CHARACTERISATION OF NUCLEOCYTOPLASMIC TRANSPORT PATHWAYS OF THE UNP DEUBIQUITINATING ENZYME

by

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Dedicated to my beloved parents
Khalima and Alex Sobolev
DECLARATION OF THE CANDIDATE

The work presented in this thesis, is to the best of my knowledge and belief, original, except as acknowledged in the text, and has not been submitted, either in whole or in part, for a degree at this or any other University.

Tatiana Soboleva (December, 2002)
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Conference papers and invited seminars


Abstract

Unp (Ubiquitous nuclear protein) belongs to a large family of deubiquitinating enzymes (DUBs). DUB function, in general, is to cleave ubiquitin from its precursor molecules, or from ubiquitin conjugates that are generally targeted to the proteasome for degradation. This latter activity can enhance or inhibit proteasome-mediated proteolysis. Very little is known about DUB function, substrate specificity, and the functional significance of each member of the DUB family.

Unp (Usp4) was first described in 1993 as a proto-oncogene, related to the Tre-2 oncogene. It was later shown that Unp causes tumours when overexpressed in 3T3 cells xenografted into immunosuppressed mice. There also was evidence at the beginning of this study that Unp interacts in vitro with the Retinoblastoma tumor suppressor protein (pRb). Since then, interaction with pRb was shown in vivo, and in vitro with the other pRb members p130 and p107. However, the mechanism of oncogenic transformation and the functional significance of pRb interaction remain unclear.

Unp subcellular localisation was also controversial. Initially Unp was characterised as a nuclear protein containing a putative nuclear localisation sequence (NLS). In a separate study, Unp was shown to localise mainly in the cytoplasm.

The aims of the work described in this thesis were thus to address the question of Unp subcellular localisation and to contribute to better understanding of the functional significance of Unp association with pRb-family members. In particular, the aim was to define subcellular targeting signals in Unp and their nuclear transport mechanisms using ectopically expressed Unp, as well as examine the subcellular localisation of endogenous Unp.

- A red fluorescent protein-Unp (RFP-Unp) fusion protein was derived for transfection studies, and used in HeLa cells in the presence and absence of Leptomycin B (LMB). Unp nuclear localisation was found to be sensitive to LMB, indicating that Unp export from the nucleus is mediated by the CRM1 nuclear export receptor. Two putative nuclear export sequences (NESs) in Unp were identified (pNES1 $^{87}$LIDELDYL $^{96}$ and pNES2 $^{133}$VEVYLLEKL $^{142}$) and tested for activity by incorporation into a nuclear-localising reporter construct. Experiments in HeLa cells showed that pNES2 is active, and partially sensitive to LMB.
- Three putative NLSs were identified in Unp (pNLS1 $^{413}$KKKP $^{416}$, pNLS2
Site-directed mutagenesis was employed to analyse activity of these putative NLSs, and NLS3 was shown to be active. ELISA-based binding assay showed that NLS3 is recognised by the Importinα/β nuclear import receptor.

- The putative NLS2 was proposed to be a cyclinA/E binding site, and in vitro phosphorylation of Unp by cyclinA/cdk2 complex was shown. Mutation of this sequence resulted in an enhanced nuclear localisation, consistent with a role for phosphorylation regulating Unp nuclear transport.

Taken together these results suggest that Unp is a shuttling nucleocytoplasmic protein, partially sensitive to LMB and containing functional NLS and NES elements.

Subsequent experiments were dedicated to generation of Unp and Usp15-specific antibodies (Usp15 is a close Unp homologue) and to the analysis of endogenous Unp and Usp15 subcellular localisation as well as transport properties of an endogenous Unp. The involvement of the Retinoblastoma (pRb) tumour suppressor protein family in Unp localisation was also examined.

- Antibodies were raised against Unp and Usp15 and demonstrated not to cross-react, whereas previously published antibodies were shown to cross-react.
- Immunostaining of HeLa and NIH3T3 cell lines with the anti-Unp and -Usp15 antibodies revealed that the proteins localised mutually exclusively. Unp was always nuclear in HeLa cells and excluded from nucleoli, while Usp15 was either nucleolar or in the cytoplasm. In NIH3T3 cells in contrast, Unp was nuclear, or cytoplasmic or throughout the cell, while Usp15 was in the cytoplasm and near the plasma membrane. Immunostaining of a range of cells with anti-Unp antibodies showed that Unp localisation varied from exclusively cytoplasmic (HepG2 cells) to exclusively nuclear (HeLa and Saos-2 cells). LMB treatment of mouse primary embryonic fibroblasts led to higher accumulation of Unp in the nucleus, indicating that endogenous Unp is able to shuttle between nucleus and cytoplasm in a physiological context.
- In order to investigate the involvement of Rb proteins in Unp nuclear transport, two attempts to create Rb-binding mutants of Unp were made. However, the first mutant, RFP-UnpC461G, was not stable, and the second mutant, RFP-UnpL459G, was still able to bind Rb in a GST pull-down assay.
- However, experiments with endogenous Unp showed that pRb
is probably not involved in Unp nuclear transport, as localisation of Unp in Rb-defective Saos-2 cells and in Rb-wild-type SR-40 cells was the same. Stable expression of human papilloma virus (HPV) E7 protein in NIH3T3 cells resulted in increased nuclear localisation of Unp, raising the possibility that Unp localisation can be controlled by the other Rb family members such as p130 or p107, as HPV E7 targets these proteins for proteasome-mediated degradation.

In summary, the work described in this thesis establishes that Unp is a nucleocytoplasmic shuttling protein, with different localisations in different cell types. This explains the previous controversy regarding Unp subcellular localisation. There appear to be roles for both the NLS/importinα/β and NES/CRM1 receptor pathways, as well as a possible piggyback mechanism involving pRb family proteins, in regulating Unp subcellular localisation. It is proposed that the shuttling characteristics of Unp are fundamental to its functions, distinct from those of Usp15.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ActD</td>
<td>actinomycin D</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)-benzenesulfonyl</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
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<td>β-Me</td>
<td>β-mercaptoethanol</td>
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<td>bp</td>
<td>base pairs</td>
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<td>cDNA</td>
<td>complementary DNA</td>
</tr>
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<td>CHX</td>
<td>cycloheximide</td>
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<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CR1/2</td>
<td>conserved region 1, 2</td>
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<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>distilled deionised H₂O</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DUB</td>
<td>deubiquitinating enzyme</td>
</tr>
<tr>
<td>E7</td>
<td>human papilloma virus E7 protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>Fn/c</td>
<td>ratio of nuclear to cytoplasmic fluorescence</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>IBB</td>
<td>importin β-binding domain</td>
</tr>
<tr>
<td>Imp</td>
<td>importin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>LMB</td>
<td>leptomycin B</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation sequence</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma tumour suppressor protein</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>T-ag</td>
<td>large T-antigen</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl)aminoethane</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>Ubp</td>
<td>ubiquitin specific protease</td>
</tr>
<tr>
<td>UCH</td>
<td>ubiquitin C-terminal hydrolase</td>
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Chapter 1
GENERAL INTRODUCTION
1.1 Introduction

The focus of this study is to characterise the nuclear-cytoplasmic transport properties of the deubiquitinating enzyme Unp (ubiquitous nuclear protein). Deubiquitinating enzymes (DUBs) remove ubiquitin molecules or chains from proteasome substrates, either before or after degradation. In addition to its enzyme activity, another important feature of Unp is its ability to interact with the Retinoblastoma family of proteins.

Given these features of Unp, and the aims of this study, the general introduction will review proteasome-mediated degradation, functions of ubiquitin, the roles of Retinoblastoma family of proteins, and the mechanisms of nuclear-cytoplasmic transport.

1.2 Proteolysis Mediated by the Ubiquitin-Proteasome Pathway

Every protein, which either enters an organism or is synthesised within a cell, sooner or later has to undergo degradation. Foreign proteins, which are consumed by the organism, are degraded in the lumen of the gastro-intestinal tract. These proteins stay “outside” of the body because the epithelial lining of the lumen does not allow the absorption of proteins until they are degraded to single amino acids. Extracellular proteins, which are synthesised by the cell and secreted, are targeted through the series of endosomes to the lysosome, where they are degraded. These extracellular proteins include some hormones, immunoglobulins, blood coagulation factors, etc. The degradation of foreign and extracellular proteins is non-specific, as long as they enter the degradation compartment (the gastro-intestinal tract or the lysosome), they are attacked by proteases and degraded at approximately the same rate. On the contrary, the half-life of intracellular proteins varies from several minutes (e.g. p53) to several hours (e.g. cyclins), and even days (muscle proteins) or years (crystalline). Needless to say, degradation of these proteins ought to be very specific and carried-out by a completely different mechanism to lysosomal proteolysis. This mechanism is called the ubiquitin-proteasome proteolytic pathway (Glickman and Ciechanover, 2002).
The ubiquitin-proteasome proteolytic pathway involves two discrete steps: (i) covalent attachment of a polyubiquitin chain(s) to a substrate and (ii) degradation of the tagged substrate by the 26S proteasome. Figure 1.1 illustrates the ubiquitin-proteasome mediated degradation cycle.

Ubiquitin, a highly conserved 76-amino acid protein, is conjugated to a substrate protein via a three-step mechanism. First, the ubiquitin-activating enzyme (E1) uses the energy of ATP hydrolysis to bind to a C-terminal 76-Gly of ubiquitin forming a high energy thiol ester intermediate, Ub-E1. Second, the ubiquitin conjugating enzymes (E2) transfers the activated ubiquitin from E1 to the internal lysine(s) of a substrate protein, which is bound to the one of ubiquitin ligases (E3). This process is repeated several times until a polyubiquitin chain, with ubiquitin molecules linked to each other through a Gly\textsuperscript{76}-Lys\textsuperscript{48} isopeptide bond, is formed. Finally, ubiquitinated proteins are degraded by the proteasome and ubiquitin molecules are recycled back into the pool of free ubiquitin.

1.2.1 The Ubiquitin-Conjugating Machinery: Enzymes E1, E2 and E3

1.2.1.1 E1, the ubiquitin-activating enzymes

The main role of E1 enzyme is to activate ubiquitin via formation of a thioester bond between Gly-76 of ubiquitin and its active site cysteine. Uba1 is a major form of E1 enzyme in yeast and human, whose inactivation in yeast is lethal (McGrath et al., 1991). Uba1 protein exists as a homodimer, composed of two 105 kDa subunits (Ciechanover et al., 1982). Uba1 has been shown to be phosphorylated and to possess a nuclear localisation signal (NLS) (Handley et al., 1991), which mediates Uba1 translocation into the nucleus in a cell cycle-dependent manner (Stephen et al., 1996).

1.2.1.2 E2, the ubiquitin-conjugating enzymes

In contrast to the few members of the E1 enzyme family, the E2 family contains several dozens of members. The E2 enzymes also possess a conserved active site cysteine, which facilitates their binding to ubiquitin again as a high energy thioester. Some E2 enzymes contain a UBC domain, for binding to ubiquitin ligase enzymes, E3s. The role of E2 enzymes is to transfer E1-activated ubiquitin directly to a substrate or, in the case of UBC-containing E2s, to E3 ligase. E2 enzymes can interact with substrate
Figure 1.1 The ubiquitin-proteasome pathway.

A ubiquitin molecule forms a high-energy thiolester bond with the ubiquitin-activating enzyme (E1). Activated ubiquitin is then transferred to a member of ubiquitin-conjugating enzyme family (E2), which in turn interacts with a substrate-bound ubiquitin ligase family member (E3). In some cases a transfer of activated ubiquitin on the E3 forms a high-energy E3-S-Ubiquitin intermediate (ie the E6-AP). The activated ubiquitin is then covalently attached to the side chain of a Lys residue of a substrate protein. In the other cases Ub is not transferred to the E3 but is directly attached by the E2 to a substrate (SCF E3). This process is repeated several times until a multi-ubiquitin chain is formed. Multi-ubiquitinated protein is recognised and degraded by the proteasome.

Deubiquitinating enzymes (DUBs) play an important role in this cascade. Firstly, they are involved in generation of mature ubiquitin by cleaving the multi-ubiquitin precursor (a). Secondly, they can act as proofreading enzymes by cleaving ubiquitin off the proteins, which are not to be degraded (b). Thirdly, DUBs are involved in disassembly of ubiquitin chains after they were released by the proteasome, thus maintaining the pool of free ubiquitin (c). DUB action is shown with green arrows.
Ubiquitin-Proteasome Proteolytic Cascade.

(a) DUB

Ub

E1

Ub

Gly 76

(b) DUB

Ub

E3

protein

(c) DUB

Ub

Ub

(proteasome)

Ub
proteins but the physiological significance of this interaction is still unclear (Kalchman et al., 1996). Thirteen E2 enzymes have been reported in Baker’s yeast. Eleven of them (Ubc1-8, 10, 11, and 13) conjugate ubiquitin molecules while two others conjugate ubiquitin-like proteins: Ubc9 conjugates SMT3 (SUMO) and Ubc12 conjugates Rub1. More E2s have been described in mammals. Members of the E2 family interact specifically with members of the E3 family. Thus yeast E2, Rad6, acts together with E3 Ubr1 in order to target N-end rule and other substrates (Xie and Varshavsky, 1999). Another yeast E2, Ubc3, together with the SCF-type E3 targets proteins for degradation (see below) (Deshaies, 1999). Mammalian E2 enzymes have been shown to perform functions other than ubiquitin conjugation. For example, E2 BRUCE, a murine ubiquitin-conjugating enzyme, contains an inhibitor of apoptosis repeat (Hauser et al., 1998), suggesting a role in the inhibition of apoptosis. Other examples, such as ER-associated yeast E2s, Ubc6 and Ubc7, are involved in degradation of proteins within the ER (Plemper and Wolf, 1999), (Bays et al., 2001).

1.2.1.3 E3, the ubiquitin-protein ligases

E3 enzymes are responsible for the specific recognition of proteasome substrates, and the interaction with E2 enzymes. E3 ligases can be divided into two types, the HECT domain E3s and the RING finger domain E3s (RING is a Really Interesting New Gene).

HECT domain E3s

The HECT (Homologous to E6-AP C-Terminus) type E3 ligases are comprised of only one protein (Figure 1.2 A). These HECT proteins contain a 350 aa domain within their C-terminus, which harbours the active site cysteine involved in the interaction with ubiquitin. The HECT domain proteins are usually large (90 to 200kDa). An extensive N-terminus of HECT proteins facilitates binding to a substrate, whilst the C-terminus transfers ubiquitin onto the surface of the substrate. NEDD4, a HECT ligase in humans, targets the epithelial sodium channel for degradation, while its yeast orthologue, Rsp5, targets the large subunit of RNA polymerase as well as uracil permease, Fur4, and other
FIGURE 1.2 A comparison of ubiquitin ligases. (E3 enzymes)
The ubiquitin ligases can be categorised as containing proteins with HECT domains, RING finger domains or one of several cullins. (a) **HECT domain E3s** bind directly to their cognate ubiquitin conjugating enzyme, or E2, and directly to substrate. (b) **RING finger E3s** might use the metal-binding RING finger domains to facilitate catalysis. The E2 enzyme can bind directly to the RING finger protein, and the E3 might bind directly to substrate. (c) **SCF complexes.** The substrate is recruited to the complex by a protein-binding domain within a protein that contains an F-box. The F-box motif itself binds to the Skp1 protein, which can bridge the F-box protein to the cullin. The cullin serves as a scaffold to bind a RING finger protein, Roc1/Rbx1, and to a specific E2. (d) **VBC complex.** The substrate binds to a protein containing a SOCS box. The SOCS box binds to the Skp1 homologue elongin C, and a ubiquitin homologue elongin B. Cul2 and the Roc1/Rbx1 RING finger protein are associated with the complex, but the E2 remains unclear. (e) **The anaphase-promoting complex (APC).** The APC is thought to associate with its substrates through the substrate-specific activators Cdc20 and Cdh1, although proof of direct binding is lacking. Cdc20 binds to mitotically phosphorylated APC, although a direct linking protein (akin to Skp1) has not been demonstrated. Key: substrates are shown in pink, adaptor proteins in blue. Linking proteins including Skp1 and elongin C are triangular and green, cullins are elongated and shown in magenta, E2s are shown in purple. RING finger domains or proteins are shown as open rings (taken from Jackson et al., 2000).
plasma membrane proteins for degradation (reviewed in Glickman and Ciechanover, 2002; and Jackson et al., 2000).

**RING finger domain E3s**

The RING finger E3s are subdivided into 4 classes: SCF complexes, VBC complexes, APC complexes, and RING-finger E3s. Members of four classes are very heterogeneous but all contain a subunit with a RING finger motif. The RING motif contains eight ordered cysteine and histidine residues, which form a cross-brace with two zinc atoms stabilising the structure (Jackson et al., 2000).

**SCF complexes**

SCF ubiquitin ligases are generally comprised of four proteins: Skp1, Cul1, Rocl/Rbx1/Hrt1, and F-box-containing protein. They form a complex by interacting with each other as shown in Figure 1.2 C. Each member of the complex performs a specific function. Thus Cul1 activates the complex and helps to recruit the E2 enzyme. Rocl/Rbx1/Hrt1 RING finger proteins, promote the association of Cul1 with the E2 and enhance overall activity of the E3 (Ohta et al., 1999). The F-box adaptor protein directs the ubiquitination of substrates. Skp1 in turn serves as a bridge to link the F-box protein to Cul1. It has been shown that SCF complexes act along with E2 Cdc34/Ubc3. Interestingly, substrate proteins have to be phosphorylated prior to being recognised and ubiquitinated by the SCF-type E3s. Several known SCF substrates are β-catenin, Ikβ, and p27 (Winston et al., 1999; Carrano et al., 1999).

**VBC ubiquitin ligases**

The VBC complexes are structurally similar to SCF complexes (Figure 1.2 D). Proteins that comprise the VBC ligases are VHL, Roc1/Rbx1, Cul2, elongin B, and elongin C. VHL, a tumour suppressor protein that is inactivated in the von Hippel-Lindau disease, interacts with a substrate, while Roc1/Rbx1 and Cul2 play the same role as in SCF complexes. Elongin C is believed to serve the same function as Skp1, while elongin B has a ubiquitin-like domain but its function remains unknown (Kaelin and Maher, 1998).
APC ubiquitin ligases

The anaphase promoting complex (APC) E3 ligases (Figure 1.2 E) are required for the degradation of proteins controlling the transition from metaphase to anaphase during mitosis. The APCs are more complex structures, compared to SCF or VBC E3 enzymes, containing 11 subunits in total. However, certain proteins within APC complexes are similar to that of SCF. For example, Apc2 is a homologue to Cul1 and Cul2; the RING finger-containing protein in APC complex is similar to Roc1/Rbx1. Interestingly, the APC ligase has to be phosphorylated for proper ubiquitination of its substrate (Zachariae and Nasmyth, 1999). The best characterised substrate of the APC E3 is cyclin B (Peters, 1998).

Single protein RING finger E3 ligases.

RING finger E3 ligases consist of only one protein. These proteins contain the RING domain and a substrate-binding site on the same molecule. Mdm2, Ubr1/E3α and Parkin are representatives of such E3 enzymes (Glickman and Ciechanover, 2002) (Figure 1.2 B).

