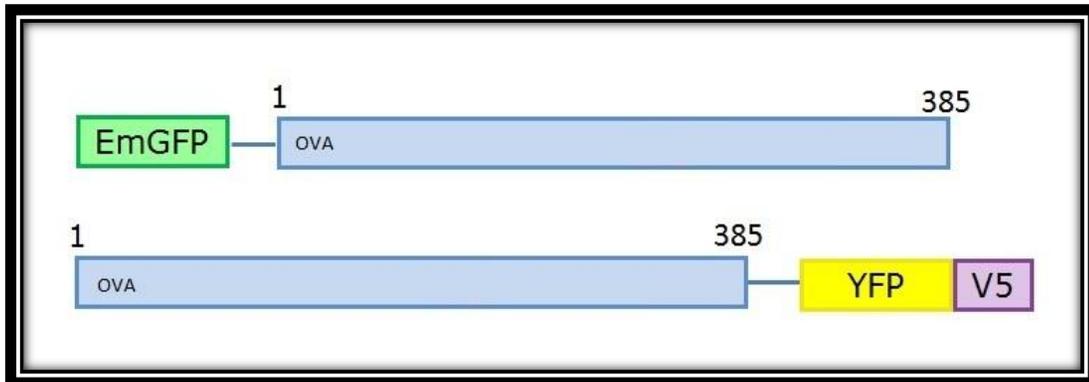
Figure 4-2: Schematic representation of OVA fusion proteins. A diagram of EmGFP- and YFP- OVA fusion protein constructs used in the nuclear accumulation experiment study as controls.
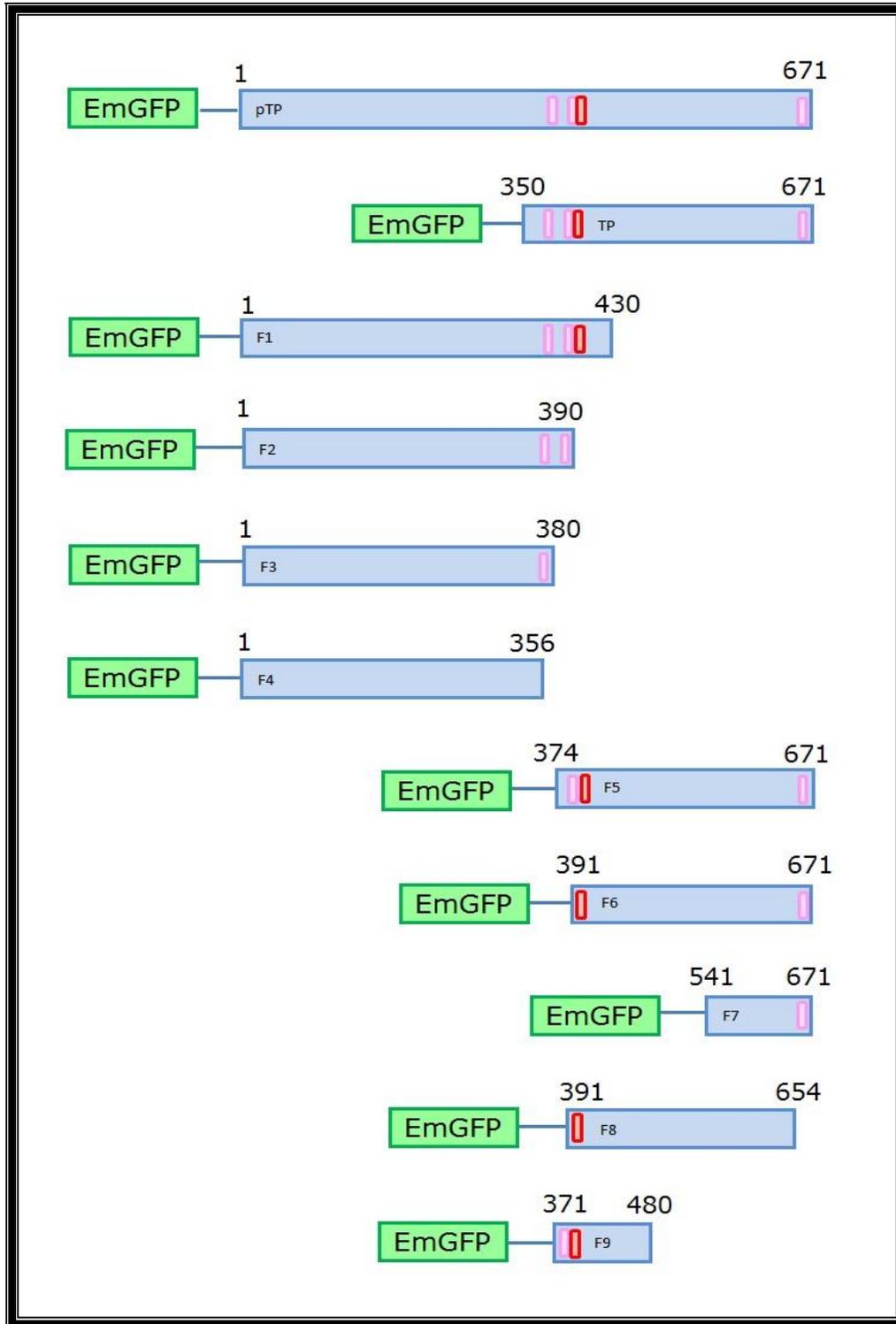
Figure 4-3: Schematic representation of EmGFP fusion proteins. A diagram of EmGFP fusion protein constructs used in this study with proposed NLS and negative rich domains in pink and red box respectively. From top to bottom: EmGFP-pTP, EmGFP-TP, EmGFP-F1, EmGFP-F2, EmGFP-F3, EmGFP-F4, EmGFP-F5, EmGFP-F6, EmGFP-F7, EmGFP-F8 and EmGFP-F9.
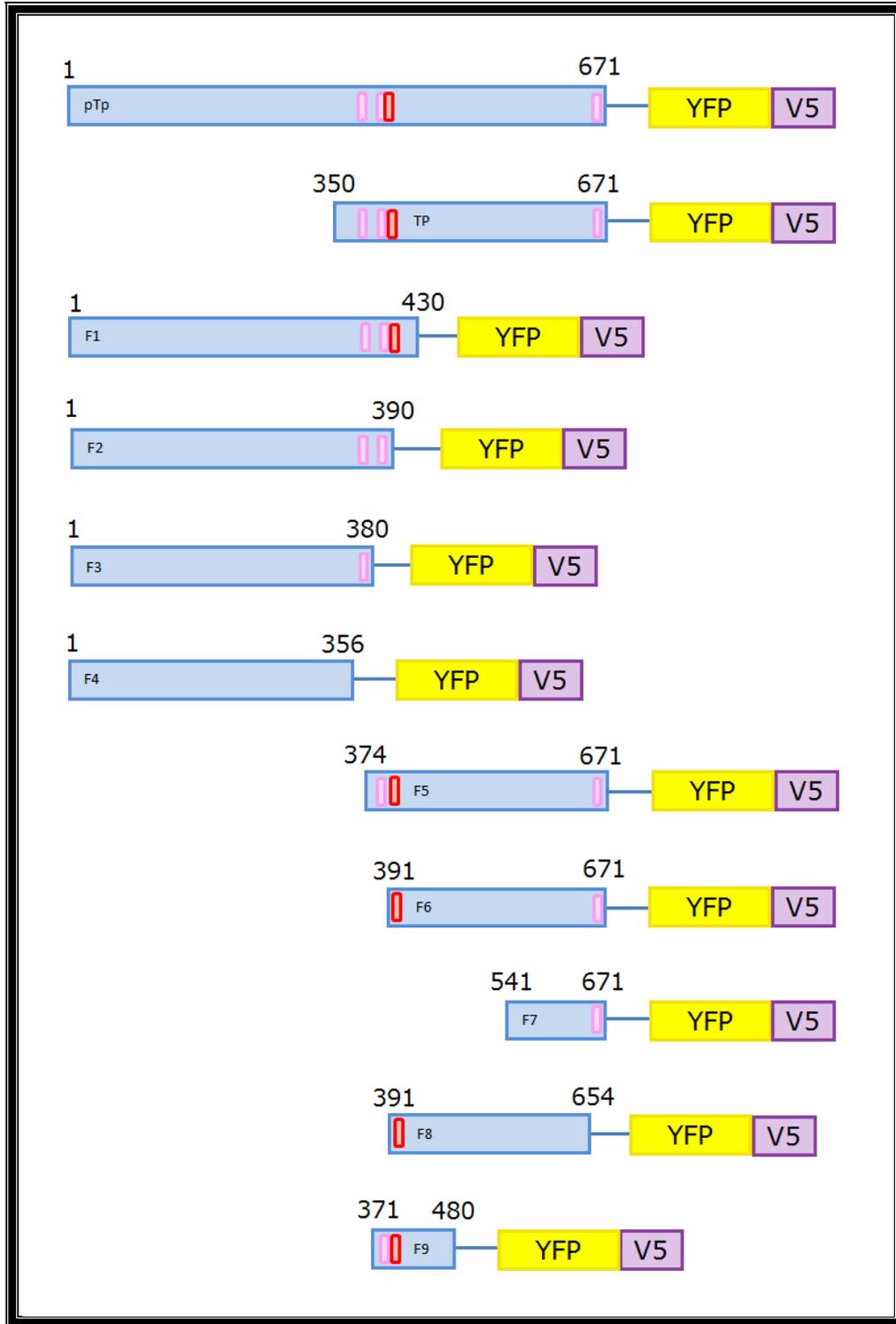
Figure 4-4: Schematic representation of YFP fusion proteins. A diagram of YFP fusion protein constructs used in this study with proposed NLS and negative rich domains in pink and red box respectively, and V5 tag as indicated. From top to bottom: YFP-pTP, YFP-TP, YFP-F1, YFP-F2, YFP-F3, YFP-F4, YFP-F5, YFP-F6, YFP-F7, YFP-F8 and YFP-F9.

Western blot was performed on the lysate of transfected HeLa cells to validate the expression of each fusion proteins. The results of EmGFP fusion proteins (against anti-GFP antibody) are shown in Figure 4-5 andFigure 4-6 shows results of YFP fusion proteins (against anti-V5 antibody).
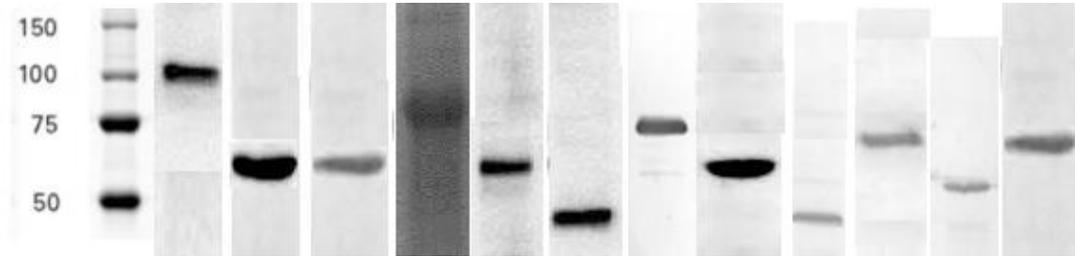
Figure 4-5: Western blot results of EmGFP-fusion proteins. Resultant bands from SDS-PAGE gel showing fusion proteins of interest. The expressed fusion proteins of interest were detected by Western blot against anti-GFP antibody. From left to right: Protein ladder, pTP (107 kDa), TP (65 kDa), F1 (62 kDa), F2 (77 kDa), F3 (62 kDa), F4 (45 kDa), F5 (73 kDa), F6 (69 kDa), F7 (42 kDa), F8 (72 kDa), F9 (57 kDa) and OVA (72 kDa).
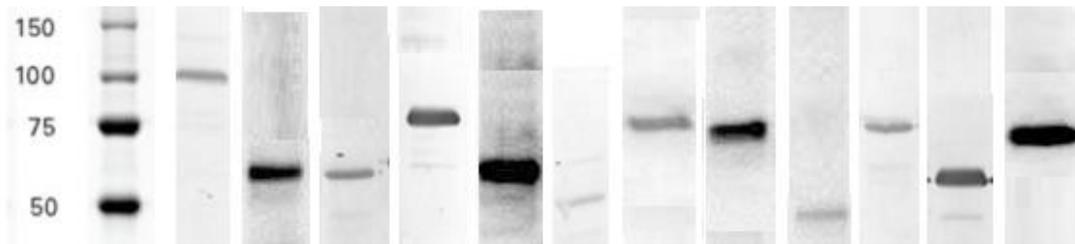
Figure 4-6: Western blot results of YFP-fusion proteins. Resultant bands from SDS-PAGE gel showing fusion proteins of interest. YFP-fusion proteins were expressed with V5 epitope tag allowing expression of each protein to be detected by Western blot against anti-V5 antibody. From left to right: Protein ladder, pTP (111 kDa), TP (69 kDa), F1 (66 kDa), F2 (81 kDa), F3 (66 kDa), F4 (49 kDa), F5 (77 kDa), F6 (73 kDa), F7 (46 kDa), F8 (76 kDa), F9 (61 kDa) and OVA (76 kDa).

Live cell imaging was performed on both HeLa and COS-7 cells expressing studied fusion proteins and the images are illustrated in Figure 4-7 and Figure 4-8 respectively. The analysed fluorescent ratio for HeLa cells and COS-7 cells are tabulated in Table 4-6 and Table 4-7 respectively. The graphical representations can be found in Figure 4-9, Figure 4-10**,** Figure 4-11, Figure 4-12 and Figure 4-13.

Table 4-6: Average calculated Fn/Fc ratio data obtained from HeLa cells for all studied fusion proteins.

Fusion Protein	n number	Average Fn/Fc Ratio	Average Fnu/Fc Ratio	Significant difference to control
EmGFP-OVA	150	1.00	-	-
EmGFP-pTP	150	9.40	15.98	***
EmGFP-TP	150	8.39	-	***
EmGFP-F1	150	8.75	-	***
EmGFP-F2	150	3.91	-	***
EmGFP-F3	150	1.62	-	**
EmGFP-F4	150	0.91	-	
EmGFP-F5	150	1.53	-	**
EmGFP-F6	150	3.54	-	***
EmGFP-F7	150	10.01	15.13	***
EmGFP-F8	150	0.99	-	
EmGFP-F9	150	2.24	-	***
YFP-OVA	150	0.87	-	-
YFP-pTP	150	12.55	15.00	***
YFP-TP	150	9.54	-	***
YFP-F1	150	8.62	-	***

YFP-F2	150	4.43	-	***
YFP-F3	150	1.81	-	**
YFP-F4	150	0.89	-	
YFP-F5	150	2.20	-	***
YFP-F6	150	4.00	-	***
YFP-F7	150	8.73	13.75	***
YFP-F8	150	0.92	-	
YFP-F9	150	2.82	-	***

Table 4-7: Average calculated Fn/Fc ratio data obtained from COS-7 cells for all studied fusion proteins.

Fusion Protein	N number	Average Fn/Fc Ratio	Average Fnu/Fc Ratio	Significant difference with control
EmGFP-OVA	150	0.92	-	-
EmGFP-pTP	150	11.37	16.7	***
EmGFP-TP	150	7.23	-	***
EmGFP-F1	150	7.63	-	***
EmGFP-F2	150	3.09	-	***
EmGFP-F3	150	1.50	-	**
EmGFP-F4	150	0.97	-	

EmGFP-F5	150	2.38	-	**
EmGFP-F6	150	3.04	-	***
EmGFP-F7	150	10.83	17.03	***
EmGFP-F8	150	1.10	-	
EmGFP-F9	150	1.96	-	***
YFP-OVA	150	0.91	-	-
YFP-pTP	150	10.78	16.26	***
YFP-TP	150	7.87	-	***
YFP-F1	150	7.40	-	***
YFP-F2	150	4.00	-	***
YFP-F3	150	1.83	-	**
YFP-F4	150	0.77	-	
YFP-F5	150	2.97	-	***
YFP-F6	150	3.50	-	***
YFP-F7	150	9.38	16.51	***
YFP-F8	150	0.94	-	
YFP-F9	150	2.47	-	***

A

B
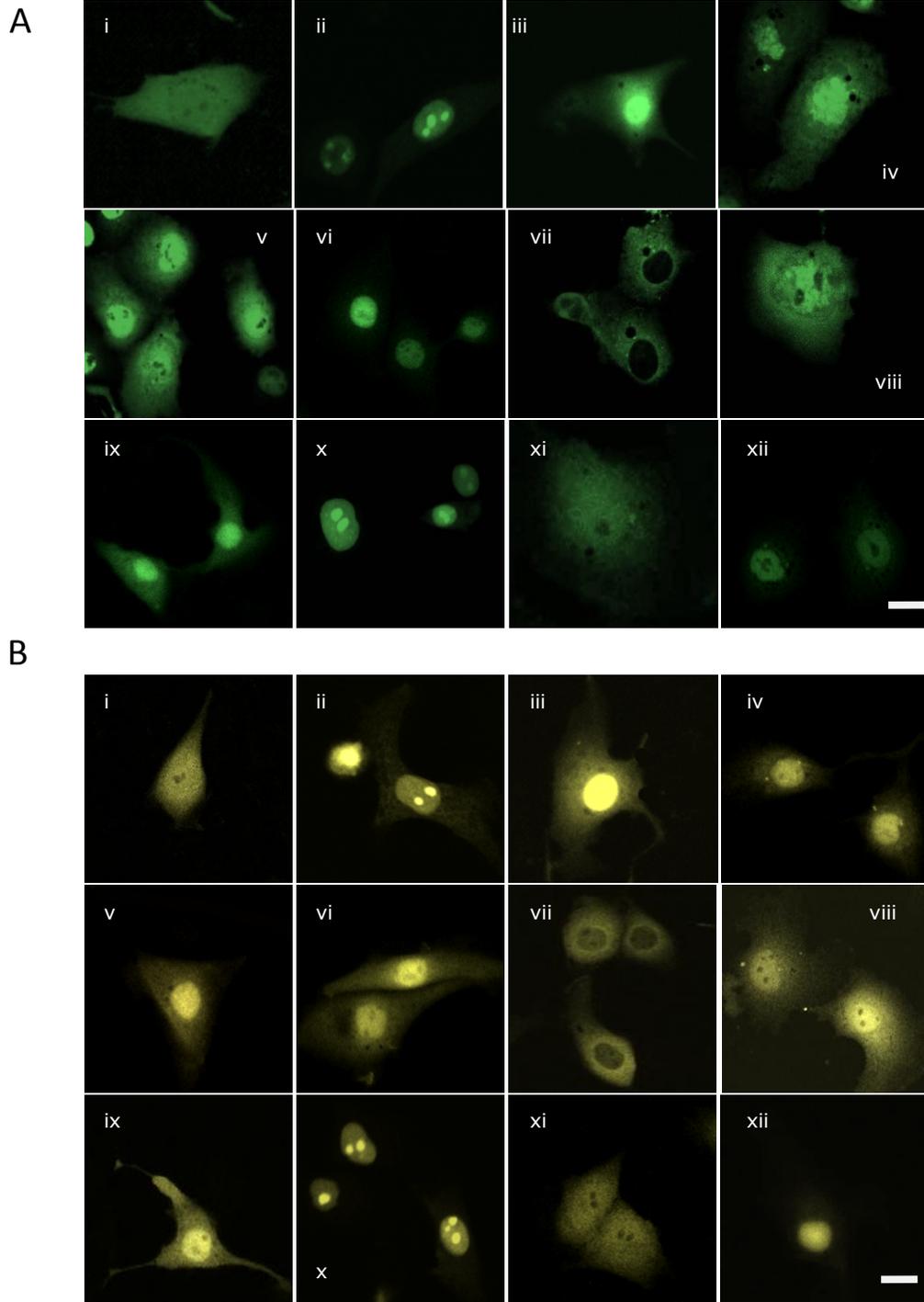
Figure 4-7: Confocal images of live HeLa cells transfected to express indicated fusion proteins at 20x magnification with 10 μm scale bar. (A) EmGFP-fusion proteins (i) EmGFP-OVA, (ii) EmGFP-pTP, (iii) EmGFP-TP, (iv) EmGFP-F1, (v) EmGFP-F2, (vi) EmGFP-F3, (vii) EmGFP-F4, (viii) EmGFP-F5, (ix) EmGFP-F6, (x) EmGFP-F7, (xi) EmGFP-F8, and (xii) EmGFP-F9. (B) YFP-fusion proteins (i) YFP-OVA, (ii) YFP-pTP, (iii) YFP-TP, (iv) YFP-F1, (v) YFP-F2, (vi) YFP-F3, (vii) YFP-F4, (viii) YFP-F5, (ix) YFP-F6, (x) YFP-F7, (xi) YFP-F8, and (xii) YFP-F9.

A

B
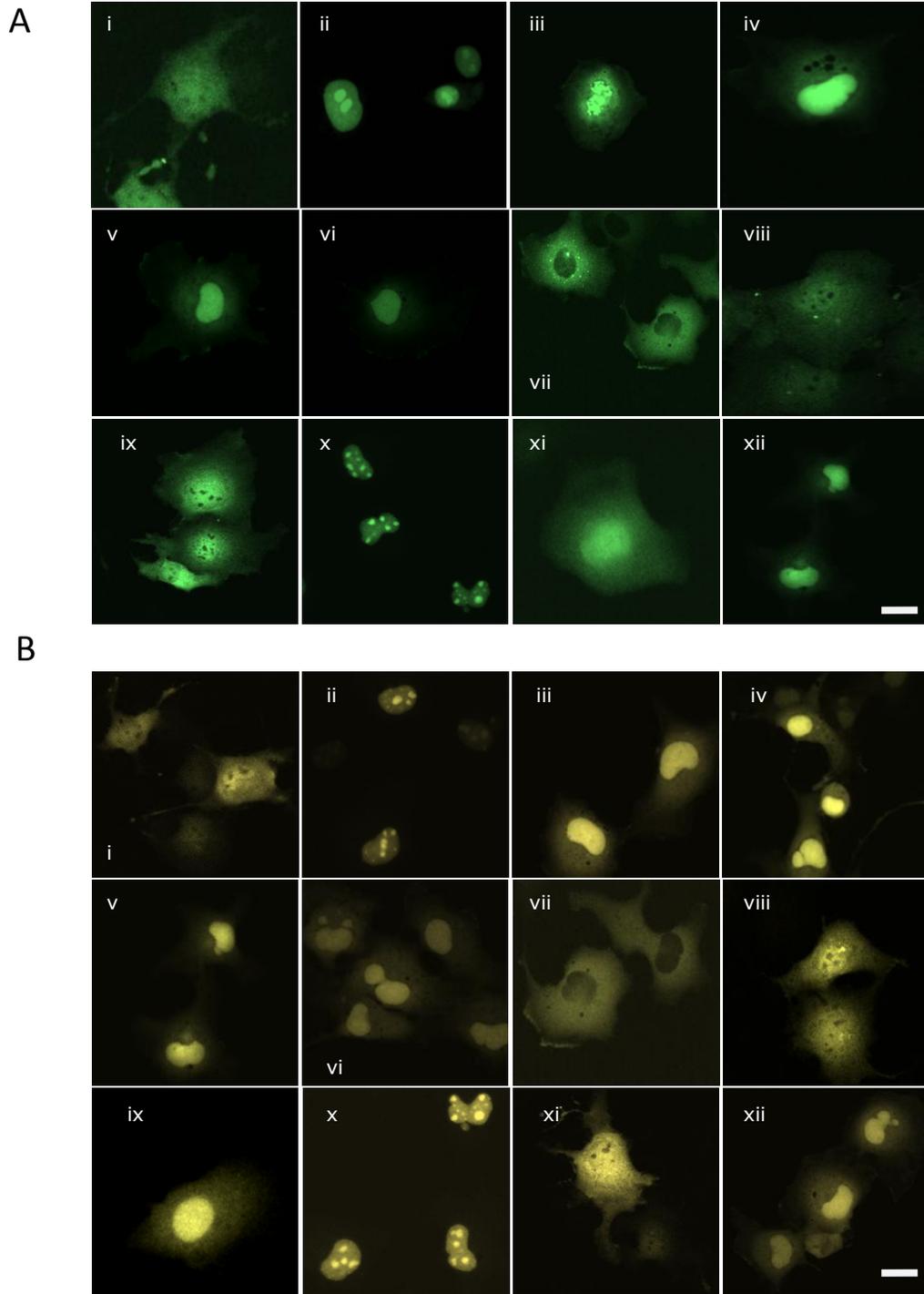
Figure 4-8: Confocal images of live COS-7 cells transfected to express indicated fusion proteins at 20x magnification with 10 μm scale bar. (A) EmGFP-fusion proteins (i) EmGFP-OVA, (ii) EmGFP-pTP, (iii) EmGFP-TP, (iv) EmGFP-F1, (v) EmGFP-F2, (vi) EmGFP-F3, (vii) EmGFP-F4, (viii) EmGFP-F5, (ix) EmGFP-F6, (x) EmGFP-F7, (xi) EmGFP-F8, and (xii) EmGFP-F9. (B) YFP-fusion proteins (i) YFP-OVA, (ii) YFP-pTP, (iii) YFP-TP, (iv) YFP-F1, (v) YFP-F2, (vi) YFP-F3, (vii) YFP-F4, (viii) YFP-F5, (ix) YFP-F6, (x) YFP-F7, (xi) YFP-F8, and (xii) YFP-F9.