1.2.1.4 E4 enzymes

A recently identified class of enzymes, termed E4, contain a modified version of the RING finger domain, called the U-box (Aravind and Koonin, 2000), and have been shown to enhance the efficiency of ubiquitin chain elongation (Koegl et al., 1999).

1.2.2 The Proteasome and Degradation of Ubiquitinated Substrates

1.2.2.1 Structure of the 26S proteasome

The 26S proteasome is a large, 2.5 MDa complex, comprised of two major subcomplexes: the 19S regulatory particle (RP) and the 20S protease catalytic core. The 20S core is made up of four rings, of seven subunits each. Subunits of the two inner β-rings have protease activity, while the two outer α-rings are regulatory parts of the 20S proteasome involved in the attachment of the 19S RP to the 20S core. The 19S RP can be subdivided into two parts: the base and the lid. The base contains 6 ATPase subunits,
Rpt1-6, and three non-ATPase subunits, Rpn1, 2, and 10. The lid of the 19S RP is made up of eight non-ATPase proteins, Rpn3, 6-9, 11, 12) (reviewed in Glickman and Ciechanover, 2002). The role of the lid is still unclear, but it has been shown that several proteins, including the deubiquitinating enzyme Doa4, are associated with it. Remarkably, most proteasome subunits exhibit more than 40% identity between yeast and humans, with some key components maintaining 70-80% identity. Such conservatism implies that proteasome activity and function is extremely important.

1.2.2.2 Substrate recognition and processing by the 26S proteasome

The Rpn10 subunit of the yeast proteasome 19S RP binds to polyubiquitin chain covalently attached to the substrate (Baboshina and Haas, 1996). Interestingly, a four-ubiquitin chain binds to the proteasome with a 100-fold higher affinity than a two-ubiquitin chain, while the addition of an extra 8 ubiquitin molecules increases binding to the proteasome by only 10-fold (Pickart, 2000). Therefore, it is the surface provided by the four-ubiquitin chain that is recognised by the proteasome, not a single ubiquitin molecule. The interaction between the ubiquitin chain and the Rpn10 subunit is facilitated by a hydrophobic patch of amino acids, LALAL, located in the C-terminus of Rpn10 (Fu et al., 1998). Interestingly, deletion of the Rpn10 gene does not cause a discernible phenotype indicating that it is not the only chain-binding subunit. It has been proposed that other base subunits, Rpn1 and Rpn2, are also responsible for the efficient binding of ubiquitin chains to the proteasome (Glickman and Ciechanover, 2002).

Binding of the polyubiquitin chain(s) to the proteasome is necessary but not sufficient for successful substrate degradation. In order to be degraded, the substrate itself has to bind to the proteasome. This association triggers substrate unfolding and feeding through the proteolytic 20S core of the proteasome. The subunits that are responsible for substrate binding are Rpn1 and Rpn2 since they contain leucine rich repeats (LRR), which have been suggested to mediate protein-protein interactions (Lupas et al., 1997). Six other subunits of the base, the Rpt ATPases, are also involved in the unfolding of the substrate, and they also bind the substrate through hydrophobic repeats similar to that of Rpn1/2 (Gottesman et al., 1997). The unfolding process requires the energy of ATP hydrolysis. Rpt ATPases, together with the α-subunits of the core, control the efficiency and specificity of degradation of substrates by the
proteasome, through the opening and the closing of the channel leading to the β-subunits of the catalytic core (Groll et al., 2000).

When the unfolded substrate reaches the β subunits, it undergoes degradation. Generally, the proteasome cleaves the substrates into peptides between 3 and 23 amino acids in length (Kisselev et al., 1999). The 20S core is capable of cleaving peptide bonds after any amino acid. However, different β-subunits preferentially cleave after different amino acid residues. Thus β1 cleaves after acidic or small hydrophobic amino acid residues, β2 cleaves after basic or small hydrophobic amino acid residues, and β5 cleaves peptide bonds after any hydrophobic amino acid residue (Dick et al., 1998). The peptides produced by the 20S core are rapidly hydrolysed by downstream proteases and aminopeptidases. Some of the peptides are presented by MHC class I molecules to the immune system, triggering the immune response (Stoltze et al., 2000).

1.2.3 Non-proteolytic functions of ubiquitin
Ubiquitin is involved in the regulation of a number of different cellular processes other than proteasome-mediated degradation. Figure 1.3 illustrates numerous ubiquitin functions discovered so far. Proteins can be modified by monoubiquitination, where ubiquitin conjugates to one or more Lys residues. Monoubiquitination of proteins plays a role in endocytosis, gene expression, and protein sorting by the late endosome. Multi-ubiquitin chains target proteins for degradation by the proteasome (see Section §1.2), but may also serve as signals for IKK activation, DNA repair, and endocytosis when are assembled via Lys-63.

1.2.3.1 Histone ubiquitination
Monoubiquitination of histones is essential for gene expression. Histones H2A and H2B are modified by mono- or short chains of ubiquitin. Approximately 10% of H2A and 1% of H2B are ubiquitinated in mammalian cells. Yeast strains that lack the ubiquitination site of H2B are unable to sporulate and they also grow slowly. Ubiquitination of histone H2A is important for meiosis. Mutations in the active site of Rad6, the E2 enzyme, which is involved in H2A and H2B ubiquitination, leads to defects in sporulation (reviewed in Hicke, 2001).
Figure 1.3 Signalling function of ubiquitin
Protein ubiquitination triggers different cellular processes. (a) monoubiquitination of target proteins is important for endocytosis, sorting into endosomes, regulating subnuclear location, and for the activation of gene expression. (b) multiubiquitination triggers protein degradation by the proteasome (K48 assembled ubiquitin chains) and is also involved in IKK activation, DNA repair, and endocytosis (K63 assembled ubiquitin chains).
1.2.3.2 Endocytosis and protein sorting

Proteins anchored in the plasma membrane are often downregulated by internalisation into the endocytic pathway. Ubiquitination of such proteins is the signal for internalisation into the lysosome. It has been shown that monoubiquitination triggers membrane protein translocation to the early endosome. Interestingly, monoubiquitination also controls the activity of the endocytic machinery. Monoubiquitination of EPS15, a protein that interacts with the Clathrin-mediated endocytic machinery, upregulates the endocytosis system. However, there are several examples of proteins, such as yeast permeases, that require the attachment of ubiquitin chains (linked to each other through Lys-63) prior to endosomal internalisation. Notably, recognition of a targeted protein itself is not required when Lys-63 ubiquitin chains are present (reviewed in Hicke, 2001).

An interesting example of ubiquitin-dependent membrane trafficking and the activation of gene expression are two yeast transcription factors, Mga2p and Spt23p. The precursors of Mga2p and Spt23p are bound to a cytosolic face of the endoplasmic reticulum (ER). Upon proteolytic activation, which involves a proteasome and ER-associated proteins, Npl4, Ufd1p, and cdc48p, the mature factors are released from the ER and translocate to the nucleus where they activate transcription of the Delta9-fatty acid desaturase gene OLE1. Mutations in either of NPL4, UFD1, and CDC48 genes block Mga2p and Spt23p maturation and inhibit OLE1 expression (Hitchcock et al., 2001).

1.2.3.3 Subnuclear trafficking

Monoubiquitination of certain proteins is required for their translocations within the nucleus. For example, Falconi Anemia (FA) is a cancer susceptibility disorder associated with DNA damage sensitivity of several genes (FA genes). Monoubiquitination of one of these gene products, known as D2, activates several upstream FA genes, and leads to a subsequent relocation of D2 within the nucleus. The monoubiquitination of D2 usually happens in response to DNA damage. In normal cells D2 is targeted to certain nuclear foci together with BRCA1, a tumour suppressor gene, involved in DNA repair. Therefore, D2 monoubiquitination is most likely linked to BRCA1-mediated DNA repair (reviewed in Pickart, 2001).
1.2.3.4 DNA repair

Rad6-dependent DNA repair is another example of a process where ubiquitination is essential. DNA damage triggers ubiquitination of a protein, called PCNA, by ubiquitin ligase Rad6 and other co-factors. PCNA, modified by Lys-63 linked polyubiquitin, promotes error-free post-replicative DNA repair. Interestingly, the modification of the same Lys residue of PNCA by SUMO inhibits such DNA repair (Hoege et al., 2002; Pickart, 2002).

1.3 Deubiquitinating Enzymes

1.3.1 Structure

Ubiquitination of proteins is generally a reversible process. The enzymes that facilitate removal of ubiquitin molecules are called Deubiquitinating enzymes or DUBs. DUBs are represented by two classes, the Ubiquitin Carboxyl-terminal Hydrolases (UCHs), and the Ubiquitin-specific Proteases (UBPs).

The UCH enzymes are small proteases (23-27 kDa) that cleave ubiquitin from small proteins or from ubiquitinated protein remnants. The crystal structure of one UCH protein, UCH-L3, has been solved (Johnston et al., 1997). The core catalytic domain of UCH-L3 has a strong resemblance to the papain-like thiol protease, Cathepsin B. The catalytic core contains a triad of Cys, His, and Asp residues. This differs from other thiol proteases as they have an Asn, not Asp, as a third residue in the triad. The triad core forms two blocks. The Cys block with the active site Cys and the oxyanion hole Gln, and the His block with His and Asp residues at the active site.

A second class of deubiquitinating enzymes (UBP) is able to cleave linear and branched ubiquitin chains, and ubiquitin molecules from large proteins. Therefore, the UBPs are believed to be involved in ubiquitin maturation by cleaving ubiquitin from polyubiquitin precursors, by deubiquitinating proteasome remnants, and by deubiquitinating proteins (thereby controlling protein stability).

UBPs are very diverse structurally (see Figure 1.4). Their size ranges from 41 kDa (chicken Ubp41) to 308 kDa (Drosophila Faf). The catalytic core domain of UBPs is approximately 350 amino acids long. The catalytic core is bounded by conserved Cys
Figure 1.4 Schematic representation of a sample of UBPs.

The UBPs, its species of origin and predicted molecular mass are listed at the left. Proteins are drawn as open rectangles with the number of residues at the right. Positions of the conserved Cys box (black box) and His box (vertical striped box) are shown. Active site Cys and His residues are numbered above each box. In most UBPs, the His box is at or near the extreme C-terminus, with the exception of Faf, Ubp15 and their orthologues (not shown). Other conserved elements are located between the Cys and His boxes, and the size of this region varies due to extra sequence insertions. The length of the N-terminus varies considerably. D.m., Drosophila; S.c., Saccharomyces cerevisiae; M.m., mouse; G.g., chicken. Mouse Usp2, the subject of this thesis, is an orthologue of the chicken (G.g.) UBP41 enzyme. (Taken from Baker, 2000).
and His boxes, and contains an Asp-box and a KRF-box. There is no significant sequence similarity between UBPs except in the conserved domains, which facilitate ubiquitin recognition and cleavage. The diversity of UBP sequences is most likely due to their substrate specificity. The structure and function of DUBs have been the subject of recent extensive reviews (Baker, 2000; Wilkinson, 1997; Wilkinson, 2000).

1.3.2 Function and Substrate Specificity

This section (see Table 1.1) summarises data from the last two to three years, regarding substrate specificity of DUBs, their involvement in regulation of certain cellular pathways, organism development, and diseases. Given the diversity of DUBs, they have been divided into groups for ease of discussion, although there is clearly some overlap between the groups.

1.3.2.1 Group 1: Positive regulators of proteasome activity

This group (see Table 1.1) comprises of DUBs whose function is to cleave ubiquitin from proteins, which already have been processed by the proteasome. These DUBs have multiple substrates, and their overall aim appears to balance the activity of all components of proteasomal degradation by maintaining a pool of free ubiquitin, and preventing proteasome clogging by ubiquitin chains.

**Doa4**

Yeast DUB Doa4 is believed to play a key role in releasing ubiquitin chains from proteolytic remnants of proteasome activity in yeast. Doa4 has been shown to interact with the yeast 26S proteasome (Papa et al., 1999). Small ubiquitinated proteolytic remnants of proteasome activity accumulate in Doa4 deficient yeast. In addition, free ubiquitin is depleted in Doa4 mutant cells, due to the accumulation of ubiquitin conjugates. The depletion of the pool of free ubiquitin leads to mis-regulation of proteasome-mediated protein degradation and loss of cell viability (Swaminathan et al., 1999).

Doa4 localises reversibly with the late endosome and is involved in the control of vacuolar protein sorting and in the endocytic pathway (Amerik et al., 2000). Doa4 has
<table>
<thead>
<tr>
<th>Name(s) of DUB</th>
<th>Subcellular localisation</th>
<th>Interacting partners</th>
<th>Substrates</th>
<th>In vivo function and other notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Positive regulators of proteasome activity</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
| Doa4 (Usp in yeast) | ? | Proteasome, Endosome | Multiple substrates | -Plays a key role in deubiquitination of remnants of proteasome activity in yeast.  
-Important for protein sorting in endosomes |
| Iso-T-1 yUbp14/ UbpA/ atUbp14 | ? | ? | ? | -yUbp14 involved in overall disassembly of free ubiquitin chains  
-UbpA - essential for development  
-AtUbp14 - essential for survival  
- Iso-T-1 expression is increased in Down syndrome |
| Iso-T3 | ? | MURF-1/2 (Ring finger proteins) | Possibly multiple substrates | |
| UbpY/Usp8 | ? | Hbp, CDC 25, ARE-mRNA | CDC 25 | -key positive regulator of the proteasome in mammals  
-hUbpY involved in cell cycle and cell proliferation  
-mUbpY interaction with Hbp causes destabilisation of Hbp  
-destabilises ARE-mRNA when overexpressed |
| **Group 2: Negative regulators of proteasome activity** |
| **Subgroup A: substrate-specific DUBs** |
| Usp7/HAUSP | Nuclear, localised in PML nuclear bodies | p53 Vmw110 - (Ring-finger protein) | p53 | -Stabilises p53 tumor suppressor  
-Binding of viral Vmw110 protein to Usp7 enhances viral gene expression |
| Avp (adenoviral DUB) | ? | Histone H2A | Histone H2A? | -removes ubiquitin and USG15 |
| Ubp-M/ Usp16 wt: cytoplasmic; Cys-mutant: Nuclear or associated with chromosomes in mitosis. | Histone H2A | Histone H2A | Is phosphorylated by cdc-2/cyclin B and unphosphorylated during mitosis |
| Usp10 (human) | ? | G3BP | ? | -Involved in Ras signal transduction through interaction with G3BP |
| Usp11 | Nuclear or throughout mitotic cell | RanBPM | RanBPM | -Involved in RanGTPase-mediated pathways  
-Highly expressed in retina tissues |
| Dot4p (yeast) | Nuclear | SIR4p, Gap1 | SIR4p | -Involved in positive regulation of transcription silencing  
-Nutrient uptake |
| Ubp3 (yeast) | ? | SIR4 | ? | -Negative regulator of transcription silencing |
| CreB | ? | CreC (contains WD40-repeat) | CreA | -Plays a regulatory role in carbon catabolism in fungus A nidulans |
### Table 1.1, continued

<table>
<thead>
<tr>
<th>Name(s) of DUB</th>
<th>Subcellular localisation</th>
<th>Interacting partners</th>
<th>Substrates</th>
<th>In vivo function and other notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subgroup B: DUBs in development</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>?</td>
<td>Lqf</td>
<td>Lqf</td>
<td>involved in eye development, may enhance apoptosis, involved in synaptic development</td>
</tr>
<tr>
<td>Fam (Mouse orthologue of Faf)</td>
<td>- Cell-cell contacts, - Dot-like structures in cytoplasm</td>
<td>AF-6 β-catenin, AF-6 β-catenin</td>
<td>-Mouse orthologue of Drosophila Faf, -Important for eye development in mice</td>
<td></td>
</tr>
<tr>
<td>UbpB</td>
<td>?</td>
<td>MEKKα</td>
<td>MEKKα</td>
<td>-control of fruiting body formation</td>
</tr>
<tr>
<td>Ubp41/Usp2</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-Rat Ubp41 is highly expressed in bone tissue, and induced by PTH, -cleaves linear and branched ubiquitin</td>
</tr>
<tr>
<td>Ubp-t Isoforms 1 and 2 (rat Usp2)</td>
<td>Ubp-t1: nuclear, Ubp-t2:perinuclear and associated with centrosome</td>
<td>?</td>
<td>?</td>
<td>-Involved in spermatogenesis, -Expressed in different stages of sperm development, -Have distinct ubiquitin substrate specificity</td>
</tr>
<tr>
<td><strong>Subgroup C: DUBs in disease and immune response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UchL-1 (UCH in yeast)</td>
<td>nuclear</td>
<td>?</td>
<td>?</td>
<td>-Mice with deletion of exons 7 and 8 of UchL-1 gene have a Gad neuro-degenerative disorder, -Ile93Met mutation of UchL-1 is associated with Parkinson disease, -Overexpressed in lung adenocarcinomas</td>
</tr>
<tr>
<td>BAPI (human UCH)</td>
<td>Nuclear, contains 2 putative NLSs</td>
<td>BRCA1</td>
<td>BRCA1 (?)</td>
<td>-Enhances BRCA1 inhibition of breast cancer cell growth, -Removes ubiquitin and USG15</td>
</tr>
<tr>
<td>VDU-1/2</td>
<td>?</td>
<td>VHL (E3 ligase)</td>
<td>?</td>
<td>-VDU1/2 are ubiquitinated and degraded upon interaction with VHL</td>
</tr>
<tr>
<td>DUB-1, -2, 2A</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-cytokine inducible DUBs, -DUB-1 is expressed in B-cells, induced by IL-3, -DUB-2 expressed in T-cells, induced by IL-2, improves cell survival</td>
</tr>
<tr>
<td>Ubp43/Usp18</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-Removes ISG15, but can remove Ubiquitin in vitro, -Expression is upregulated by LPS, -Involved in immune response to bacterial infection</td>
</tr>
<tr>
<td>Usp21/ Usp23</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-Removes Ub as well as NEDD8, -When overexpressed inhibits growth of U2OS cells</td>
</tr>
<tr>
<td>hUBH1/ Usp12</td>
<td>Contains two putative NLSs</td>
<td>?</td>
<td>?</td>
<td>-Highly expressed in cells of neural origin</td>
</tr>
</tbody>
</table>
been shown to deubiquitinate a plasma membrane protein, uracil permease (Fur4) prior to its endosomal degradation (Dupre and Haguenauer-Tsapis, 2001). Several other membrane proteins, such as carboxypeptidase S (Cps1), polyphosphatase (Phm5), and oxygenase (Hmx1) are mis-sorted in Doa4 mutant cells and accumulate in the vacuolar membrane instead of being transported inside of an endocytic vesicle (Reggiori and Pelham, 2001).