A

B
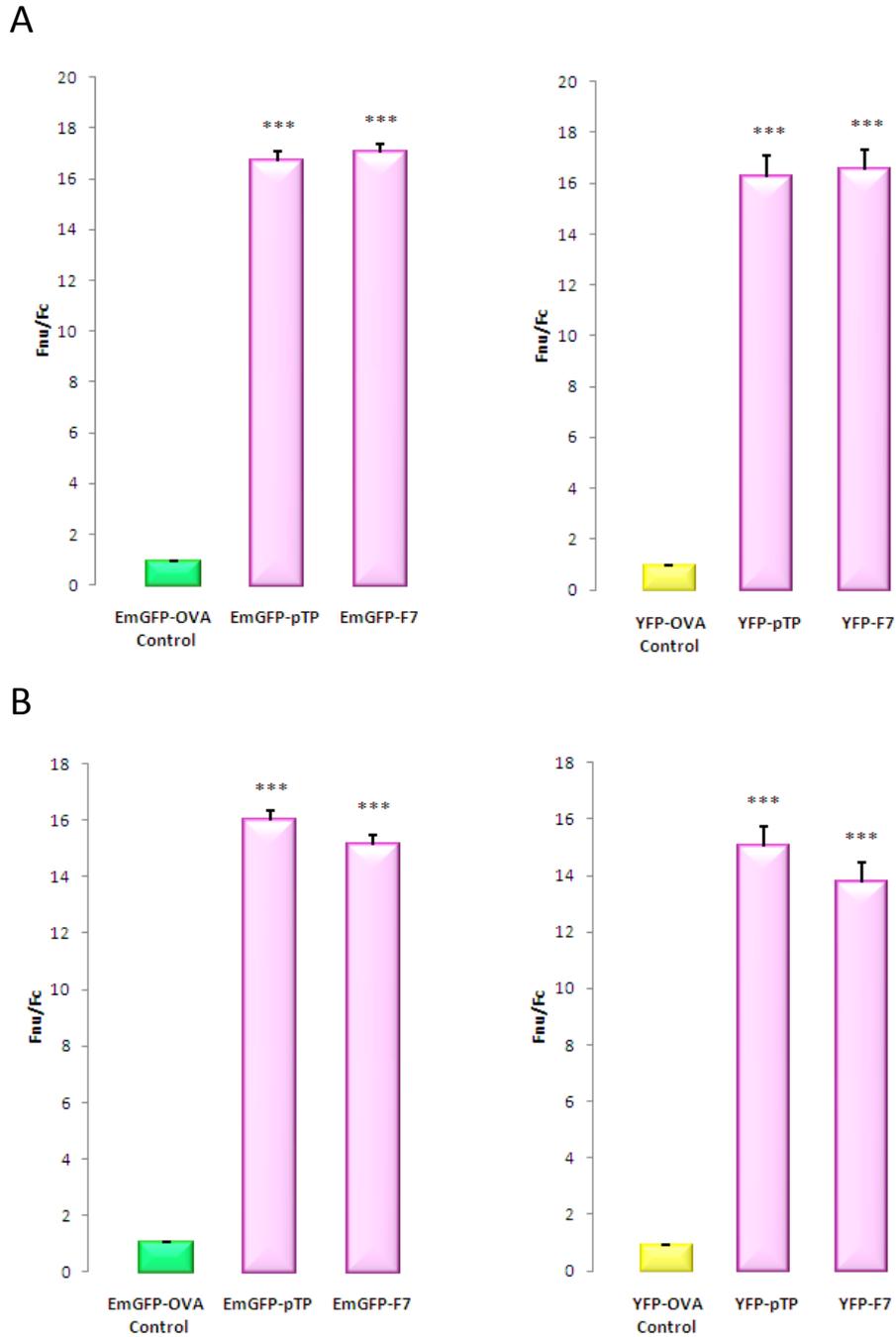
Figure 4-9: Quantitative analysis results for the levels of nucleolar accumulation (Fnu/c: ratios of the nucleolar to the cytoplasmic fluorescence after the subtraction of background fluorescence) in (A) COS-7 and (B) HeLa cells expressing the indicated EmGFP and YFP fusion proteins. Quantitative Fnu/Fc analysis was performed on 150 cells and analysed by one way ANOVA. The difference between the sample and control is considered to be * significant when P < 0.05, ** significant when P < 0.01 and *** significant when P < 0.001.
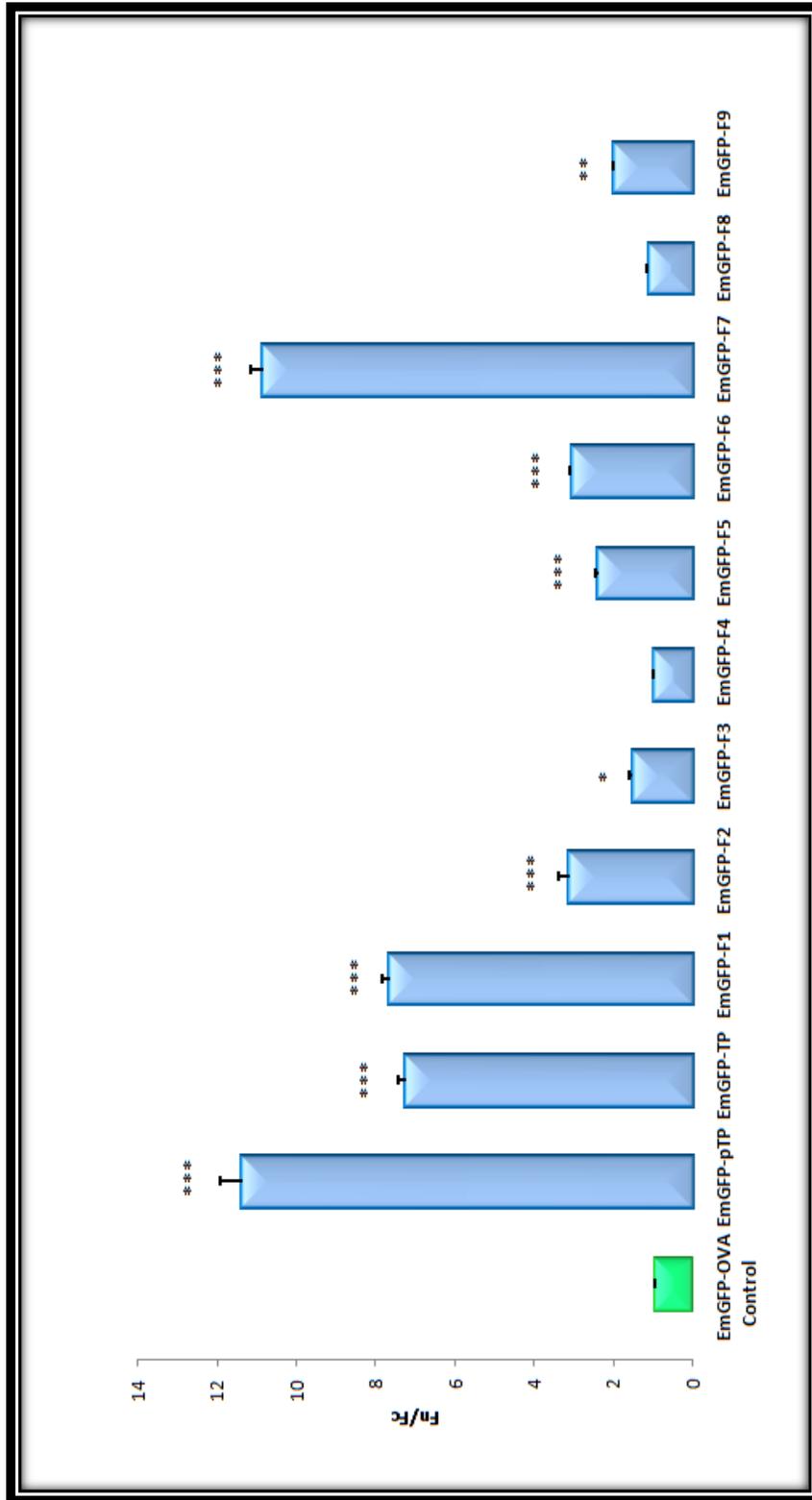
Figure 4-10: Quantitative analyss results for the levels of nuclear accumulation (Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence) **in COS-7 cells expressing the indicated EmGFP fusion proteins.** Quantitative Fn/Fc analysis was performed on 150 cells and analysed by one way ANOVA. The difference between the sample and control is considered to be * significant when P < 0.05, ** significant when P < 0.01 and *** significant when P < 0.001.
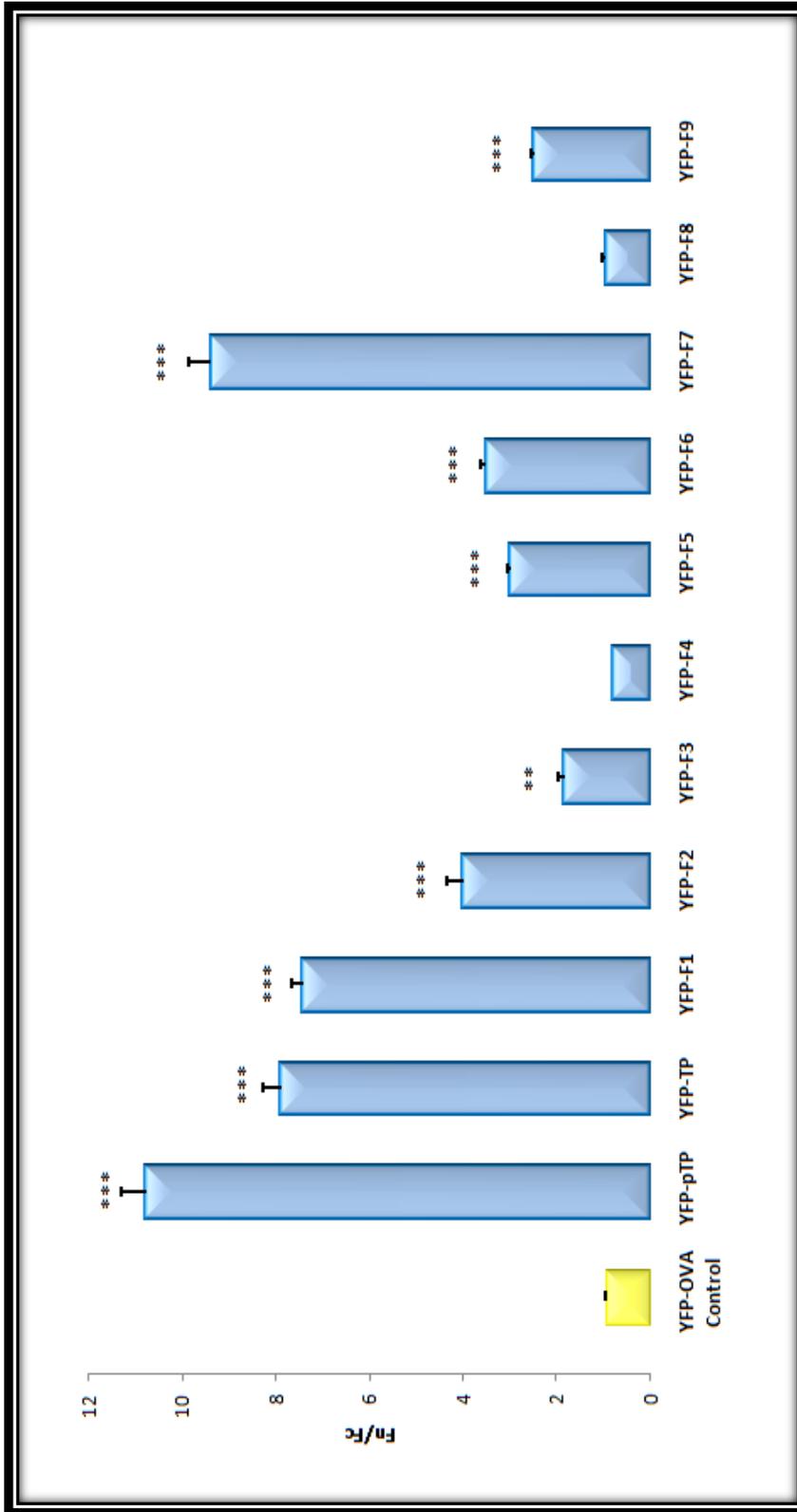
Figure 4-11: Quantitative analysis results for the levels of nuclear accumulation (Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence) **in COS-7 cells expressing the indicated YFP fusion proteins.** Quantitative Fn/Fc analysis was performed on 150 cells and analysed by one way ANOVA. The difference between the sample and control is considered to be * significant when P < 0.05, ** significant when P < 0.01 and *** significant when P < 0.001.
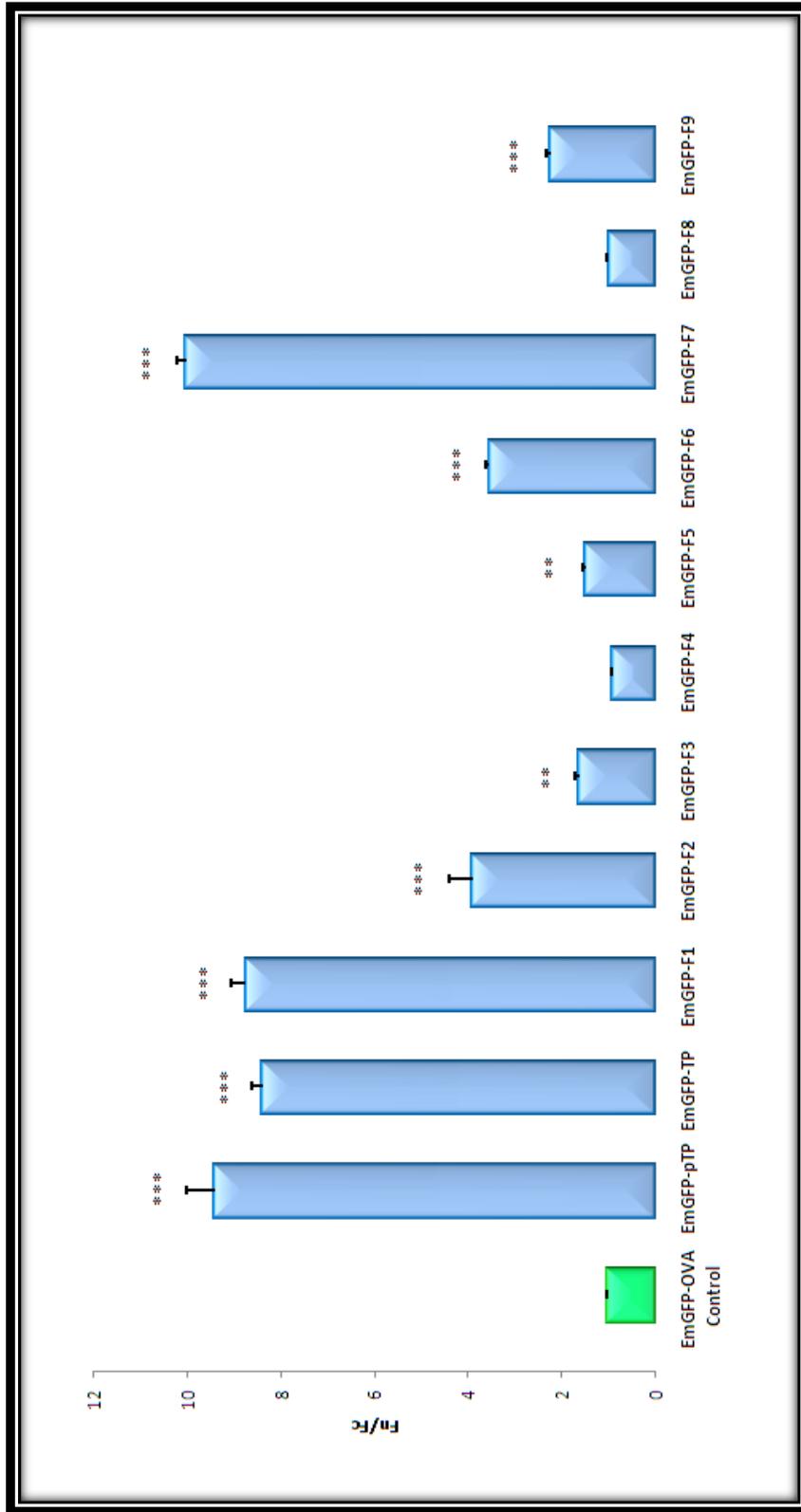
Figure 4-12: Quantitative analysis results for the levels of nuclear accumulation (Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence) **in HeLa cells expressing the indicated EmGFP fusion proteins.** Quantitative Fn/Fc analysis was performed on 150 cells and analysed by one way ANOVA. The difference between the sample and control is considered to be * significant when P < 0.05, ** significant when P < 0.01 and *** significant when P < 0.001.
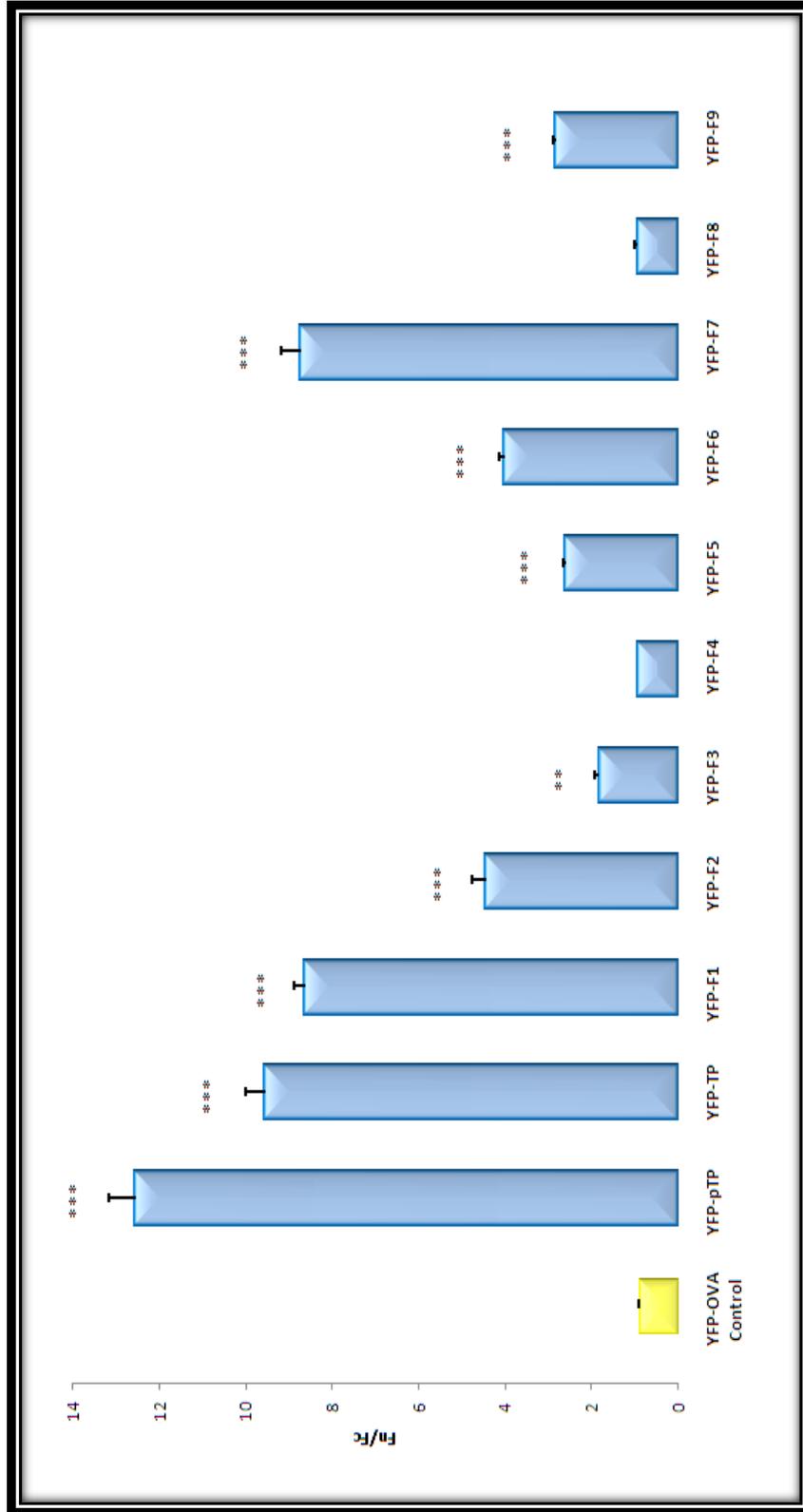
Figure 4-13: Quantitative analysis results for the levels of nuclear accumulation **(Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence)** in HeLa cells expressing the indicated YFP fusion proteins. Quantitative Fn/Fc analysis was performed on 150 cells and analysed by one way ANOVA. The difference between the sample and control is considered to be * significant when P < 0.05, ** significant when P < 0.01 and *** significant when P < 0.001.

The position of the NLS and its content in the transported protein can be important (Silver, 1991). To investigate whether the position of fluorescent protein would influence the localisation of studied proteins, both N- and C- terminus coupled proteins were created. Quantitative analysis to determine the nuclear to cytoplasmic ratios (Fn/Fc) of EmGFP- and YFP- proteins revealed that generally the function of each NLS was not influenced to a great extent by whether the fluorescent protein was coupled to the N- or C- terminus. This indicates that localisation of the TP fragments was not artificially influenced by the position of the EmGFP or YFP. Table 4-8 and Table 4-9 summarise the fluorescent ratio between EmGFP and YFP of studied fusion proteins.

Table 4-8: A Table showing the nuclear/cytoplasmic fluorescent ratio between EmGFP and YFP expression vectors in HeLa cells.

Protein fragments	EmGFP Fn/Fc	YFP Fn/Fc	EmGFP/YFP
OVA	1	0.87	1.15
pTP	9.4	12.55	0.75
TP	8.39	9.54	0.88
F1	8.75	8.62	1
F2	3.91	4.43	0.88
F3	1.62	1.81	0.9
F4	0.91	0.89	1
F5	1.55	2.2	0.70
F6	3.54	4	0.89
F7	10.01	8.73	1.15

F8	0.99	0.92	1.08
F9	2.24	2.82	0.79

Table 4-9: A Table showing the nuclear/cytoplasmic fluorescent ratio between EmGFP and YFP expression vectors in COS-7 cells.

Protein fragments	EmGFP Fn/Fc	YFP Fn/Fc	EmGFP/YFP
OVA	0.92	0.91	1
pTP	11.37	10.78	1.05
TP	7.23	7.87	0.92
F1	7.63	7.4	1.03
F2	3.09	4	0.77
F3	1.5	1.83	0.82
F4	0.97	0.77	1.23
F5	2.38	2.97	0.8
F6	3.04	3.5	0.87
F7	10.83	9.38	1.15
F8	1.1	0.94	1.17
F9	1.96	2.47	0.79

pTP, the truncated derivatives F1 and F7 had significantly higher levels of nuclear accumulation, estimated by Fn/c, than the control *(p ≤ 0.001).* The nuclear expression level produced by these truncated derivatives was the same as the level detected for native TP. This implies that aa1-430 and aa541-671 have NLS activity which suggests that multiple NLSs are functional in TP. Although the truncated derivatives F2 and F5 *(p ≤ 0.001)*, F3, F6 and F9 *(p ≤ 0.005)* showed significant increase in nuclear accumulation it was not to the same level as the native TP, suggesting that additional sequences of the TP are required for optimal nuclear import efficiency. The truncated derivatives F8 showed no increased in nuclear accumulation in comparison to that of the control, indicating the absences of NLS on these fragments (aa391-654). Strikingly, the truncated derivates of F4 were excluded from the nucleus in transfected cells, implying that aa1-356 does not code for NLS. The data indicates that the NLSs can be found between aa357-430 and aa655-671.

Interestingly, only the full length pTP (aa1-671) and the truncated derivative F7 (aa541-671) showed nucleolar accumulation. This indicates that the NuLS is present on TP and the region likely to encode for this signal is aa541-671. However, F8 (aa391-654) did not show any nucleolar localisation, suggesting that the nucleolar signal encoding region is aa655-671. It was noted that there is an absence of nucleolar accumulation of the TP (aa322-671), the truncated derivatives F5 (aa374-671) and F6 (aa391-671) which is unexpected as these fragments contains the region of proposed NuLS (aa655-671).

Since the position of fluorescent tag does not influence the function of studied NLSs, only YFP- fusion proteins were used to investigate whether the expression of TP is cell specific. Live cell imaging was performed on AD293, Caco-2 and PC-3 cells expressing the YFP-TP. The nuclear accumulation results obtained across all 5 different cells type suggested that the expression of TP is not cell specific.

Figure 4-14 represents AD293, Caco-2 and PC3 cells expressing YFP-TP fusion protein.
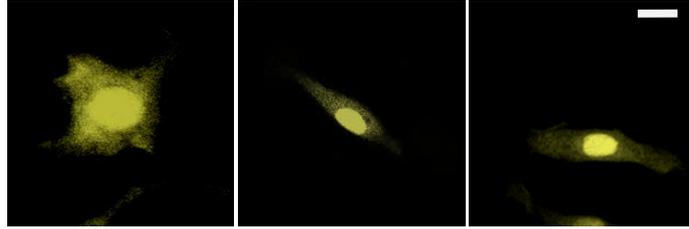
Figure 4-14: Transfection results of YFP-TP. Confocal images of live AD293, PC-3 and Caco-2 cells transfected to express YFP-TP fusion proteins at 20x magnification with 10 μm scale bar.

4.9.2 All three cationic clusters in TP contribute to its nuclear localisation

It was identified that the aa357-430 and aa655-671 of TP encode for NLSs which indicates that there are at least 2 potentially significant NLS clusters on TP. Site-directed mutagenesis was performed within aa357-430 and aa655-671 to determine the NLS sequences responsible for nuclear import. The aa357-430 contains two basic amino acids separated by a linker of 11 residues which resemble a classical bipartite NLS. Mutations were created within these basic cluster residues to produce TP mutant derivatives. COS-7 cells were transfected to express the mutants as well as the wild type YFP-TP (aa350-671) as control. The transfected images were captured 48 hours after transfection and typical images are illustrated in Figure 4-15. Tabulated results of nuclear accumulation of the studied mutated fusion proteins can be found in Table 4-10 and graphical representation can be found in Figure 4-16.
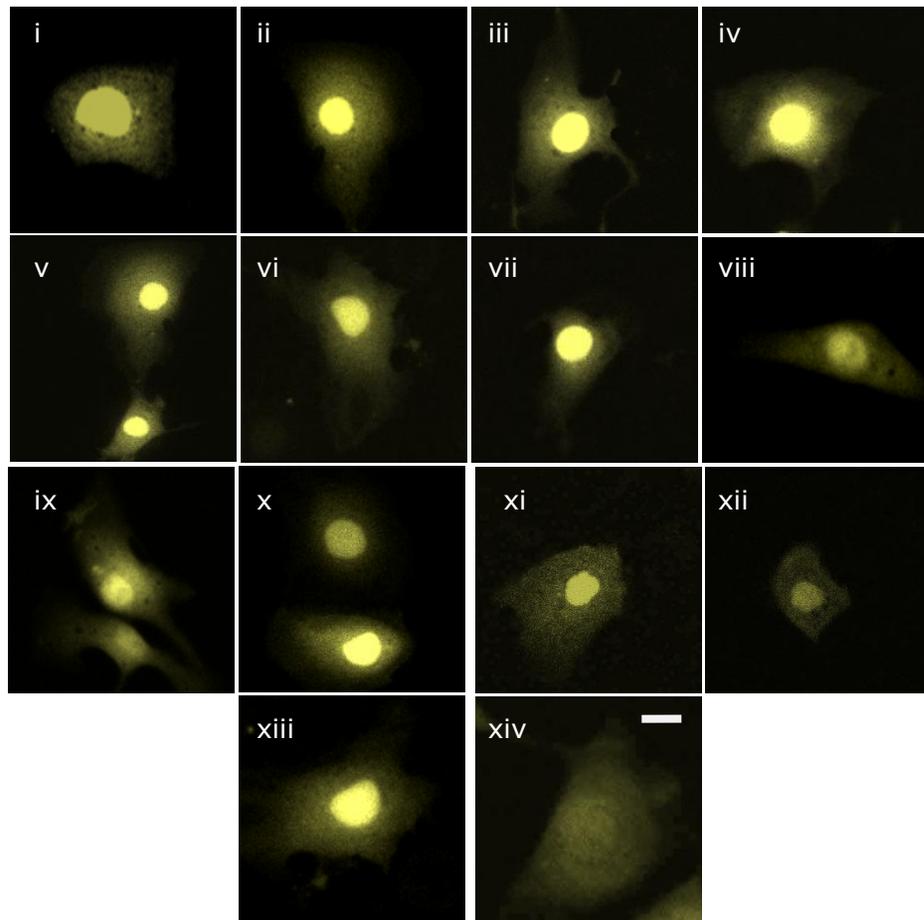
Figure 4-15: Transfection results of mutated fusion proteins in COS-7 cells. Confocal images of live COS-7 cells transfected to express indicated YFP-fusion proteins at 20x magnification with 10 µm scale bar. (i) YFP-TP, (ii) MUT-1, (iii) MUT-2, (iv) MUT-3, (v) MUT-4, (vi) MUT-5, (vii) MUT-6, (viii) MUT-7, (ix) MUT-8, (x) DEL-1 (xi) DEL-2, (xii) DEL-3, (xiii) DEL-4 and (xiv) DEL-5.