**USP5**

Yeast Ubp14 (USP5/IsoT-1 in humans, Ubp14 in yeast, UbpA in Dictyostelium, and AtUbp14 in Arabidopsis thaliana) acts downstream of Doa4 and is involved in overall disassembly of free ubiquitin chains after they have been cleaved from remnants of proteasome substrates. Yeast mutants lacking Ubp14 have defects in ubiquitin chain disassembly and accumulate free ubiquitin chains. Isopeptidase IsoT-1, which performs the same function in human cells, can substitute for Ubp14 in yeast cells (Amerik et al., 1997). It was recently discovered that IsoT-3, a close homologue of IsoT-1, interacts with muscle-specific RING-finger proteins 1 and 2 (MURF-1/2) (McElhinny et al., 2002). Previously it was shown that the RING domain of MURF-1 is sumoylated with SUMO-3 (Dai and Liew, 2001). It can be suggested that IsoT-3 is required for desumolation of MURF-1 or MURF-1 downstream targets. This is an example of IsoT-3 action as a substrate-specific protease.

UbpA plays an essential role in Dictyostelium development. Dictyostelium amoebae grow as separate, independent cells but interact to form multicellular structures when challenged by adverse conditions such as starvation. Deletion of UbpA from cells prevents cell aggregation and fruiting body formation (Lindsey et al., 1998). Doelling and co-workers have shown that AtUbp14 is essential for the survival of Arabidopsis thaliana (Doelling et al., 2001).

**UBPY**

Human DUB UBPY (USP8) can remove Ub from multiple proteolytic remnants of the proteasome. De-regulation of Ubpy in cells causes a substantial overall increase in protein ubiquitination (Naviglio et al., 1998). Consequently, UBPy may play a role
similar to both Doa4 and Ubp14. UBPY has also been shown to be involved in cell cycle regulation and cell proliferation (Naviglio et al., 1998).

Studies by Gnesutta et al. (2001) revealed that mouse UbpY can also act as a substrate-specific enzyme by interacting with Ras guanine nucleotide exchange factor, CDC25, in vivo. CDC25 is known to be ubiquitinated and degraded by the proteasome. Simultaneous overexpression of CDC25, UbpY and HA-tagged Ubiquitin in Hek-293 cells stabilises CDC25. In addition, the same enzyme has been shown to play a regulatory role in endosome-mediated protein degradation by associating with the Hbp protein, which targets growth factor-receptor complexes for degradation through the early endosome (Kato et al., 2000).

A recent report by Laroia et al. (2002) described human UBPY as a DUB, which is able to enhance degradation of mRNAs containing AU-rich destabilising elements (ARE-mRNA) when overexpressed in mammalian cells. It seems most likely that UbpY mediates degradation of proteins that protect ARE-mRNA from degradation.

1.3.2.2 Group 2: Negative regulators of proteasome activity

Subgroup A: Substrate specific DUBs.

USP7

USP7 (HAUSP) has been shown to interact in vivo and stabilise one of the key tumour suppressor proteins, p53. Low p53 levels in the cell are maintained by Mdm2 E3 ligase-mediated ubiquitination. Overexpressed USP7 is able to stabilise p53 even in the presence of overexpressed Mdm2. USP7 thus mediates stabilisation of p53 through its intrinsic deubiquitinating activity (Li et al., 2002a).

Earlier work from Everett and co-workers established that USP7, in herpes simplex virus infected cells, is bound to a viral Ring finger protein Vmw110. Vmw110 is a strong non-specific activator of gene expression playing a critical role in the balance between the viral latent and lytic states. The interaction of Vmw110 with Usp7 stimulates viral gene expression and lytic growth (Everett et al., 1997). It seems likely that binding of Usp7 to Vmw110 inhibits its interaction with p53 thus preventing p53 stabilisation.
Ubp-M (USP16)

Ubp-M is a mammalian USP, which interacts with histone H2A. Ubp-M can be phosphorylated \textit{in vitro} by cdc-2/cyclin B and dephosphorylated during metaphase/anaphase transition. It has been shown \textit{in vitro} (Cai et al., 1999) and \textit{in vivo} (Mimnaugh et al., 2001) that Ubp-M is able to deubiquitinate histone H2A. Interestingly, the wild type Ubp-M is localised in cytoplasm, while the mutant form, lacking the active cite cysteine was not able to deubiquitinate H2A \textit{in vivo}, and was found associated with mitotic chromosomes in the nucleus in all stages of cell cycle.

USP10

The human DUB USP10 has been shown to specifically interact with Ras-GAP SH3 binding protein (G3BP) (Soncini et al., 2001). G3BP most likely is not a substrate for USP10 as it is a long-lived protein, which appears not to be ubiquitinated. However, G3BP and Usp10 are associated with ubiquitinated species, and also were shown to be part of a high molecular weight complex. This implies that G3BP may serve as a bridge between USP10 and its substrate. Interestingly, G3BP is able to inhibit \textit{in vitro} USP10 DUB activity (Soncini et al., 2001).

USP11

USP11 was first identified as a human ubiquitin carboxy-terminal hydrolase, whose gene is localised on the X chromosome, and was therefore named UHX1. It has been shown that USP11/UHX1 is expressed 5-10 fold higher in the retina than in other tissues. Even though no mutations of the \textit{UHX1} gene were detected in patients with retinal disorders, UHX1 was linked to disease states because of its localisation to a region of the X chromosome that contains other genes involved in retinal disorders (Swanson et al., 1996). Another group of researchers has shown that the X chromosome locus of \textit{UHX1} is linked to neurogenetic disorders (Thiselton et al., 2002).

USP11/UHX1 has recently been shown to stabilise RanBPM, the RanGTPase binding protein required for correct nucleation of microtubules (Ideguchi et al., 2002).

Dot4 and Ubp3

Dot4 and Ubp3 yeast DUBs are involved in the regulation of transcription silencing through interaction with the transcription silencing protein Sir4. Sir4 is
required for establishment and maintenance of silencing at the silent mating loci and at telomeres in yeast. Mutant cells lacking Dot4p exhibit reduced silencing and have a decreased level of Sir4 protein (Kahana and Gottschling, 1999). Interestingly, yeast which lack Ubp3 show markedly improved silencing of genes regulated by Sir4 indicating that, in contrast to Dot4, Ubp3 is an inhibitor of gene silencing (Moazed and Johnson, 1996).

More recent work by Kahana (2001) has revealed a role for Dot4p in the regulation of nutrient uptake. This regulation is based on Dot4-mediated stabilisation of the membrane-bound nutrient transporter Gap1, whose activity and concentration is depleted in Dot4p mutant cells.

**CreB**

CreB is a deubiquitinating enzyme from fungus *Aspergillus nidulans* and is related to the human DUB UBH1 (Lockington and Kelly, 2001). CreB has been shown to interact *in vivo* with a WD40-repeat containing protein, CreC. Both proteins are involved in carbon metabolite repression. Mutation of CreB affects the expression of many genes involved in both carbon catabolite repression and derepression pathways. Interestingly, CreC is thought to stabilise and activate the deubiquitinating function of CreB. The DNA-binding protein CreA may be a substrate for CreB. When CreB is not present, CreA gets ubiquitinated and is subsequently degraded, which leads to carbon catabolite derepression (Lockington and Kelly, 2002).

**Subgroup B: DUBs involved in development.**

**Fat Facets (Faf).**

The Faf deubiquitinating enzyme in *Drosophila* is essential for early eye development. Its action limits the number of photoreceptors in each facet to eight. Activity of Faf antagonises that of the proteolytic machinery by preventing degradation of certain proteolytic substrates. One recently identified substrate of Faf is the endocytic protein Liquid Facets (Lqf), a homologue of vertebrate epsin (Chen et al., 2002b). Other findings suggest that Faf, together with several other proteins, is involved in ubiquitin-mediated proteolysis, because it is involved in grim-reaper protein associated apoptosis in *Drosophila* (Wing et al., 2002).
Faf also appears to be involved in the regulation of synaptic development at the *Drosophila* neuromuscular junction (NMJ). The overexpression of Faf leads to a profound disruption of synaptic growth control (DiAntonio et al., 2001).

Interestingly, mutation of the Faf gene can be suppressed by a mutation in the 60-kDa primase subunit of DNA polymerase although the link between these two genes is poorly understood (Chen et al., 2000).

**USP9X and Y**

Human USP9X and USP9Y genes were first discovered as human orthologues of *Drosophila* Faf and were termed DFFRX and DFFRY (*Drosophila* Fat Facets Related X/Y genes) (Jones et al., 1996). Both genes were mapped to regions of sex chromosomes involved in fertility of men and women. Interestingly, a report from Vogt and colleagues have shown that USP9Y encodes for a human male-specific minor transplantation antigen, which facilitates rejection of a bone marrow graft when transplanted from a male to a female (Vogt et al., 2000).

**Fam**

Fam, the mouse orthologue of Faf, also plays an important role in murine eye development. In addition, it interacts and stabilises AF-6 the effector of Ras small GTPase. In turn, AF-6 controls epithelial cell polarity in primitive ectoderm cells. Fam and AF-6 colocalise in cell-cell contacts of epithelial cells (Taya et al., 1998), (Kanai-Azuma et al., 2000).

It has been shown that Fam stabilises another protein, β-catenin. β-catenin plays a dual role in controlling cell-cell adhesion, and facilitating signal transduction to the nucleus during embryogenesis. Fam and β-catenin colocalise in dot-like structures in the cytoplasm of mouse L cells (Taya et al., 1999).

**USP2 (UBP41, UBP45, UBP69, Ubp-t1, Ubp-t2)**

Usp2 was initially isolated from chicken skeletal muscle and was named Ubp41 reflecting its molecular weight (Baek et al., 1997). Ubp41 is the smallest known Ubp consisting only of a catalytic core with no N- or C-terminal extension. Ubp41 is able to cleave linear as well as branched poly-Ubiquitin chains, suggesting a possible role in
recycling free ubiquitin from 26S proteasome proteolytic products as well as generating mono-Ub form linear poly-Ub precursor. Subsequently, a family of three more closely related proteins with distinct N- and C-terminal extensions were identified in chick skeletal muscle (UBP46, UBP52, UBP66; Baek et al., 1998). Another report from the same group described two other UBPs from rats, UBP45 and UBP69, which appear to be the alternatively spliced isoforms with a catalytic core very similar to chicken Ubp41, which have a role in muscle differentiation (Park et al., 2002).

The same two rat Usp2 isoforms (termed Ubp-t1 and Ubp-t2) have been reported to be testis specific. Here, their divergent N-termini target them to different subcellular locations. Ubp-t1 (UBP45) is localised in the nucleus while Ubp-t2 (UBP69) is predominantly in the perinuclear region of cytoplasm and associated with the centrosome. The two isoforms are also differentially expressed during sperm development. Thus, Ubp-t1 is expressed from step 16 to 19 spermatids and Ubp-t2 is from steps 18 to 19 spermatids (Lin et al., 2000).

The divergent N-terminal sequences of Ubp-t1/2 also modulate substrate specificity. The Ubpt-catalytic core removes ubiquitin from testis ubiquitinated proteins more rapidly than Ubp-t1 or -t2. In addition, Ubp-t2 increases the ability of the core to cleave branched triubiquitin in vitro compared to Ubp-t1 (Lin et al., 2001).

A recent report by Miles and co-workers has shown that expression of Ubp41 protein is very high in bones of rats and mice (Miles et al., 2002). Moreover, the Ubp41 mRNA level in bones in rats is stimulated upon treatment with parathyroid hormone (PTH). PTH is a well-known modulator of bone metabolism. The effect of PTH in bone involves the expression of numerous genes such as c-fos, fra-2, c-myc, and IL-6. The end product of PHT action is new bone formation. Clearly, Ubp41 may have an important role in regulating the anabolic and catabolic effects of PTH.

**Subgroup C: DUBs with a role in disease and immune response.**

**Uchl-1, a mammalian ubiquitin carboxy-terminal hydrolase.**

Mice with gracile axonal dystrophy (gad mice) have a mutation in the Uchl-1 gene, which results in deletion of exons 7 and 8, encoding part of the catalytic segment of the protease. Uchl-1 is selectively expressed in the brain and testis in mice. No
expression of Uchl-1 in the nervous system of gad mice was observed, suggesting that the truncated protein is rapidly degraded after translation. Due to the lack of Uchl-1, gad/gad homozygous mice have ubiquitinated dot-like deposits of proteasomes in brain tissues, not present in wild type mice (Saigoh et al., 1999). Therefore, Uchl-1 appears to be essential for proper function of the ubiquitin degradation system, with disruption of a Uchl-1 function leading to neurodegenerative disorders.

Interestingly, an unrelated mutation in Uchl-1p protein (Ile93Met) was detected in a German family with Parkinson disease (Winterneyer et al., 2000), and another recent study revealed elevated expression of UCHL-1 in lung adenocarcinomas in humans (Chen et al., 2002a).

**BAP1 (human UCH, BRCA1-binding protein).**

BAP1 is a ubiquitin carboxy-terminal hydrolase (UCH), which possesses 2 putative NLSs, and localises exclusively in the nucleus of rhabdomyosarcoma cells (Rh30). BAP1 interacts in vivo and in vitro with the Ring finger domain of the breast/ovarian cancer susceptibility protein, BRCA1. The BAP1/BRCA1 interaction enhances BRCA1-mediated inhibition of breast cancer cell growth, probably through BAP1-mediated stabilisation of BRCA1 or BRCA1 interacting proteins. The latter implies that BAP1 may be a tumour suppressor functioning through BRCA1 (Jensen et al., 1998). Interestingly, BAP1 does not interact with naturally occurring mutants of Ring finger domain of BRCA1 (Lorick et al., 1999).

**VDU-1 and VDU-2, human UBPs**

VDU-1 and VDU-2 are 59% identical deubiquitinating enzymes of the UPB family. Both VDUs interact with a component of E3 ubiquitin ligase, VHL, mutations of which are associated with von Hippel-Lindau disease. Both proteins interact with the β-domain of pVHL, leading to their ubiquitination and degradation by the proteasome. Interestingly, the β-domain of pVHL is the region that harbours the naturally occurring mutations in von Hippel-Lindau disease. Some of these mutations have been shown to disrupt VDU-1/2 interaction with pVHL, implying an important role for VDU-1/2 in von Hippel-Lindau syndrome (Li et al., 2002b; Li et al., 2002c).

**DUB1 and 2 (cytokine-inducible murine UBPs)**
Murine DUB1 and -2 play regulatory roles in cytokine-mediated responses. DUB-1 is expressed mainly in B-lymphocytes in response to interleukin-3 (IL-3), peaking in early G1 phase and then rapidly declining. Interestingly, the continuous expression of DUB-1 arrests cells in G1 phase (Zhu et al., 1996). DUB-2 is induced by IL-2 in T lymphocytes (Zhu et al., 1997). DUB-2 expression prolongs IL-2-induced gene activation and suppresses apoptosis induced by cytokine withdrawal, therefore allowing cells to survive (Migone et al., 2001).

**USP18**

The main role of USP18 (Ubp43) appears to be to cleave ubiquitin-like protein ISG15. Deletion of Ubp43 in mice leads to accumulation of ISG15 conjugates in cells, indicating that Ubp43 is the main ISG15-specific proteolytic enzyme (Malakhov et al., 2002).

Ubp43 expression is stimulated by treatment with bacterial cell wall lipopolysaccharide (LPS) which induces monocyte/macrophage inflammatory responses. LPS treatment also increases the level of intracellular ISG15 conjugates. Interestingly, LPS treated Ubp43-/- macrophages exhibited massive accumulation of ISG15 conjugates, suggesting that Ubp43 has a crucial role in maintaining the proper balance of ISG15 conjugation (Malakhova et al., 2002). These findings suggest an important role for Ubp43 in a number of cellular processes including inflammation, stress, and the immune response.

**1.4 Unp Deubiquitinating Enzyme**

**1.4.1 History of Unp**

Mouse Unp (Usp4) was first characterised in 1993, when it was isolated as a cDNA clone during a study of mouse retroviral insertion sites (Gupta et al., 1993). An anti-peptide antibody was raised, which detected a protein of 180 kDa molecular weight with exclusively nuclear localisation with broad tissue distribution, leading to the original name Unp (*Ubiquitous nuclear protein*; (Gupta et al., 1993)d). The original
cDNA sequence led to a deduced protein sequence of 794 amino acids, which was subsequently corrected to be 962 amino acids (Di Fruscio et al., 1998; Frederick et al., 1998). Although Unp was not noted at the time to have sequence similarity to the UBP family of DUBs (the consensus sequence having only recently been derived from yeast UBPs; Baker et al., 1992), it was noted to have sequence similarity to a human proto-oncogene tre-17 (previously known as tre-2; now USP6). Subsequent testing of the Unp cDNA clone revealed that it is an oncogene because it could cause tumours when overexpressed in nude mice (Gupta et al., 1994).

In a study of primary lung tumour tissue, Gray et al. (Gray et al., 1995) observed that the human orthologue of Unp, UNP (USP4), had consistently elevated gene expression levels in small cell tumours and adenocarcinomas of the lung, suggesting a possible causative role for USP4 in neoplasia. UNP was originally reported as a 854-residue protein (Gray et al., 1995) but has subsequently been shown to be 963 residues (Frederick et al., 1998). In a separate study using cell lines rather than primary tumour tissue, UNP protein levels were shown to be slightly but consistently reduced in these cell lines, suggesting that UNP may in fact be a candidate tumour-suppressor gene (Frederick et al., 1998). These authors used a different anti-peptide antisera to that used by Gupta et al. (Gupta et al., 1993), from a region of UNP that is almost identical to the subsequently identified USP15 (see below), and so it is possible that both proteins were being detected.

1.4.2 Unp Homologues

USP15 - human USP15 was originally reported in 1999 and was identified as a close homologue of UNP in expressed sequence tag (EST) databases (Baker et al., 1999). This report also proposed a systematic nomenclature for human UBPs based on the acronym USP (Ubiquitin-specific protease). USP15 is 981 amino acids, and is 61% identical to USP4. The sequence similarity between Usp15 and Usp4 proteins is not uniform over their whole lengths. While the N-terminal ~520 residues of Usp4 and Usp15 are 69% identical, the following ~230 residues are only 30% identical. The remaining C-terminal ~190 residues are 66% identical. This ~230 amino acid "variable region" may contribute to functional differences between Usp4 and Usp15. It may mediate the interaction between different substrates or proteins, and/or alter the
subcellular localisation of Unp and Usp15. A rat orthologue of USP15 has been reported (UBP109; Accession number AF106657) (Park et al., 2000), Orthologues of USP4 can be identified in rat and chicken EST databases (Angelats et al., 2002).

1.4.3 Gene Structure

The mouse Unp gene has been sequenced and found to contain 22 exons distributed over 47.4kb, on a region of mouse chromosome 9 that is syntenic with human chromosome 3p21.3, the location of the human UNP gene (Di Fruscio et al., 1998; Gray et al., 1995). The Unp promoter is very GC-rich and lacks a consensus TATA element, hallmarks of a 'housekeeping' gene promoter, and consistent with the broad range of tissues in which Unp is expressed (Gray et al., 1995). The promoter contains putative binding sites for known transcription factors including several SP1, API and Oct-1 sites, as well as a single E2F site; however, factor binding studies have not yet been performed. The UNP gene undergoes alternative splicing events of exon 7, leading to the presence or absence of 47 amino acids in UNP/Unp, in both human and mouse (Di Fruscio et al., 1998); (Frederick et al., 1998). Thus, in humans, UNP isoforms of either 963 (109kDa) or 908 (104kDa) amino acids are produced, and both exhibit DUB activity in vitro (Frederick et al., 1998).

1.4.4 Unp Biochemical Characteristics

Unp/UNP contain two conservative Cys and His boxes, a feature of ubiquitin specific proteases (Ubps). Unp/UNP has been shown to exhibit peptidase activity against linear natural and artificial ubiquitin fusions in vitro. These include the natural ubiquitin-ribosomal protein fusions (cleavage sites Gly76#Ile in yeast Ubi2p and human UbCEP52; Gly76#Gly in yeast Ubi3p (Layfield et al., 1999; Gilchrist and Baker, 2000), the natural linear polyubiquitin (cleavage site Gly76#Met1, Gilchrist and Baker, 2000), and ubiquitin-human GSTP1 (cleavage site Gly76#Met, Baker et al., 1999). Unp can also cleave the Gly76#Arg bond in a Ub-β-gal fusion protein when the two proteins are co-expressed in E. coli (Gilchrist and Baker, 2000).

It is likely, though not rigorously tested, that Unp is unaffected by the amino acid in the P' position and can cleave ubiquitin followed by any amino acid in a linear fusion, as has been observed for yeast UBPs (Bachmair et al., 1986). One exception is when
proline occurs in the P' position; the ubiquitin-Pro bond is resistant to cleavage in yeast (Bachmair et al., 1986).

Unp/UNP is able to cleave the Gly76#Pro bond in a linear ubiquitin-GSTP1 fusion in vitro, whereas yeast Ubp1p or Ubp2p cannot (Gilchrist et al., 1997); (Baker et al., 1999). Unp is also able to cleave the Gly76#Pro bond in a linear Ub-β-gal fusion protein both in vitro, and when co-expressed in yeast with this substrate (Gilchrist et al., 1997). However, these were non-quantitative assays, and the relative efficiency of Usp4 in cleaving the Gly76#Pro bond versus the Gly76#Xaa bond has not been determined. Usp4 has also been shown to have an isopeptidase activity against a chemically-synthesised Ub-α-amino-Lys fusion (Layfield et al., 1999), and thus has the potential to control the extent of protein ubiquitination.

1.4.5 Unp Interaction with Pocket Proteins

The original report on mouse Unp identified the 'CR1' and 'CR2' sequence elements (LHE and LXCXE, respectively) that are present in proteins that bind to the family of retinoblastoma proteins, pRb, p130, and p107, collectively called, pocket proteins (Gupta et al., 1993). These motifs are also present in human UNP, and human, mouse and rat USP15/UBP109 (Baker et al., 1999; Gray et al., 1995; Park et al., 2000; Angelats et al., 2002). It has recently been demonstrated that Unp/UNP does indeed interact with Rb, p107, and p130 both in vitro and in whole cells (Blanchette et al., 2001; DeSalle et al., 2001). While the physiological consequences of the UNP/Rb family interactions are not fully understood, the interaction is significant, given that Unp acts as an oncogene when overexpressed in mice, and that UNP expression is elevated in specific human lung tumours (Gray et al., 1995; Gupta et al., 1994). There is some evidence that Rb and p130 can be degraded by the ubiquitin pathway (Boyer et al., 1996; Prince et al., 2002), and thus UNP could associate with Rb family members to deubiquitinate and stabilise them. UNP overexpression may disrupt Rb function, in a manner analogous to viral oncoproteins, and displace the E2F transcription factors from Rb.
1.4.6 Unp Subcellular Localisation

The original report on mouse Unp described it as an exclusively nuclear protein and suggested a putative nuclear localisation signal (NLS) (Gupta et al., 1993). However, a subsequent study using an antibody raised against human UNP, employing a cell fractionation approach, and also expressing epitope tagged USP4 protein, led to the conclusion that UNP was predominantly a cytoplasmic protein (Frederick et al., 1998). While Gupta et al (1993) used NIH3T3 cells for fractionation, Frederick et al (1998) used HeLa cells for fractionation and observed that the majority, if not all, of endogenous UNP was located in the cytoplasmic fractions. Frederick et al also transfected myc-tagged UNP into IMR90 cells and observed primarily a whole-cell (cytoplasmic plus nuclear) localisation, and never exclusively nuclear. While these discrepancies can be explained in part by different antibodies, cell lines, artefacts of different cell fractionation techniques, and overexpression of transfected proteins, the trafficking of Unp remains unclear.

1.5 Rb Family of Proteins

The Retinoblastoma protein (pRb) and its close homologues, p107 and p130, are eukaryotic proteins that control cell cycle progression. Collectively, they are called the Rb family of proteins or Pocket proteins (see explanations below). pRb was first identified as a tumour suppressor gene, mutations or deletions of pRb result in the formation of various cancers (Sellers and Kaelin, 1997; Strauss et al., 1995). The tumour suppressor activity of the other two members of the Rb family is less well defined. However, mutations of the p130 gene were identified in several cancers (Cinti et al., 2000; Claudio et al., 2000a; Claudio et al., 2000b). pRb is essential for the survival of mammals since pRb-/- knock out mouse embryos die before birth on day 11-13 of neonatal development (reviewed in Riley et al., 1994). The Rb family of proteins have recently been reviewed (Classon and Dyson, 2001; Kaelin, 1999; Tonini et al., 2002; Yee et al., 1998). Here, I will briefly describe several features of the Rb family including their structure, role in cell cycle control, involvement in other cellular processes, and subcellular localisation.
1.5.1 Structure of Rb Proteins

All three members of the Rb family are closely related, with regions of homology throughout their lengths. The Rb family of proteins also has the name “pocket proteins” because of two “pocket” structures, which are present in all three proteins. A and B pockets respectively, are the defining characteristic of Rb family of proteins. The pocket motifs have high homology between all members of the Rb family of proteins and serve as binding sites for E2F transcription factors as well as for proteins containing an LXCXE conserved motif, which is present in many oncoproteins. All three proteins contain multiple phosphorylation sites, and phosphorylation and dephosphorylation controls the activity of Rb family of proteins. p130 and p107 have higher sequence similarity to each other than to pRb. Thus, when amino acid sequences of Rb family of proteins are aligned, p130 and p107 share approximately 50-55% identity, while pRb and p130 or p107 have only 30% identity to each other. The B pocket of p130 and p107 is separated by a spacer. In addition, both p130 and p107 contain a high-affinity binding site for cyclinA/ckd2 and cyclinE/ckd2 situated between A and B pockets, which is not present in pRb. It is suggested that this cyclin binding site assists in formation of a stable complex with cyclins-ckds. Another feature shared by p130 and p107, but absent from pRb, is a conserved patch of amino acids near the N-termini, which is thought to serve as a cdk inhibitor (see Figure 1.5 A. for a schematic presentation of pRb family of proteins).

1.5.2 Role in the Cell Cycle Control

All three Rb family proteins control cell cycle progression. This control is achieved by blocking the activity of the E2F family of transcription factors. E2F transcription factors activate transcription of certain genes required for entry into S phase of the cell cycle. pRb interacts with E2F factors 1-4, while p130 interacts mainly with E2F4 and 5, and p107 interacts only with E2F4 (see Figure 1.5 B). When pRb binds to E2F factors, transcription is inhibited. Hyperphosphorylation of pRb, by cyclins A/E-ckd2 kinases, in late G1 phase releases E2F factors from the complex enabling free E2F factors to activate transcription (Figure 1.5 D). A similar mechanism controls the activity of the E2F factors, which interact with p130 and p107. However, all three Rb family proteins influence the cell cycle differently. The expression patterns
Figure 1.5 The Retinoblastoma family of proteins.

Panel A represents a schematic diagram of the structure of the pocket proteins. Functional domains and protein binding sites are marked (modified from Yee et al., 1998). Panel B shows interaction between pocket proteins and E2F family members (modified from Johnson and Schneider-Broussard, 1998). Panel C: expression pattern of pRb, p130, and p107 during G1 progression (modified from Classon and Dyson, 2001). Panel D: The interaction of pRb and E2F is regulated by cell cycle-dependent phosphorylation. pRb is underphosphorylated in G0 and G1 and forms stable complexes with E2F/DP heterodimers thus repressing transcription. In late G1 at or near the restriction point (R), pRb becomes hyperphosphorylated and releases E2F/DP complex, which subsequently can activate transcription. Later in S-phase DP becomes phosphorylated which results in inactivation of E2F/DP heterodimer (modified from Kaelin, 1999).
A  

**E2F FAMILY BINDING**

**LXCXE PROTEINS BINDING**

- **pRb**
  - A
  - B

- **p107**
  - A
  - B
  - B

- **p130**
  - A
  - B
  - B

- Cdk INHIBITOR FUNCTION
- CYCLIN A/B KINASE BINDING SITE

B  

**DP1**
- E2F1
- E2F2
- E2F3
- E2F4
- E2F5

- pRb

**DP2**
- p107
- p130

C  

- p107
- p130

Graph showing the relationship between G0, G1, and S phases with p107 and p130 proteins.

D  

- **pRB**
- **Cyclin D**
- Early S
- Late S

Graph showing the progression through the cell cycle with Cyclin D, Cyclin E, and Cyclin A.
of pRb, p130 and p107 during cell cycle progression varies: pRb concentration stays stable throughout the cell cycle with a slight increase towards S phase, whereas the concentration of p107 is very low in G0 stage and it reaches its maximum during S phase. In contrast, p130 is highly expressed during G0 stage and its concentration rapidly decreases and reaches its minimum in S phase (see Figure 1.5 C). Therefore, it is not surprising that p130 stimulates terminal differentiation of cells, while p107 stimulates cell proliferation processes.

1.5.3 Control of Other Cellular Processes by the Rb Family

pRb, p130 and p107 interact with numerous cellular proteins other than E2F factors and cyclin-dependent kinases. pRb alone has more than 100 binding partners, among them histone deacetylases 1-3 (HDAC1-3). This interaction implies that pRb has a role in the chromatin condensation process regulating gene transcription (Cinti et al., 2000). Interaction with differentiation-specific transcription factors like MyoD, NF-IL6 and HBPl (Wang et al., 1997) suggests a role for pRb in the terminal differentiation of tissues such as muscle and nerve. pRb also interacts with p53, a nuclear DNA-binding protein, which controls DNA repair, apoptosis and the cell cycle.

All Rb family members interact with proteins that contain the LXCXE motif. These proteins are either of viral origin (T-ag of SV-40, E7 of HPV, adenoviral E1A) or are cellular oncoproteins, like Unp, or non-oncogenic cellular proteins, such as cyclin E/D/A and HDAC1/2. Interestingly, interaction of E7 or T-ag with the Rb family of proteins disrupts Rb protein function, which significantly alters cell fate. E7 binding to Rb proteins prevents their interaction with E2F factors. This triggers an uncontrolled activation of transcription. Moreover, E7 also targets p130 and p107 for degradation by the ubiquitin-proteasome machinery. The resulting loss of Rb family protein function, in cells expressing E7 or T-ag viral proteins, leads to the formation of cancers (reviewed in McMurray et al., 2001).
1.5.4 Subcellular Localisation of Pocket Proteins.

pRb is known to be a nuclear protein, but it is also detected in the cytoplasm (Chestukhin et al., 2002). Its transport into the nucleus is mediated by a bipartite NLS (amino acids 860-877) (Zacksenhaus et al., 1993) as well as by other NLS-containing proteins associating with its A/B pockets (a piggy-back mechanism) (Zacksenhaus et al., 1999).

Recently it has been shown that p130 is a shuttling protein. It contains multiple NLS signals and a Leu-rich stretch of amino acids, which is able to export a GFP reporter construct from the nucleus to the cytoplasm. Interestingly, this Leu-rich motif is not sensitive to Leptomycin B (see Section §1.6.3.2 below) treatment. (Chestukhin et al., 2002). It has also been shown that subcellular localisation of p130 is cell cycle-dependent since it accumulates in the cytoplasm in early S phase (Cinti et al., 2000).

At present, there is no direct evidence that p107 is also a shuttling protein. However, it has been shown that p107 is one of the cytoplasmic factors involved in the import of the E2F4 transcription factor to the nucleus during cell cycle progression from G1 to S phase (Puri et al., 1998). In addition, Zini and colleagues have reported that p107 and p130 associate with the nuclear matrix. Both proteins are localised in the nucleolus of Saos-2 cells in a complex with E2F4. Interestingly, p130/E2F4 complexes are more abundant during the G0/G1 stage of the cell cycle, while p107/E2F4 complexes are more common in S phase (Zini et al., 2001). This is not surprising since p130 and p107 concentrations peak at G0/G1 and the S phase, respectively.

Amino acid sequence analysis of p107 reveals that it contains a putative bipartite NLS (amino acids 360-375 of human p107) and a Leu-rich motif (amino acids 163-212 of human p107) almost identical to that of p130 discussed above. In addition, I have noted several Rev-like putative NES motifs. Therefore, this raises the distinct possibility that p107 may also be a shuttling protein.

1.6 Nuclear Transport

1.6.1 Structure of Nuclear Pore Complexes

Nuclear pore complexes (NPCs) are the key regulators of the traffic of macromolecules between the nucleus and the cytoplasm of the cell. NPCs are large
proteinaceous structures with molecular weights of 125 MDa in vertebrates and 66 MDa in yeast (Reichelt et al., 1990; Rout and Blobel, 1993). The vertebrate cell nucleus contains approximately 2,000-5,000 NPCs, while the smaller yeast nucleus contains about 200 NPCs (Rout and Blobel, 1993). As the exclusive site for macromolecule transport between the nucleus and cytoplasm, NPCs provide an important control point for proper gene expression, cell growth and progression through the cell cycle. NPC structures of strikingly similar appearance have been identified in all eukaryotes from yeast to human.

During interphase, the only way of entrance to the nucleus is through the NPC. Vertebrate NPCs (vNPCs) are octagonal symmetric structures composed of a cylindrical channel that is attached to an outer rim by eight spokes (Pante and Aebi, 1996; Rout et al., 2000; Wente, 2000). The central channel, spokes, and rim are sandwiched between two rings. From the ring that faces the cytoplasm, eight fibrils of 2-3 nm in diameter and approximately 50 nm in length extend. The nucleoplasmic side of the vNPC is comprised of eight long filaments, about 100 nm in length, that are linked to the another ring structure, forming a basket structure. The basket ring is able to open and close, like an iris, in response to changes in calcium ion concentrations.

The yeast NPC (yNPC) is simpler in structure. Compared to the vNPC, it lacks both cytoplasmic and nuclear rings as well as the lumenal ring of the central spoke domain. Instead, the central transporter is surrounded by an inner spoke ring and a membrane-associated ring. Like vNPC, yNPC contains cytoplasmic fibrils and nucleoplasmic fillaments, in a basket structure. The difference is that the fibrils and filaments of the yNPC are shorter (Figure 1.6).

The proteins which comprise the NPC are called nucleoporins (Nups). Interestingly, in spite of NPCs massive molecular weights, there are only about 30 distinct protein components of yNPC (Fahrenkrog et al., 2000) and vNPC contains about 50 individual Nups (Fahrenkrog et al., 1998). The Nups can be divided into 3 classes: those that span the nuclear membrane (NPC structure-mantaining Nups), non-membrane proteins which contain FG-repeats (F and G are single letter amino acid code), which are involved in trafficking; and non-membrane proteins without FG-repeats.
Figure 1.6 A comparison of the structures of (a) vertebrate and (b) yeast nuclear pore complexes
The spoke complex of the vertebrate NPC is composed of three sections: cytoplasmic and nuclear rings (in blue) and the central spoke ring (green; some details of the spoke ring are omitted from the figure, for simplicity, but are described in the text). The yeast NPC lacks the cytoplasmic and nuclear rings. The red structure in the middle of each NPC is the central transporter. The relative sizes of each component are approximate (figure is taken from Adam, 2001).
Chapter 1

General Introduction

About one-third of NPC mass is composed of members of the FG-family of nucleoporins. Each FG Nup harbours a domain with several GLFG, FXFG, or FG amino acid repeats separated by polar amino acid spacers. 160 copies of FG-repeat domains are present in each NPC. The FG-repeats interact directly with specific HEAT repeats of the mobile transporters discussed in detail below (Bayliss et al., 2000). Although the interaction between one FG repeat and one HEAT repeat is of relatively low affinity, the multiplicity of FG repeats provides many interaction sites.

1.6.2 The Ran GDP/GTP cycle

Ran provides the energy for the nuclear transport of macromolecules. Interestingly, the energy for Ran-facilitated transport comes in GTP not ATP form (Ohno et al., 1998) (Mattaj and Englmeier, 1998). Ran is a member of the Ras family of small monomeric GTPases. Ran-GTPase proteins act in both nuclear import and export. To ensure the directionality of nucleocytoplasmic transport, it is essential that the nuclear form of Ran is always GTP bound (Ran-GTP), and that cytoplasmic Ran is GDP bound (Ran-GDP). This important asymmetry is achieved through Ran regulatory proteins. The nuclear regulator is RCC1, the GDP-GTP exchange factor for Ran (RanGEF). As soon as Ran-GDP enters the nucleus, RCC1 assists the GDP dissociation from Ran allowing subsequent GTP binding (Klebe et al., 1993, 1995). RCC1 is exclusively nuclear through binding to histones H2A and H2B in nucleosomes (Nemergut et al., 2001). Thus, Ran is mostly in GTP-bound form in the nucleus of the interphase cell.

When Ran-GTP leaves the nucleus and reaches the cytoplasm, three other proteins convert Ran-GTP into Ran-GDP. Two Ran binding proteins (Ran-BP1/2) are involved together with the Ran GTP-ase activating protein Ran-GAP. All three cooperate to induce hydrolysis of GTP by Ran. As a result, the cytoplasmic concentration of Ran-GDP is high and of Ran-GTP is low (Figure 1.7).

An enormous number and volume of macromolecules shuttle between the cytoplasm and the nucleus. It is estimated that in a rapidly growing cell about 1 million translocation events happen every minute, most of which are facilitated by Ran. Therefore, Ran-GTP in the nucleus has to be maintained constantly. Ran is small enough to be able to diffuse through the NPC but passive diffusion is too slow to
Figure 1.7 Nucleotide-bound state of Ran is determined by the asymmetric distribution of its regulators. Chromatin-bound RCC1 is the GDP-GTP exchange factor for Ran (RanGEF). RCC1 promotes GDP dissociation from Ran and thereby allows GTP to bind. Because RCC1 binds to chromatin, this results in Ran near chromatin being predominantly in its GTP-bound form. Once Ran leaves the nucleus, two related RanGTP-binding proteins, RanBP1 and RanBP2, cooperate with the Ran GTPase-activating protein (RanGAP) to induce GTP hydrolysis by Ran. The result is that the cytoplasmic RanGTP concentration is low (figure is taken from Kuersten et al., 2001).
maintain the required volume of transport. Ran thus has its own specific transport factor, called Nuclear Transport Factor 2 (NTF2). NTF2 has high affinity for the GDP-bound form of Ran, and can simultaneously bind FG-repeats of nucleoporins, thus providing efficient translocation of Ran into the nucleus (Clarkson et al., 1996; Paschal et al., 1996) (Figure 1.8).