Table 4-10: Average calculated Fn/Fc ratio data obtained from COS-7 cells for all studied fusion proteins.

Fusion Protein	N number	Average Fn/Fc Ratio	Significant difference with control
YFP-TP	150	8.29	
MUT-1	150	8.10	
MUT-2	150	8.20	
MUT-3	150	7.61	
MUT-4	150	8.22	
MUT-5	150	8.04	
MUT-6	150	7.93	
MUT-7	150	6.33	
MUT-8	150	5.73	**
DEL-1	150	7.52	
DEL-2	150	5.88	*
DEL-3	150	3.27	***
DEL-4	150	7.44	
DEL-5	150	2.20	***

Figure 4-16: Quantitative analysis results for the levels of nuclear accumulation (Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence) **in COS-7 cells expressing the indicated YFP fusion proteins.**

MUT-1, MUT-2, MUT-3, MUT-4, MUT-5, MUT-6, DEL-1 and DEL-4 showed no significant reductions in nuclear accumulation compared to wild type. The nuclear transport of MUT-7 and MUT-8 were reduced indicating that the arginine residues within the PVRRRRRV[390] cluster has a significant NLS function (MUT-8, $p \leq 0.01$). Remarkably even when all of cationic arginine residues in cluster PVRRRRRV[390] were replaced by tyrosine (Y) the protein was still localised to the nucleus to a significant extent, indicating that other putative NLSs are functional. DEL-2 ($p \leq 0.05$) also showed reduction in nuclear accumulation which further emphasis the importance of arginine clusters within the PVRRRRRV[390]. This cluster has previously been assumed to be the only NLS on TP, but this study indicates that significant nuclear accumulation takes place in its absence. The reduced nuclear accumulation found in DEL-3 *(p ≤ 0.001)* indicates that both the MRRRR[370] and PVRRRRRV[390] clusters are required for optimal enhancement of TP nuclear import suggesting that these clusters form a bipartite NLS system. DEL-5 *(p ≤ 0.001)* shows significant reduction in nuclear expression implying that PGARPRGRF[671] is also involved in TP nuclear delivery. Interestingly, DEL-4 showed no significant reduction in nuclear transport of TP implying that the MRRRR[370] and PVRRRRRV[390] are capable of transporting TP independent of PGARPRGRF[671]. However, the nucleolar accumulation was not detected in DEL-4 indicating that the PGARPRGRF[671] may also play a crucial role in nucleolar transportation of pTP.

4.9.3 Adenovirus Terminal Protein does not encode for nuclear export signal

Based on the results described in the previous section, the truncated F4 does not localise in the nucleus. It was hypothesised that this was due to the lack of NLS on F4. However, to ensure the absence of F4 in the nucleus is not as a result of nuclear export, an experiment to investigate the effect of leptomycin B (LMB) was conducted to determine whether the F4 fragment contains a NES. LMB is a specific nuclear export inhibitor for CRM1/EXP 1, which is required for nuclear export of proteins containing a NES (Jang *et al.*, 2003). The typical COS-7 cells images from LMB experiment can be found in Figure 4-17. The graphical illustration of Fn/Fc is shown in Figure 4-18.

A

B
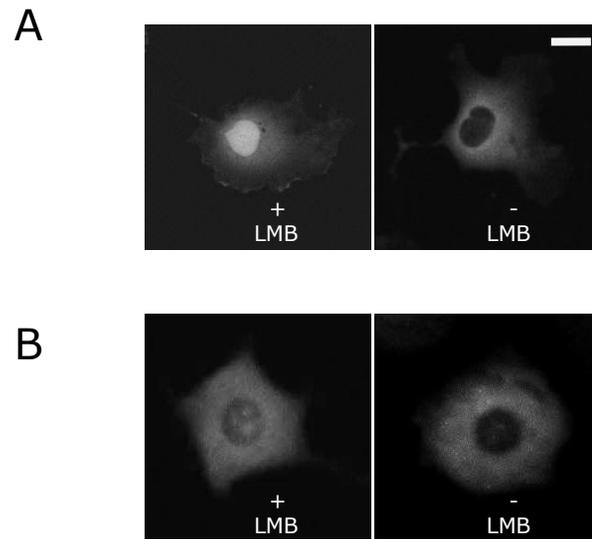
Figure 4-17: Images of COS-7 cells expressing NES-GFP and EmGFP-F4 in the presence and absence of LMB at 20x magnification with 10 µm scale bar. COS-7 cells expressing (A) NES-GFP control and (B) F4 truncated fragment in the presence and absence of LMB.
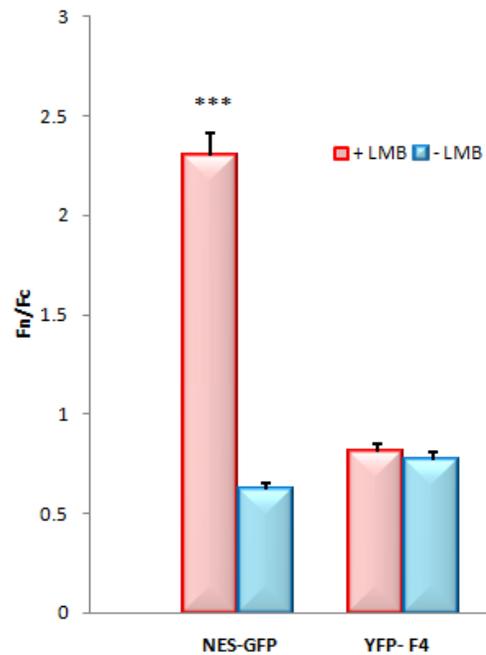
Figure 4-18: Quantitative analysis results for the levels of nuclear accumulation (Fn/c: ratios of the nuclear to the cytoplasmic fluorescence after the subtraction of background fluorescence) in COS-7 expressing the indicated fusion proteins in the presence and absence of LMB.

NES-GFP was used as a control for LMB experiment. The NES-GFP vector contains NES sequence (ALQKKLEELELDE) which is a classical NES found in MAPKK (mitogen-activated protein kinase kinase). In the absence of LMB, the protein was excluded from the nucleus. However, after the LMB treatment NES no longer has access to its export receptor and the protein retains in the nucleus. In the case of F4, there was no difference in Fn/Fc ratio in the presence or absence of LMB, indicating that the F4 fragment does not contain any NES activity. This confirms that the F4 fragment was unable to localise to the nucleus because it does not encode for NLS.

4.10 **Discussion**

All transport of macromolecules between the cytoplasm and the nucleoplasm occurs at the nuclear pore complex (Schlenstedt, 1996). It is known that the major pathway for active uptake of proteins into the nucleus is by interaction of importins with the nuclear localisation signal (Whittaker *et al.*, 1998). This interaction occurs prior to the active uptake of the protein complex via the nuclear pore complex (Whittaker *et al.*, 1998). Previous research has shown that adenoviral terminal protein, which is a protein coupled to the 5' end of the viral DNA strand, contains an NLS (Zhao *et al.*, 1988). However the specific sequence(s) which encode for NLS had not been investigated in detail prior to the present study, and as yet its role in nuclear delivery of the adenovirus DNA has not been established. One of the aims of this PhD was to identify the NLS encoding sequence(s) within TP. Based on the findings of this study, it seems quite plausible that nuclear import of the viral DNA could rely on the import of TP, and that coupling TP to DNA could be used to enhance gene delivery.

4.10.1   Identification of Multiple NLSs in Adenoviral Terminal Protein

Proteins enter the nucleus through NPC, which provides a channel between the nucleus and cytoplasm. Although it is possible for small proteins to diffuse through the NPC, the import of large proteins is an active process and is dependent on NLS (Silver, 1991). The

majority of NLSs can be classified into two types, the bipartite or the monopartite but there are also few other identified NLSs that do not belong within either categories (Silver, 1991).

Adenovirus TP is known to localise to the nucleus, presumably as pTP after its translation in the cytoplasm. TP has various roles in the cell nucleus and has shown to be associated with DNA replication. TP may also have a role in initial infection of cells by the Adv, when it would be present coupled to the Adv genome as the cleavage product (Fredman *et al.*, 1993; Mysiak *et al.*, 2004; Pronk *et al.*, 1993; Smart *et al.*, 1982). In 1988, Zhao and Padmanabhan showed that pTP localised in the nucleus and identified RLPV(R)$_6$VP as its NLS (Zhao *et al.*, 1988). However, this is the first study that has investigated other putative NLS sequences encoded within the TP. As a first step to identify the regions of TP which encoded the NLS, a range of truncated fragments were created, making no assumption about the significance of the previously identified NLS.

Wild type pTP and TP localised in the nucleus with no significant differences in the extent of protein delivery between the five mammalian cell lines studied (HeLa, COS-7, Caco-2, PC-3 and AD293 cells) suggesting that TP nuclear import is not cell-specific. Currently one of the factors limiting success of non-viral gene therapy is the relative inability of the vector to target genes at a desired cell type (Vacik *et al.*, 1999). As nuclear import of TP is not cell-specific, NLSs of TP may be incorporated into any vector to target a specific cell with no cell type limitations (Vacik *et al.*, 1999).

The position of NLS within a protein may be an important factor for protein delivery in that the NLS needs to be presented to IMPs in an appropriate manner. For example in SV40 large tumour T antigen, the flanking regions of its NLS play an important role in modulating the interaction between the NLS and IMP by either enhancing or masking it (Jans, 1995; Wagstaff *et al.*, 2006; Xiao *et al.*, 1997). Therefore fusion proteins (EmGFP and YFP) may influence the protein folding structure and change recognition of NLSs by IMPs. Hence, the precaution was taken to produce fusion proteins at both the C and N termini in all cases. However, folding of the fusion proteins had little influence on nuclear localisation in this

case because very similar Fn/Fc values were determined for both C-terminal EmGFP and N-terminal YFP fusion constructs, for all of the corresponding fusion proteins.

The truncated derivative fragments (F1, F2, F3, F5, F6, F7 and F9) were shown to localise to the nucleus, however, only F1 and F7 showed the same nuclear localisation level as that of the wild type TP. In HeLa cells, the Fn/Fc of EmGFP-TP, EmGFP-F1 and EmGFP-F7 were 8.39, 8.76 and 10.01 respectively while Fn/Fc of YFP-TP, YFP-F1 and YFP-F7 were 9.54, 8.62 and 8.73 respectively. In COS-7 cells, the Fn/Fc of EmGFP-TP, EmGFP-F1 and EmGFP-F7 were 7.23, 7.63 and 10.83 respectively while Fn/Fc of YFP-TP, YFP-F1 and YFP-F7 were 7.89, 7.40 and 9.38 respectively. As F1 (aa1-430) and F7 (aa541-671) contained different members of the three putative TP sequences, this implied that TP contained multiple active NLSs. Based on the nuclear localisation level produced by the fusion proteins, it can be deduced that a fully operational NLS sequence are found in F1 and F7 while the other 5 truncated fragments only contained for part of the full NLS activity. All of the putative NLS sequences were absent in F4 (aa1-356) and F8 (aa391-654) and their nuclear localisation levels were not different to that produced by the OVA-control. In HeLa cells, the Fn/Fc of EmGFP-OVA, EmGFP-F4 and EmGFP-F8 were 1, 0.91 and 0.99 respectively while Fn/Fc of YFP-OVA, YFP-F4 and YFP-F8 were 0.87, 0.89 and 0.92 respectively. In COS-7 cells, EmGFP-OVA, EmGFP-F4 and EmGFP-F8 were 0.92, 0.97 and 1.10 respectively while Fn/Fc of YFP-OVA, YFP-F4 and YFP-F8 were 0.91, 0.77 and 0.94 respectively. It was concluded that TP aa357-430 and aa655-671 were responsible for full NLS activity. Further study was conducted on the MRRRR[370], PVRRRRRRV[390] and PGARPRGRF[671] regions within aa357-430 and aa655-671 as these fragments fit the putative consensus sequences of the known classical NLSs. MRRRR[370] and PVRRRRRRV[390] fit both the classical monopartite NLSs (K-K/R-X-K/R) and bipartite NLSs ((K/R)(K/R)X$_{10-12}$(K/R)$_{3/5}$, with (K/R)$_{3-5}$ represents at least 3 of either lysine or arginine of five consecutive amino acids) while PGARPRGRF[671] fits the sequence of monopartite NLSs (Bayliss *et al.*, 2000; Kosugi *et al.*, 2009; Lange *et al.*, 2007).

Further investigation by mutagenesis provided evidence of the presence of multiple NLSs. The NLS found between residues 357 and 430 are of classical bipartite sequence (MRRRR[370] and PVRRRRRRV[390]) since the absence of one or the other reduced the overall nuclear

expression level. As most known NLSs are rich in positively charged amino acids, it was expected when arginine residues within the PVRRRRRRV^{390} sequence were found to be critical to the function of the NLS. The PGARPRHRF^{671} sequence was thought to be a possible NLS found between residues 655 and 671 but the deletion mutation (DEL-4) did not show any reduction in nuclear delivery. One possible explanation is that PGARPRHRF^{671} does not code for NLS, but instead NLS is found between 655 and 662 residues. However, this is not likely because PGARPRHRF^{671} is the only sequence found within the 655-671 fragment that includes characteristics of known NLSs. A more plausible explanation is that PGARPRHRF^{671} can code for a NLS but the presence of the strong bipartite NLS was enough to deliver TP into the nucleus. This was confirmed by the reduction in nuclear expression of DEL-5 (MRRRR^{370} → MYYYY^{370}, PVRRRRRRV^{390} → PVYYYYYYV^{390} and PGARPRHRF^{671} → PG------F^{671}), when all of the proposed NLS were mutated. In the absence of the bipartite NLS sequences (DEL-3), the protein with PGARPRHRF^{671} was able to enter the nucleus resulting in a higher Fn/Fc ratio than that of DEL-5. This suggested that PGARPRHRF^{671} did indeed have NLS activity and that this NLS acts independently of the bipartite cluster to deliver TP into the nucleus. The dominance of bipartite NLS, over the existing monopartite NLS, in delivering TP to the nucleus was not unexpected as similar result was observed in parafibromin (Lin *et al.*, 2007). A site-directed mutagenesis of the NLSs within parafibromin showed a dominant bipartite NLS over a secondary monopartite NLS (Lin *et al.*, 2007),

To investigate the potential function of the negatively rich domain with regard to nuclear delivery, comparison analysis was performed between F1 and F2 (p < 0.001), and between F6 and F7 (p < 0.001). Although the negatively rich domain is shown to influence the level of nuclear import, its role still remains unclear. F1 (aa1-430; with negatively rich domain) has a higher nuclear accumulation than F2 (aa1-390; without negatively rich domain), suggesting that the presence of negatively rich domains increased the nuclear delivery. However, F7 (aa541-671) shows a higher level of nuclear accumulation than F6 (aa391-671) which contains negatively rich domain. One possible explanation for this outcome is that the proximity of the negatively rich domain to the NLS influences the folding structure of the protein and hence the exposure of the NLS to the receptor. When its presence is distant, in the case of F6, the negatively rich domain may bind to the NLS signal masking it from the environment leading to the reduction in nuclear expression. However, in close

proximity, as in the case of F1, it may not be able to completely mask the NLS but instead may influence the protein folding to expose the signal for receptor recognition. Further investigation on the folding structure of the TP will be required to draw a conclusion on these observations.

4.10.2 Adenovirus Pre-Terminal Protein encodes for nucleolar localisation signal

It has been reported that nucleolar localisation is part of virus strategy to regulate translation process of both virus and host subgenomic RNA and as a result regulate DNA replication (Hindley *et al.*, 2007a; Li *et al.*, 2011). Many NuLSs have been discovered in viral proteins, for example Herpes simplex virus regulatory protein ICP27 (aa110-152) and Pseudorabies virus early protein UL54 (aa45-57) (Li *et al.*, 2011; Mears *et al.*, 1995). In the case of Adv, both protein V and protein VII were shown to localise to the nucleolus (Lee *et al.*, 2003b; Matthews, 2001). Interestingly, the full length pTP (aa1-671) and the truncated derivative F7 (aa541-671) also show nucleolar accumulation, suggesting that pTP also coded for at least one or multiple NuLSs. The result was not completely unexpected as the three most central proteins for Adv DNA replication are the viral polymerase (Advpol), pTP and DNA-binding protein (DBP) (Hindley *et al.*, 2007a). Previous study has also identified interaction between pTP and B23.1, which is one of the two isoforms of nucleolar protein B23 (Hindley *et al.*, 2007a; Okuwaki *et al.*, 2001). However, no mention of NuLS was made.

The region coding the nucleolar signal is clearly present in both the pTP and F7, which implies that the potential signal coding region is aa541-671. However, it was observed that the nucleolar expression is absence in F8 (aa391-654), suggesting that the nucleolar signal coding region must be found between 655 and 671 residues. It was noted, that the nucleolar expression is absence in TP (aa322-671), F5 (aa374-671) and F6 (aa391-671) although the proposed nucleolar signal encoding region is present. One possible explanation is that the folding structure of the protein influences the signal exposure to the environment. The main difference between F7 and the other three fragments are the length of the sequence found prior to the nucleolar localisation signal. In the case of TP, F5

and F6, there are long sequences of at least 250 residues which could possibly mask the signal preventing it from being recognised by the appropriate nucleolar receptor. For F7, only around 100 residues can be found prior to the signal which may be too short to mask the signal, hence, the signal is readily exposed to the environment. Currently there are no accepted consensus sequences for NuLSs. This aspect of the current project is worthy of further investigation to find out whether PGARPRGRF[671] can deliver other proteins to the nucleoli.

4.10.3   Adenovirus Terminal Protein does not encode for nuclear export signal

Some proteins, such as heterogeneous ribonucleoproteins (hnRNP) A1 protein, have been identified to encode for both NLS and NES, allowing them the ability to shuttle between the nucleus and cytoplasm (Guil *et al.*, 2006; Siomi *et al.*, 1995). To ensure that the absence of F4 in the nucleus was not due to NES activity, LMB experiments were conducted.

NES-GFP was used as a control for LMB experiments. In the absence of LMB, the NES interacts with CRM1/EXP 1 for its nuclear export and the protein was excluded from the nucleus. However, after LMB treatment NES no longer has access to its export receptor and the protein is retained in the nucleus. The Fn/Fc ratio for F4 shows no differences in the presence or absence of LMB, indicating that the F4 fragment does not contain any NES activity and its cytoplasmic localisation is due to the lack of NLS.

4.11 **Conclusion**

TP was found to contain multiple NLSs which include the classical bipartite sequence (MRRRR[370] and PVRRRRRRV[390]) and monopartite sequence (PGARPRGRF[671]). These two sequences were able to deliver TP to the nucleus independently of one another, although for intact TP the bipartite sequence was dominant. The arginine residues within the MRRRR[370] and PVRRRRRRV[390] clusters were found to be critical part of the NLS as there is a

noticeable reduction in nuclear import in their absence. The PGARPRGRF[671] sequence was shown to have a nucleolus delivery property when not masked by the flanking regions. Furthermore, the negatively rich domain within TP may play a role in protein folding to mask or expose the NLS to the appropriate IMP receptor, influencing the nuclear delivery of TP.

CHAPTER 5

**Characterisation of
The Adenovirus Terminal Protein
Nuclear Import Pathway**

CHAPTER 5   Characterisation of the Adenovirus terminal protein nuclear import pathway

5.1 Overview

The success of non-viral gene therapy depends on the development of gene delivery methods with similar efficiencies as viruses. In order to design novel, clinically relevant gene delivery approaches that mimic viral gene transfer, characterisation of viral nuclear delivery systems is of fundamental importance. The nuclear import of viruses/viral particles is crucial for pathogenecity of many types of viruses (Whittaker, 2003; Whittaker *et al.*, 1998; Whittaker *et al.*, 2000). Viruses take advantage of host cellular signals and factors, and transport processes. However the mechanisms by which each type of virus interacts with the host cell nuclear import machinery vary considerably (Whittaker *et al.*, 1998; Whittaker *et al.*, 2000).

Cells use several pathways to import proteins into the nucleus, but the most common general trafficking route for both proteins and viruses is dependent upon the recognition of NLSs by members of the importin (IMP) superfamily. Most commonly this involves the IMPα/β heterodimer, IMPβ alone or an IMPβ homologue (Chook *et al.*, 2001; Christophe *et al.*, 2000; Jans, 1995; Jans *et al.*, 2000; Lange *et al.*, 2007). The cargo protein complex then translocates through the nuclear pore complex (NPC) and is released within the nucleus (Chook *et al.*, 2001; Christophe *et al.*, 2000; Jans, 1995; Jans *et al.*, 2000; Lange *et al.*, 2007). Different consensus sequences of NLSs bind specifically to particular IMP receptors (Kosugi *et al.*, 2009).

This thesis focuses on the nuclear trafficking of Adv proteins. Understanding how TP reaches the nucleus is an important aspect of the Adv life cycle and may also provide information on the mechanisms involved in nuclear delivery of the Adv DNA during the process of infection. This chapter concentrates on the mechanisms of nuclear import of Adv TP and its truncated derivatives. To understand how a particular protein enters the nucleus, it is necessary to study how NLS consensus sequences are recognised. The aim of

the chapter is to determine the binding activities between the TP and their derivatives, and three members of the IMP superfamily. For the purpose of binding experiments, IMPα, IMPβ and IMPα/β were expressed in *E.coli*.

5.2 **Nucleocytoplasmic Transport**

The direct access of proteins to the nucleus is limited by a double lipid bilayer nuclear envelope and the only transport route is through the NPC (Christophe *et al.*, 2000; Jans *et al.*, 2000; Kosugi *et al.*, 2009; Silver, 1991). Molecules which are smaller than 45 kDa can diffuse passively through the NPC, to different extents depending on their size and diffusivity, however transportation of larger molecules takes place by way of an active process dependent on the presentation of NLSs (Christophe *et al.*, 2000; Gasiorowski *et al.*, 2003; Jans *et al.*, 2000; Lange *et al.*, 2007; Schlenstedt, 1996; Silver, 1991).

The initiation of nuclear import involves the recognition of the targeting signal, NLS by karyopherins or IMPs (Izaurralde *et al.*, 1998; Kosugi *et al.*, 2009; Moroianu, 1998; Moroianu, 1999). There are 6 IMPα and over 20 different IMPβ homologues in humans which can mediate nuclear import, but basic NLS are most commonly recognised by the IMPα/β heterodimer, IMPβ or IMPβ homologues (Ribbeck *et al.*, 1998). In all cases, IMPβ homologue docks with the NLS-protein cargo complex. With the help of monomeric GTP-binding protein Ran (GTPase Ran), the cargo complex is translocated through the NPC into the nucleus (Schlenstedt, 1996).

5.2.1 Nuclear Pore Complex (NPC)

The NPC is surrounded by the NE and consists of three substructures: the cytoplasmic filaments, a central core and the nuclear basket (Bayliss *et al.*, 2002; Forwood *et al.*, 2002; Forwood *et al.*, 2001b; Ribbeck *et al.*, 1998; Stewart, 2007; Stewart *et al.*, 2001). The NPC has a mass of approximately 125 Mda in vertebrates and 88 MDa in yeast (Hoelz *et al.*, 2011). The NPC contains eight spokes which form three main rings surrounding a central core connecting the cytoplasm and nucleus (Bayliss *et al.*, 2002; Forwood *et al.*, 2002;

Hoelz *et al.*, 2011; Jans *et al.*, 2000; Ribbeck *et al.*, 1998). The inner ring is found between two outer rings located on the cytoplasmic and nuclear sides. The NPC has a central diameter of approximately 30nm which serves as a channel for macromolecular transport (Forwood *et al.*, 2002; Forwood *et al.*, 2001b; Stewart *et al.*, 2001). The NPC is composed of approximately 30 different nucleoporin proteins (Nups), belonging to either transmembrane, core scaffold (inner and outer ring), linker or Phenylalanine-Glycine (FG) class (Bayliss *et al.*, 2002; Forwood *et al.*, 2002; Ribbeck *et al.*, 1998; Stewart *et al.*, 2001). Each nucleoporin occurs in multiple copies, resulting in approximately 500-1000 protein molecules in a single fully assembled NPC (Hoelz *et al.*, 2011).

Transmembrane Nups constitute a lumenal ring and are responsible for anchoring the NPC to the NE (Ribbeck *et al.*, 1998). The core scaffold Nups made up the outer and inner rings of NPC while the linker Nups are responsible for attaching the central core with FG Nups (Forwood *et al.*, 2002; Ribbeck *et al.*, 1998). FG Nups interact with the transport receptors (IMPs and EXPs) to facilitate the movement of protein cargoes through the NPC (Bayliss *et al.*, 2002; Hoelz *et al.*, 2011; Jans *et al.*, 2000; Ribbeck *et al.*, 1998).
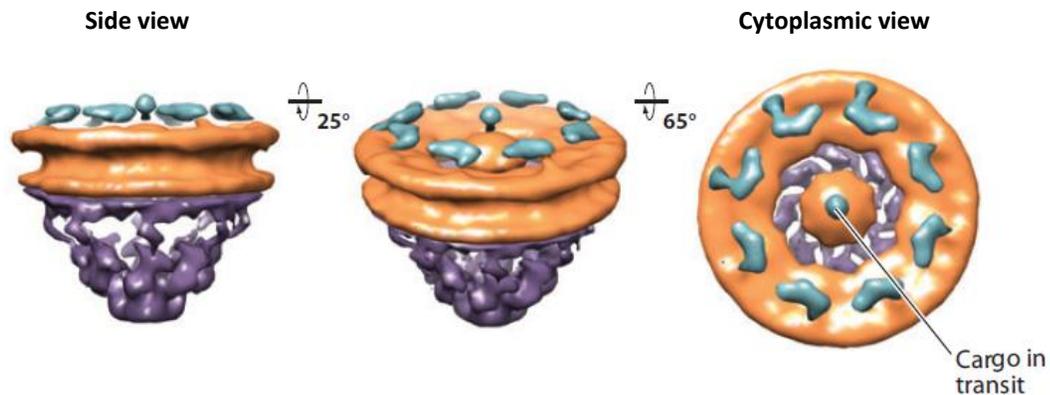
Figure 5-1: A cryo-electron tomographic reconstruction of the *Dictyostelium discoideum* NPC. The cytoplasmic filaments are coloured in cyan, the symmetric core in orange and the nuclear basket in purple [taken from (Hoelz *et al.*, 2011)].