Energy consumption (GTP hydrolysis) is not associated with the translocation through the NPC. The energy of GTP hydrolysis is used to direct the export and import against a concentration gradient. While binding of cargo to RanGDP in the cytoplasm does not require energy, release of the cargo in the nucleus is driven by the energy of binding Ran-GTP. A second energy-dependent process is the disassembly of RanGTP-cargo-importin complexes in the cytoplasm. As a result of the cooperative function of RanGEF, RanBPs, RanGAP and NTF2, the speed of transport is accelerated by 500,000-fold, compared to passive diffusion (Macara, 2001).

While as described above, Ran is involved in nuclear transport during interphase, it also plays an essential role in assembly of the mitotic spindles during mitosis (Nemergut et al., 2002; Clarke and Zhang, 2001). There is also evidence that Ran is involved in formation of the nuclear envelope at the end of mitosis (Hetzer et al., 2000; Zhang and Clarke, 2000). The most unexpected recent finding suggested that Ran plays a role in spindle formation in cells that do not have a nucleus (metazoan cells in mitosis) (Dasso, 2001; Clarke and Zhang, 2001).

1.6.3 Cargo Transporters (Importins and Exportins)

Although its main role is related to the transport of cargo proteins through the NPC, Ran does not interact with the cargo directly. A family of cargo transporters perform this function. The cargo transporters are involved either in import of proteins into the nucleus ("importins") or in export of proteins to the cytoplasm ("exportins").

The importins and exportins are able to form a complex with a cargo on one hand and with Ran-GTP on the other. Another common feature of importins/exportins is that they can interact with the FG-repeats of nucleoporins.
Figure 1.8 NTF2 is a dedicated import receptor for RanGDP.
RanGTP is exported together with an importin-β-like receptor, NTF2, to the cytoplasm, where Ran dissociates and GTP hydrolysis occurs. NTF2 binds specifically to RanGDP and mediates efficient interaction with the nuclear pore complex and translocation into the nucleus. Abbreviation: β-like, importin-β-like (figure is taken from Kuersten et al., 2001).
1.6.3.1 Cargo transporters involved in Import

Importin α

Importin α (Impα), also known as Karyopherin α in yeast (Kap 60), is a 60 kDa adaptor protein, which recognizes classical Nuclear Localisation Sequences (NLSs), (see Section §1.6.4 for more detail). Only one gene encodes Kap α in yeast but there are 6 isoforms in humans, which have different affinities for different NLSs (Malik et al., 1997). Impα interacts with Impβ1 (Kap 95 in yeast) and together they facilitate nuclear import of NLS-containing proteins through the NPC (Chook and Blobel, 2001).

The crystal structure of Impα indicates three structural regions: a central NLS-binding domain with ten Armadillo (ARM) repeats; a smaller hydrophilic C-terminal domain of unknown function; and a positively charged, autoinhibitory N-terminal IBB domain (after Importin β Binding domain) that binds either the central ARM domain or Impβ1. There are two NLS-binding motifs within the central ARM domain of Imp α. The major one is between ARM repeats 2-4, which can accommodate 6-7 residues of the NLS, and the minor one spans ARMs 6-8 (Figure 1.9 B, D-F) (Conti and Kuriyan, 2000; Conti et al., 1998; Fontes et al., 2000). Conserved Trp-XXX-Asn motifs (where X is any residue) are involved in NLS-binding. In the absence of Impβ1, an NLS-like segment of the N-terminal domain binds intramolecularly to the ARM domain, significantly reducing the probability of binding an exogenous NLS (Moroianu et al., 1996). When Impβ1 is present, the N-terminus of Impα binds to it, thus allowing binding of exogenous NLS to ARM domain. Taken together, this data shows that an Impα/β1 heterodimer has much higher affinity for NLS-containing proteins than Impα alone. When the cargo/ Impα/β1 complex enters the nucleus, Ran-GTP dissociates the Imp α/β1 heterodimer, releasing the N-terminal domain of Imp α. The pseudo-NLS of IBB domain replaces the exogenous NLS by intramolecular competition, freeing the cargo (Figure 1.10). The empty Impα has to be exported back to the cytoplasm through a specific mechanism involving the importin β homologue CAS (see Section §1.6.3.2 below).
Figure 1.9 Structures of Impα and Impα-NLS complexes. (a) Ribbon diagram of N-terminally truncated yeast Impα (yImp88-530). Ten ARM repeats are shown in a different colour. (b) Yeast Imp88-530 complexed with two SV40 T-antigen NLSs (NLSs shown in grey). NLS peptides bind at two sites on the concave groove. (c) Ribbon diagram of full-length mouse Impα. Residues 44-54 of the N-terminal Imp domain (in green) form an internal NLS that binds the N-terminal NLS-binding site of the ARM domain. (d) The N-terminal NLS-binding site of yeast Imp88-530 bound to the SV40 T antigen NLS. The NLS peptide is drawn in ball and stick, and its side chains are denoted P1-P5. Interacting Imp residues are presented by the H3 helices (represented by cylinders) of ARM repeats 2-5. (e) The NLS site of the yeast Imp88-530-nucleoplasmin NLS complex. Bipartite nucleoplasmin NLS spans both the major N-terminal and minor C-terminal monopartite NLS-binding sites. (f) The N-terminal NLS-binding site of the yeast Impα88-530-c-myc NLS complex. (g) Autoinhibited mouse Impα, with its internal NLS (Impα residues 44-54) bound in the N-terminal NLS-binding site. All ARM repeat colours are as in (a). Representation and colour scheme for (e) to (g) as in (d) (figure is taken from Chook and Blobel, 2001).
Figure 1.10 Reactions involving Impα, Impβ1, NLS and Ran
Impα is divided into three structural units: a basic autoinhibitory N-terminal domain, a central NLS-binding domain with ten tandem ARM repeats and a small hydrophilic C-terminal domain of unknown function. The Impα autoinhibitory N-terminal domain binds Kap1, relieving autoinhibition and allowing NLS peptides to bind the ARM domain. In the presence of Ran-GTP, a high-affinity Impβ1-Ran-GTP complex is formed, Impα is dissociated from Impβ1, returned to its autoinhibitory state and NLS peptides are released. Impβ1 is unique among the Impβ family in its use of Impα as an adaptor protein. Other members of the Impβ family bind their substrates directly (taken from Chook and Blobel, 2001).
Chapter 1  General Introduction

**Transportin 1 (importin β2)**

There are at least 14 importins β homologues in yeast and more than 20 in mammalian cells. Some of them import proteins to the nucleus (importins) or export proteins to the cytoplasm (exportins). Of these, the crystal structures of two importins: Transportin 1 (also called, Kapβ2) and Impβ1 have been defined.

The crystal structure of the full length transportin 1 bound to Ran:GppNHp, a slowly hydrolyzing analogue of GTP, has been solved (Chook and Blobel, 1999). Transportin 1 is entirely α-helical and consists of 18 HEAT repeats. HEAT repeats are named after proteins in which they were first identified: huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, and the Tor1 kinase, (HEAT). The HEAT repeats bear a striking resemblance to ARM domains of Kap α. This similarity supports the idea that all HEAT and ARM domains are evolutionarily related. Each HEAT domain contains two α-helices that are joined by a sharp turn to form a hairpin structure. The HEAT domains are twisted in a superhelix like an S in which the bottom arch is rotated forward from the surface by 90°. Ran interacts with the N-terminal arch, while the NLS binds to the C-terminal arch. One very important feature is that binding of Ran-GTP and NLS-cargo is mutually exclusive. As a result, cargo binds to the Impβ in the cytosol only (where Ran-GTP concentration is low) and Ran-GTP binds to Impβ in the nucleus (where Ran-GTP concentration is high). This creates a very efficient directionality of nuclear transport.

**Importin β1**

Impβ1 is a more complex type of importin. It is the only importin that is able to transport cargo to the nucleus in conjunction with an adaptor (Impα), while other importins β bind their cargos directly. However, Impβ1 itself has several substrates, like HIV-Rev, GAL4, cyclin B1, parathyroid hormone-related protein (PTHRP), and T-cell tyrosine phosphatase, which it binds directly without Impα (Lam et al., 1999). The crystal structures of the complex between the IBB domain of Impα bound to Impβ1, and a fragment of Impβ1 with Ran have been solved (Vetter et al., 1999; Cingolani et al., 1999); (Figure 1.11). Remarkably, despite the sequence similarity of only 14%, the tertiary structures of transportin 1 and Impβ1 are very similar. Impβ1 is also totally helical, composed of HEAT repeats twisted into a right-handed superhelix. Transportin
Figure 1.11 Structures of carrier complexes with Ran (A), the N-terminal domain of Imp α (B), and a bipartite NLS (C). H1 to H18 identify the HEAT motifs of transportin1 and importinβ. L7 is the loop within HEAT motif 7. Ran (bound to GppNHp, a non-hydrolysable analogue of GTP), Imp α N-terminal domain, and the NLS are shown in white. Note how the HEAT motifs H11 to H18 are twisted up and around the Imp α N-terminal domain, compared to their positions in panel A (39, 263). A1 to A10 identify the ARM motifs, which are closely related to HEAT motifs. Note that the superhelical twist is in the same direction in all three structures and that the ligands all interact with the concave inner surface of the carriers (figure is taken from Macara, 2001).
1 and Impβ1 bind Ran through the first three HEAT repeats and through an acidic loop within HEAT repeat 7 (transportin 1) or 8 (Impβ1). However, the interaction of Ran with either importin does not happen through the same amino acid residues. Even more interesting is that the contact residues on Ran itself differ between the two importins. This binding diversity may confer different affinity of interaction of Ran with importins, which can also regulate the accumulation of cargo against a concentration gradient.

1.6.3.2 Cargo transporters involved in export

Export receptors identified thus far include exportin-t, Msn5p, CAS, Crm1, and Calreticulin. Exportin-t (Los1p in S. cerevisiae) is the export receptor for tRNA (Arts et al., 1998; Kutay et al., 1998). Msn5p is the export receptor for several transcription factors in yeast, including the phosphorylated form of Pho4p (Kutay et al., 1998). CAS (Cse1p in S. cerevisiae) is a dedicated export receptor for importin α (Kutay et al., 1997). Crm1 (Xpo1p in S. cerevisiae) is receptor that mediates export of proteins that contain leucine-rich Nuclear Export Sequences (NESs), originally characterised in Rev and protein kinase inhibitor (PKI) (Fischer et al., 1995). Calreticulin’s primary structure is unrelated to importin β-type exportins, although it has some functional similarity to CRM1 (Holaska et al., 2001).

CRM1

CRM1 is the best-studied mammalian export receptor, although its structure has not yet been solved. Its function is to bring cargo proteins from the nucleus to the cytoplasm. CRM1 requires Ran-GTP for cargo binding, since alone it has a very low affinity to NESs as well as to Ran-GTP. Together all three proteins form a tight trimeric complex (cargo/CRM1/Ran-GTP) (Askjaer et al., 1998). This complex can translocate through the NPC and dissociate in the cytosol through hydrolysis of Ran-GTP. Free CRM1 then returns to the nucleus. It has been shown that CRM1 alone has a lower affinity for FG-Nups, compared to the CRM1/Ran-GTP complex. It is proposed that low affinity of CRM1 alone to FG-Nups stops the receptor from an idle shuttling between the nucleus and the cytoplasm.

The CRM1 specifically recognizes leucine-rich Nuclear Export Signals (NESs) within export cargo. Recognition can be inhibited by an antifungal agent, Leptomycin B. Leptomycin B binds irreversibly to Cys residue (Cys 529) within the NES-binding
domain of CRM1 preventing NES binding and thus completely abolishing CRM1-mediated export from the nucleus. Interestingly, the \textit{S} \textit{cerevisiae} yeast orthologue of CRM1, Xpo1, does not possess an analogous cysteine, enabling resistance of the receptor to Leptomycin B treatment.

In addition, CRM1 has recently been found to use several cofactors. For example, eukaryotic initiation factor 5A (eIF-5A) is essential for Rev and Rev-mediated viral RNA export, mediated by CRM1 (Hofmann et al., 2001). RanBP3 is another CRM1 cofactor, which enhances the binding affinity of CRM1 to NESs as well as to Ran-GTP (Lindsay et al., 2001).

\textit{Calreticulin}

Calreticulin was identified about 25 years ago as an abundant calcium-binding protein of the Endoplasmic Reticulum (ER) (Ostwald and MacLennan, 1974). Recently, however, calreticulin has been shown to function as an export receptor. Structurally unrelated to CRM1, Calreticulin was able to export HIV-Rev and PKI proteins from the nucleus via binding to their Leucine-rich NESs. Like CRM1, Calreticulin assembles into a trimeric complex containing NES-possessing cargo and Ran-GTP, but unlike CRM1, calreticulin-mediated export is not inhibited by Leptomycin B. It has been found that calreticulin mediates the nuclear export of the glucocorticoid receptor (GR) as well. Here, calreticulin binds to the DNA-binding domain of GR, which is believed to act as an NES (Holaska et al., 2001).

Figure 1.12 illustrates import and export cycles of cargo nucleocytoplasmic transport mediated by importin-like transport receptors as well as by Ran-GTPase.

\textit{1.6.4 NLSs, NESs, And Other Cargo Signals}

The transporters described above recognize special stretches of amino acids on the surfaces of their specific cargoes in order to translocate cargo through the central channel of the NPC. If these sequences are hidden or not present, the cargo will not be recognized and therefore will not be translocated to the other side of the NPC.

The signals for nuclear import, NLSs, are recognized by importins, whilst those for export, NESs, are recognized by exportins. Shuttling Signals, which can be
Figure 1.12 Import and export cycles mediated by importin--like transport receptors. Importins (left) bind to cargo molecules in the cytoplasm and mediate interactions with the nuclear pore complex to translocate the import complex into the nucleus. RanGTP in the nucleus binds to the importin and induces cargo release from the complex. The importin/RanGTP complex is then recycled to the cytoplasm, where RanGTP is displaced from the importin by RanBP1 or RanBP2, followed by RanGAP-induced GTP hydrolysis (Fig. 1). An export cycle (right) is similar, the crucial difference being that RanGTP induces cargo binding in the nucleus. Upon removal of RanGTP from the complex by GTP hydrolysis in the cytoplasm, the exportin dissociates from the cargo, and the empty receptor recycles back to the nucleus (figure is taken from Kuersten et al., 2001).
recognized by both export and import receptors, mediate transport in both directions through the NPC.

NLSs can be divided into 2 classes: those which interact with the Impα/β heterodimer, and those which interact directly with importin β family members.

The first characterised NLSs were those recognized by Impα/β complex. They in turn can be subdivided into 3 groups: monopartite, resembling the Simian virus SV-40 T large antigen (T-ag) NLS, bipartite NLSs, and NLSs that resemble that of yeast Mata2. Table 1.2 lists selected examples of these NLSs. Generally, monopartite and bipartite NLSs comprise clusters of lysine and arginine residues, but in bipartite NLSs these stretches are separated by a 7-13 amino acid spacer. The third group is less well-characterised and less well-defined with basic amino acids interspersed with nonpolar residues (Jans et al., 1998).

Importin β1 can use adaptor proteins to bind an NLS, different from importin α. These adaptors in vertebrates include snurportin 1, which is involved in import of m3G-capped small ribonucleoproteins, snRNPs (Huber et al., 1998), and XRIPα, which is involved in the nuclear import of replication protein A, RPA (Jullien et al., 1999). Importin β1 can also form a complex with another importin-α-like factor, importin 7, in order to transport linker histone H1 into the nucleus (Jakel et al., 1999).

In addition, importin β1 is able to interact directly with a large variety of different cargoes, including the human immunodeficiency virus (HIV) TAT and Rev proteins (Truant and Cullen, 1999), ribosomal proteins L23a, S7, and L5 (Jakel and Gorlich, 1998), cyclin B1 (Moore et al., 1999), (Takizawa et al., 1999), Smad (Xiao et al., 2000), PTHrP; (Lam et al., 1999, 2001), CREB and AP-1 transcription factors (Forwood et al., 2001b), and telomere elongation suppressor TRF1 (Forwood and Jans, 2002). Table 1.3 illustrates several examples of the NLSs of proteins recognized by importin β alone.

Remarkably, unlike importin α, importin β does not seem to have a single defined region for NLS binding. Thus, residues 1-380 of Impβ are involved in binding to TRF1 (Forwood and Jans, 2002), while CREB (Forwood et al., 2001b) and the HMG-box-containing chromatin remodelling factor SRY (Forwood et al., 2001a) require the Impβ C-terminus for binding, and PTHrP appears to bind to the central region of Impβ (residues 380-623) (Lam et al., 2001).
Table 1.2 The NLSs recognized by Kapα/Kapβ heterodimer (Jans et al., 1998)

A) Selected Examples of Conventional NLSs Resembling That of T-ag

<table>
<thead>
<tr>
<th>Protein</th>
<th>NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40 T-ag</td>
<td>PKKKRKRV</td>
</tr>
<tr>
<td>h p53</td>
<td>PQPKKKPL</td>
</tr>
<tr>
<td>h c-myc</td>
<td>PAAKRVKLD</td>
</tr>
<tr>
<td>Lamin LI</td>
<td>RQRRNELKRSF</td>
</tr>
<tr>
<td>Lamin B2</td>
<td>VRRTKGKRKRDIV</td>
</tr>
<tr>
<td>Polyoma T-ag</td>
<td>RSSRGKRRRIE</td>
</tr>
<tr>
<td>NF-kB p50</td>
<td>QRKRQK</td>
</tr>
<tr>
<td>NF-kB p65</td>
<td>EEKRRKR</td>
</tr>
<tr>
<td>Yeast histone 2B</td>
<td>GKKRSKA</td>
</tr>
<tr>
<td>hnRNP B1</td>
<td>KTLETVPRLKKREK</td>
</tr>
<tr>
<td>h DNA ligase</td>
<td>PKRRTARKQLPKRT</td>
</tr>
</tbody>
</table>

B) Selected Examples of Bipartite NLSs

<table>
<thead>
<tr>
<th>Protein</th>
<th>NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRb (p110)</td>
<td>KR — 11 a.a. spacer — KKL R</td>
</tr>
<tr>
<td>x nucleoplasmin</td>
<td>KR — 9 a.a. spacer — KKKKL</td>
</tr>
<tr>
<td>Herpes ICP-8</td>
<td>RKR — 14 a.a. spacer — KK</td>
</tr>
<tr>
<td>h c-fos</td>
<td>KRRIRR — 12 a.a. spacer — KRRRL</td>
</tr>
<tr>
<td>hGlucocorticoid receptor</td>
<td>RK — 10 a.a. spacer — RKTKK</td>
</tr>
<tr>
<td>hProgestosterone receptor</td>
<td>RK — 10 a.a. spacer — RKFKK</td>
</tr>
</tbody>
</table>

C) Selected Examples of Conventional NLSs Resembling That of Yeast Mata2

<table>
<thead>
<tr>
<th>Protein</th>
<th>NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mata2</td>
<td>MNKIPIKDLLNPQ</td>
</tr>
<tr>
<td></td>
<td>VRILESWFAKNI</td>
</tr>
<tr>
<td>Hepatitis B virus core protein</td>
<td>SKCLGWLWG</td>
</tr>
<tr>
<td>VirD2 protein (octopine)</td>
<td>EYLSRKGGKLEL</td>
</tr>
<tr>
<td>Influenza virus nucleoprotein</td>
<td>AAFEDLRVLS</td>
</tr>
<tr>
<td>MyoD</td>
<td>VNEAFETLKRC</td>
</tr>
<tr>
<td>Maize R protein</td>
<td>MISESLRAIGKR</td>
</tr>
</tbody>
</table>

h, human; x, Xenopus laevis. Single letter amino acid code used
Proteins, which are to be exported from the nucleus, contain NESs. These sequences are usually rich in leucine or isoleucine residues, but recently many NESs have been discovered which contain other amino acids, like methionine, phenylalanine, and valine. NESs are recognized by export receptors, such as CRM1, calreticulin and others. Several examples of leucine-rich NESs are presented in Table 1.4.

Another class of targeting sequences, called shuttling signals are able to facilitate binding to import as well as export receptors, therefore enabling to be transported in both directions through the NPC. They do not resemble either classical basic NLSs or leucine-rich NESs. The selected shuttling signals of RNA-binding proteins hnRNPs are presented in the Table 1.5.

### 1.6.5 Phosphorylation as a Mechanism to Regulate Nucleocytoplasmic Transport

Nucleocytoplasmic transport can be regulated not only by transport factors and signals within the cargo but also by posttranslational modification of the cargo. One of the most common modifications is phosphorylation, a very powerful mechanism to control the cargo/transport factor complex formation. Phosphorylation can either inhibit or enhance passage through the NPC.

The first evidence for the enhancement of nuclear import by phosphorylation was the observation that the efficiency of nuclear import of SV40 large tumour antigen (T-ag) depends not only on its NLS, but also on the phosphorylation of residues close to the NLS (Rihs and Peters, 1989). Later it has been shown that dual phosphorylation of Ser-112 by protein kinase CK2 and Ser-120 by double-stranded DNA-dependent protein kinase is responsible for 100-fold increase of T-ag nuclear import (Hubner et al., 1997); (Xiao et al., 1997). Nuclear import of another protein, *Drosophila* transcription factor Dorsal, is also enhanced by phosphorylation. In this case, phosphorylation of Ser-312 by cAMP-dependent kinase, 22 amino acids away from the Dorsal NLS, both enhances importin α/β binding affinity to the Dorsal NLS as well as nuclear transport efficiency (Briggs et al., 1998).

Another example of nuclear transport control by phosphorylation is cyclin B1. In this case, however, phosphorylation of cyclin B inhibits nuclear transport. During most of the cell cycle, cyclin B1 resides in the cytoplasm and translocates to the nucleus only
Table 1.3 NLSs Recognized directly by the Importin β (Jans et al., 1998)

<table>
<thead>
<tr>
<th>Protein</th>
<th>NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev</td>
<td>KAVRLIKlyqsnnppnpnegtrqarRNRRRRWR</td>
</tr>
<tr>
<td>Gal4</td>
<td>RLKKLKeckpekckcklknwecRYSRKTKR</td>
</tr>
<tr>
<td>TCPTP</td>
<td>RKRIREDKattaqvqmkqrlneneRKRKR</td>
</tr>
</tbody>
</table>

Table 1.4 Examples of Leucine-Rich Nuclear Export Sequences (Jans et al., 1998)

<table>
<thead>
<tr>
<th>Protein</th>
<th>NES</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 Rev</td>
<td>LQLPPLERLTL</td>
</tr>
<tr>
<td>Adenovirus E4</td>
<td>MVL TREELVI</td>
</tr>
<tr>
<td>NF-AT</td>
<td>GLGAC TLLGSPQ</td>
</tr>
<tr>
<td>MEK (MAPKK)</td>
<td>ALQKKLEELEL</td>
</tr>
<tr>
<td>Gie1</td>
<td>LP LGKLTL</td>
</tr>
<tr>
<td>HTLV Rex</td>
<td>LSAQLYSSSLTL</td>
</tr>
<tr>
<td>TFIIIA</td>
<td>LPVLENLTL</td>
</tr>
<tr>
<td>PKI</td>
<td>LAL KLALDLI</td>
</tr>
</tbody>
</table>

Leucine/isoleucine residues are in bold type.

Table 1.5 Shuttle Sequences of hnRNP Proteins (Jans et al., 1998)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Shuttling Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>h hnRNP A1</td>
<td>NQSSNFGPMKGGNFGGRSGLGGGQYYFAKPRNQGGY</td>
</tr>
<tr>
<td>h hnRNP A2/B1</td>
<td>QQPSNYGPMKSGNFGSRNMGGPYGGNYPQGSQSSGY</td>
</tr>
<tr>
<td>x hnRNP A1</td>
<td>SQSSSFOMFGMGRSNGRSGPGYGGGYGGYGGYGGSSGY</td>
</tr>
<tr>
<td>x hnRNP A2</td>
<td>QQSSNYGPMKSSGSRNMGGPYGGGYGGYGGYGGYGNSQSSGY</td>
</tr>
<tr>
<td>h hnRNP K</td>
<td>YDRRGRPGDRYDGMVQFGSADETWDSAYTWSPSEWQHAMY</td>
</tr>
</tbody>
</table>

h, human; x, Xenopus laevis Bold letters indicate residues whose deletion/mutation oblates targeting function. Single letter amino acids code used.
at the beginning of mitosis. It was first thought that cyclin B possessed a cytoplasmic retention signal (CRS), which anchors it within specific cytoplasmic structures (Pines and Hunter, 1994). Later it was discovered that cyclin B contains a nuclear export signal. Therefore, every time it enters the nucleus, CRM1 exports it back to the cytoplasm, until before mitosis, when cyclin B becomes phosphorylated, which prevents cyclin B/CRM1 interaction (Yang et al., 1998).

### 1.6.6 Translocation through the Nuclear Pore

How exactly transport substrates translocate through the NPC remains unclear. While small molecules and ions can passively diffuse through the NPC channel, it is a rather slow process (estimated about 2 minutes per one passive translocation). For faster rates and larger molecules passive diffusion is not the option. There are 3 models of aided protein translocation through the nuclear pore proposed so far (Rabut and Ellenberg, 2001):

#### 1.6.6.1 The affinity gradient model

The model explains translocation through the NPC by binding of protein complexes to FG-nucleoporins in stepwise manner with increased affinity. Ben-Afraim and Gerace (2001) showed that selected FG-nucleoporins on the cytoplasmic side, central part and nucleoplasmic side of the NPC have increasing affinity to an importin β-IBB complex in vitro with apparent dissociation constants of 200 nM, 100 nM and 10 nM, respectively. If that is correct, then it means that the translocation itself is directional, which disagrees with evidence provided by several independent investigators (see references in Rabut and Ellenberg, 2001) that the passage through the NPC is fully reversible. Furthermore, the high affinity binding of FG-nucleoporins to cargo complexes would slow down the translocational process. Another concern is that in vitro measurements may not reflect the in vivo situation.

#### 1.6.6.2 The Brownian affinity gate model

This model (reviewed in Rabut and Ellenberg, 2001) does not rely on high-affinity interaction (Figure 1.13 A). Instead, Brownian motion and a simple diffusion are proposed to be the translocational forces. The entrance channels on both sides of the NPC are quite narrow so that it is unlikely that large molecules are able to enter the
Figure 1.13 Schematic illustration of Brownian affinity gate (A) and selective phase translocation models (B and C). (A) In the Brownian affinity gate model, translocation occurs through a narrow aqueous channel. A hypothetical import substrate (green), interacting with FG repeat nucleoporins on cytoplasmic filaments is concentrated at the entrance of the channel by constant binding and dissociation (dotted arrows). By contrast, inert molecules (red) that diffuse randomly (solid arrows) are unlikely to enter the channel and are thus excluded from translocation. (B) In the selective phase model, FG repeat nucleoporins (thin black lines) form a meshwork linked by hydrophobic interactions (dark green spots). If a hypothetical large import substrate (blue) (C) can recruit transport receptors or have hydrophobic surface properties (light green), they can dissolve into the FG repeat mesh and be translocated (taken from Rabut and Ellenberg, 2001).
channel, unless they can bind the FG-repeats of nucleoporins. Binding would greatly increase the probability of entering the channel and diffuse through it. The model thus supports the selectivity idea (binding to FG-repeats) and does not rely on high-affinity binding. However, it does not explain the translocation through the NPC of very large particles of up to 36 nm diameter, as well as the fast rate of transport.

1.6.6.3 The selective hydrophobic phase model

Ribbeck and Gorlich (2002) (Figure 1.13 B) propose that the central channel of the NPC is filled with the selective phase, formed by hydrophobic interaction of FG-repeats of nucleoporins with each other. The interacting nucleoporins form a flexible meshwork, which allows small molecules to diffuse freely through the pore but stops larger particles. The larger particles can move through the mesh only if they also can interact with FG-repeats, thus dissolving within the mesh. This model implies that, unlike the Brownian affinity gate model, interaction of transporting complex with the proteins of NPC happens all the way through the channel. What makes the model attractive is that it is based on selectivity, does not contradict high rates of flux of molecules going through the NPC, and can explain the transport of very large particles through the NPC. In a recent report, Ribbeck and Gorlich (2002) showed that nuclear transport receptors are generally more hydrophobic than average cytosolic proteins. They also found that large cargoes could drastically delay the passage through the NPC, unless they are bound to several hydrophobic receptors. Finally, they used small molecules to reversibly disrupt the nucleoporin hydrophobic meshwork, and showed that selectivity in such NPCs no longer existed. All these findings are consistent with the idea of hydrophobic interactions within the NPC.

1.7 Scope of this thesis

The degradation of proteins by the proteasome has been extensively studied over the last 10-15 years which has led to the function of many proteins involved in this pathway being elucidated. The Unp protein, the subject of this thesis, is involved in the regulation of the ubiquitin-proteasome pathway. Being a deubiquitinating enzyme, it is proposed to stabilise proteasome substrates by removing multi-ubiquitin chains attached to their Lys residues. Even though certain biochemical and physiological aspects of Unp
function have been characterised (Baker et al., 1999; Blanchette et al., 2001; DeSalle et al., 2001; Frederick et al., 1998; Gilchrist et al., 1997; Gilchrist and Baker, 2000; Gray et al., 1995; Gupta et al., 1993), very little is known about its subcellular localisation and the mechanism of intracellular transport. Moreover, controversial findings have been reported in the literature concerning Unp subcellular localisation. Initially, Unp was described as being a nuclear protein (Gupta et al., 1994). Subsequently, it was shown to be cytoplasmic (Frederick et al., 1998).

The overall aim of this thesis was thus to resolve this issue and determine the subcellular localisation of Unp in different cell lines. The strategy adopted was to elucidate the mechanisms that regulate Unp nucleocytoplasmic transport by introducing fluorescently tagged Unp into cells and by generating new antibodies that can identify endogenous Unp. The specific aims addressed in this thesis were to: (i) identify and characterise specific modules within Unp involved in Unp trafficking within the cell (NESs and NLSs); (ii) raise antibodies against Unp and its homologue, Usp15, and determine the subcellular localisation of these endogenous proteins, (iii) characterise the transport properties of endogenous Unp; and (iv) investigate whether Unp-binding proteins, pRb, p130, or p107, are involved in Unp nucleocytoplasmic transport. The findings indicated for the first time that Unp is able to shuttle between the nucleus and cytoplasm through distinct NLS and NES sequences, implying specific roles for Unp in both nuclear and cytoplasmic compartments of the cell.
Chapter 2

MATERIALS AND METHODS
2.1 Sources of Reagents, Kits, Bacterial strains and solutions

Common reagents used in media and solutions and their suppliers are listed in Table 2.1. Kits used for this study are listed in Table 2.2. All reagents were of analytical or A grade quality. Bacterial strains and solutions used for bacterial work are listed in Tables 2.3 and 2.4 respectively.

2.2 Standard Laboratory Procedures

Sterilisation of solutions was performed by autoclaving at 121°C/100kPa for 15 min or by filtration through 0.2µm membranes. Plastic disposable laboratory-ware was autoclaved prior to use and sterile glassware was baked at 180°C overnight. Distilled deionised water (ddH₂O) was used for all solutions. All recombinant DNA procedures were performed under PC2 laboratory conditions as stipulated by the Genetic Manipulation Advisory Committee of Australia or the office of the Gene Technology Regulator, and radioactive substances were used and disposed of in accordance with the Australian National University Radiation Safety Handbook.

2.3 DNA Manipulation methods

2.3.1 Bacterial Cultures

Bacterial strains were grown from glycerol stocks by streaking onto a Luria broth (LB) plate (containing the appropriate antibiotic) and grown for approximately 16 hours at 37°C. Single colonies were selected for liquid culture (5ml-400ml depending on preparation) and grown for 8-16 hours at 37°C, selected again by the appropriate antibiotic (100 µg/ml ampicillin or 25 µg/ml kanamycin).
2.3.2 DNA Preparations

All plasmids used here and their descriptions are listed in Table 2.10. DNA vectors were purified from bacterial strains using Geneworks Minipreps, Wizard Maxiprep, and Wizard PureFection DNA Midi/Maxiprep according to the manufacturer's instructions.

2.3.3 Restriction Endonuclease Digestions, Phosphorylation and Dephosphorylation of DNA

T4 Polynucleotide Kinase and Calf Intestinal Phosphatase were supplied by Pharmacia Biotech; restriction enzymes were supplied by New England Biolabs and Pharmacia Biotech. All these enzymes were used with the supplied buffers under the suggested incubation conditions (incubation at 37°C for 15-30 minutes).

2.3.4 Agarose Gel Electrophoresis

Electrophoretic separation of DNA was carried out using 0.7-2.0% agarose gels in 1xTAE containing 0.5µg/ml ethidium bromide, or 1/4xTBE buffers (see Table 2.5). Purification of DNA excised from the gel was carried out using the QIAquick Gel extraction kit (Qiagen).

The concentration of DNA solutions was approximated by comparison to the ethidium bromide fluorescence of standards of known concentration and molecular weight run on the same agarose gel or by measuring the absorbency of solutions at 260nm (1 A_260 unit = 50mg/ml DNA) using a CARY 1 UV-Visible Spectrophotometer (Varian, Australia).

2.3.5 Basic Cloning

Ligation reactions typically used 100ng of vector at vector to insert molar ratio of 1:1 to 1:5. Ligation reactions were carried out by first incubating DNA at 65°C for 2 minutes, followed by cooling on ice. T4 DNA ligase (3 units, Pharmacia Biotech), and 5X ligation buffer (250mM Tris/HCl, 50mM MgCl_2, 25% w/v PEG_8000, 5mM ATP, 5mM DTT, pH7.6) were added to the DNA, made to 10-15µl with ddH_2O and incubated at room temperature for 2-4 hours or overnight at 16°C.
**2.3.6 Oligonucleotide Primers**

Oligonucleotide primers for PCR, sequencing, and mutagenesis were synthesised by the GenSet Pacific Oligos (Australia), and purified using Reverse Phase Cartridge or desalting columns. Primers were supplied in distilled water, ready to use. All primer sequences and their descriptions are provided in the Table 2.11.

**2.3.7 Annealing double-stranded oligos**

The primers designed for creating of double-stranded oligos were first phosphorylated as described in §2.3.3. To anneal the oligos to each other, the mixture (40pMol of each oligo in 1x NEB#2 restriction enzyme buffer (New England Biolabs) in a total volume of 120 /µl) was incubated for 5 minutes at 72°C and gradually cooled down to RT. The double-stranded, phosphorylated oligos were ligated into appropriate restriction enzyme digested and de-phosphorylated vector.

**2.3.8 Automated Sequencing**

Double stranded DNA sequencing was carried out using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The Reaction mixture (final volume 15 µl) contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>200-500 ng</td>
<td>DNA template</td>
</tr>
<tr>
<td>6.0 µl</td>
<td>Terminator Ready Reaction Mix (labelled dye terminators, dNTPs, MgCl₂, AmpliTaq DNA polymerase, Tri₅-HCl pH9.0)</td>
</tr>
<tr>
<td>2.0 pmol</td>
<td>Primer</td>
</tr>
<tr>
<td>Sterile ddH₂O</td>
<td></td>
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</tbody>
</table>

Reactions were cycled on a Corbett Research PC960C Cooled Thermal Cycler at: 25 cycles of 96°C for 30secs, 50°C for 15secs, 60°C for 4mins; end at 4°C.

Extension products were purified by adding 2.0µl of 3M NaOAc (pH4.6) and 50µl of 95% EtOH. The mixture was vortexed briefly then placed on ice for 10mins. The tubes were centrifuged for 30mins at 12000rpm and the pellet washed with 250µl 70% EtOH, vortexed and centrifuged again for 5mins. The pellet was dried at room temperature for 10mins.
Sequencing products were separated and analysed at the Biomolecular Resource Facility, JCSMR, Australian National University using the ABI Model 377 Automated DNA Sequencer.

2.3.9 Polymerase Chain Reaction (PCR)

Amplification of DNA was conducted in 50µl reaction volumes performed on a Corbett Research PC960C Cooled Thermal Cycler. Typical reactions contained *Pfu* Turbo DNA polymerase (2.5 units), 1X *Pfu* turbo DNA polymerase buffer, 0.2mM of each dNTP, 1-3 pMoles of each primer, and 5-10 ng plasmid DNA. Optimal conditions for each primer-set were determined experimentally. Typical amplification cycles were:

- 95°C 2 mins
- 1 cycle
- 95°C 30 secs *(denature)*
- 52-65°C 30 secs *(annealing)*
- 72°C 1-5 mins *(extension)*
- 30-35 cycles
- 72°C 2 mins
- 4°C end

Variation of annealing temperature depended on primer sets. Variation of extension times depended upon the length of product. Oligonucleotide primers used for PCR are listed in Table 2.11

2.3.10 Site Directed Mutagenesis

Site directed mutagenesis was performed using the Quikchange Mutagenesis Kit (Stratagene). This technique utilises the double stranded vector containing the insert and two synthetic complementary oligonucleotides containing the desired mutation. The oligonucleotides extend during PCR using *Pfu* Turbo DNA polymerase. The incorporation of the nucleotides based on the primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with the endonuclease *Dpn* 1, which is specific for methylated and hemimethylated DNA and therefore only digests the template DNA. The nicked vector mutant DNA is then transformed into *E.coli* (XL-1 blue strain) which repairs the nick and replicates the
DNA. *E.coli* transformed with the plasmid is selected by plating on LB plates containing specific antibiotic.

Successful colonies were cultured and the vector DNA isolated by minipreps. Successful nucleic acid mutagenesis was confirmed by sequencing over the site of the mutations.

**Mutagenesis reaction mixture:**

- 5-10 ng DNA template
- 1X Reaction Buffer (supplied)
- 2-5 Units *Pfu turbo*
- 125 ng Each primer
- 1 µl dNTP Mix (25mM)
- To 50 µl ddH$_2$O

Thermal cycling performed on a Corbett Research PC960C Cooled Thermal Cycler under the following conditions:

- $\triangleright$ 95°C 30secs
- 1 cycle
- $\triangleright$ 95°C 30secs
- $\triangleright$ 55°C 1min
- $\triangleright$ 68°C 2mins/kb
- 12-18 cycles
- $\triangleright$ 4°C end

*DpnI* digestion and transformation of XL-1 Blue *E.coli* were carried out following the kit instructions.