5.3 **Nuclear Import Pathways**

There are several nuclear import pathways, where NLS-carrying proteins are recognised by distinct IMPs (Kosugi *et al.*, 2009). However, in most aspects the import process is essentially similar: in the cytoplasm NLS-containing cargo is recognised by IMPs to form a complex. The complex is translocated through the NPC, and subsequently the cargo is released from the IMP in the cell nucleus upon binding of the monomeric guanine nucleotide binding protein Ran (Jans *et al.*, 2000; Kosugi *et al.*, 2009). This section will focus on IMPα/β heterodimer mediated pathway and IMPβ mediated pathway.

5.3.1 IMPα/β Heterodimer Mediated Pathway

The IMPα/β heterodimer mediated pathway is the best understood nuclear import pathway to date. IMPα serves as an adapter protein that binds to the NLS-containing protein, while IMPβ mediates the translocation of the complex through the NPC (Kobe, 1999; Kosugi *et al.*, 2009). The rate at which this pathway mediates the nuclear import is approximately 100-1000 cargo molecules per minute per NPC (Ribbeck *et al.*, 2001).

The pathway is initiated when IMPα binds to IMPβ to form a IMPα/β heterodimer (Kobe, 1999). IMPα and IMPβ interaction is achieved through the N-terminal IMPβ binding (IBB) domain found on IMPα (Kobe, 1999). Following the IMPα/β heterodimer formation, the IMPα/β heterodimer complex binds to a NLS-containing cargo protein to form a ternary complex (Kobe, 1999). The ternary complex is then translocated across the NPC to the nucleus which is mediated by interactions between IMPβ with Nup's FG sequence repeats found in the core centre of the NPC (Bayliss *et al.*, 2000; Bayliss *et al.*, 2002; Hoelz *et al.*, 2011). The cargo protein is released from the IMPα/β heterodimer complex into the nucleus when RanGTP binds to IMPβ and Nup50 to IMPα (Stewart, 2007). Once free, both IMPβ and IMPα are recycled back to the cytoplasm to take part in another nuclear import cycle (Stewart, 2007). The schematic illustration of the pathway is shown in Figure 5-2.
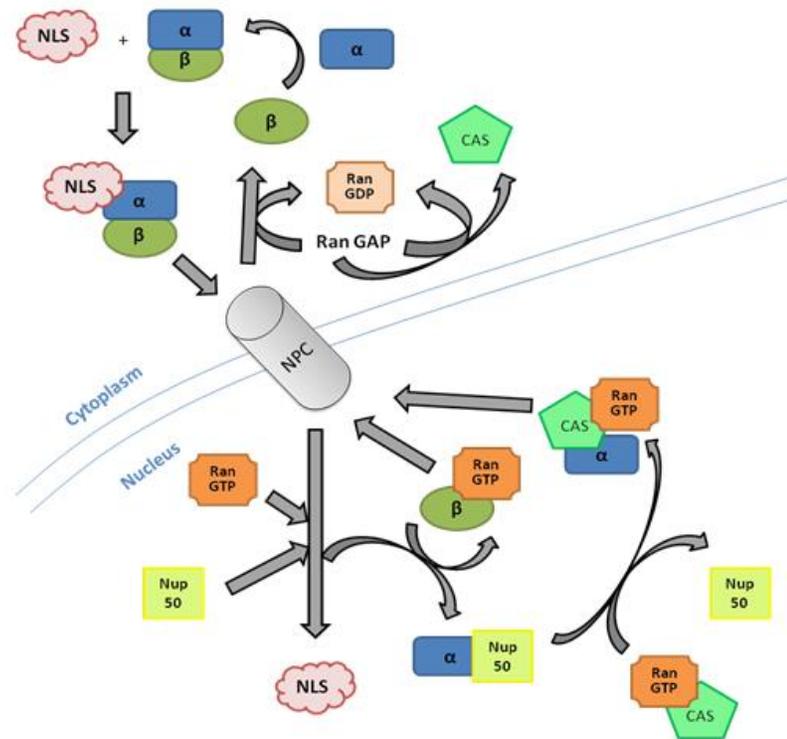
Figure 5-2: IMPα/β heterodimer mediated nuclear protein import [adapted from (Quimby *et al.*, 2001)]. Nuclear imports of NLS-containing proteins are recognised by the IMPα/β heterodimer. Through the IMPα adaptor, NLS-containing cargo protein binds to the IMPα/β heterodimer and the complex is translocated through NPC into the nucleus. In the nucleus, release of the NLS-containing protein is achieved through the bindings of RanGTP to IMPβ and Nup50 to IMPα. IMPα is recycled back to the cytoplasm by its nuclear export factor, CAS, complexed with RanGTP. The IMPs are released into the cytoplasm after hydrolysis of GTP by Ran catalysed by RanGAP.

The interaction between NLS cargo proteins and IMP receptors is controlled by Ran, specifically by its two nucleotide binding states: RanGTP and RanGDP (Ribbeck *et al.*, 1998; Smith *et al.*, 1998). The nucleotide state of Ran is regulated by nuclear Ran guanine nucleotide-exchange factor (RanGEF) and the cytoplasmic Ran GTPase activating protein (RanGAP) (Ribbeck *et al.*, 1998; Smith *et al.*, 1998). The Ran cycle is illustrated in Figure 5-3. Ran is a member of the Ras superfamily of monomeric GTPases and can cycle between nucleus and cytoplasm depending on its nucleotide binding state. The cytoplasmic RanGAP catalyses the hydrolysis of RanGTP to form RanGDP, which is the form that is transported into the nucleus through its own nuclear transport factor-2 (NTF-2) (Ribbeck *et al.*, 1998). In the nucleus, RanGEF catalyses nucleotide exchange of RanGDP for RanGTP (Quimby *et*

al., 2001; Stewart *et al.*, 2001). RanGTP then binds to IMP to release cargo proteins within the nucleus (Quimby *et al.*, 2001; Stewart *et al.*, 2001). Consequently, the concentration of RanGDP is higher in the cytoplasm, where IMPs will bind to NLS-containing cargo proteins, and lower in the nucleus, where Ran is predominantly found in its GTP-bound form, promoting dissociation of IMP and the cargo protein complex (Quimby *et al.*, 2001; Stewart *et al.*, 2001).
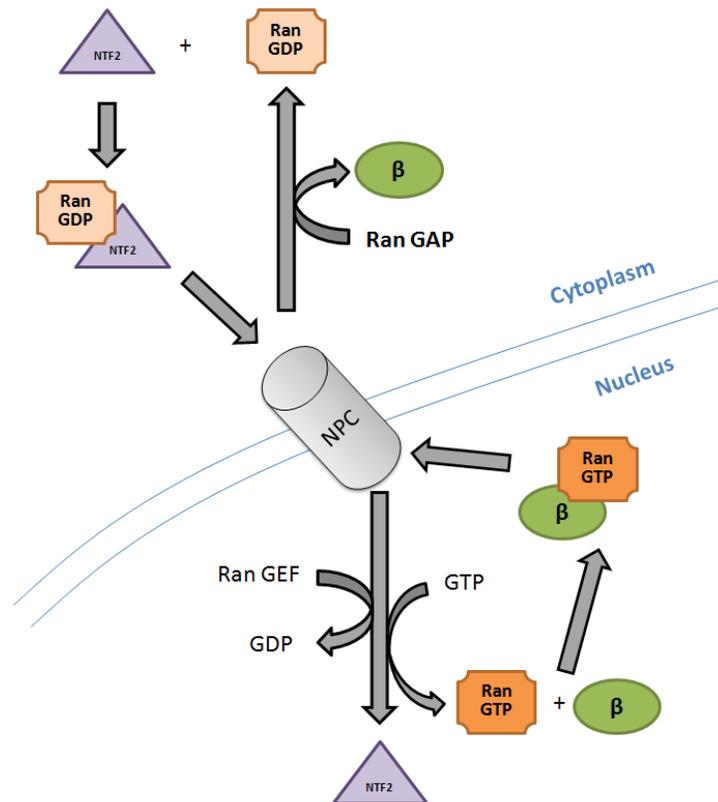
Figure 5-3: Ran cycle between nucleus and cytoplasm [adapted from (Quimby *et al.*, 2001)]. Cytoplasmic RanGAP facilitates hydrolysis of GTP to GDP by Ran. RanGDP is imported into the nucleus by the nuclear transport factor-2 (NTF2). Once in the nucleus, guanine nucleotide-exchange factor (RanGEF) catalyses nucleotide exchange of RanGDP for RanGTP. RanGTP is free to bind to IMPs to dissociate them from cargoes prior to recycling back into the cytoplasm.

5.3.2 IMPβ Mediated Nuclear Pathway

In the absence of IMPα, IMPβ1 or one of a number of its homologues can mediate nuclear protein import. Some proteins that are known to utilise IMPβ1 alone as an import receptor include; sterol response element binding proteins (SREBP-1a and SREBP-2), c-AMP response element binding protein (CREB), HIV-1 protein Rev, telomere repeat binding factor 1 (TRF-1), and parathyroid hormone related protein (PTHrP) (Forwood *et al.*, 2002; Forwood *et al.*, 2001b; Henderson *et al.*, 1997; Lam *et al.*, 1999; Nagoshi *et al.*, 1999). Of the IMPβ homologues utilising the same basic pathway, IMPβ2 (transportin) is responsible for transport of mRNA-binding proteins into and out of the nucleus, whereas IMPβ2, IMPβ3, IMPβ4 and IMPβ7 can all mediate nuclear import of ribosomal proteins (Aitchison *et al.*, 1996; Bonifaci *et al.*, 1997; Jäkel *et al.*, 1998; Pollard *et al.*, 1996; Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). IMPβ3 and IMPβ7 can also mediate nuclear import of some transcription factors (Kaffman *et al.*, 1998; Nakielny *et al.*, 1999). Figure 5-4 illustrates a schematic diagram of the IMPβ mediated pathway. The fundamental principle of the IMPβ mediated pathway is the same as for the IMPα/β heterodimer mediated pathway. However, in the IMPβ mediated pathway the NLS-containing cargo protein binds directly to IMPβ. The cargo protein-IMPβ complex is translocated across the NPC and dissociates in the nucleus upon the binding of IMPβ to RanGTP (Moroianu, 1999; Quimby *et al.*, 2001; Stewart *et al.*, 2001). Afterwards IMPβ is recycled back into the cytoplasm for another cycle of nuclear import (Moroianu, 1999; Quimby *et al.*, 2001; Stewart *et al.*, 2001).
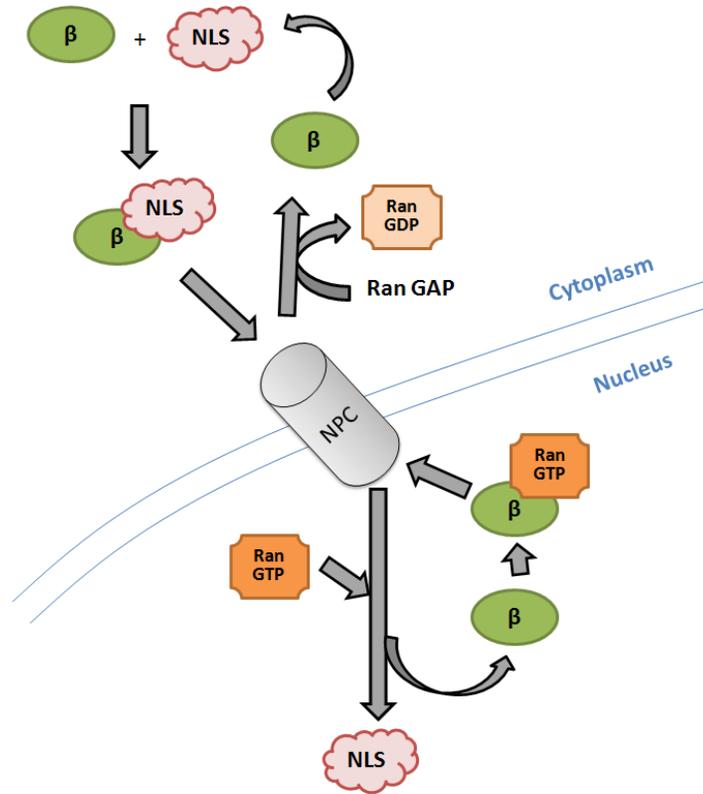
Figure 5-4: IMPβ mediated nuclear protein import [adapted from (Quimby *et al.*, 2001)]**.** The transport process is essentially the same as described in Figure 5-2, with exception of NLS-containing protein binding directly to IMPβ instead of the IMPα/β heterodimer. The protein cargo is translocated through the NPC and released in the nucleus upon binding of RanGTP to IMPβ. IMPβ is recycled back into the cytoplasm for another cycle of nuclear import.

5.4 Structural Properties of Importins

5.4.1 Importinα (IMPα)

IMPα is a 55kDa protein composed of a small basic N-terminal domain, termed the IMPβ binding (IBB) domain, and a large 50 kDa NLS-binding domain connected by a flexible linker (Stewart, 2007; Stewart *et al.*, 2001). There are 6 distinct IMPα isoforms in humans, all containing a conserved N-terminal IBB domain and able to interact with IMPβ specifically (Jans *et al.*, 2000; Köhler *et al.*, 1999). However, each isoform carries distinct properties for NLS recognition and differential expression in different tissues (Jans *et al.*, 2000; Köhler *et*

al., 1999; Miyamoto *et al.*, 1997; Sekimoto *et al.*, 1997). This allows the 6 isoforms to provide a mechanism for a broad range of regulation by controlling the trafficking of particular cargoes in distinct cell types (Jans *et al.*, 2000; Köhler *et al.*, 1999; Miyamoto *et al.*, 1997; Sekimoto *et al.*, 1997). A structural diagram of IMPα is shown in Figure 5-5.
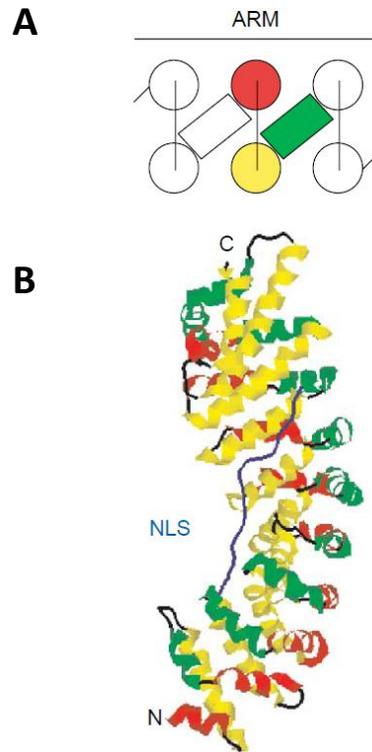
Figure 5-5: Structural diagram of importin α [taken from (Goldfarb *et al.*, 2004)] (a) A figure showing the H1, H2 and H3 helices in green, red and yellow respectively. (b) A model of yeast importin α bound to the bipartite NLS of nucleoplasmin, with the H1, H2 and H3 helices of the ARM protein showing in green, red and yellow, respectively. The bound ligands are shown in purple. The model is oriented with its N-termini at the bottom and C-termini at the top.

The NLS-binding domain consists of ten armadillo (ARM) repeats which are 40 amino acid motif of three α-helices (H1, H2 and H3) forming superhelical twist (Smith *et al.*, 1998; Stewart, 2007; Stewart *et al.*, 2001; Wagstaff *et al.*, 2006). The site of NLS recognition is found on the inner surface of IMPα and contains two binding pockets (major and minor groove) (Smith *et al.*, 1998; Stewart, 2007). The NLS binding site is formed from an array of tryptophan (Trp), asparagine (Asn) and acidic residues (Smith *et al.*, 1998; Stewart, 2007).

The monopartite NLS binds to both the major and minor grooves. In the case of bipartite NLS, the larger cationic cluster binds to the major groove while the smaller cluster binds to the minor groove (Fontes *et al.*, 2003; Stewart, 2007; Stewart *et al.*, 2001; Wagstaff *et al.*, 2006). Both NLSs bind to IMPα in an extended conformation. The polypentide backbone forms hydrogen bonds with Asn residues, the NLS peptide side chains insert into a series of Trp residues, and the positively charged tips of the NLS Lys residues interact electrostatically with the surrounding acidic residues of IMPα (Ribbeck *et al.*, 1998; Stewart, 2007). In the case of the bipartite NLS, the linker sequence makes limited contacts with the IMP, consistent with its tolerance to mutation (Fontes *et al.*, 2000; Stewart *et al.*, 2001).

5.4.2 Importinβ (IMPβ)

IMPβ is a 95 kDa flexible super-helix composing of 19 tandem HEAT repeats (Huntingtin, elongation factor 3, protein phosphatise 2A and the yeast PI3-kinase TOR1) (Stewart *et al.*, 2001). Each HEAT repeats contain two helices (α and β) connected by a loop (Stewart *et al.*, 2001). The α helices are found on the outside of IMPβ, forming a convex surface, whereas β helices are located inside, and form a concave surface (Stewart *et al.*, 2001). The Nup FG sequences interact with IMPβ outer surface, while the IMPα IBB domain interacts with C-terminal (HEAT 7-19) and N-terminal (HEAT 1-8) regions on the inner surface of IMPβ (Stewart *et al.*, 2001). A structural diagram of IMPβ is illustrated in Figure 5-6.

In contrast to IMPα, that recognises relatively short and linear NLS, the flexibility of IMPβ allows it to accommodate cargoes of different sizes and shapes (Fontes *et al.*, 2000; Fontes *et al.*, 2003; Stewart, 2007; Stewart *et al.*, 2001; Wagstaff *et al.*, 2006). For example, the NLS of PTHrP peptide binds to the N-terminus of the IMPβ, resembling interactions between classical NLS and IMPα but the NLS of SREBP-2 interacts with the long α helices of IMPβ (Cingolani *et al.*, 2002; Lee *et al.*, 2003a). Besides its ability to bind and transport NLS-containing cargo proteins, the IMPβ family members also contain Ran and NPC-binding activities (Stewart, 2007; Stewart *et al.*, 2001). In the S shaped unbound conformation, IMPβ1 is able to bind to the IBB domain of IMPα (Stewart *et al.*, 2001).
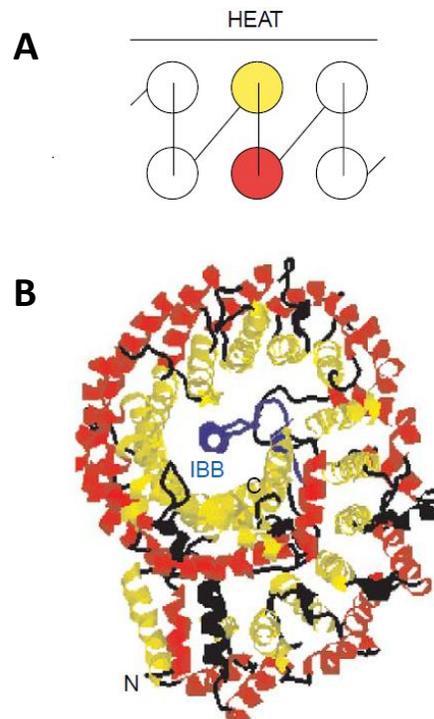
Figure 5-6: Structural diagram of importin β [taken from (Goldfarb *et al.*, 2004)] (a) A figure showing the α and β helices in red and yellow respectively. (b) A model of human importin β bound to the importin-β-binding domain of importin α, with α and β helices of the HEAT protein showing in red and yellow, respectively, and the bound ligands showing in purple. The model is oriented with its N-termini at the bottom and C-termini at the top.

5.5 Cytoskeleton-facilitated transport

Sub-cellular transport in cells can also be facilitated by two components of the cytoskeleton: actin filaments and MTs. Actin filaments generally facilitate slower and short-range transportation, whereas MT networks provide high-speed and long-range transportation and are responsible for most intracellular transport (Campbell *et al.*, 2003; Rogers *et al.*, 2000).

Nuclear localisation of a protein is dependent primarily on members of the nuclear import machinery (Poon *et al.*, 2005; Pouton *et al.*, 2007). However, recent study suggests that the cytoskeleton, particularly the MT network, may play a central role in both nuclear targeting

of viral proteins and also in modulating nuclear localisation of cellular proteins as well as facilitating nuclear protein import (Döhner *et al.*, 2005; Phung-Koskas *et al.*, 2005; Roczniak-Ferguson *et al.*, 2003; Ziegelbauer *et al.*, 2001).

5.6 **Aims**

The aims of this chapter are:

1. To investigate the interactions that characterise the nuclear trafficking pathway of Adv TP.

2. To determine the binding activities between the full length TP, truncated derivatives of TP containing the bipartite NLS or just a monopartite NLS, with three components of the IMP superfamily: IMPα, IMPβ and the complex IMPα/β.

5.7 **Method**

Otherwise stated, the detailed experimental procedures used in this chapter are described in Chapter 3.

5.7.1 Transfection and Drug Treatments

cDNA sequences of TP (aa322-671), F2 (1-430), F1 (391-671), F7 (371-480), were each inserted into mammalian expression plasmid pcDNA6.2/N-YFP (fused in frame to the N-terminus of the YFP) using the Gateway recombination system.

HeLa cells were maintained in DMEM, supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C. Cells were transfected at 70-80% confluence using Lipofectamine 2000, according to manufacturer's instructions. The cells were imaged 48 hours post-transfection using confocal microscopy after incubation for 1 hour in the absence or presence of 25 µM ivermectin to investigate potential inhibition of the IMPα/β pathway.

Incubation with 5 μg/ml nocodazole (NCZ) for 4 hours was also used in some experiments to investigate the effect of disrupting MT function. GFP-Tg, obtained from the Jans laboratory group at Monash University, was used as a control in the NCZ experiments.

5.7.2 Image Analysis

Image analysis was carried out on the digitalised confocal files using Image J 1.42q public domain software, to measure relative fluorescence of nuclear (Fn), nucleolar (Fnu) and cytoplasm (Fc) (background fluorescence was subtracted). This allowed the calculation of, nucleolar to cytoplasmic (Fnu/c), and nuclear to cytoplasmic (Fn/c) ratios.

Statistical analysis was performed on raw data using the analysis tools provided in Microsoft Excel. Student's t test was carried out to determine the significance of the differences between untreated and treated cells. In all cases, the difference between the untreated and treated cells is considered to be significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

5.7.3 Construction of Expression Plasmids

cDNA sequences of full length TP (aa 322-671) and 3 of its truncated derivatives: 1-430 (F2), 1-390 (F5), and 541-671 (F4), were each inserted into the bacterial expression plasmids, pDEST15 (fused in frame to the N-terminus of the GST) and pDEST24 (fused in frame to the C-terminus of the GST) using the Gateway recombination system. GST-IMPα and GST-IMPβ fusion proteins were expressed in E coli and purified using methods used previously in the Jans laboratory.

5.7.4 Protein Expression and Purification

Fusion proteins GST-IMPα, GST-IMPβ GST-TP (322-671), GST-F2 (1-430), GST-F5 (1-390) and GST-F4 (451-641), were purified from bacteria as GST-tagged proteins using glutathione affinity chromatography under native conditions.

5.7.5 ALPHAScreen® Assay

ALPHAScreen® assays were carried out to study the binding affinity between the selected IMP family members and proteins of interest. The ALPHAScreen® assay technique was performed in solution with proteins in native conformation and the energy transfer between binding partners was achieved via an excited oxygen singlet diffusing through solution over distances of up to 200 nm (Wagstaff *et al.*, 2006). The assay does not require the binding partners to be fluorescently labelled, but instead uses biotinylated and GST-tagged moieties, both of which only present small modifications that are unlikely to interfere with protein folding or function. A schematic illustration of the assay is shown in Figure 5-7.
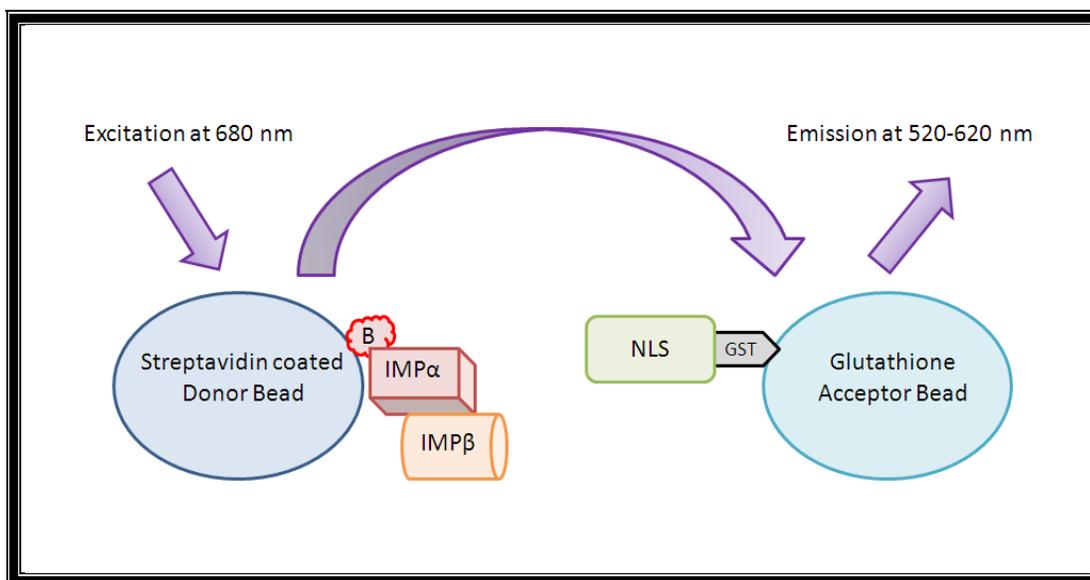
Figure 5-7: Schematic representation of the ALPHAScreen-based assay [adapted from (Wagstaff *et al.*, 2006)]. The diagram shows the binding between the IMPα/β heterodimer and NLS-containing proteins. The IMPα/β heterodimer is covalently linked to the streptavidin-coated donor beads through the biotinylated IMPα. The NLS-containing protein has a GST tag and binds to the glutathione acceptor beads. When the binding between the NLS-containing protein and IMPα/β heterodimer happens, the two beads are brought into close proximity. Excitation of the donor bead at 680 nm causes an excited oxygen singlet to be generated and if the acceptor bead is within this range (up to 200 nm), when the binding occurs, it will produce a detectable emission spectrum at 520-680 nm.

5.7.5.1 Protein Expression and Purification

The expression and purification for the proteins required in the assay are discussed in Section 3.12 of the Method Chapter.

5.7.5.2 Thrombin Cleavage

GST was cleaved from GST fusion IMP proteins using thrombin prior to biotinylation of IMP proteins. A reaction mixture containing 1 mg GST fusion IMP protein, 35 units thrombin, 0.5 mM DTT in 4.5 ml of intracellular buffer was incubated for 4 hours at 4°C. To remove the thrombin, the reaction mixture was combined with 66 µl binding buffer and incubated with pre-washed 300 µl benzanidine sepharose 6B beads overnight at 4°C on a rotating shaker. The next day, the reaction mixture underwent centrifugation at 14000 rpm for 10 minutes, and the supernatant was collected and incubated with pre-washed Econocolumn containing 100 µl glutathione sepharose 4B beads. The flow through was collected.

5.7.5.3 Biotinylation of IMP Proteins

IMP proteins were biotinylated using the Sulfo-NHS-Biotin reagent. Briefly, 1 mg of sulfo-NHS-biotin was diluted in 150 µL of water. A reaction mixture containing 350 µL of IMP proteins, diluted biotin (1 µL per 4145 pmol protein) and water for the total volume of 400 µL were incubated for 2 hours at 4°C. Excess biotin was removed using the PD-10 column as per the manufacturer's instructions and the resultant biotinylated proteins were concentrated using an Amicon 30 concentration device.

5.7.5.4 ALPHAScreen® Binding Assay

The ALPHAScreen® assay was performed in triplicate in 384-well white opaque plates. 2 µL of GST-tagged proteins (30 nM final concentration) was added to each well, followed by 20 µL of the appropriate IMP concentration (0, 2.25, 7.5, 15, 30 or 60 nM) prepared by serial dilution in PBS and incubated for 30 minutes at room temperature. 1 µL of a 1:10 dilution

(in PBS) of the glutathione acceptor beads and 1 μL of 2.5% BSA were added simultaneously and incubated for 90 minutes in the dark at room temperature. Then 1 μL of a 1:10 dilution of the streptavidin donor beads was added to give a final sample volume of 25 μL. The mixture was incubated in the dark at room temperature for 2 hours. The assay was measured on a Perkin Elmer Fusion Alpha plate reader (USA). Average values for the triplicate were taken and titration curves (three-parameter sigmoidal fit) were plotted using Sigma Plot®. The quenching values of the signal were excluded from the final plot. Student's T test statistical analysis was performed on the values.

5.8 Experimental Results

5.8.1 Nuclear accumulation of TP does not require functional/intact MTs

To investigate whether TP employs a MT-facilitated nuclear import pathway to reach the nucleus, HeLa cells were analysed for the level of nuclear accumulation of wild type TP in the absence or presence of the MT perturbing drug, NCZ, which results in depolymerisation of MTs. The results are illustrated in Figure 5-8. GFP-Tg was used as a control vector, containing NLS derived from SV40. SV40 NLS is known to not utilise MT for its transport. The nuclear accumulation of TP was unaffected by NCZ treatment, implying that nuclear import of TP is not dependent upon intact and functional MTs.
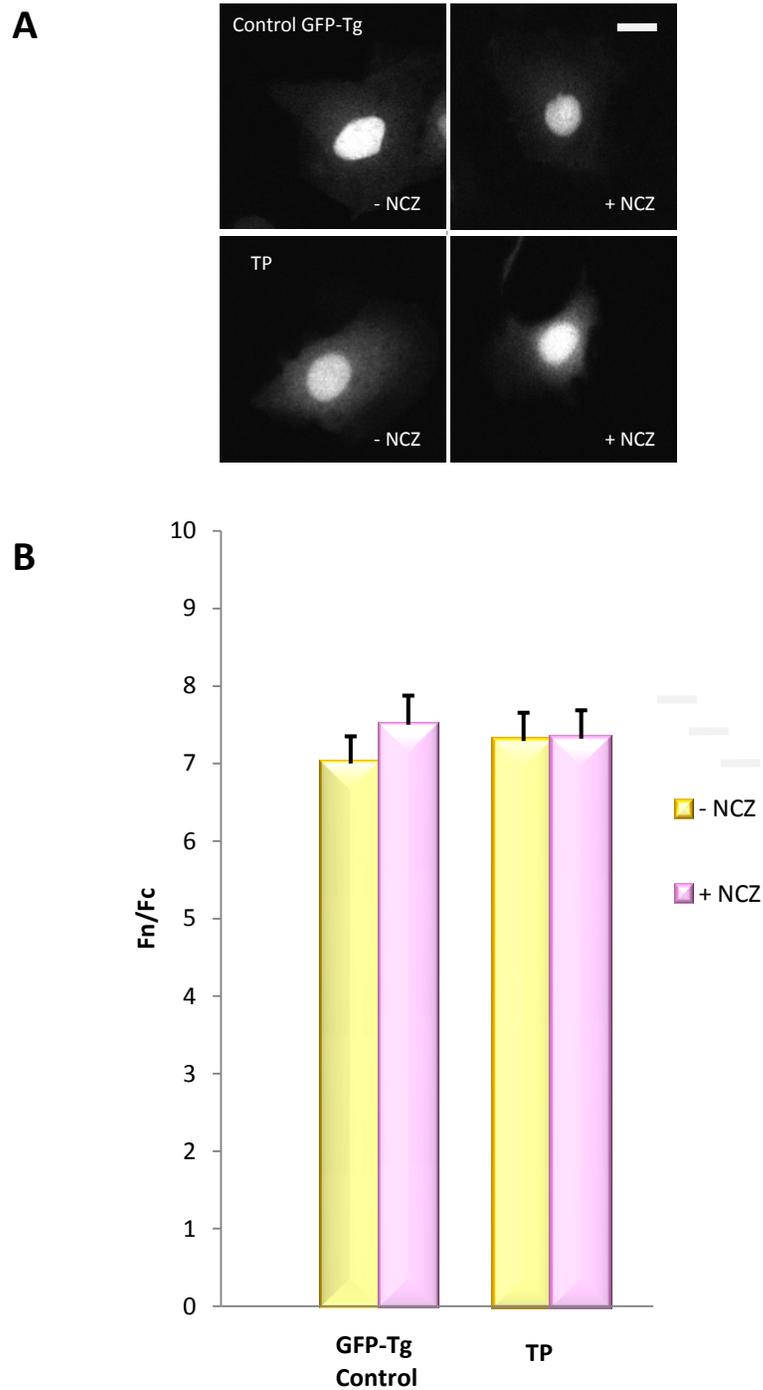
Figure 5-8: Nuclear accumulation of adenovirus TP is not dependent on functional/intact MTs. (A) Confocal microscope images of HeLa cells treated for 4 hours with or without 5 μg/ml NCZ at 20x magnification with 10 μm scale bar. (B) Results for quantitative analysis of transfected HeLa images, for nuclear accumulation expressed as the Fn/c ratio [nuclear fluorescence (fn) divided by the cytoplasmic fluorescence (Fc), above the background fluorescence] in the presence and absence of NCZ. Results represent the mean ± SEM (n = 150).

5.8.2 NLSs in TP uses different importin pathway

As indicated in Chapter 4, TP encodes for multiple NLSs which act independently of one another. For proteins to be delivered into the nucleus there are several import pathways where NLSs are recognised by distinct IMPs. To test whether the multiple NLSs found on TP uses the same importin pathway to reach the nucleus, HeLa cells were analysed for the level of nuclear accumulation of TP in the absence and presence of the IMPα/β pathway inhibitor, ivermectin (Wagstaff *et al.*, 2012). The results are illustrated in Figure 5-9. Nuclear accumulation of YFP-TP, YFP-F1 (1-430) and YFP-F2 (1-390) was significantly reduced (*p<0.0001*) after ivermectin treatment. In the absence of ivermectin, the nuclear accumulation of YFP-TP and YFP-F1 were approximately 8 times higher (Fn/c value of 9 and 8.5 respectively) while YFP-F2 was approximately 4 times higher (Fn/c values of 4), than in the treated cells, indicating that nuclear import of TP is dependent on the importin α/β pathway. Interestingly, nuclear accumulation of YFP-F7 (541-671) was unaffected by ivermectin treatment which strongly implies that the C-terminal NLS on TP is recognised by different importin receptor(s).

The ALPHAScreen binding assay was performed using GST-TP, GST-F1 (1-430), GST-F2 (1-390), and GST-F7 (541-671) with either IMPα, IMPβ or IMPα/β, to investigate the binding partners that could be involved in nuclear delivery of TP. GST-IMPα was used as a positive control.

A

B
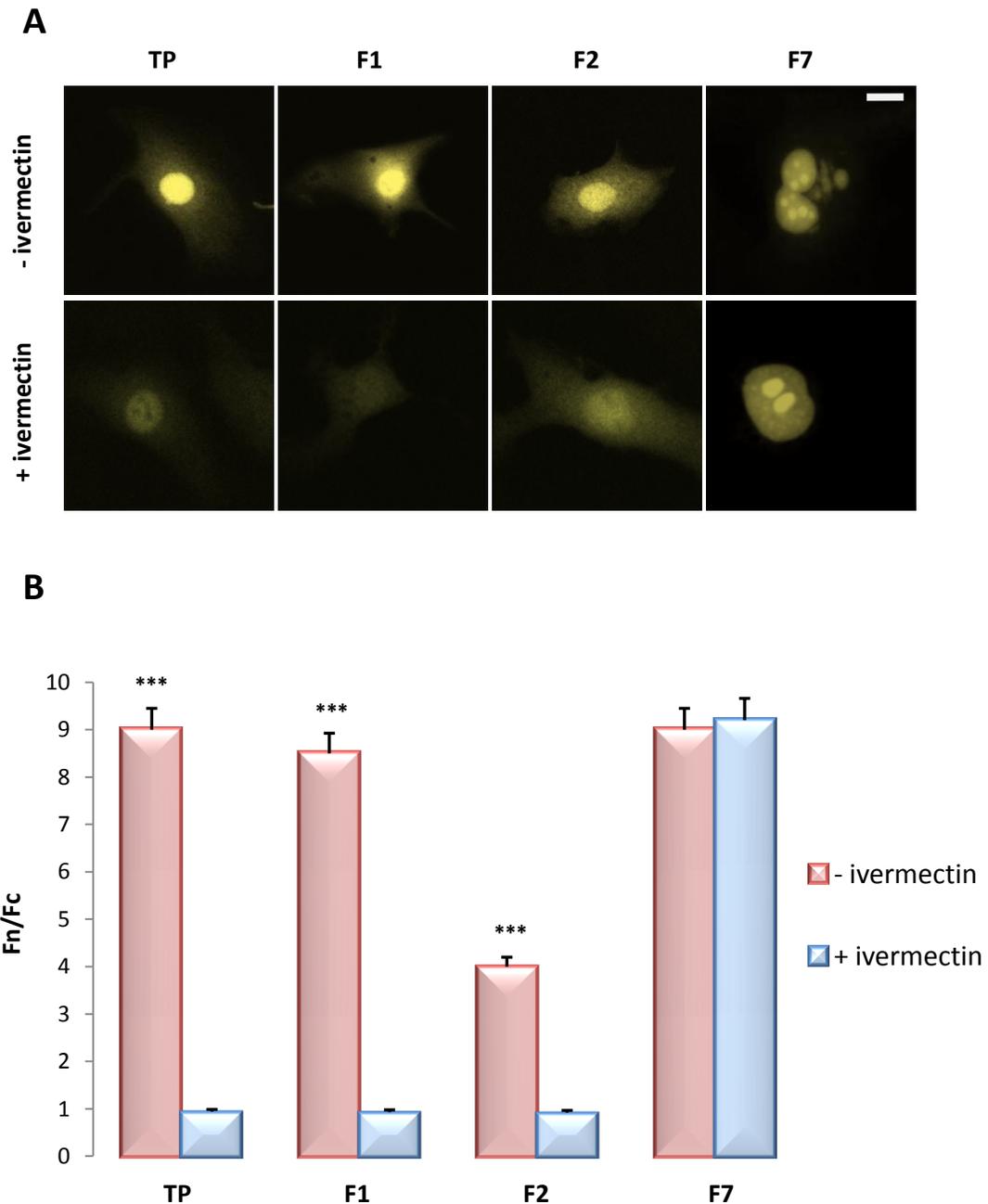
Figure 5-9: Inhibition of the Importin α/β pathway impairs TP nuclear accumulation dependent on the bipartite NLS (MRRRR370 and PVRRRRRRV390). (A) Confocal microscope images of HeLa cells treated for 1 hour with or without 25 µM ivermectin at 20x magnification with 10 µm scale bar. (B) Quantitative analysis of transfected HeLa cells images, for nuclear accumulation expressed as the Fn/c ratio. Results represent the mean ± SEM (n = 150), where significant differences between treated and untreated cells are denoted by * when $p < 0.05$, ** when $p < 0.01$ and *** when $p < 0.001$.

5.8.3 Interactions with IMPα/β complex

The ivermectin transfection experiments suggested that IMPα/β complex is required for nuclear accumulation dependent on elements of the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]; found on TP, F1 and F2) but not for nuclear accumulation dependent on the monopartite NLS (PGARPRGRF[671]; found on F7).

All four proteins tested were found to bind to IMPα/β with high affinity (Figure 5-10), with dissociation factor [K_d] between 1.5nM and 5.9nM. The binding affinity of IMPα/β for GST-TP was 3.6 nM (maximum binding [B_{max}] of 4950 arbitrary units). GST-F1 (1-430) is recognised by IMPα/β with high affinity (B_{max} of 3476; K_d of 1.5 nM) comparable to that of GST-IMPα control (B_{max} of 37938; K_d of 1.0 nM). GST-F2 (1-390) showed a high but slightly reduced binding affinity to IMPα/β (B_{max} of 6706; K_d of 5.9 nM), compared to GST-F1, indicating that the negatively rich domain (391-430) is required for optimal binding between the bipartite NLS and IMPα/β complex. Interestingly, GST-F7 (541-671) showed high binding affinity to IMPα/β (B_{max} of 4553; K_d of 2.2 nM), although its nuclear import was unaffected by the ivermectin experiment. The apparent number of binding sites was greater for the control than any of the TP fragments examined. The apparent number of binding sites, however, is dependent upon the number of molecules that are bound to the beads and there could be differences between the IMPα control and the GST proteins examined.

A

B
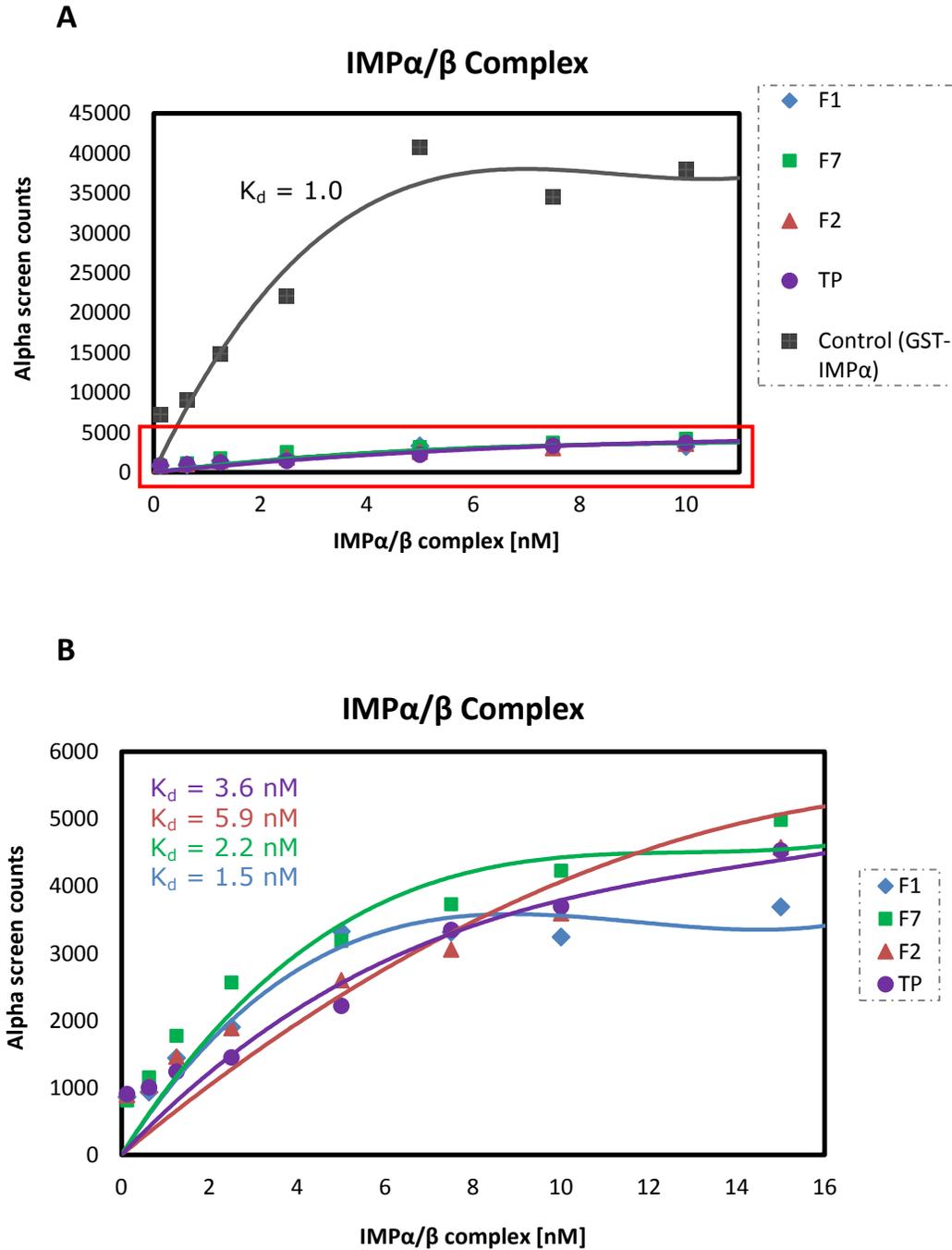
Figure 5-10: The presence of domain rich in negatively charged amino acids, adjacent to the bipartite sequence, is required for optimal IMPα/β binding. (A) GST fusion of wild type TP and truncated proteins, as indicated, were incubated with increasing concentrations of biotinylated IMPα/β complex and an ALPHAscreen assay performed. SigmaPlot software was used to fit Sigmoidal curves to determine B_{max} and K_d values. Each point represents the average of triplicate results of three separate experiments. (B) Enlarged of the area highlighted by red box in A.

Table 5-1: Summary of K_d and B_{max} values* obtained from ALPHAscreen assays using IMPα/β complex

GST-	K_d (nM)	B_{max}	B_{max} relative to TP 322-671 (%)
F1 1-430	1.5 ± 0.04	3,476 ± 36	70
F2 1-390	5.9 ± 0.47	6,706 ± 255	135
F7 541-671	2.2 ± 0.4	4,553 ± 214	92
TP 322-671	3.6 ± 0.03	4,950 ± 85	100
IMP-α control	1.0 ± 0.04	37,938 ± 564	-

Abbreviations: B_{max}, maximum binding; K_d, dissociation constant, *Values shown are the average of 3 individual experiments ± SEM as per Figure 5-10.

5.8.4 Interactions with IMPα

In a follow up experiment, interactions of TP and its truncated frangments (F1, F2 and F7) with only IMPα were determined. Both GST-F2 (B_{max} of 19397; K_d of 1.9 nM) and GST-F7 (B_{max} of 16734; K_d of 1.5 nM) bound IMPα with highest affinity. In comparison GST-F1 and GST-TP were recognised by IMPα with reduced affinity (B_{max} of 11352; K_d of 2.6 nM and B_{max} of 19802; K_d of 2.7 nM, respectively).
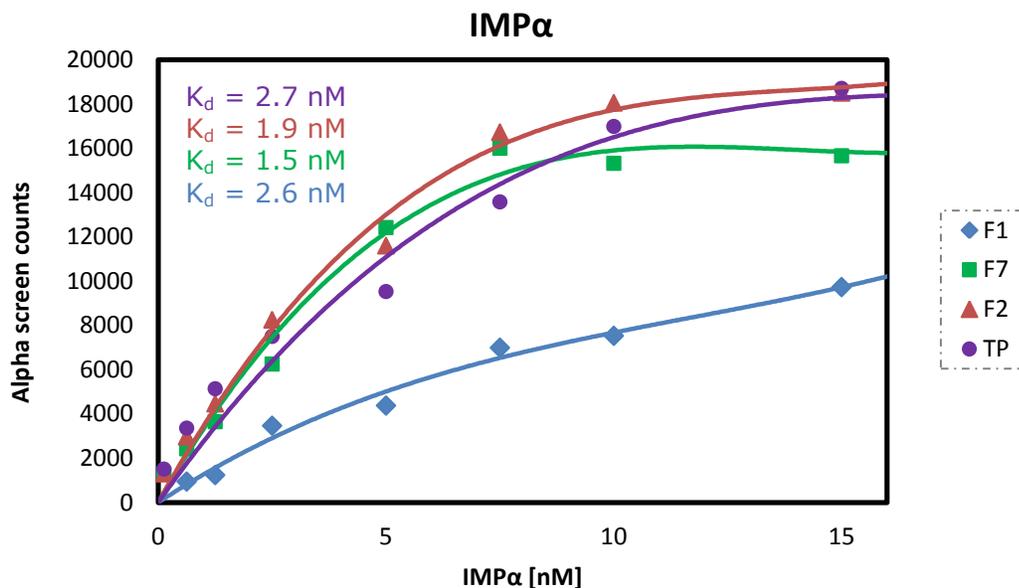
Figure 5-11: Both bipartite (MRRRR370 and PVRRRRRRV390) and monopartite (PGARPRGRF671) binds to IMPα. GST fusion of wild type TP and truncated proteins, as indicated, were incubated with increasing concentrations of biotinylated IMPα and an ALPHAscreen assay performed. SigmaPlot software was used to fit Sigmoidal curves to determine Bmax and Kd values. Each point represents the average of triplicate results of three separate experiments.

Table 5-2: Summary of K_d and B_{max} values* obtained from ALPHAscreen assays using IMPα

GST-	K_d (nM)	B_{max}	B_{max} relative to TP 322-671 (%)
F1 1-430	2.6 ± 0.2	11352 ± 283	57
F2 1-390	1.9 ± 0.06	19397 ± 166	98
F7 541-671	1.5 ± 0.07	16734 ± 173	85
TP 322-671	2.7 ± 0.09	19802 ± 1072	100

Abbreviations: B_{max}, maximum binding; K_d, dissociation constant, *Values shown are the average of 3 individual experiments ± SEM as per Figure 5-11.

5.8.5 Interactions with IMPβ

All of the proteins examined were found to bind to IMPβ with high affinity. The calculated data has been summarised in Table 5-3.
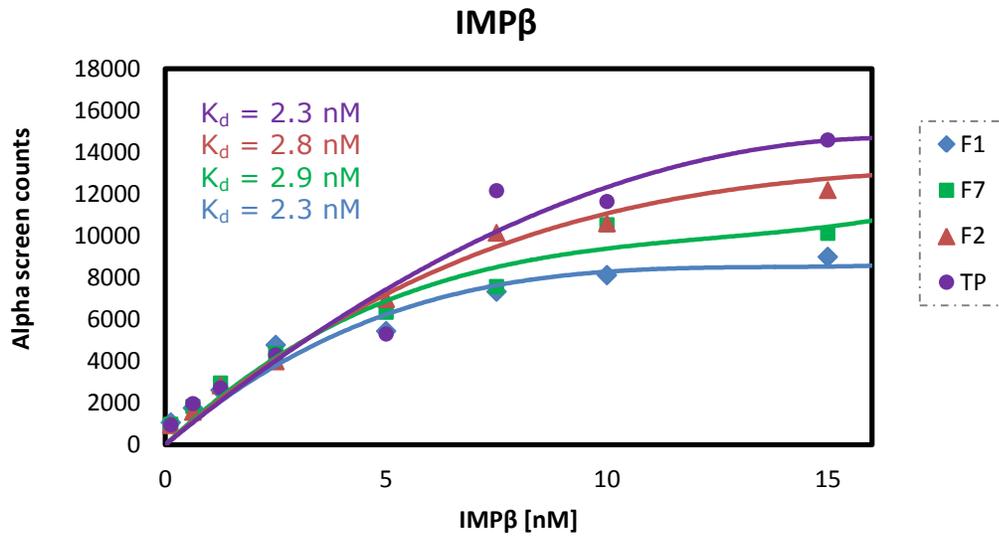
Figure 5-12: Both bipartite (MRRRR370 and PVRRRRRRV390) and monopartite (PGARPRGRF671) showed no differences in binding affinity to IMPβ. GST fusion of wild type TP and truncated proteins, as indicated, were incubated with increasing concentrations of biotinylated IMPβ and an ALPHAscreen assay performed. SigmaPlot software was used to fit Sigmoidal curves to determine Bmax and Kd values. Each point represents the average of triplicate results of three separate experiments.

Table 5-3: Summary of K_d and B_{max} values* obtained from ALPHAscreen assays using IMPβ

GST-	K_d (nM)	B_{max}	B_{max} relative to TP 322-671 (%)
F1 1-430	2.3 ± 0.03	8919 ± 15	62
F2 1-390	2.8 ± 0.12	14060 ± 78	97
F7 541-671	2.9 ± 0.1	11910 ± 18	82
TP 322-671	2.3 ± 0.07	14490 ± 701	100

Abbreviations: B_{max}, maximum binding; K_d, dissociation constant, *Values shown are the average of 3 individual experiments ± SEM as per Figure 5-12.

5.9 Discussion

TP has been reported to localise in the nucleus, however the nuclear import pathway of TP and in particular which IMPs may be involved has not been examined. This chapter investigates how Adv TP achieves its nuclear transport and determined the binding affinity of Adv TP to the three members of IMP superfamily (IMPα, IMPβ and IMPα/β).

5.9.1 Cytoskeleton-facilitated transport

There have been several studies suggesting a link between Adv nuclear import and the MTs network, particularly during early infection (Bremner *et al.*, 2009b; Mabit *et al.*, 2002). To investigate the role of MTs in protein trafficking, a number of studies have utilised MT perturbing drugs, such as the MT depolymerising agent nocodazole (NCZ) and/or MT stabilising drug taxol (Giannakakou *et al.*, 2000; Mabit *et al.*, 2002; Malki *et al.*, 2005). The NCZ experiment was conducted to identify whether nuclear trafficking of TP is influenced by MTs as TP is produced during early infection of the Adv life cycle. In the presence of NCZ, MTs are depolymerised disrupting the MT-facilitated transport.

NCZ treatment had no effect on the levels of nuclear accumulation of control protein, GFP-Tg, which does not use MTs for nuclear import (Roth *et al.*, 2007). NCZ treatment also had no effect on the levels of nuclear accumulation of TP indicating that nuclear import of TP does not rely on functional MTs.

5.9.2 Nucleocytoplasmic transport

There are several nuclear import pathways that allow NLS-carrying proteins to be recognised by distinct IMP receptors. Most commonly, basic NLSs are recognised by either the IMPα/β heterodimer or IMPβ alone. Ivermectin is an inhibitor of IMPα/β pathway, with no effect on a range of other nuclear import pathways including that mediated by IMP β1 alone (Wagstaff *et al.*, 2012)

In the presence of ivermectin, the nuclear accumulation of TP, F1 and F2 were impaired indicating that IMPα/β pathway is required for their nuclear trafficking. However ivermectin had no effect on the nuclear import of F7. This shows that F7 was able to utilise an alternative pathway to achieve its nuclear delivery. The results suggested that the Adv pTP has evolved to take advantage of several import pathways using the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]; found on TP, F1 and F2) and monopartite NLS (PGARPRGRF[671], found on F7). The ALPHAscreen test was conducted to quantify the binding affinities between each protein and the three members of the IMP superfamily: IMPα, IMPβ and IMPα/β. The results obtained shows that each protein is recognised by IMPα/β, IMPα and IMPβ, all with high affinity interactions, which further support that Adv TP employs several nuclear trafficking routes.