Oligonucleotide primers used for mutagenesis are listed in Table 2.11.

**2.3.11 GATEWAY cloning technique**

A number of the DNA constructs used in this study were created by using the GATEWAY technology. The GATEWAY cloning system (GibcoBRL), an alternative to the classic cloning, uses phage Lambda-based site-specific recombination instead of restriction endonuclease digestion and ligation. The key DNA recombination sequences (*att* sites) and enzymes (recombinases) that mediate the recombination reactions are the foundation of the GATEWAY technology. The main difference between GATEWAY
and classical cloning is that once the DNA segment is in the system, it can be transferred between vectors in one-step reaction. The high level of positive clones is achieved through the combination of the antibiotic selection and use of the ccdB-gene, which is lethal for bacteria strains that do not contain a ccdA gene (eg DH5α).

Briefly (see Figure 2.1), the DNA fragment is firstly amplified by PCR reaction using primers, which contains the 25 base pairs (bps) of attB-sites on 5’-end and 18-25 gene-specific bp on their 3’-ends. The PCR reaction and all following reactions (BP and LR) were done as described in the Instruction Manual for GATEWAY cloning (GibcoBRL)

Secondly, the PCR product is mixed with the Donor vector (pDONR201) in appropriate buffer with addition of appropriate enzyme mix (BP reaction). The pDONR201 vector contains a ccdB gene flanked by attP sites, and Kanamycin resistance gene. During the BP reaction the ccdB gene recombines/switches with PCR product, creating a so-called Entry vector (a Donor vector, containing a PCR product instead of a ccdB cassette). The product of the BP reaction is transfected into DH5α cells and seeded on LB/Kanamycin plates. Only cells containing the recombination product can grow, all others are not able to grow either because of the antibiotic selection or ccdB gene expression.

Thirdly, the Entry vector is recombined with ccdB cassette of a number of Destination vectors (pDESTs), creating the final product, the Expression clone (a desired DNA fragment in the appropriate expression vector). This reaction is called LR. The pDESTs contain ampicillin resistance gene (AmpR) and the ccdB cassette. The DH5α cells are again transformed with the LR mix and plated on LB/Ampicillin plates. Only Expression clone-containing cells can grow as they have AmpR and no ccdB gene.

The vectors and primers used in GATEWAY cloning are listed in Tables 2.10 and 2.11, respectively.
**Figure 2.1 GATEWAY cloning system.**
The gene of interest is amplified by a PCR with attB primers to create a GATEWAY-compatible PCR product planked by attB sites (a). Site-specific recombination between the PCR product and a Donor vector (pDONR201) (b) in BP reaction, creates an Entry clone (c) and a by-product. Transfection of bacterial cells, like DH5α, with the BP-reaction mix, and their growth on kanamycin-containing agar results in survival of Entry clone-containing bacteria only (d) (see text for more details). The entry clone can be recombined in LR reaction with a Destination vector (e) (see text for details) in order to obtain the final product, the Expression clone (f). Transfection of DH5α cells with the LR mix and subsequent growth of bacteria on ampicillin-agar provides selection of cells containing the expression vector (g).
(a) **PCR product**

(b) **Donor vector**

(c) **Entry clone**

(d) 

(e) **Destination vector**

(f) **Expression clone**

(g) 

**Transfect DH5α cells, Select on Km**

**Transfect DH5α cells, Select on Amp**
2.4 Protein manipulation methods

2.4.1 Purification of recombinant proteins

2.4.1.1 Small-scale protein expression

A single colony containing a plasmid, encoding a gene of interest was grown in 5 ml of LB+ Amp overnight. Next morning 100µl of the overnight culture was inoculated into 2ml fresh LB+ Amp, and incubated at 37°C for 1-2 hours with shaking. To induce the protein expression IPTG was added to the culture at different concentrations and the bacteria incubated further for 0.5-3 hours at different temperatures, depending on the experiment (see Section §4.3.2.3). Then the cells were spun down and either resuspended in 3x sample loading buffer (the total cell lysate) or resuspended in 200µl of PBS/ 0.25mg/ml lysozyme / 1mM DTT, and incubated on ice for 5 mins prior to ultrasonication. The sonicated lysates were spun down, with supernatants representing the soluble fraction, pellets represented the insoluble fraction. All fractions were analysed by SDS-PAGE.

2.4.1.2 Large-scale protein expression and purification

The bacteria containing plasmids encoding GST-Unp(var) or -Usp15 (var) recombinant proteins were grown overnight in 10 mls of LB+Amp at 37°C, then all of the overnight cultures were inoculated into 1 L of LB+ Amp and grown another night at 37°C. Next morning 1 L cultures were inoculated into 10 L of pre-warmed (27°C) LB+ Amp and grown for 3 hours. Then IPTG was added to a final concentration of 0.01mM and cultures were incubated at 27°C another 2 hours. The spun cells were resuspended in 1 L of TN buffer (see Table 2.7) each with protease inhibitors added (Table 2.7a). A large volume of resuspension buffer was required because the pellets were very viscous and it was impossible to resuspend them in a smaller volume. The cells were lysed by a French press. Lysates were cleared by centrifugation at 5,000 g and the supernatants were incubated with GSH agarose beads (3 mls of 50% slurry) with slow stirring for 3 hours at 4°C. The beads were washed 5 times with 50 mls of PBS/1mM β-Me and the bound proteins were eluted 3 times with 2 mls of 5mM GSH in TN buffer. The eluates were analysed by SDS-PAGE.
2.4.1.3 Medium Scale protein purification

2.4.1.3.1 Purification of pGST-Rb (379-928)

The purification of GST-Rb was based on a method described by Kato et al and modified by Zarkowska, et al (Kato et al., 1994; Zarkowska et al., 1997).

The plasmid expressing the truncated Rb protein (amino acids 379-928), fused to the C-terminus of GST in pGEX-2T plasmid) was transfected into protease deficient bacterial strain BL21(DE3). A single colony was grown in 400 ml (LB+Amp) media at 37°C until OD$_{600}$=2. Then cells were induced with fresh IPTG to final concentration of 100 µM and grown at 23°C for 18 hours. The pellet was resuspended in “GST-Rb Lysis” buffer (see Table 2.7) and lysed by repeated freezing thawing. Clarified lysate was bound to GSH beads, which were prewashed in buffer “GST-Rb Lysis”. Following 2-hour binding on a wheel at 4°C, the beads were washed 5 times with “GST-Rb Washing” buffer (see table 2.7) and protein was left on beads at 4°C for several weeks. To prevent bacterial growth, Na-azide was added to the solution.

2.4.1.3.2 Purification of β-galactosidase fusion proteins

The MC1060 bacterial strain was transformed with pPR2all-based construct in order to express β-galactosidase fusions. The bacteria were grown all day in 5 ml of (LB+Amp), then inoculated in 500ml (LB+Amp) media, induced with freshly made IPTG (1mM final concentration) and incubated overnight in a 37°C shaker. The pellet was resuspended in 5 mls of “β-gal Lysis” buffer without NaCl (see Table 2.7). The cells were lysed by repeated ultrasonication (3 times, 4 minutes each) and lysed solution was clarified by centrifugation. After NaCl (dry powder) was added to a final concentration of 1.6 M, the lysate was added to pre-washed APTG-agarose (4-aminophenyl-β-D-thiogalactopyranoside, Boehringer Mannheim) and incubated 30 minutes at RT. The beads with bound β-galactosidase-fusion proteins were washed overnight with 2 litres of “β-gal Washing” buffer (see Table 2.7) and 15-20 fractions were eluted with “β-gal Elution” buffer (see Table 2.7). 250 µls of 2M Tris, pH7.0 were added to each 750 µl-fraction of eluted protein to neutralise the high pH of the elution buffer and the fractions were stored at -20°C or dialysed against Dialysis buffer (see Table 2.7) overnight at 4°C.
2.4.1.3.3 Purification of 6xHis-fusion proteins

The 6xHis fusion proteins were expressed in BL21(DE3) cells. All buffers used for this procedure are listed in the Table 2.7. A single colony was enoculated in 20mls of (LB+Amp) and grown overnight at 37°C with shaking. Overnight culture was then added to 400mls of 30°C-warm (LB+Amp) and grown at 30°C until OD$_{600}$=0.6-0.8. Induction of fusion protein expression with 0.1-0.5mM IPTG was performed for 4-5 hours at 30°C. The spun down cells were frozen overnight at -70°C and resuspended next morning in “6xHis Lysis” buffer (10 ml per 1g of wet cells) with addition of dry lysozyme at 1/11 g/ml. To lyse cells completely, ultrasonication was performed (10sec, 6 times, with 50sec rest between sonications). Clarified lysate was bound to pre-washed Ni$^{2+}$-agarose for 2 hours at 4°C. The protein bound to the agarose was washed 6 times with “6xHis Washing” buffer, eluted with “6xHis Elution” buffer, and then dialysed overnight against PBS/1mM β-Me-Ethanol. The dialysed protein was stored at -20°C.

2.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis and Western blotting of recombinant proteins and cell lysates were performed using buffers and solutions listed in the Table 2.6.

SDS-PAGE was performed according to Laemmli et al (Laemmli, 1970). Gels consisted of differing resolving gel concentrations with a 5% stacking gel and the components of the resolving and stacking gel buffers are given in Table 2.6. Samples were boiled for 5 min in Laemmli loading buffer prior to loading and electrophoresis was carried out using the X-Cell II™ Mini-Cell (Novex, USA). To visualise separated proteins, gels were stained in Coomassie-blue stain and destained in the Distaining solution (see Table 2.6). For visualisation of radiolabelled proteins, gels were fixed in 10% (v/v) acetic acid/25% (v/v) methanol for 30 min, soaked in EN$^{3}$HANCE™ (DuPont NEN®, USA) for 60 min then rinsed in ddH2O for 30 min before being dried at 70°C under vacuum and exposed to X-ray film. Immunoblotting of SDS-PAGE gels was carried out as described below (§2.5.3).
2.4.3 In vitro phosphorylation of Unp by Cyclin A/cdk-2 complex

Phosphorylation of 6xHis-Unp in vitro was performed in a 30 µl consisting of 20 mM Tris–HCl pH 7.5, 0.2 mM DTT, 10 mM MgCl2, 20 µM ATP and 10 µCi of \( \gamma^{32P} \)ATP for 30 min at 37°C in the presence or absence of GST-cyclinA/cdk2 complex (the details of GST-cyclin A and cdk2 cloning and purification are described in Sarcevic et al., 1997). Reactions were terminated by the addition of 15 µl of 3x stop buffer (187 mM Tris–HCl pH 6.8, 30% (w/v) glycerol, 6% SDS, 15% β-mercaptoethanol). The samples were then heated at 100°C for 2 min, centrifuged and electrophoresed on a 12% SDS–polyacrylamide gel. Following electrophoresis, proteins were stained with 0.5% Coomassie Blue, the gel dried under vacuum and exposed to X-ray film for autoradiography.

2.4.4 GST-pRb pull-down assay

Interactions between the pRb and RFP-Unp wt, or RFP-UnpL459Gmut proteins were determined in vitro using GST-pRb fusion protein. GST-pRb bound to GSH-agarose beads was prepared as described above (Section §2.4.1.2). 100µL of 50% slurry (beads to buffer ration is 1:1) beads were mixed with total HeLa cell lysate prepared as described in Section §2.6.3 and diluted to 1 ml with HeLa lysis buffer and rocked for 1-2 hours at 4°C. Following binding, beads were washed 1 time with 1ml of ice-cold PBS with added protease inhibitors, then washed 2-4 times with PBS alone. The beads were then recovered by centrifugation, the supernatant was discarded and beads were resuspended in 20 µL of Laemmli loading buffer and analysed by separation on 9% SDS-PAGE and Western blotting. Blots were probed with rabbit anti-Unp antibodies (1:1000 dilution), followed by anti-rabbit IgG HPR-conjugated (1:4000 dilution) and ECL detection as described in Section §2.5.3.

2.5 Immunohistochemistry methods

2.5.1 Rabbit immunisation

New Zealand female rabbits were immunised with antigen as described below:
Chapter 2

**Materials and Methods**

<table>
<thead>
<tr>
<th>Event</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary injection (day 1)</td>
<td>150 μg of antigen mixed with Freund’s Complete solution in ratio 1:1</td>
</tr>
<tr>
<td>First booster (day 8)</td>
<td>150 μg of antigen mixed with Freund’s Incomplete solution in ratio 1:1</td>
</tr>
<tr>
<td>Second Booster (day 15)</td>
<td>150 μg of antigen mixed with Freund’s Incomplete solution in ratio 1:1</td>
</tr>
<tr>
<td>First Bleeding (day 22)</td>
<td>5 ml of blood collected.</td>
</tr>
<tr>
<td>Second Bleeding (day 29)</td>
<td>10 mls of blood collected</td>
</tr>
</tbody>
</table>

Serum was separated from the collected blood and used for either western blotting or for affinity purification of the antibody.

2.5.2 Affinity purification of antibodies from crude antiserum by using antigen immobilised on Immobilon-P transfer membrane

The purified recombinant 6xHis-Unp (2-3 μg) was run on 10% SDS-PAGE gel and transferred to Immobilon-P transfer membrane as described in Chapter 2. The membrane was then soaked in 0.5% Ponceau S / 1% acetic acid solution to reveal the band of the antigen protein (full-length 6xHis-Unp). The strip containing the antigen protein was cut out and blocked in 3% BSA/0.02% sodium azide/PBS for 1 hr at RT. The crude serum containing the Unp-specific antibody was then incubated with pre-blocked membrane on a rocking platform overnight at 4°C. To wash off unbound proteins, the membrane was rinsed in 0.15M NaCl for 20 minutes and then with PBS for 20 minutes on a rocking platform at RT. Then a small volume (200-400 μl) of elution buffer (0.2 M glycine (pH2.8) / 1mM EGTA) was added to the membrane for 20 minutes at RT. The elution buffer containing the antibody of interest was then neutralised by adding 1/10 volume of 1M Tris base. The pH was confirmed to be approximately 7.0 and 1/10 volume of 10xPBS was added to the solution. The purified antibodies were stored in 0.02% Sodium Azide at 4°C or in 50% glycerol at -20°C. The same procedure was utilised to purify the Usp15-specific antibody, where 6xHis-Usp15 was used as the antigen protein to purify antibody from the Usp15-specific crude serum.
2.5.3 Western Immunoblotting

Western blots were prepared by electroblotting of proteins from SDS-PAGE gels onto Immobilon™-P Transfer Membrane, wetted with methanol followed by transblot buffer, using the X-Cell II™ Blot Module (Novex, USA) filled with transblot buffer. Transfer was conducted at 30V for 1.5 to 2 h. Following transfer the filter was rinsed in PBS buffer and air-dried for storage overnight at 4°C. All the following procedures were performed at RT and incubations done using a rocking platform. The buffers used for Western blotting are listed in the Table 2.5. The filter was re-wet with methanol and rinsed with PBS prior to immunological detection. In order to block unspecific binding of antibodies the membrane was incubated in Blocking buffer for 1 hour. The primary antibodies were diluted 1:1000 in Antibody Incubation buffer and added to the membrane for 1 hour. The washing was performed in the Washing buffer with NaCl for 50 minutes with vigorous shaking and then in Washing buffer without NaCl for three times, 5 minutes each. Secondary antibodies (anti-rabbit, HRP-conjugated, DAKO) were diluted in Antibody Incubation buffer in 1:4000 ratio and added to the membrane for 1 hour. The washing was performed 3 times in Washing buffer without NaCl, 15 minutes each. The detection was achieved by using an ECL reagent 1 and 2 (Amersham) mixed in 1:1 ratio and applied to the membrane for 1 minute. After the membrane was blotted dry it was exposed to the X-ray film for 1 minute to 2 hours, depending on strength of the signal.

2.5.4 ELISA protein binding assay

The binding of bacterially expressed GST-tagged mouse importins (a gift from David Jans, Nuclear Signalling Laboratory, JCSMR, ANU) to Unp NLS-β-galactosidase fusion proteins, was assessed using the ELISA-based binding assay (Efthymiadiis et al., 1998; Efthymiadiis et al., 1997; Hubner et al., 1997; Xiao et al., 1997). The β-galactosidase fusion proteins were incubated in 50 mM NaHCO₃ (pH 9.8) and coated into the wells of polystyrene microtiterplates (Nunclon) in triplicate at 0.5 μg/well for 16 h at 4°C. The non-specific binding to the wells was blocked by addition of 400 μl of 1x IB buffer (110 mM KCl; 5 mM NaHCO₃; 5 mM MgCl₂; 1 mM EGTA; 0.1 mM CaCl₂; 20 mM HEPES, pH 7.4) containing 1% BSA for 1 h at RT with shaking (Platform rocker). The unbound protein was then washed off 4 times with 1x IB with the
third wash being incubated for 1 h at RT as above. Serial dilutions of importins were added to the microtiterplates in 1X IB/1 mM DTT/1% BSA buffer for 16 h at 4°C. After extensively washing the microtiterplates with 1x IB containing 1% BSA, goat GST-specific antibody (500 ng/well) was incubated for 3 h and then washed 10 times with PBS containing 0.3% Tween 20 (Bio-Rad). Alkaline phosphatase-conjugated rabbit anti-goat IgG (0.025 units of enzyme/well) was incubated for 1 h at RT and washed as described for that of the primary antibody. Binding activity was determined using the chromogenic substrate para-nitrophenyl phosphate (p-NPP, 1 mg/ml, Sigma) dissolved in 10% diethanolamine and 0.5 mM MgCl₂ (pH 9.8). The change of absorbance at 405 nm was followed over 90 min using a plate reader (Molecular Devices, Menlo Park, Ca, USA), with values corrected by subtracting both the absorbance at 0 min, and that in wells incubated without importins. Data were collected and stored using Softmax software program (Macintosh).

To correct for differences in coating, UnpNLS β-gal, T-ag-NLS-β-gal fusion proteins, and β-gal were subject to a parallel β-galactosidase ELISA assay using an anti-β-gal specific mouse monoclonal antibody (40µg/well, Promega) together with an alkaline phosphatase conjugated anti-mouse antibody (0.0158 units of enzyme/well, Sigma). All manipulations were comparable to those for GST fusion proteins (see above). The correction factor per pmol of NLS carried by β-gal fusion proteins coated on the plate was obtained by subtracting its absorbance at 0 minutes of β-gal followed by correction for the molecular weight of each β-gal fusion protein. The correction factor was used to modify the absorbance values from the above ELISA assay and enable correction of any differences in coating to make a true estimate of bound importins per pmol of β-gal fusion protein.

Processing of the ELISA raw data and correction of values (see above) were carried out using Microsoft Excel software (Eftymiadiis et al., 1997; Hubner et al., 1997). Binding affinity of proteins or peptides to importins subunits were evaluated by plotting the corrected absorbance for different importin concentrations against time using the Kaleida Graph 2.13 software (Macintosh). The data in the linear range were used to obtain the OD change per min (OD/min) which was plotted against the concentration of importins. Curves obtained were fitted using the function as shown:

\[ B(x) = B_{max}(1 - e^{-kx}) \]
Kd the apparent dissociation constant, representing the concentration of importins yielding half maximal binding, was calculated from the above equation.