5.9.3 Binding affinity to IMP

Facilitated nuclear import is mediated by members of IMP superfamily, which recognise NLSs within proteins destined for the nucleus. The binding affinity results are tabulated in Table 5-1, Table 5-2 and Table 5-3 for IMPα/β, IMPα and IMPβ respectively.

From the ivermectin experiment, it was shown that TP reaches the nucleus through IMPα/β mediated pathway and therefore would have a high binding affinity with IMPα which is a receptor that recognises NLSs within the IMPα/β heterodimer. The binding affinity of IMPα to GST-TP was K_d of 2.7 nM. Furthermore, GST-TP binds to IMPα/β with K_d of 3.6 nM, higher than that of the T-ag (K_d = 5.8 ± 0.7 nM) which is known to employ the IMPα/β mediated route (Fontes *et al.*, 2003; Wagstaff *et al.*, 2006). This implies that IMPα/β is TP's nuclear import receptor. It is interesting to note that there is no significant difference in the binding affinity of GST-TP to IMPα or IMPβ (K_d = 2.3 nM), with GST-TP binding to IMPβ at an affinity comparable to that of IMPβ-recognised chromatin remodelling factor SRY (K_d = 1.8 ± 0.3 nM) (Lam *et al.*, 1999; Wagstaff *et al.*, 2006). However, the binding was significantly reduced (*p<0.003*) from B_{max} of approximately 19800 for IMPα to B_{max} of approximately 14500 for IMPβ. This suggests that the folding structure of TP influences the recognition of NLS by IMPs, in this case favouring for the IMPα receptor. This explains why TP was unable to utilise an alternative pathway when the primary IMPα/β route becomes inaccessible, as specificity for IMPβ may further reduced *in vivo,* preventing TP from binding to the IMPβ receptor. By studying the folding structure of TP further, as well as the TP NLS-IMP binding structure, it may be possible to alter the TP folding to allow it to access IMPβ receptor with higher specificity. This may allow TP to adapt to alternate nuclear trafficking pathway when its primary IMPα/β mediated pathway is impaired. Some of the possible alternative pathways could be the IMPβ mediated pathway or the transportin facilitated pathway as transportin is an IMPβ-related homologue (Hindley *et al.*, 2007b; Saphire *et al.*, 2000; Trotman *et al.*, 2001). Transportin has already been shown to be a primary import receptor for Adv protein VII and Adv DNA and is required for subcellular localisation of Adv protein V (Hindley *et al.*, 2007b).

The binding affinity of IMPα for GST-F1 (1-430) was approximately 3 nM comparable to that of the full length TP (K_d = 2.7 nM). This suggests that the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]) is recognised by IMPα. Interestingly, the binding affinity of IMPα for the bipartite NLS increased in the absence of a domain rich in negatively charged amino acids (GST-F2 (1-390), K_d = 1.9 nM). The domain also influences the binding of IMPα in terms of reducing the B_{max}. In GST-F2, when a domain rich in negatively charged amino acids is absent, the B_{max} is comparable to that of GST-TP (B_{max} of 19397 and 19802, respectively)

however it was reduced significantly (*p<0.005*) in GST-F1 (B_{max} of 11352). The additional amino acids (391-430) referred to as negatively rich domain, exert effects on the NLS, modulating recognition by IMPs. The implication is that the surrounding residues of an NLS are of critical importance to the NLS-IMP interaction and influence the specificity of binding.

GST-F1 binds more strongly to IMPα/β (K_d = 1.5 nM) than does GST-F2 (K_d = 5.9 nM), further stressing the importance of surrounding residues of an NLS as it may mask or enhance NLS-IMP interaction. In this case the negatively rich domain reduces the binding affinity between NLS and IMPα/β, while in the case of IMPα binding the negatively rich domain enhanced affinity. However, the binding specificity of the NLS to IMPα/β was doubled in the absence of the negatively rich domain, similar to the IMPα findings. The reduction in binding specificity between NLS and its IMP partner caused by the additional amino acids (391-430), referred to as negatively rich domain, is most likely due to its charge. NLS is typically positively charged, when a stretch of opposite charge is present in close proximity, it may attract the NLS and as a result reduce NLS specificity to its primary partner.

To investigate the IMP partner for the monopartite NLS (PGARPRGRF[671]), GST-F7 (541-671) was tested. GST-F7 shows high binding affinity to all the IMPs tested with highest affinity for IMPα (K_d = 1.5 nM). Given that the nuclear accumulation of F7 was not effected when the IMPα/β pathway was blocked, the data implies that the monopartite NLS can utilise an alternative pathway when exposed but this pathway may be overridden by the bipartite NLS in the full length TP. This would also explain the high binding affinity of GST-F7 to all the IMPs. A study on the folding structure of TP would reveal whether the monopartite NLS, which is present at the C-terminus of the protein, is masked in nature, further supporting that the primary nuclear trafficking route is determined by the bipartite NLS.

5.10 **Conclusion**

The nuclear accumulation of TP was not dependent on the functional microtubules. The nuclear accumulation of TP fragments coded for the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]) is dependent on the IMPα/β mediated pathway, whereas the monopartite NLS (PGARPRGRF[671]) employs alternative nuclear trafficking pathway. The pathway in which PGARPRGRF[671] utilises is most likely involved IMPβ and/or its homologues. The negatively rich domain may mask or enhance NLS-IMP interaction influencing the binding affinity, however it clearly reduce the binding specificity between NLS and its IMP binding partner.

CHAPTER 6

General Discussion

CHAPTER 6   General Discussion

6.1 **Introduction**

The success of non-viral gene delivery systems is currently hindered by generally low levels of transgene expression, particularly when compared to the viral systems. It has been identified that a major contributory factor to this limitation is due to inefficient transfer of DNA from the cytoplasm to the nucleus (Brunner *et al.*, 2002; Capecchi, 1980; Li *et al.*, 2000; Zabner *et al.*, 1995). One way of enhancing non-viral gene delivery system is to develop vector systems that mimic the highly efficient processes of viral nuclear delivery.

The passive arrival of a DNA plasmid to the nucleus is a rare event due to barrier effect of lipid membranes and diffusional barriers within the cell, therefore a specific mechanism is required for the therapeutic DNA to be delivered efficiently to the nucleus (Johnson-Saliba *et al.*, 2001). The nuclear membrane is a barrier that prevents the uptake of most macromolecules with a size of greater than 70 kDa into the nucleus, unless they are able to interact with the nuclear pore active transport system (Busch, 1974; Moroianu, 1999; Strambio-De-Castillia *et al.*, 2010). Many viruses have evolved mechanisms to overcome the intracellular barriers of eukaryotic cells.

In order to mimic viral delivery systems, it is important to first characterise their delivery system and to identify viral proteins involved. The primary objective of the project was to improve the understanding of how an adenovirus achieves its nuclear delivery, with focus on the role of Adv terminal protein. The Adv TP was shown to include more than one NLS, both the monopartite NLS (PGARPRGRF[671]) and the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]). It was established that the nuclear trafficking of TP protein (as a GFP or YFP fusion) is not dependent on the functional microtubules, and that both of the NLSs are functional but probably employ different pathways in reaching the nucleus. The bipartite NLS utilises the IMPα/β mediated pathway while the monopartite NLS is also able to utilise an alternative pathway involving either IMPβ and/or its homologues.

Improved understanding of the mechanism involved in Adv nuclear delivery, may allow researchers to assemble particles with the precision that is achieved by the Adv, to improve the efficiency of non-viral gene delivery systems. Such particles could potentially be assembled synthetically, using the main protein components of Adv, to allow active nuclear transport to be utilised. This approach could prove to be a critical pathway for the success of non-viral gene delivery.

6.2 Adenoviral Vectors and Gene Therapy

The ideal gene therapy vector system would be administered to patients by a non-invasive route, would target only the desired cells within the target tissue, and would express therapeutic gene products with the desired onset and duration of expression. It is therefore unlikely that a single vector system will be optimal for all potential gene therapy applications. Adenoviruses have been among the most commonly used vectors for gene therapy, because they offer the possibility of controlling gene expression in a transient manner. Their potential has not been realised in practice, mainly due to immunogenicity and a severe adverse reaction that occurred in a clinical study. The development of Adv technology is described below.

The adenoviruses have several features that make them suitable for therapeutic use. First, they are ubiquitous – 57 unique serotypes have been reported in human (Buckwalter *et al.*, 2012; Fields *et al.*, 2007). Most adults have been exposed to the Adv serotypes that are commonly used in gene therapy (serotype 2 and 5) without adverse effects, leading to measureable titres of anti-Adv antibodies, which limits the success of multiple doses (Bauer *et al.*, 2005; Vorburger *et al.*, 2002; Zhang *et al.*, 2013). Second, Adv infection is not specific to cell types or proliferating cells, thus, adenoviral vectors can be used to target a broad range of human cells. Furthermore, adenoviral vectors tend to yield higher levels of gene delivery in comparison to other currently available vectors (Hendrie *et al.*, 2005; Mah *et al.*, 2002; Vorburger *et al.*, 2002). Third, the low pathogenicity of Adv in humans – causing only mild symptoms associated with the upper respiratory tract (Fields *et al.*, 2007; Vorburger *et al.*, 2002). Forth, due to the large genome of Adv, adenoviral vectors can accommodate up to 7.5 kb of DNA segment or more when genomic regions have been deleted (Benihoud

et al., 1999; Fields *et al.*, 2007; Giacca *et al.*, 2012; Tatsis *et al.*, 2004; Vorburger *et al.*, 2002; Wilson, 1996). Fifth, a low rate of viral genome rearrangement and, thus, inserted therapeutic DNA expression cassettes are maintained without change through the process of viral replication (Fields *et al.*, 2007; Vorburger *et al.*, 2002). Finally, recombinant DNA techniques can be used to manipulate adenoviral vectors relatively easily (Anderson *et al.*, 2000; Chartier *et al.*, 1996; Choi *et al.*, 2012; Vorburger *et al.*, 2002).

The therapeutic DNA can be either inserted or substituted in three regions of the Adv genome to generate adenoviral vector; (1) a region in E1, (2) a region in E3 and (3) a short region between E4 and the end of the genome (Gabitzsch *et al.*, 2010; Vorburger *et al.*, 2002). The genes E1 to E4 are expressed during early phase of Adv replication and are responsible for initiating the Adv genome replication process (Fields *et al.*, 2007; Giacca *et al.*, 2012; Nemerow *et al.*, 2012). Once the replication has started, the major late promoter is responsible for expression of the major late transcription unit which encodes for most of the Adv proteins (Fields *et al.*, 2007; Giacca *et al.*, 2012; Parks *et al.*, 1997). The *cis* and *trans* elements are then responsible for the packaging of Adv genome (Fields *et al.*, 2007; Gräble *et al.*, 1992). The *cis* genes must be carried by the Adv itself, however, the *trans* gene may be inserted or replaced by the foreign DNA (Fields *et al.*, 2007; Gräble *et al.*, 1992; Xing *et al.*, 2003).

The major drawback of the first-generation of Adv gene therapy was caused by the cellular and humoral immune response (Hendrie *et al.*, 2005; Somia *et al.*, 2000; Vorburger *et al.*, 2002; Wilson, 1996; Yang *et al.*, 1995). In the first-generation Adv vectors, the E1 region was removed to prohibit Adv replication and replaced by the therapeutic DNA (Hendrie *et al.*, 2005; Vorburger *et al.*, 2002; Zoltick *et al.*, 2001). Although the removal of E1 gene resulted in no Adv replication, there was still a low level of transcription of the remaining Adv genes generating immune responses from the host (Ghosh *et al.*, 2005; Giacca *et al.*, 2012; Hendrie *et al.*, 2005; Vorburger *et al.*, 2002). This resulted in a loss of the therapeutic gene expression and inflammation, caused by the cell-mediated apoptosis of the transduced cells (Wilson, 1996). To overcome these immunological problems, the second-generation Adv vectors have deletions of various E1, E2 and E4 genes as these genes have been shown to encode for proteins that trigger the host immune response (Engelhardt *et*

al., 1994; Fields *et al.*, 2007; Gabitzsch *et al.*, 2010; Giacca *et al.*, 2012; Schiedner *et al.*, 1998; Wang *et al.*, 1996; Wen *et al.*, 2000). Despite elimination of these regions, these vectors still cause inflammation of both targeted and surrounding cells as they continue to trigger both adaptive and innate immune response (Giacca *et al.*, 2012; Kaufmann *et al.*, 2012; McConnell *et al.*, 2004). The pre-existing humoral response also causes clearance of Adv vectors resulted in undetectable level of therapeutic proteins within few weeks (McConnell *et al.*, 2004). Furthermore, the vectors are unable to facilitate efficient gene transfer upon second administrations due to the boost in antibody titres (Benihoud *et al.*, 1999; Sharma *et al.*, 2009; Wilson, 1996; Wu *et al.*, 2009; Yang *et al.*, 1995). Therefore, until the problem of immunogenicity is solved, Adv vectors are better suited to conditions requiring short-term activation of a gene, for example to induce apoptotic stimuli. A third generation of Adv vectors are known as gutless, helper-dependent or high capacity vectors (Alba *et al.*, 2005; Brunetti-Pierri *et al.*, 2008; Giacca *et al.*, 2012; Sato *et al.*, 2001). These vectors contain only the genomic regions for viral DNA replication and packaging (ITR and ψ respectively) with transgene and/or non-coding "stuffer" DNA replacing any unused spaces (Alba *et al.*, 2005; Brunetti-Pierri *et al.*, 2008; Giacca *et al.*, 2012; Sato *et al.*, 2001). Stuffer DNA is required to build the vector to a size suitable for packaging (Ehrhardt *et al.*, 2002; Hardy *et al.*, 1997). The sequence of stuffer DNA has been shown to influence the role in production and in transient expression level (Ehrhardt *et al.*, 2002; Parks *et al.*, 1999). Helper-dependent Adv (HDAdv) vectors display a major reduction in toxicity and immunogenicity, and are able to deliver large therapeutic DNA of up to approximately 37kb (Rosewell *et al.*, 2011). The usage of third generation Adv vectors have been hindered due to low production yields of clinical-graded vectors until recently when a high-throughput system has been developed (Choi *et al.*, 2012; Józkowicz *et al.*, 2005). Although HDAdv vectors hold a great promise in clinical research, there are complications in selecting appropriate stuffer DNA for optimal transgene expression.

Despite differences in designs between generations of Adv vectors, they all are able to produce high level of transgene expression. We believe that an effective non-viral delivery vector should resemble Adv to mimic its trafficking mechanism for optimal transgene expression. It is, therefore, critical to understand how the Adv delivery system works in

order to either 1) incorporate only the vital parts of the Adv genome into the vector, or 2) engineer a non-viral system with substantially increased delivery efficiency. Our study has characterised the ability of Adv TP to serve as a transport adaptor. We have identified two independent regions of Adv with NLS activity: the monopartite NLS (PGARPRGRF[671]) and the bipartite NLS (MRRRR[370] and PVRRRRRV[390]) (Chapter 4). We showed that EmGFP and YFP fusions containing either full length TP or of each individual NLS region of TP are able to efficiently translocate to the nucleus of infected cells (Chapter 4). Although only full length pTP (aa1-671), F1 fragment (aa 1-430) and F7 fragment (aa541-671) were able to produce the same level of nuclear delivery as that of the native TP, other fragments (F2 (aa1-390), F3 (aa1-380), F5 (aa374-671), F6 (aa391-671) and F9 (aa371-480)) containing either of the NLS's were able to reach the nucleus more effectively than the control (Chapter 4). Furthermore, the PGARPRGRF[671] sequence was shown to facilitate delivery to the nucleolus when not masked by the flanking regions as shown by TP and F7 (Chapter 4). NLSs have been shown to enhance delivery of several vector systems (Jeyarajan *et al.*, 2010; Matschke *et al.*, 2012; Zanta *et al.*, 1999). An ability of TP NLS to delivery therapeutic DNA should be explored further as it may be used to enhance existing non-viral delivery system. As TP are found coupled to viral DNA, it may be worth exploring whether the same coupling could be reproduced between TP and therapeutic DNA. The actual role of TP in Adv nuclear delivery also deserves a deeper investigation, particularly with focus on the nucleolar property.

However, there are many proteins involved in the Adv trafficking and the findings from this project is only a stepping stone towards generating a new vector system. Hexon is thought to be involved in microtubule trafficking and protein VII in endosomal escape. It is clear that more research is needed to fully understand their functions and to identify the minimal encoding regions required for production of functional proteins. By incorporating only the critical encoding sequences of vital proteins into non-viral delivery system, the vectors will efficiently transfer therapeutic DNA without immunological problems of the virus.

6.3 Applications of NLS in non-viral gene therapy

The delivery of therapeutic genes into the cell nucleus through the use of non-viral vectors is extremely inefficient due to many limitations in overcoming intracellular barriers, such as the crowded cytoplasm and the NE, with only 0.1% of naked DNA or 1% of polyplex DNA reaching the nucleus following microinjection (Capecchi, 1980; Pollard *et al.*, 1998; Zabner *et al.*, 1995). NLS has been incorporated into multiple non-viral systems to enhance transgene expression (Wagstaff *et al.*, 2007). The coupling of NLS to the DNA can be achieved through an electrostatic charge between negatively charged DNA to relative positively charged NLS or through a covalently attachment of NLS to DNA directly or via components of the vector (Aa *et al.*, 2006; Chowdhury, 2009; Guo *et al.*, 2011; Wagstaff *et al.*, 2007)

NLSs are short peptide sequences responsible for facilitating the nuclear import of large proteins. Conjugation of NLS peptide(s) to DNA could thus increase nuclear entry of DNA and thereby improve the transfection efficiency of non-viral gene delivery systems. In 1999, it was reported that conjugation of a single NLS peptide to linear DNA enhanced the transfection in non-viral gene delivery (Zanta *et al.*, 1999). In the study, 300-fold increase in transfection was observed when SV40 derived NLS peptide was covalently attached to the target DNA (Zanta *et al.*, 1999). Recently, Matschke *et al*. reported for the first time the use of bipartite NLS in non-viral delivery system when Ku70-NLS was shown to increase plasmid DNA uptake (Matschke *et al.*, 2012). However, several studies have found that conjugated of a single NLS peptide to DNA is insufficient to increase the nuclear delivery (Jeyarajan *et al.*, 2010; Neves *et al.*, 1999; Roulon *et al.*, 2002; van der Aa *et al.*, 2005). Ludtke and colleagues identified that two or more NLSs are required for enhanced DNA nuclear delivery and protein expression (Ludtke *et al.*, 1999). There are also studies in which NLS peptide(s) coupled to DNA did not result in significant increase in transfection. In one of the studies, the attachment of SV40 derived NLS peptide to a specific region in the plasmid DNA resulted in only 5- to 8- fold increased in transgene expression, which is very low in comparison to 300- fold obtained by Zanta *et al.* (Brandén *et al.*, 1999; Zanta *et al.*, 1999). Ciolina *et al.* found that NLS peptides helped promote proteins binding to importin but observed no transgene expression in the nucleus after microinjection (Ciolina *et al.*, 1998).

Nagasaki *et al.* required total of 5 NLS peptides to be coupled to plasmid DNA before observing a 4-fold increased in nuclear delivery, with no observation of increased protein expression when NLS peptides were directly coupled to the DNA (Nagasaki *et al.*, 2003). Furthermore, Roulon *et al.* were unable to identify an increase in transgene expression when NLS peptides were coupled to DNA (Roulon *et al.*, 2002). These results indicate that coupling of currently known NLS peptide(s) to either linear or plasmid DNA does not necessarily result in an enhanced transfection efficiency. Possible explanations could be that the size of DNA is too large and/or a strong interaction of the positively charged NLS peptides with the negatively charged DNA masked the activity of NLS. The number of NLS peptides required to obtain sufficient nuclear delivery enhancement is also debatable, with studies supporting both single NLS and multiple NLS peptides as discussed. Although further investigation is necessary to identify the number of NLS peptides required for optimal delivery of large DNA, NLSs may be used to apply to gene therapy strategies using smaller DNA molecules. For larger DNA, non-viral system may require mechanisms to facilitate early translocation route before NLSs deliver DNA through the NPC. It is also interesting to note that most of the studies on NLS-mediated delivery system have been on the use of monopartite NLS with only one study on the use of bipartite NLS (Matschke *et al.*, 2012; Wagstaff *et al.*, 2007). Researches on the use of bipartite NLS are worth conducting.

Our study has characterised the ability of Adv TP to serve as a transport adaptor. The Fn/Fc of EmGFP and YFP fusion proteins coding for either of the identified NLS was between 4- to 8- folds higher in comparison to the control (Chapter 4). However, examination on the ability of these NLSs in increasing transgene expression when coupled to either plasmid or linear DNA was not conducted and further research is required with regard to construction of advanced DNA delivery systems. Given that covalently coupled NLSs are not usually capable of increasing nuclear delivery of therapeutic DNA, it will be interesting to determine whether TP has a role to play in delivery of the Adv genome, in particular because TP is found covalently attached to the 5' ends of the viral DNA. It will be interesting to find out if TP or fragments of TP may be able to deliver DNA in a similar way to the Adv, to improve delivery efficiency of non-viral systems. When the TP fragments contain both bipartite NLS (MRRRR[370] and PVRRRRRRV[390]) and the monopartite NLS

(PGARPRGRF[671]), the nuclear delivery determined as Fn/c was at least 8-fold higher than the control (OVA-EmGFP/YFP) which did not contains an NLS (Figure 4-10, Figure 4-11, Figure 4-12 and Figure 4-13 of Chapter 4). Independently, both the bipartite and N-terminal NLSs were able to retain the 8-fold increased of the nuclear expression as shown by fragment 1 (contains bipartite NLS) and fragment 7 (contains the N-terminal NLS). It was observed that the highly negative domain, adjacent to the bipartite NLS, is required in order for the bipartite NLS to retain its effectiveness (Chapter 4). In its absence, the bipartite NLS was only able to increase the Fn/c by 3-fold as shown by fragment 2. Since a single NLS cannot always increase transgene expression, having multiple NLSs that are known to work cooperatively may be advantageous. Although it is possible to use several NLSs from the same source, it is not known how many NLSs would be required for optimal delivery. NLS peptides might be expected to interfere with the secondary structure of DNA by interacting with negatively charged phosphate backbone of DNA resulting in reduced interaction with transport receptor(s). It is known that the positive charges of the lysine and arginine residues of many NLSs are critical for interaction with the transport protein and neutralisation of these charges by electrostatic interaction with DNA reduces cargo recognition. Our identified NLSs exist together in nature, therefore when engineered into a vector its optimal delivery function may be retained and the influencing of DNA structure will be at the minimal. The highly negative domain which is a critical part to the function of bipartite NLS may minimise the positive charges of the NLS reducing the electrostatic force between NLSs and DNA. Condensation and neutralisation of the bulk of the DNA is also likely to be critical. In the Adv, DNA condensation is achieved by complexation with three cationic proteins; protein VII, protein V and mu (Wodrich *et al.*, 2006).

We also found specific binding between recombinant GST fusions of either full length TP or of each fragment containing NLSs to multiple IMPs; IMPα/β heterodimer, IMPα alone and IMPβ alone (Chapter 5). The ability of TP to use several IMP receptors could reflect a similar adaptation of Adv genome. Our study shows that the bipartite NLS (MRRRR[370] and PVRRRRRRV[390]) employed the IMPα/β mediated pathway, the same route employed by the T-ag (Fontes *et al.*, 2000; Fontes *et al.*, 2003) (Chapter 5). Interestingly, the monopartite PGARPRGRF[671] NLS are able to utilise both the IMPα/β mediated pathway and an alternative pathway (Chapter 5). Although the specific alternating route has yet been

identified, it is likely to involve IMPβ and/or its homologue(s) (Chapter 5). When both NLSs are presented the primary nuclear trafficking route of the TP is determined by the bipartite NLS as the nuclear accumulation of TP is reduced significantly when the IMPα/β mediated pathway is blocked (Chapter 5). One explanation could be that the monopartite NLS is masked in the native TP and hence unable to interact with transport receptors to deliver TP using the alternative pathway. This is supported by the finding that when the monopartite NLS is exposed in F7 (aa541-671), the nuclear accumulation was not hindered by the inhibition of IMPα/β mediated pathway (Chapter 5). The ability for this monopartite NLS to employ different trafficking route is beneficial as it would increase the versatility of the recombinant vector coupling with such sequence as currently most of the known NLSs employed only specific import pathway. For further understanding on the interactions between NLS and IMP receptors, a structural study of TP should be conducted. The folding structure of TP would reveal the location of each NLS and their binding to the partner IMP.

6.4 The role of TP in Adv life cycle

pTP is found covalently coupled to the replicating DNA and is cleaved to TP by an Adv protease during a late step of virus DNA packaging. This cleavage results in the covalently attached TP (C-terminal half of pTP) and viral DNA being packaged into virions (Fredman *et al.*, 1993). The attachment between the Adv DNA and TP is through a phosphodiester bond between the β-hydroxyl group of a 232 serine residue of TP and the 5'-terminal deoxycytidine residue of the Adv DNA (Bayliss *et al.*, 2000; Challberg *et al.*, 1981b; Desiderio *et al.*, 1981; Ribbeck *et al.*, 2001; Smart *et al.*, 1982). pTP functions as a primer for DNA synthesis by forming a complex with dCMP facilitated by Adv DNA polymerase and (Lichy *et al.*, 1982; Tamanoi *et al.*, 1982). Although TP enters the nucleus with the viral DNA, the processing of pTP to TP is not required for the Adv DNA replication (Challberg *et al.*, 1981b). Its presence, however, increases the efficiency of the Adv replication process by stabilising origin binding of the pTP-Adv polymerase complex and induces changes in the origin structure (Angeletti *et al.*, 1998; Pronk *et al.*, 1993). TP is also involved in attachment of the DNA to the nuclear matrix which is significant for efficient replication and transcription of Adv DNA (Fredman *et al.*, 1993).

In Adv infected cells, pTP exists in the form of a heterodimer tightly bound to Adv polymerase (pol) ((Lichy *et al.*, 1982; Stillman, 1989). The genome of Adv is maintained in a highly condensed form by small basic proteins (protein VII, protein V and mu), however after infection, the virion is uncoated allowing viral DNA to be released into the cell nucleus (Anderson *et al.*, 1989; Fields *et al.*, 2007; Hay, 1996; Liu *et al.*, 2003). DNA replication is initiated at either terminus of the covalent attachment between α-phosphoryl group of dCMP and β-OH group of a serine residue of the pTP (Chowrira *et al.*, 1991; Hay, 1996; Liu *et al.*, 2003). This reaction is catalysed by Adv pol and a cellular CCAAT-binding protein, nuclear factor I (NFI) (Chowrira *et al.*, 1991). A cellular type I DNA *topoisomerase* (NFIII) is responsible for elongation of newly synthesised DNA strand and is catalysed by Adv pol (Chowrira *et al.*, 1991; Hay, 1996; Liu *et al.*, 2003). Assembly of Adv particles occurs in the nucleus with DNA entering the particles after immature capsids are formed followed by maturation process of the capsids prior to virions escape through cell lysis (Ostapchuk *et al.*, 2011; Ostapchuk *et al.*, 2005).

Although pTP is cleaved to form TP, their functions in Adv life cycle may differ as discussed. This correlates with the data obtained in the PhD study which shows that pTP and TP have different tropisms within the nucleus. pTP does not only localise to the nucleus but it also enters the nucleolus, however, this property is lost in TP. A similar result was described with Adv protein VII when the pre-protein VII localised to the nucleus but protein VII could not (Lee *et al.*, 2003b). The difference in tropism between the precursor and mature protein may be as a result of the NuLS being masked by differences in the folding structure of mature protein compared to the precursor. Unlike NLS, there is currently no known common template for NuLS. The NuLS can range from a few amino acids to over a hundred. However, the truncated fragment 7 also shows nucleolar homing property suggesting that NuLS is likely be found between aa541-671 (Chapter 4). It is interesting, however, that no nucleolus delivery was shown by F5 (aa374-671), F6 (aa391-671) and F8 (aa391-654) although they contain the proposed regions (Chapter 4). One explanation for the result of F8, could be that NuLS is encoded between aa654-671. The different results between F5, F6 and F7 supported the statement that folding structure of protein influences the location of NuLS (Chapter 4). The folding structure of F5 and F6 may result in NuLS

being masked due to electrostatic force between the NuLS and the negatively rich domain while in its absence in the case of F7 NuLS is exposed and recognised by transport receptor(s) (Chapter 4). This would also explain the absence of nucleolar activity in TP as it also encode for the highly negative regions. Nucleoli are the centres of ribosome biogenesis where rRNA is synthesised and processed (Raška *et al.*, 2004). Adv infection is shown to inhibit the formation of both 18S and 28S of rRNA (Castiglia *et al.*, 1983). During late infection, Adv also disrupt nucleoli (Puvion-Dutilleul *et al.*, 1993; Rodrigues *et al.*, 1996). It is unclear why pTP would need to enter nucleolus but one explanation could be that nucleolus contain resources that pTP needs to be coupled to newly replicated DNA. Alternatively, pTP may simply disrupt the ribosome biogenesis to promote Adv replication. This is plausible as Adv protein V, which is also found in a core of Adv, is known to enter the nucleolus to disrupt rRNA biogenesis to releases resources for production of Adv mRNA (Matthews, 2001). It does this by redistributing nucleolin, which is known to interfere with Adv replication, to cytoplasm (Matthews, 2001; Yang *et al.*, 1994). Similar actions are also seen in other viruses, suggesting that the virus may exploit nucleolar function to alter host cell transcription, translation and disruption of the host cell cycle to facilitate viral replication. Coronavirus nucleoproteins (N proteins) have been revealed to interact with two nucleolar components, fabillarin and nucleolin (Chen *et al.*, 2002). NS1 protein of avian influenza virus was also found to interact with host protein that acts as a chaperone between cytoplasm and nucleolus (Zhu *et al.*, 2013). Retroviral Gag protein also shows nucleolar activity (Beyer *et al.*, 2013). In each case, it is speculated that proteins interact with nucleolar components to disrupt their normal functions to promote viral production. Further study on the nucleolar property of pTP may provide more insight into its role in Adv life cycle.

pTP and TP clearly have important roles to play in the Adv life cycle but it is not yet clear whether TP plays a critical role in internalisation of Adv DNA. Experiments to determine whether TP is essential will be important to aid the design of non-viral systems.

6.5 **Future generation non-viral vector**

6.5.1 Introduction

To enhance transgene expression of non-viral vectors, many researchers have attempted to exploit the trafficking mechanism of viruses particular with the use of NLS for nuclear delivery. However, the role of microtubules in trafficking is often overlooked and may be a necessary component to produce an efficient non-viral system. Many viruses are thought to use the existing cellular transport systems inside endosomes and/or via microtubules to efficiently delivery their genomes to the nucleus as part of their life cycle (Whittaker *et al.*, 2000). Usually, the viral DNA is either within the capsid or associated with a number of other viral proteins. In many cases, the nuclear trafficking has been shown to occur through the NPC including the 36 kb genome of Adenovirus (Mudhakir *et al.*, 2009; Trotman *et al.*, 2001). However before the Adv DNA can be imported into the nucleus, the virion needs to be transported to its vicinity. This study identified that Adv TP does not require functional microtubule networks to reach the nucleus, however, the intact virus is thought to rely on microtubular transport once it has escaped the endosome (Bremner *et al.*, 2009b; Corjon *et al.*, 2011). Thus when considering the delivery of non-viral systems to the nucleus it is important to recognise that non-viral particles may also require mechanisms to facilitate early events before the final step of delivery through the nuclear pore can be accomplished.

We believe that HDAdv is an excellent template for a new generation Adv vectors. It is a replication-defective vector that is safe, stable and produces high expression levels of transgene (Dudek *et al.*, 2006). However, care needs to be taken when removing large sections of viral coding sequences to ensure that viral proteins responsible for providing signals for cellular trafficking are retained. It is vital that such functions are preserved when using the minimum genomic sequence. As tertiary structure of protein is important for its function, it is essential for each of the proteins in the vector to retain their structure so as not to interfere with the overall structure of vector system. With vital proteins encoded, the vector will be able to mimic the complete Adv trafficking process, instead of perhaps retaining some but not all aspects of the Adv delivery system. The route of Adv trafficking

resembles that of a classical clathrin-mediated endocytotic pathway (Leopold *et al.*, 2007). It involves five distinct stages; binding to a cell receptor, cell entry, endosomal escape prior to degradation, microtubule trafficking and nuclear import. The vector system must contain at least the minimum number of Adv proteins required to achieve all of the delivery stages without immunological problems or reduction in transfection efficiency. The sections below discuss potential aspects of the design of future Adv vectors and non-viral gene delivery systems.

6.5.2 Evasion of pre-existing immunity

To overcome the potential adverse effects associated with viruses, it is important to design a vector without a reproductive genome to reduce and/or eliminate the possibility of viral production. Viral vectors have been engineered to be replication-defective when it was shown that removal of gene(s) responsible for replication does not interfere with the transfection efficiency.

A replication-defective Bluetongue virus (BTV) was able to maintain viral protein expression (Matsuo *et al.*, 2011). No reduction in transfection efficiency was found when replication-defective lentivirus human immunodeficiency virus (HIV) was used in *in* vivo gene delivery with high expression level of the transgene in target tissues (Dull *et al.*, 1998; Miyoshi *et al.*, 1997; Naldini *et al.*, 1996). A similar result was also seen in the vector H6R28LEP, which is a vector based on a human herpesvirus 6 (HHV-6) without the expression of U2 through U8 genes that are responsible for viral replication (Shimizu *et al.*, 2013). The H6R28LEP vector shows a reduced viral production without compromising transgene expression (Shimizu *et al.*, 2013). All replication-defective viral vectors retain its infection ability, including integration of its DNA into the host chromosomes (Shimizu *et al.*, 2013). Vector integration may cause tumours by up-regulating cellular oncogenes adjacent to the integration site (Bokhoven *et al.*, 2009). For example, patients with common gamma-chain deficiency treated with a gammaretroviral vector have developed leukaemia following vector insertion in oncogenic loci (Hacein-Bey-Abina *et al.*, 2003). Lymphoid leukaemia was also reported in X-linked severe combined immunodeficiency trial when γ-

retroviral vector integrated into the target cell genome (Howe *et al.*, 2008). Therefore, replication-defective vectors based on Adv are probably more suitable for gene therapy as Adv does not require its DNA to be integrated into the host chromosome (Dudek *et al.*, 2006). To eliminate Adv replication, the E1, E2, E3 and/or E4 genes can either be mutated or removed (Dudek *et al.*, 2006; Giacca *et al.*, 2012; Lai *et al.*, 2002; Tatsis *et al.*, 2004; Wersto *et al.*, 1998). Their effectiveness in clinical use, however, is currently limited by anti-vector immunity causes by pre-existing antibodies against Adv (Shiver *et al.*, 2004; Xiang *et al.*, 2002).

Adv serotype 5 is the most abundantly found in human and is also the most widely used as recombinant gene transfer vectors. It has been reported that the dominant Adv5 neutralising antibodies are directed primarily against hexon proteins particularly their HVRs (Bradley *et al.*, 2012; Kaufmann *et al.*, 2012; Roberts *et al.*, 2006; Sumida *et al.*, 2005). One possibility to circumvent the immunogenicity challenge is to replace the hexon HVR of Adv5 with HVR from an Adv with low seroprevalence in humans. It is possible to replace seven HVRs on the surface of hexon proteins, without perturbing its core structure (Crawford-Miksza *et al.*, 1996). It was shown that a chimeric Adv5 vector with the seven HVRs exchanged from the corresponding regions of Adv 48 evaded majority of anti-Adv 5 immunity (Roberts *et al.*, 2006). Recently, neutralising antibodies against the fibre protein have been detected (Bradley *et al.*, 2012). The study revealed that dominant vector-specific neutralising antibodies are directed primarily against the hexon HVRs, but there are also subdominant neutralising antibodies directed against the fibre knob (Bradley *et al.*, 2012). As the immunity against fibre is only subdominant, it may be possible to avoid immunogenicity by replacing just the hexon HVRs. More studies to identify key neutralising epitopes on Adv capsids will lead to a better understanding of Adv immunology and will aid the selection of polypeptide fragments for use in non-viral delivery systems.

6.5.3 Improving specificity of cell binding

Adv achieves efficient gene delivery as a result of specific binding to the target cell and intra-cytoplasmic translocation of virions to the nucleus (Kaufmann *et al.*, 2012; Miyazawa

et al., 1999; Wu *et al.*, 2003). Adv from different subgroups are known to have different characteristics of intra-cytoplasmic trafficking. Although Adv 5 vectors demonstrate very efficient gene transfer, their tropism is limited by receptor expression on target cells (Huang *et al.*, 1996). One approach to enhance gene therapy is to introduce a novel tropism to the capsid to allow for cell specific binding (Miyazawa *et al.*, 1999; Schoggins *et al.*, 2006). The fibre protein, found at the apexes of the icosahedral capsid of Adv, is responsible for interacting with CAR for initial cell entry (Russell, 2009). Once inside the cell, the RGD motif in the penton base at the N-terminus of the fibre protein interacts with either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins to activate virus internalisation and to promote membrane penetration (Lyle *et al.*, 2010; Nicklin *et al.*, 2005; Wickham *et al.*, 1994).

To enable vectors to target a broader range of cells, including ones that do not normally express receptors for Adv, the fibre protein may be altered and/or replaced with other proteins. It is possible to create a fibreless particle without compromising the Adv translocation machinery which would allow different proteins to be used as targeting ligands (Daniels *et al.*, 2012; Von Seggern *et al.*, 1999). When the fibre of Adv 7 was used in an Adv 5 vector, the vector did not target CAR and had intracellular trafficking characteristic of Adv 7 (Miyazawa *et al.*, 1999). In another study, a vector was no longer dependent on the CAR attachment when fibre was altered with the addition of an RGD-motif to provide an alternative cell binding ligand (Magnusson *et al.*, 2001). Insertion of immunoglobulin binding domains at the extremity of short-shafted, knobless fibers, also resulted in CAR independent translocation (Henning *et al.*, 2005). Currently specific delivery roles of the fibre protein in post-internalisation stages have not been identified and further studies are required to ensure modified vectors retains adequate delivery efficiency. It would also be worth investigating the role of penton base RGD in vector cell entry in the absence of fibre to gain a better understanding of Adv intracellular mechanisms.

6.5.4 Escaping endosomal degradation

Following virus attachment, Adv is internalised via receptor-mediated endocytosis. During endocytosis, the vector is surrounded by the cell plasma membrane which engulfs it into a vesicle that then pinches off from the cell membrane and is assumed to approach and fuse with the endosome. The low capacity of vectors to escape from endosomes is thought to be one of the major factors causing poor transfection efficiency in non-viral delivery. We believe that future non-viral vector should comprise a mean to escape the endocytotic vesicle to enhance transported along microtubules to the perinuclear region of the cell for optimal transgene expression. Protein VI was shown to play a role in Adv endosomal escape however it is speculated that penton base may also be involved. Better understanding of the translocation pathways of the Adv capsid proteins will allow further improvements on the vector. The transgene expression of a non-viral vector can then be enhanced by encoding for the appropriate proteins.

Upon fibre interaction with CAR, the RGD motif of the penton base binds to $\alpha_v\beta_3$, $\alpha_v\beta_5$ or $\alpha_v\beta_1$ integrins mediating viral entry into clathrin-coated pits and endosomes (Li *et al.*, 2001; Lyle *et al.*, 2010). The majority of internalised penton base was found to escape the lysosomes. It was also reported that there is a small nuclear accumulation of penton base suggesting that at least a certain subpopulation of free penton base is able to penetrate the endosomal membrane (Rentsendorj *et al.*, 2006). Penton base was also shown to assist with the early release of vesicle contents from endosomes (Rentsendorj *et al.*, 2006; Seth, 1994). It has been proposed that the RGD loop of penton base is responsible for the endosome escape mechanism (Russell, 2009; Shayakhmetov *et al.*, 2005). The precise functions of penton base on Adv cell entry and intracellular trafficking still remains to be described and once this is fully understood it may help to improve the design of non-viral systems. Protein VI has been thought to be involved in mediating disruption of endosomal membrane for virion to escape degradation (Maier *et al.*, 2010; Russell, 2009; Wiethoff *et al.*, 2005a). It was shown that the dissociation of protein VI from Adv capsid is required for efficient membrane disruptions (Wiethoff *et al.*, 2005a). Adv encoding a single point mutation in protein VI resulted in a 10-fold reduction in infectivity due to impaired endosomal escape. The amino-terminal end of protein VI has an amphipathic helix that is

responsible for inducing positive curvature in the inner leaflet of the lipid bilayer causing partial raptures of the endosomal membrane (Moyer *et al.*, 2011). It is thought that reduced association of protein VI and inner endosomal membrane is responsible for failure to disrupt the endosome (Moyer *et al.*, 2011). The function of protein VI may provide a foundation for the engineering of non-viral vector to mediate endosomal escape resulting in enhanced delivery.

In chemical vector, in attempt to increase its low transfection efficiency, PEI has been manipulated to incorporate virus-like properties to improve its efficacy. It was shown that compacting DNA with the cationic polymer PEI helped to facilitate endosomal escape as the modified PEI compacts and releases DNA efficiently from the endosomes into the cytosol (Chan *et al.*, 2012; de Ilarduya *et al.*, 2010; Midoux *et al.*, 2009). Despite their high potential for gene transfer, PEI applications remain limited *in vivo* due to its propensity to aggregate (Ogris *et al.*, 2012; Wightman *et al.*, 2001). Furthermore, PEI can cause significant toxicities both *in vitro* and *in vivo*. PEI was found to influence the pattern of epithelial cells as well as induces multiple cellular responses (Godbey *et al.*, 2001; Ragnarsson *et al.*, 2003). Better understand of the underlying mechanisms of PEI need to be achieved before PEI-base vector becomes suitable for non-viral delivery system. There have also been investigations into the use of amphipathic peptides in vector to facilitate release of delivered particle from intracellular vesicles to the cytoplasm following endocytosis (Plank *et al.*, 1998). It is thought that amphipathic peptides facilitate the destabilisation of membranes causing greater disruption of the lipid packaging (Plank *et al.*, 1998). The cationic amphipathic peptide KLA has been shown to induce apoptosis in cancer cells. It is speculated that KLA depolarised mitochondrial membranes releasing proapoptotic proteins into the cytoplasm which resulted in activation of apoptosis (Costantini *et al.*, 2000; Ellerby *et al.*, 1999; Rege *et al.*, 2007). However, currently there is a limited understanding of the gene delivery mechanisms facilitated by amphipathic peptide. More researches need to be conducted before amphipathic peptide may be used for the generation of efficient synthetic peptide-based delivery system.

6.5.5 Utilise intracellular trafficking

Other than Adv, many other viruses are also known to require microtubule-mediated translocation as part of their infection pathway. The herpesvirus nucleocapsid requires the activity of the microtubule-associated molecular motor cytoplasmic dynein to achieve nuclear localisation (Lyman *et al.*, 2009). The human immunodeficiency virus (HIV-1) also relies on the host cytoskeleton, both actin and microtubules, to initiate infection (Yoder *et al.*, 2011). Microtubule-dependent motility appears to be a broadly utilised mechanism by which the efficiency of viral infection is enhanced and we believe that it may be a necessary component of the design of an efficient non-viral system.

Following receptor-mediated uptake into endocytic vesicles and escape from the endosome, Adv is transported along microtubules to the perinuclear region of the cell. Adv capsids undergo microtubule-dependent transport as part of their evolved machinery to deliver the viral genome to the nucleus (Bailey *et al.*, 2003; Yea *et al.*, 2007). Hexon was shown to facilitate intracellular trafficking through interaction with dynein towards MTOC (Bremner *et al.*, 2009a; Carlisle *et al.*, 2001; Samir A *et al.*, 2004). The dynein motor complex links Adv to cytoskeleton as well as promotes microtubule trafficking during viral infection. The attachment between dynein and Adv has been reported to depend on interactions between the dynein IC and LIC_1 subunits and the capsid protein hexon (Bremner *et al.*, 2009a; Bremner *et al.*, 2009b). To produce a non-viral vector that mimics this translocation process, it may be possible to include the hexon protein, however, the use of full length hexon is to be avoided. As discussed previously, hexon is the major contributor to Adv immunogenicity but it may be possible to design a system capable of repeated use by using fragments of hexon. There are diseases that will require multiple administration of therapeutic DNA, therefore it is essential that the delivery system evades the unwanted induction of an immune response. Although it is possible to replace seven HVRs of the hexon without disturbing its core structure, influences on the dynein interactions remain to be investigated. For successful engineering of hexon into future non-viral vectors, the precise regions of hexon responsible for binding the dynein complex need to be established. Furthermore, live cell analysis of virions after endosomal escape has reveals that capsid transport along MTs also occurred towards plus-end directions,

presumably driven by cytoplasmic kinesin motors (Bremner *et al.*, 2009a; Bremner *et al.*, 2009b). Hexon interactions with kinesin deserve to be explored for a better understanding of microtubular trafficking in Adv infection.

6.5.6 Enhancement of nuclear delivery

Enhancement on nuclear delivery has been the primary focus of non-viral delivery systems for improvement on transfection efficiency. NLSs have been used to couple to many vectors to promote nuclear uptake. Inclusion of NLS to TAT-peptide based vectors resulted in at least 200-fold increased in gene expression (Yi *et al.*, 2011). SV40 based NLS also helped enhance nuclear entry of Her (Heregulin-α) moiety and CTHD (C-Terminal disulfide homodimer) peptide (Jeyarajan *et al.*, 2010; Kim *et al.*, 2012). It is interesting to note that most of the NLSs employed to enhance gene expression are based on the SV40 NLS with very limited exploration on the use of bipartite NLS. Although employment of NLS has shown successful increased in transfection efficiency of non-viral gene delivery, optimal coupling of NLS-DNA still remains a complication with many investigations undergoing to find the best method (Wagstaff *et al.*, 2007).

It is known that in the final trafficking stage, Adv must leave the microtubule and establish association with the NE for DNA delivery through NPC. Adv proteins closely associated with viral DNA are thought to be involved in the nuclear uptake mechanism. TP is found covalently attached to the 5' terminal of DNA and is likely to be an adaptor for linking viral DNA to the import machinery. Our study has identified $MRRRR^{370}$, $PVRRRRRRV^{390}$ and $PGARPRGRF^{671}$ as regions encoding for NLS in TP (Chapter 4). Further experiments suggested that $MRRRR^{370}$ and $PVRRRRRRV^{390}$ are bipartite sequence with an 11 amino acid spacer, as absence of either sections resulted in reduction of nuclear expression level. The mutagenesis experiments revealed that $MRRRR^{370}(x_{11})PVRRRRRRV^{390}$ is the primary NLS employed by TP as significant reduction in nuclear expression level was detected it its absence (Chapter 4). It needs to be noted that the nuclear expression level still remains 3-folds higher than the control indicating $PGARPRGRF^{671}$ is able to retain some nuclear delivery mechanism. The ability of TP NLSs to use several import receptors (IMPα/β heterodimer, IMPα and IMPβ) was also observed suggesting TP adaptation that could

further aid nuclear transport of viral genome (Chapter 5). TP NLSs ability to enhance DNA delivery is yet to be investigated but they remain as strong candidate for enhancing future non-viral system. It will be interesting to find out the ability of both bipartite and monopartite NLS sequence in enhancing vector transfection efficiency. Furthermore, as TP is covalently attached to viral DNA, it will also be interesting to investigate whether a similar coupling system between protein-DNA can be developed for enhancement of non-viral vector. Another possible candidate for improvement on nuclear uptake is protein VII, which also coded for NLS (aa25-54) (Lee *et al.*, 2003b; Wodrich *et al.*, 2006). Due to its close association with DNA, protein VII is also likely to facilitate nuclear transport of viral genome. Protein VII also found to interact with multiple IMP receptors. This finding, together with ours, could reflect an optimised mechanism of Adv to access the transport machinery, similar to that of ribosomal proteins or core histones where import process is mediated by several IMP members (Jäkel *et al.*, 1998; Mosammaparast *et al.*, 2001; Wodrich *et al.*, 2006). Further studies will be necessary to determine precise role of protein VII and TP in nuclear uptake as it may be possible that for optimal Adv nuclear uptake, NLSs of both proteins are required. It will be important to investigate whether TP is essential for import of viral DNA and whether import of the DNA-protein complexes can be achieved by multiple receptor pathways. The findings will be valuable for improvement of non-viral delivery systems. It has also been proposed that protein V may play a role in Adv nuclear import as this protein is able to enter the nucleus and its NLS has been identified. However, due to its weaker association with viral DNA, it may be dissociated from DNA prior to DNA import (Matthews, 2001; Matthews *et al.*, 1998b).

Although currently there is no obvious reason to develop non-viral vectors to target the nucleolus, nucleolar localisation sequences appear to be present in protein V, pTP and the precursor of protein VII; proteins that are all closely associated with viral DNA. This suggested that the nucleolus may play a role in early stage of Adv infection before maturation. We have identified aa654-671 of TP to encode for NuLS. It is likely that the identified PGARPRGRF[671] NLS is also a NuLS but further analysis is needed before any conclusion can be made. While NuLs of protein VII is found between aa93-112 it is not known whether nucleolar tropism will be retained when the proteins are incorporated into a synthetic delivery system (Lee *et al.*, 2003b). Establishment of the precise role of the

nucleolus in Adv infection will improve our understandings on Adv life cycle. If the nucleolus plays a significant role, it will be interesting to investigate whether NuLS can be used to enhance transfection efficiency of non-viral system.

6.5.7 Protection of therapeutic DNA

Enhanced transgene expression cannot be achieved even with the best non-viral delivery vector if therapeutic DNA is degraded before reaching its target. Therefore protection of DNA is one of the main strategies for the next generation of non-viral system. It is known that cationic polymers condense DNA into small particles and prevent DNA from degradation. The condensation process is achieved through electrostatic force between positively charged cationic peptides and the negatively charged phosphate backbone of DNA (Saccardo *et al.*, 2009). poly-l-lysine (PLL) peptide was shown to condense plasmid DNA into compact nanostructures and a hexammine cobalt(III) cation complex was shown to effectively induced DNA condensation of calf thymus DNA (Kankia *et al.*, 2001; Mann *et al.*, 2008). However complication remains as there is a limited understanding on the influences of condensing molecules in cellular trafficking of vector system.

We believe that incorporating Adv proteins for DNA condensation is a step towards designing an effective non-viral delivery vector. Adv is known to condense DNA for its protection but it is yet clear whether the condensed DNA is involved in the trafficking process. If one wanted to use Adv proteins to construct a non-viral system, to protect therapeutic DNA from cellular degradation, the delivery should contain protein VII which is known to help condense DNA (Lee *et al.*, 2003b). It has been established that mixing protein VII and DNA results in compact DNA-protein structures when viewed under electron microscopy (Johnson *et al.*, 2004). Furthermore, microinjection of protein VII into *Xenopus oocytes* shows protein VII to efficiently associate with lampbrush chromosomes and condense DNA (Johnson *et al.*, 2004). As protein VII also encoded for NLS as previously discussed, incorporating protein VII into a vector will not only protected DNA from immature degradation but could also enhance nuclear uptake.

It would also be worth exploring whether μ and protein V play a role in DNA condensation as these proteins are also found in Adv core closely associated with Adv DNA (Nemerow *et al.*, 2012).

6.6 **Conclusion**

Next-generation non-viral gene delivery systems will need careful design, so that a variety of attributes are present after their self-assembly. To produce an efficient non-viral gene delivery vector, one will needs a combination of abilities, including DNA binding, DNA condensation/protection, cellular entry and nuclear delivery without suppressing transcriptional activity of the gene product. It is known that some viruses utilise cellular trafficking mechanisms for efficient delivery of their DNA. Adenovirus is a non-enveloped virus that utilised cathrin-mediated endocytosis for internalisation prior to employing microtubular network to reach the nucleus. Better understanding of how Adv achieves its delivery at a molecular level will underpin the development of non-viral vectors that are able to mimic the highly efficient processes of viral infection.

In this PhD study, a nuclear delivery stage of Adv was explored by examining function of TP found covalently attached to viral DNA. Three regions on TP were identified as NLSs: $MRRRR^{370}$, $PVRRRRRV^{390}$ and $PGARPRGRF^{671}$ (Chapter 4). All three regions were able to significantly increased nuclear expression of EmGFP- and YFP- fusion protein in both HeLa and COS-7 cells. Truncated experiments revealed that $MRRRR^{370}$ and $PVRRRRRV^{390}$ are bipartite NLS as both sequences need to be present for optimal nuclear delivery. Further experiments using mutagenesis technique, identified that the bipartite NLS is a primary NLS employed by TP for nuclear uptake. A significant reduction in nuclear expression of YFP-fusion proteins was detected when only $PGARPRGRF^{671}$ is presented although the expression level is still at least 3-folds higher than the control. An ivermectin experiment further supported this finding when significant reduction in nuclear expression is seen when an IMPα/β mediated pathway is blocked, a pathway utilised by the bipartite NLS. Nuclear delivery by $PGARPRGRF^{671}$ was not affected by the presence of ivermectin suggesting that the NLS uses different importin route (Chapter 5).

An ALPHAScreen® assay was used to identify importin receptors of the NLSs (Chapter 5). It was found that TP's NLSs are able to use several IMP receptors, showing high binding affinity to IMPα/β heterodimer, IMPα and IMPβ. Based on this finding, and our previous finding from ivermectin experiment, it was concluded that IMPα/β mediated pathway is the importin pathway of the bipartite NLS. PGARPRGRF[671] shows highest binding affinity to IMPβ and is thought to employ pathway involving IMPβ and/or its precursor. Further studies on interaction of PGARPRGRF[671] with other binding receptors will be of great interest in understanding Adv nuclear delivery of TP. The ability of TP NLSs to use several import receptors may suggests TP adaptation to further aid nuclear transport of viral genome.

An interesting discovery was made when nucleolar localisation was observed for pTP and a truncated fragment containing PGARPRGRF[671] but not for TP. This suggested that nucleolus may be involved in early stage of Adv infection prior to maturation when pTP is cleaved to form TP. As PGARPRGRF[671] is also presented in TP, it is speculated that the tertiary structure of protein may inhibit recognition of PGARPRGRF[671] by receptor. This will also explain why TP did not utilise alternate nuclear import pathway when IMPα/β mediated pathway was blocked. Further investigation into folding structure of TP will lead to better understanding on the location of NLSs and their interactions with IMP partners.

After treatment of the cells with nocodazole, it was concluded that TP nuclear delivery is independent of intact microtubular network. This finding suggested TP is not involved in the intracellular trafficking of Adv and therefore not responsible for bringing the virion to close proximity to NE for nuclear uptake.

Identification of TP's NLSs and their import pathway is a small step towards better understanding of the TP contribution to the overall Adv's nuclear delivery. The NLSs of TP may themselves be used to enhance therapeutic DNA delivery. Exciting future studies to dentify whether TP's NLSs could enhance both large and small DNA, as well as determining the NLS-IMP binding structure, will be of great interest in non-viral gene delivery approaches/gene therapy applications.

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Appendices

Taken from the Clinical Trials Worldwide Database:

http://www.wiley.co.uk/genmed/clinical
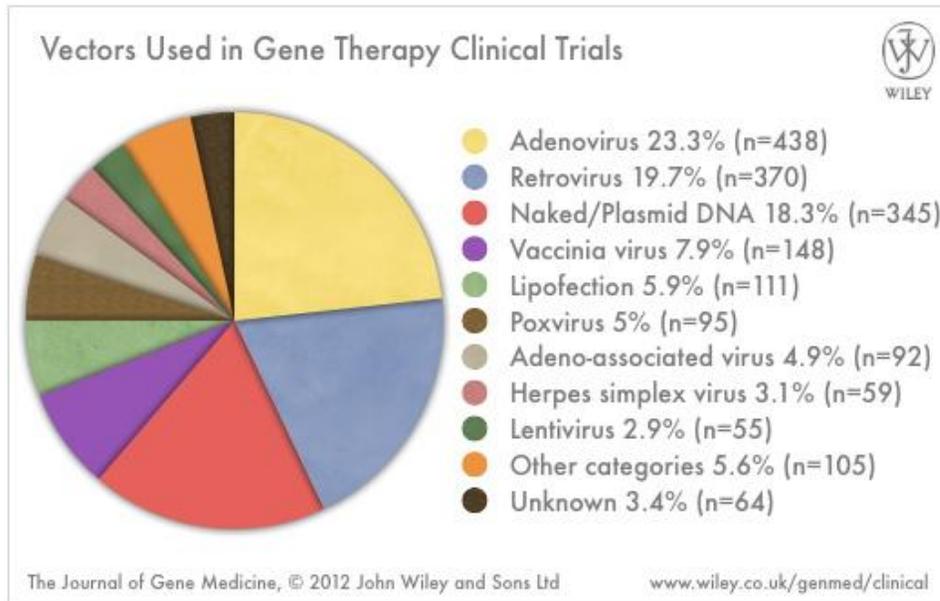
Vector	Gene Therapy Number	Clinical Trials %
Adeno-associated virus	92	5
Adenovirus	428	23.2
Adenovirus + Modified vaccinia Ankara virus (MVA)	1	0.1
Adenovirus + Retrovirus	3	0.2
Adenovirus + Vaccinia virus	3	0.2

Antisense oligonucleotide	6	0.3
Bifidobacterium longum	1	0.1
E. coli	2	0.1
Flavivirus	8	0.4
Gene gun	5	0.3
Genomic DNA (no vector)	1	0.1
Herpes simplex virus	59	3.2
Lactococcus lactis	5	0.3
Lentivirus	55	3
Lipofection	111	6
Listeria monocytogenes	6	0.3
Measles virus	6	0.3
Modified Vaccinia Ankara virus (MVA)	2	0.1
mRNA Electroporation	1	0.1
Naked/Plasmid DNA	341	18.5
Naked/Plasmid DNA + Adenovirus	3	0.2
Naked/Plasmid DNA + Vaccinia virus	1	0.1
Newcastle disease virus	1	0.1
Poliovirus	1	0.1
Poxvirus	67	3.6

Poxvirus + Vaccinia virus	28	1.5
Retrovirus	367	19.9
RNA transfer	27	1.5
RNA virus	5	0.3
Saccharomyces cerevisiae	7	0.4
Salmonella typhimurium	3	0.2
Semliki forest virus	2	0.1
Sendai virus	2	0.1
Shigella dysenteriae	1	0.1
Simian virus 40	1	0.1
Sleeping Beauty transposon	4	0.2
Streptococcus mutans	1	0.1
Vaccinia virus	116	6.3
Venezuelan equine encephalitis virus replicon	3	0.2
Vesicular stomatitis virus	2	0.1
Vibrio cholerae	1	0.1
Unknown	64	3.5
Total	1843	

Appendix B

As per the Clinical Trials Worldwide Database:

http://www.wiley.co.uk/genmed/clinical

Trial ID	Title
BE-0013	A phase III, multi centre, open-label, randomised study to compare the overall survival and safety of bi-weekly intratumoural administration of INGN 201 versus weekly methotrexate in 240 patients with refractory squamous cell carcinoma of the head and neck (SCCHN)
BE-0014	A phase III, multi centre, open-label, randomised study to compare the effectiveness and safety of intratumoural administration of INGN 201 in combination with chemotherapy versus chemotherapy alone in 288 patients with recurrent squamous cell carcinoma of the head and neck (SCCHN)
CN-0009	Gendicine intratumoral injection combined with radiotherapy for advanced nasopharyngeal carcinoma
CN-0010	Gendicine intratumoral injection combined with radiotherapy for advanced cervical carcinoma
DE-0037	A Phase III, Multi-Center, open-label, randomized study to compare the Overall Survival and Safety of Bi-weekly intratumoral administration of INGN-201 versus weekly Methotrexate in 240 patients with chemotherapy refractory Squamous Cell Carcinoma of the Head and Neck (SCCHN) - INGN-201 Trial 301
DE-0072	A Phase III, multi-center, open-label, randomized study to compare the Effectiveness and Safety of intratumoral administration of INGN-201 in combination with chemotherapy versus chemotherapy alone in 288 patients with recurrent Squamous Cell Carcinoma of the Head and Neck (SCCHN) - INGN-201 Trial 302

RU-0002	A Randomized, Controlled, Parallel Group, Multicenter Phase 3 Study to Evaluate the Efficacy and Safety of Ad5FGF-4 Using SPECT Myocardial Perfusion Imaging in Patients With Stable Angina Pectoris
US-0366	A Phase III Multi-Center, Open-Label, Randomized Study to Compare the Overall Survival and Safety off Bi-Weekly Intratumoral Administration of RPR/INGN 201 Versus Weekly Methotrexate in 240 Patients with Refractory Squamous Cell Carcinoma of the Head and Neck (SCCHN)
US-0412	A Phase III, Multi-Center, Open-Label, Randomized Study to Compare the Effectiveness and Safety of Intratumoral Administration of RPR/INGN 201 in Combination with Chemotherapy Versus Chemotherapy Alone in 288 Patients with Recurrent Squamous Cell Carcinoma of the Head and Neck (SCCHN). Sponsor: Aventis Pharmaceuticals - Gencell Division
US-0821	A Randomized, Double-Blind, Placebo-Controlled, Parallel Group, Multicenter Study to Evaluate the Efficacy and Safety of Ad5FGF-4 in Female Patients with Stable Angina Pectoris Who Are Not Candidates for Revascularization
US-0842	A Randomized, Controlled Phase III Trial of Replication-Competent Adenovirus-Mediated Suicide Gene Therapy in Combination with IMRT Versus IMRT Alone for the Treatment of Newly-Diagnosed Intermediate-Risk Prostate Cancer
US-0854	A Phase III, Randomized, Controlled, Open Label, Multicentre Study of the Efficacy and Safety of Trinam? (EG004); an Assessment of Vascular Access Graft Survival in Hemodialysis Patients
US-1083	A Randomized Controlled Trial of ProstAtak? as Adjuvant to Up-Font Radiation Therapy for Localized Prostate Cancer

Publication

Adenovirus: a blueprint for non-viral gene delivery

V Ann Chailertvanitkul and Colin W Pouton

Although adenoviral vectors may not find a direct clinical role in gene therapy, an understanding of the mechanisms of DNA delivery that adenoviruses use is of vital importance to the design of next-generation non-viral gene delivery systems. Adenoviruses overcome a series of biological barriers, including endosomal escape, intracellular trafficking, capsid dissociation, and nuclear import of DNA, to deliver their genome to the host cell nucleus. The understanding of these processes at the molecular level is progressing and is set to inform the design of synthetic gene delivery systems.

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Introduction

The future of gene therapy is dependent on the development of vectors that can selectively and efficiently deliver therapeutic DNA to target cells with minimal toxicity. By 2009 over 350 protocols had been approved for clinical trials of gene therapy using attenuated adenoviral vectors, 210 of which were open, but only 5 of which were Phase III trials (for details see the Clinical Trials Worldwide Database at http://www.wiley.co.uk/genmed/clinical). Widespread interest in the use of adenoviruses (Adv) developed primarily because of their ability to deliver double-stranded DNA to the nucleus efficiently. In addition, their large genome allows for extensive modification and incorporation of therapeutic genes. Although Adv vectors remain promising, clinical development of Adv constructs has been disappointing. Development into Phase III has been limited owing to: 1) the incidence of an unexpected severe inflammatory reaction [1–3]; 2) the fact that, once recognized by the immune system, Adv vectors are cleared rapidly, which prevents repeat dosing of the same product; 3) the safety concerns that are common to all viral vectors, that is the

possibility that their manufacture or use could generate replication-competent viruses (i.e. rare but finite incidences of DNA recombination that take place during biosynthesis in packaging cells or *in vivo* after administration [3,4]). During the past 20 years research on the molecular mechanisms of cell entry of Adv has continued. The basic understanding of how Adv delivery systems work, how they overcome each biological barrier, is fundamental to ensure further development and enhancement of the non-viral gene therapy. In short, the next-generation non-viral systems will need attributes that mimic adenoviral DNA delivery.

Structure of adenoviruses

The *Adenoviridae* family consists of over 50 distinct serotypes and can be categorized into six subgroups based on their DNA sequence homology (A–F). The human Adv are non-enveloped, double-stranded DNA viruses that contain a linear genome of approximately 36 kb [5,6•]. The Adv virion consists of an outer icosahedral capsid surrounded by an inner nucleoprotein core as illustrated in Figure 1 [1,2]. Hexon, a structural protein, is the most prevalent protein of the viral capsid with other minor protein components being protein IIIa, protein VI, protein VIII, and protein IX. At each of the twelve vertices found on the capsid are penton base proteins that are non-covalently attached to the fiber proteins [5,6•,7]. The adenovirus core consists of viral DNA, protein V, protein VII, Mu, and 2 copies of terminal protein (TP). TP is initially synthesized as pre-terminal protein (pTP) and can be found covalently coupled to the inverted terminal repeat (ITR) at the 5′ ends of each strand of the viral genome [5,6•,8,9].

Adenovirus trafficking

The majority of mechanistic information about Adv has been derived from studies on subgroup C [10•]. This review will focus on the trafficking of this subgroup, in particular on the closely related serotypes 2 and 5. Trafficking can be divided into five different stages as illustrated in Figure 2. These include: cell receptor binding, cell entry, endosomal escape before degradation, microtubule translocation and nuclear import. The route of Adv cell entry is consistent with a classical clathrin-mediated endocytic pathway [6•,11].

Binding to the cell receptor

Cell entry is initially mediated by high affinity binding between the virus fiber protein and the specific receptor present on the surface of the host cell [10•]. The receptor binding domain of the fiber protein is found in the C-terminal segment which folds into a globular knob [7]. All

Figure 1
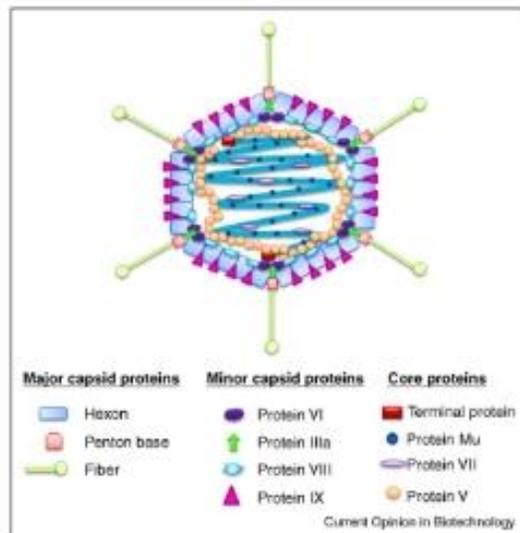
Schematic diagram of an adenovirus. Adenovirus particles include eleven polypeptides that condense adenoviral DNA and assemble into the viral capsid (adapted from Nemerow, G. R. et al., Ref. [13*]). The viral capsid also contains a cysteine protease that is involved in post-translational modification of viral proteins after nuclear export of the viral DNA.

subgroups of the Adv, except subgroup B, use Coxsackie and Adenovirus Receptor (CAR) as their extracellular receptor [12,13*,14,15]. The majority of Adv subgroup B particles bind to CD46 [14,16]. Adv subgroup C initiates cell uptake through two specific receptor interactions. The first interaction is the high affinity interaction

between the fiber knob and CAR [15,17]. CAR is a 46-kDa single transmembrane protein in the immunoglobulin (Ig) superfamily that acts as a cell-to-cell adhesion molecule on the surface of epithelial cells [18,19,20*]. The extracellular domain of CAR contains D1 and D2 regions. Although D1 alone is able to sufficiently bind to the fiber knob, both D1 and D2 interact with Adv2 and Adv12 fiber knobs [21,22]. It is thought that the amino acid at position 140 on the fiber knob plays a role in CAR-binding by way of its presentation to a negatively charged cluster on the receptor (Asp^{56} and Glu^{58}) [5,15,20*,21,23]. CAR-binding Advs contain serine, threonine, or proline, which are all neutral amino acids, at the key binding position [5,15,17,20*,21]. However, Adv serotype 3 that is a non-CAR binding Adv, instead contains a glutamate residue which is negatively charged [21]. Thus charge repulsion may prevent high-affinity binding of Adv type 3 to CAR. The structural model of CAR-fiber binding from X-ray diffraction analysis shows that each fiber knob can bind to a maximum of three CAR molecules [13*]. The initial fiber-CAR interaction is unable to promote efficient virus uptake and a lower affinity secondary interaction, between the cell surface integrins ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) and a conserved viral arginine-glycine-aspartate (RGD) motif, is an important requirement [7,13*,20*,22]. The RGD motif is located in the variable region of the exterior surface of the penton base, at the end of a flexible loop. This interaction is thought to be responsible for virus internalization [21,24–26]. The internalization of Adv requires the presence of free cholesterol in the plasma membrane, which is a feature of clathrin-mediated endocytosis [27]. In the absence of cholesterol, Adv is internalized by way of a slow and less efficient pathway with limited virus particle delivery to the cytosol [27]. The fiber protein may also play a role in directing the virus into a specific intracellular environment, as

Figure 2
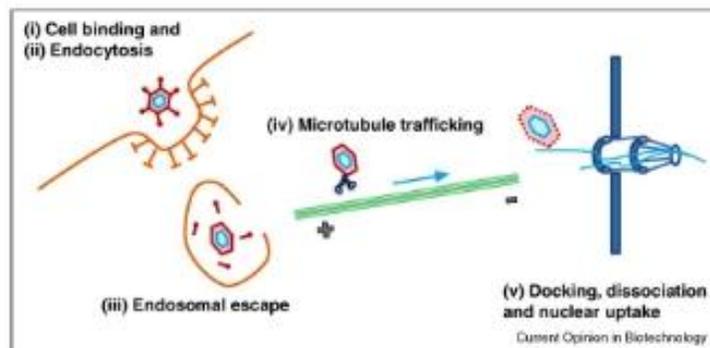
Schematic diagram of the steps involved in delivery of adenovirus DNA to the nucleus. Binding is followed by internalization into clathrin-coated vesicles (for the well studied Adv2 and Adv5 serotypes). For structural detail on binding and internalization see Ref. [13*]. Endosome escape involves loss of vertex proteins and subsequently the partially dissociated capsid binds to dynein and reaches the nucleus where it binds to Nup 214 [57*]. The viral DNA is taken up by a process that is yet to be described in detail.

demonstrated by an interesting fiber swap experiment in which a modified Ad5f7 vector, which is an Adv serotype 5 vector carrying Adv serotype 7 fibers, emulates the trafficking of Adv serotype 7 [6*,10*]. This implies that the fiber influences a post-internalization aspect of Adv trafficking. A recent report suggests that when Adv serotype 5 infects cells that lack CAR, it can use lactoferrin as a bridge to enhance binding with the target cells [12]. This observation was contradictory to the antiviral effects of lactoferrin, which is known to inhibit the virus entry into cells [12]. It has been suggested that the concentration of lactoferrin may influence its role in either inhibiting or promoting Adv infection [12]. Adv entry can also take place in the absence of CAR binding, when the key integrins may serve as both the primary cell receptor and activator of endocytosis [26]. This supports the idea that RGD-integrin binding may be a convenient mechanism for facilitating endocytosis of non-viral delivery systems, given that RGD motifs could be grafted relatively easily to the surface of a DNA delivery particle. Indeed this approach has been used previously to facilitate cellular uptake of non-viral gene delivery systems [28,29].

Endocytosis and endosome escape

Once bound at the cell surface, Adv is taken up into clathrin-coated pits by receptor-mediated endocytosis. The interactions of the penton base with the cell surface, promote integrins to aggregate, enhancing the downstream intracellular signaling pathway, which in turn induces endocytosis [12,22,30*]. An NPXY motif, which is an endocytotic sorting consensus sequence, can be found in the cytoplasmic tails of β3 and β5 integrin subunits [31,32,33*]. This motif is responsible for mediating the localization of receptors to coated pits [34]. Adv internalization is facilitated by activation of phosphatidylinositol 3-OH kinase (PI3K) that in turn activates Rac and CDC42 GTPases [6*,34,35]. This process promotes polymerization of actin filaments and subsequent endocytosis of Adv by way of clathrin-coated vesicles [6*,27,34,35]. For the Adv to gain access to the cytoplasm, it needs to escape the endosome before the degradation by lysosomal proteases; the latter being the default path for the contents of late endosomal vesicles, after fusion with lysosomes. The acidic pH in the lumen of the endosome is maintained by the proton pump enzyme H+ATPase. As the endosome matures, the pH falls to approximately pH 5 [34,35]. This acidification induces conformational changes in the Adv capsid causing the endosomal vesicle membrane to be disrupted, releasing partially disassembled Adv into the cytoplasm [21,24,34–37]. The mechanism by which the escape occurs is of considerable interest because synthetic gene delivery systems also need to be provided with an endosomal escape system. The vertex regions of Adv, including protein VI, appear to be released during the first stage of viral disassembly [25,38**]. It is thought that exposure of protein VI helps mediate endosomal membrane disruption by inducing curvature stress, which releases the remains of the Adv capsid [12,38**,39]. Other factors also contribute to the endosomal escape [37,40–42]. Integrins, particularly αvβ3, αvβ5, α5β1, and αvβ1, promote Adv permeabilization of the plasma membrane by way of attachment with the Adv penton base protein [18,43,44]. The Adv fiber protein is thought to be involved in the timing of endosomal escape by acting as a pH sensor during the progress of endocytosis [10*,34]. After endosomal escape approximately 80% of the capsid is still intact, and retains its hexon and penton proteins [45]. It appears that sometime during endocytosis or endosomal escape, the fiber protein and other capsid-stabilising proteins are lost [45]. Protein VI remains behind in endosomes in a role that may mediate Adv endosomal escape [25,38**,46]. Since hexon and penton remain associated with the Adv particle at this stage, it has long been assumed that one or both proteins may facilitate translocation toward the nucleus [45]. The molecular mechanisms of endosomal escape are partially understood but are worthy of further investigation because the escape systems used in synthetic gene delivery systems, making use of weakly basic polymers such as polyethyleneimine or cationic lipids, are known to cause endosomolysis [47]. From a practical viewpoint viral escape systems may be too complex to replicate or simulate as part of a synthetic gene delivery system, but it would be useful to know if they are less damaging to the host cell.

Microtubule trafficking

The crowded nature of the cytoplasm prevents the Adv capsid from freely diffusing toward the nucleus. It is thought that the Adv capsid reaches the nucleus by intracellular translocation along the microtubules by binding to the cytoplasmic dynein complex [48**,49–51]. Dynein is a microtubule-dependent molecular motor that aids the movement of protein cargoes toward the microtubule organization center (MTOC) and the nucleus [49]. The strength by which the Adv interacts with MTOC is similar to that between the Adv and nuclear envelope [49]. An Adv capsid surface protein is assumed to be responsible for the interaction with dynein [48**,49]. One possible candidate is the hexon protein since it has been shown to be associated with the microtubular network [48**,50]. The L3/p23 protease of Adv is another potential candidate since it contains a light chain dynein consensus binding site similar to that identified in the other viral proteins [50,52]. Although the capsid still contains the penton base during trafficking toward the nucleus, it has been reported that this protein does not show any evidence of microtubular interaction and is therefore not likely to be involved in the translocation process [50].

It is not yet evident what mechanism Adv uses to interact with the cytoplasmic dynein. It is possible that Adv may

bind directly with the dynein motor or indirectly through help of other accessory proteins from existing transport pathways [53]. A dynactin protein complex, which interacts with dynein, is thought to be one of the potential candidates, since an overexpression of this complex results in disruption of Adv translocation [50]. Further study is also required to identify the mechanism by which the Adv capsid is uncoupled from the dynein to form a more favorable interaction with the nuclear envelope.

Nuclear binding and uptake of Adv DNA

The molecular details of the nuclear delivery of Adv DNA are yet to be elucidated [45,54]. However, it is clear that Adv DNA is delivered by an active process through the nuclear pore complex (NPC) [54]. Several studies suggest that the import factors; importin α, importin β, and importin 7 may be responsible for the Adv DNA delivery to the nucleus [54]. Upon reaching the nucleus, Adv capsid protein hexon forms a stable association with the NPC via a nucleoporin, CAN/Nup214, which is located in the region of the cytoplasmic filaments [55,56,57*,58]. The Adv capsid then disassembles by an unknown mechanism, leaving the majority of capsid proteins at the perinuclear envelope, including most or all the copies of hexon. It has been estimated that only about 5% of hexon enters the nucleus [46,59]. The dissociation of the capsid enables the Adv DNA, possibly with its associated DNA binding proteins, to reach the nucleus where it can employ the host cell nuclear enzymes for transcription [49,55,56,60]. Histone H1 is thought to be an important factor in the disassembly of Adv2 and Adv5 capsids. Once the Adv capsid is docked at the NPC, the capsid disassembly process is thought to be triggered by H1 and/or H1 import factors with the help of the additional cytosolic factors, including Hsp/Hsc70 [45,46,54]. Hsp/Hsc70 is a classical nuclear protein import factor [45,46,54]. Its direct function in the Adv capsid disassembly is unclear but it may have a role before Adv docking at the NPC [45,56,59,61]. The L3/p23 protease that is included in Adv particles may be a requirement to degrade internal protein VI for a full capsid dissociation [59]. The nuclear export factor CRM1 is also involved in Adv nuclear delivery by acting as a positional indicator of the nucleus [58]. Adv detachment from the microtubules and the binding between the Ad capsid and the nuclear envelope also requires the presence of CRM1 [46,58]. Another factor that influences Adv nuclear targeting is the concentration of intracellular calcium. It has been shown that depletion of intracellular calcium inhibits nuclear import of Adv DNA [34,56]. It is possible that calcium induces conformational changes in the NPC to allow passage of macromolecular complexes. Although the contents of Adv core are well studied, it is yet to be established which of the core proteins enter the nucleus with the DNA. Owing to the covalent attachment of TP to the 5′ ends of the Adv DNA, TP is assumed to be imported into the nucleus coupled to the Adv DNA [46,62,63]. The degree to which DNA is condensed by core proteins during nuclear uptake, and the role these proteins might play in uptake is not known. pVII is thought to enter the nucleus as it is tightly associated with the Adv DNA and a basic nuclear localization signal (NLS) sequence has been identified in its precursor [25,54,64]. However, more research needs to be conducted to identify the complete list of Adv nuclear localized proteins and their potential functions in Adv nuclear delivery. The cationic proteins are indeed localized in the nucleus after host cell expression but accumulation would be expected to occur simply as a result of their polycationic nature. Their uptake may also be needed to facilitate export of the replicated Adv genome, but as yet the degree of condensation required for export of Adv DNA has not been reported.

Conclusions and outlook

Attempts to target plasmid DNA to the nucleus, by physical or chemical coupling to NLS, has met with limited success [65,66], thus the precise mechanism by which Adv DNA is imported will provide valuable insight. Key questions are: Is the TP (which has a known NLS [62]) essential for efficient import? Is Adv DNA imported in condensed form involving proteins VII, mu, and possibly V? It will be necessary to investigate each barrier in isolation and subsequently piece together a non-viral delivery system that has the necessary attributes to overcome all barriers. Currently the mechanism by which Adv enters mammalian cells is well understood. However, the mechanisms relating to endosomal escape, microtubule translocation, and nuclear import need to be further explored. More research also needs to be conducted to identify the Adv proteins involved in these mechanisms and how crucial they are to nuclear delivery of Adv DNA. Understanding these mechanisms at a molecular level will underpin the development of non-viral vectors that are able to mimic the highly efficient processes of viral infection.

Next-generation synthetic gene delivery systems will need careful design, so that a variety of attributes are present after their self-assembly. Cationic surface charge is generally a disadvantage for biodistribution, so at the surface the particles should be neutral, probably shielded by PEGylation and ideally should include a ligand for cellular recognition. An endosomal escape system will be required that could be provided by weakly basic polymers such as PEI or polypeptides rich in histidine; both of these options have been shown to facilitate escape. A microtubule-binding element will be required on the surface of the particles that are subsequently released from the endosome. As yet there are no oligopeptide motifs available which have been shown to facilitate efficient microtubular trafficking, so the establishment of this element of the design is a priority for future research. The condensed core of the DNA delivery system will need to gain access to the nucleus by active

uptake, a process which could be facilitated by the stable binding or chemical coupling of a polypeptide which displays a nuclear localization sequence. Viral particles provide an understanding of how these steps can be achieved, but synthetic systems that are intended for repeated use will need to be designed to avoid the unwanted induction of an immune response to their surface. For this reason it may not be possible to use full length viral proteins in their construction. Finally, synthetic DNA delivery systems will need to be physically stable before cellular uptake, which may require them to assemble into the type of geometric structures used by viruses, which themselves provide stability to disruption by proteins in the serum and extracellular fluids.

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