### 2.6 Mammalian Cell Culture Methods

#### 2.6.1 Cell Culture

Mammalian cell lines were maintained in the culture media, described in Table 2.8, at 37°C in 5% CO₂ with a humidity solution of 0.025% (w/v) benzalkonium chloride (Sigma Chemical Co.) and 0.0025% SDS in sterile H₂O. Cells were grown in 10ml, 25ml or 75ml in appropriate sized flask (Nunc Brand products, Nalge Nunc International, Denmark).

Cell viability was determined by the percentage of live to dead cells using the vital dye, trypan blue, observed on a haemocytometer under a light microscope. All cells were centrifuged at 1000rpm for 5 minutes unless otherwise stated. All cells were frozen at −70°C initially then transferred to liquid nitrogen with cell numbers of 5x10⁶ to 1x10⁷ per ml in media supplemented with 10% serum and 10% DMSO. To recover cells from freezing they were thawed quickly and resuspended in medium, centrifuged and washed once before resuspending all the cells in culture media.

Upon reaching confluence, the cells were washed with PBS then treated with 0.25% trypsin solution until the cells detached. Cells were either split at a ratio of 1:3 to 1:9 for continuing culture or frozen.

#### 2.6.2 Optimisation of HeLa mammalian cells transfections with RFP-Unp (wt), NLS mutants, and Rev(1.4)-GFP-based constructs

Cytofectene reagent (GibcoBRL) was used for transient transfections of HeLa cells. The optimal transfection conditions with Cytofectene reagent were assessed according to the manufacture’s manual (GibcoBRL). Firstly, the amount of transfection reagent was optimised with the DNA amount kept constant, secondly, using the optimal amount of the cytofectene reagent, the best DNA amount was found for RFP-Unp(wt) and RFP-Unp NLS mutants. It was determined that 2 μg of DNA and 4 μl of
cytofectene reagent per 1 ml of media were the best conditions for RFP-Unp wt plasmid, all NLS mutants as well as for Rev(1.4)-GFP plasmids.

Cells transfected with RFP-containing plasmids, were incubated in the 37°C incubator with 5% CO₂ supply for 40-48 hours prior to detect fluorescence. Cells transfected with GFP construct were incubated in the same incubator for 12-24 hours post-transfection.

### 2.6.3 Preparation of total HeLa cell lysate

HeLa cells grown in flasks or in Petri dishes either transfected or not were trypsinised, spun down and resuspended in a “HeLa lysis buffer” (see table 2.9) to obtain the total cell lysate. To minimise dilution of the total cell lysate, the HeLa lysis buffer was added to the cells in the 1:5 ratio (pellet: buffer). The lysate was then a subject to either western blotting with appropriate antibodies or to GST pull-down with GST-pRb.

### 2.6.4 Cell fixation

Cells grown on coverslips in 12 well plates were fixed with 4% para-formaldehyde (see Table 2.9) as follows: the cells were washed twice with PBS, then 4% para-formaldehyde solution was applied and plates were incubated in 37°C incubator for 15-30 minutes. Then the cells were washed twice in PBS again.

### 2.6.5 Coverslip Mounting on glass slides

To make the cells suitable for microscopy analysis, the coverslips with fixed cells on them were mounted on glass slides. A drop of the antifading solution (see Table 2.9) was first placed on the slide, the coverslip was carefully inverted on top of the drop, cells facing down, slightly pressed against the slide and sealed with nailpolish. For long-term storage the slides were kept in the dark at -20°C.

### 2.6.6 Immunofluorescence

To detect the subcellular localisation of endogenous proteins (Unp, Usp15 and pRb), the immunostaining of cells was performed. The antibodies used for these experiments were: rabbit anti-Unp, rabbit anti-Usp15, both were raised and purified as
described in Sections §4.3.1 and §4.3.3. To detect the endogenous pRb, the commercial mouse α-pRb antibodies (Pharminen) were used.

Antibody detection was achieved as follows: cells were seeded on coverslips in 12 well plates. When the cells reached 70-90% confluency, they were fixed with 4% paraformaldehyde as described above. The fixed cells then were permeabilised with 0.1% SDS in PBSB (1% BSA in PBS) at RT for 15-20 minutes, and blocked with PBSB at RT for 45-60 minutes. Primary antibodies were applied at 1:50 to 1:200 dilutions in PBSB, and the plates were incubated overnight at 4°C. The coverslips were washed with PBS five times to ensure that unbound antibodies were not present. After the fourth wash the coverslips were gently lifted up to that the PBS washed the bottom of the wells. The commercial secondary antibodies (either anti-rabbit FITC-conjugated, or anti-mouse Texas-Red-conjugated, Jackson laboratory) were applied in 1:100 ratio, diluted in PBSB. The secondary antibodies were kept for 1 hour at RT in the dark. Washing was performed as for the primary antibody to ensure that any unbound antibodies are not present. The cells then were mounted on glass slides as described above.

2.6.7 Confocal laser scanning microscopy (CLSM)

The subcellular localisation of fluorescently tagged or immunostained endogenous proteins were monitored by CLSM. This technique is fundamentally similar to standard fluorescence microscopy. However, has several important differences. Firstly, the information in a biological sample is converted into a video image. Secondly, the laser is used to excite fluorescence at specific wavelength, providing higher sensitivity. Thirdly and most importantly, the special optics of the confocal microscope mean that the path of the light exciting fluorescence is exactly the same as that for the fluorescence emitted. These advantages of CLSM allow obtaining much higher resolution of the images, compared to that of conventional fluorescent microscope. Therefore, analysis of living cells, optical sectioning, and double and triple staining are possible.

2.6.8 The image analysis

The analysis of cell images was performed using confocal laser scanning microscopy (CLSM) or a mercury lamp to visualise GFP signal.
Images of RFP-fusion proteins expressed in HeLa cells obtained from CLSM were used to determine the ratio of fluorescence in the nucleus relative to the cytoplasm (Fn/c) by the NIH Image 1.62 public domain software. Mean pixel density was assessed for manually cropped areas of uniform intensity in the nucleus (Fn), cytoplasm (Fc) and the untransfected cells intensity was measured as the background fluorescence (Fb). The ratio (Fn/c) was determined as follows:

\[ \frac{Fn}{Fc} = \frac{Fn - Fb}{Fc - Fb} \]

0.9 < Fn/c < 1.1 was assumed equal throughout the cell and designated as “N=C”

Fn/c > 1.1 was assumed as more nuclear and designated as “N>C”

Fn/c < 0.9 was assumed as more cytoplasmic and designated as “N<C”

When the exact ratio Fn/c was not required (NES assay with GFP-fusion proteins) a visual examination using a mercury lamp was carried out and subcellular distribution of GFP-fusion proteins was determined. The cells expressing the GFP-fusion proteins were divided into groups according to whether the staining pattern was predominantly nuclear (N), nuclear and cytoplasmic (N+C), or predominantly cytoplasmic (C).

**2.7 Statistical analysis**

The difference between Fn/c ratios of whole cell populations expressing RFP-fusion proteins was confirmed by Student’s test (T-test). The statistical significance was determined by comparison of ln(Fn/c) values [natural logarithm of Fn/c] by T-test, the two-tailed p value was considered significant when p < 0.01.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M HEPES tissue culture grade</td>
<td>Life technology (USA)</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide</td>
<td>National Diagnostics (USA)</td>
</tr>
<tr>
<td>- Protogel™ (29:1)</td>
<td></td>
</tr>
<tr>
<td>- Sequagel™ (19:1)</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>Sigma</td>
</tr>
<tr>
<td>Agarose</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Univar, Aust.</td>
</tr>
<tr>
<td>Deoxy-nucleotide Triphosphates (dATP, dCTP, dGTP, dTTP)</td>
<td>Pharmacia Biotech (Sweden)</td>
</tr>
<tr>
<td>EN3HANCE™</td>
<td>DuPont NEN® (USA)</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>Commonwealth Serum Laboratories Ltd. (Australia)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Pronalys</td>
</tr>
<tr>
<td>Immobilon™-P Transfer Membrane</td>
<td>Millipore (USA)</td>
</tr>
<tr>
<td>Isopropylthio-β-D-galactoside (IPTG)</td>
<td>Progen Industries (Australia)</td>
</tr>
<tr>
<td>Leptomycin B</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Li:e technology (USA)</td>
</tr>
<tr>
<td>Nonidet P-40</td>
<td>ICN Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>pDEST17 GATEWAY</td>
<td>Li:e technology (USA)</td>
</tr>
<tr>
<td>pDONOR GATEWAY</td>
<td>Li:e technology (USA)</td>
</tr>
<tr>
<td>pGEX-4T</td>
<td>Pharmacia Biotech (Sweden)</td>
</tr>
<tr>
<td>Phenol</td>
<td>WACO</td>
</tr>
<tr>
<td>Protein molecular weight markers:</td>
<td>Amersham (UK)</td>
</tr>
<tr>
<td>- Rainbow Markers (Low and High-range)</td>
<td>Novex (USA)</td>
</tr>
<tr>
<td>Radiochemicals:</td>
<td>ICN Pharmaceuticals (USA)</td>
</tr>
<tr>
<td>- [35S]-methionine (1000 Ci/mmol)</td>
<td>Amersham (UK)</td>
</tr>
<tr>
<td>- [33P]-dATP (1000 Ci/mmol)</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>X-ray film (Rx)</td>
<td>Fuji (Japan)</td>
</tr>
<tr>
<td>λ DNA cut with HphIII</td>
<td>New England Biolabs (USA)</td>
</tr>
</tbody>
</table>
Table 2.2 Kits and their suppliers

<table>
<thead>
<tr>
<th>Kits</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quickchange Site directed mutagenesis Kit</td>
<td>Stratagene</td>
</tr>
<tr>
<td>ABI prism Big Dye terminator cycle Sequencing Ready Reaction Kit</td>
<td>Pelkin Elmer</td>
</tr>
<tr>
<td>Miniprep DNA purification Kit</td>
<td>Bresatec</td>
</tr>
<tr>
<td>Wizard PCR preps DNA purification System kit</td>
<td>Promega Corporation (USA)</td>
</tr>
<tr>
<td>Wizard PureFecion plasmid DNA purification System (midi/maxi)</td>
<td>Promega Corporation (USA)</td>
</tr>
<tr>
<td>TNT Quick Translation Transcription</td>
<td></td>
</tr>
<tr>
<td>Cytofectene transfection reagent</td>
<td>GibcoBRL</td>
</tr>
</tbody>
</table>

Table 2.3 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>E.coli strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F-, ø80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, l-, thi-l, gyrA96, relA1</td>
</tr>
<tr>
<td>XL1 blue</td>
<td>recA1 endA1 gyrA96 thi-l hsdR17 supE44 relA1 lac [F' proAB lacIqZM15 Tn10(TetR)]</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F-, ompT, hsdSB(rB-mB-), gal, dcm (DE3)</td>
</tr>
<tr>
<td>MC1060</td>
<td>D(lacIPOZYA) X74 galU galK strA2 hsdR</td>
</tr>
</tbody>
</table>

Table 2.4 Solutions and media used to work with bacteria:

<table>
<thead>
<tr>
<th>Name of media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB)</td>
<td>10 g/l bacto-tryptone; 5 g/l bacto-yeast extract; 10 g/l NaCl(^a)</td>
</tr>
<tr>
<td>LBA</td>
<td>as above; 100 µg/ml Ampicillin</td>
</tr>
<tr>
<td>LBK</td>
<td>as above; 25 µg/ml Kanamycin</td>
</tr>
<tr>
<td>LB plates</td>
<td>LB + 1.5% (w/v) agar + appropriate antibiotic when needed</td>
</tr>
</tbody>
</table>

\(^a\) Autoclaved 121°C for 15 min
### Table 2.5 Buffers and solutions to work with DNA

<table>
<thead>
<tr>
<th>Name of media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE (50X)</td>
<td>2M Tris, 5.7% glacial acetic acid, 50 mM EDTA</td>
</tr>
<tr>
<td>TBE (10X)</td>
<td>0.9 M Tris; 1.125 M boric acid; 20 mM EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>DNA loading dye (6X)</td>
<td>2.5 mg/ml bromophenol blue, 400 mg/ml sucrose</td>
</tr>
</tbody>
</table>

### Table 2.6 Buffers and solutions for SDS-PAGE/Tricine gels and Western blotting.

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie stain</td>
<td>0.2% Coomassie Brilliant Blue R-250; 45% methanol; 10% glacial acetic acid</td>
</tr>
<tr>
<td>Destain solution</td>
<td>45% methanol; 10% glacial acetic acid</td>
</tr>
<tr>
<td>Laemmli loading buffer (3x)</td>
<td>375mM Tris-HCl, 4% (w/v) SDS, 30% (v/v) glycerol,</td>
</tr>
<tr>
<td></td>
<td>0.25% (w/v) bromophenol blue, 1mM β-mercaptoethanol, pH 6.8</td>
</tr>
<tr>
<td>SDS-PGE Resolving (4x)</td>
<td>1.5M Tris-HCl, 0.4% (w/v) SDS, pH 8.8</td>
</tr>
<tr>
<td>SDS-PAGE Running buffer</td>
<td>25mM Tris-HCl, 192mM glycine, 0.1% (w/v) SDS, pH 8.3</td>
</tr>
<tr>
<td>SDS-PAGE Stacking (4x)</td>
<td>0.5M Tris-HCl, 0.4% (w/v) SDS, pH 6.8</td>
</tr>
<tr>
<td>Tricine Anode (Running)</td>
<td>0.2M Tris, pH 8.</td>
</tr>
<tr>
<td>Tricine Cathode (Running)</td>
<td>0.1M Tris; 0.1M tricine; 0.1% SDS</td>
</tr>
<tr>
<td>Tricine gel buffer (x4)</td>
<td>3M Tris, pH 8.45; 0.3% (w/v) SDS</td>
</tr>
<tr>
<td>Transblot</td>
<td>12mM Tris; 96mM Glycine; 20% (v/v) Methanol</td>
</tr>
<tr>
<td>Blocking (Western)</td>
<td>5% skim milk or 5% BSA in 1xPBS; 0.05% Tween-20</td>
</tr>
<tr>
<td>Antibody incubation</td>
<td>1xPBS; 0.05% Tween-20</td>
</tr>
<tr>
<td>Washing (Western)</td>
<td>1xPBS; 0.5% Tween-20; (+/-) 0.5M NaCl</td>
</tr>
</tbody>
</table>
### Table 2.7 Buffers and solutions to work with recombinant proteins

<table>
<thead>
<tr>
<th>Name of a buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6xHis-Fusion proteins</strong></td>
<td></td>
</tr>
<tr>
<td>6xHis Elution</td>
<td>PBS pH7.4; 300mM NaCl; 250mM Imidazole</td>
</tr>
<tr>
<td>6xHis Lysis</td>
<td>PBS pH7.4; 300 mM NaCl; 20 mM Imidazole; 1mM EDTA; 1 mM β-Me-Ethanol; protease inhibitors*</td>
</tr>
<tr>
<td>6xHis Washing*</td>
<td>PBS pH7.4; 300 mM NaCl; 10% Glycerol; 20 mM Imidazole; 1 mM β-Me-Ethanol; protease inhibitors*</td>
</tr>
<tr>
<td><strong>GST-Fusion proteins</strong></td>
<td></td>
</tr>
<tr>
<td>GST Elution</td>
<td>50 mM Tris, pH9.0; 20 mM GSH, +/-protease inhibitors*</td>
</tr>
<tr>
<td>GST Lysis</td>
<td>1xPBS or NT 1mM β-Me-Ethanol; +/- protease inhibitors*</td>
</tr>
<tr>
<td>GST Washing</td>
<td>1xPBS or NT 1mM β-Me-Ethanol</td>
</tr>
<tr>
<td>GST-pRb Elution</td>
<td>100 mM Tris, pH 8.0; 250 mM NaCl; 0.1% Triton X-100; 25 mM GSH; 1mM DDT; protease and phosphatase inhibitors*</td>
</tr>
<tr>
<td>GST-pRb Lysis</td>
<td>20 mM Tris, pH 7.5; 150 mM NaCl; 0.5% Tween 20; 1mM DTT; protease and phosphatase inhibitors*; Dnase-1</td>
</tr>
<tr>
<td>GST-pRb Washing</td>
<td>GST-Rb Lysis buffer, with AEBSF only, without Dnase-1, 1xPBS</td>
</tr>
<tr>
<td>TN</td>
<td>50 mM Tris-HCl, 150 mM NaCl, pH 7.5</td>
</tr>
<tr>
<td><strong>β-galactosidase-Fusion proteins</strong></td>
<td></td>
</tr>
<tr>
<td>β-gal Elution</td>
<td>100mM Boric acid, pH10.1; 10mM β-Me-Ethanol</td>
</tr>
<tr>
<td>β-gal Lysis</td>
<td>20mM Tris, pH7.4; 10mM MgCl₂; 10mM β-Me-Ethanol (PI buffer)</td>
</tr>
<tr>
<td>β-gal Washing</td>
<td>The same as β-gal Lysis with 1.6M NaCl</td>
</tr>
<tr>
<td>Dialysis</td>
<td>1xPBS; 1mM β-Me-Ethanol;</td>
</tr>
</tbody>
</table>

*protease and phosphatase inhibitors are listed in the Table2.7a

### Table 2.7(a) protease and phosphatase inhibitors used in this study

<table>
<thead>
<tr>
<th><strong>Protease Inhibitor</strong></th>
<th><strong>Working Concentration</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>2µg/mL</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>2µg/mL</td>
</tr>
<tr>
<td>AEBSF</td>
<td>1mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Phosphatase Inhibitors</strong></th>
<th><strong>Working Concentration</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>1mM</td>
</tr>
<tr>
<td>Na orthovanadate</td>
<td>1mM</td>
</tr>
</tbody>
</table>
### Table 2.8 Cell lines used and culture media.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue origin</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>F15 MEM, 10% Foetal bovine serum (FBS), 2mM L-Glutamine,</td>
</tr>
<tr>
<td>MPEF</td>
<td>Mouse primary fibroblasts (extracted on 13-th day of embryogenesis)</td>
<td>DMEM, 15% FBS, 2mM L-Glutamine</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human hepatoma</td>
<td>F15 MEM, 20% FBS, 1x amino acids, 1x non-essential amino acids, 1x vitamins,</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Human osteosarcoma, (Retinoblastoma protein is truncated and found only in the cytoplasm)</td>
<td>MacCoy’s 5a, 15%FBS, 2mM L-Glutamine, PSN*, 1 mM Sodium Pyruvate</td>
</tr>
<tr>
<td>SR-40</td>
<td>Saos-2 cells infected with full-length pRb plasmid</td>
<td>MacCoy’s 5a, 15%FBS, 2mM L-Glutamine, PSN*, 1 mM Sodium Pyruvate</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Mouse epithelial cells</td>
<td>DMEM, 10% FBS, 2mM L-Glutamine</td>
</tr>
<tr>
<td>NIH3T3/E7</td>
<td>Mouse epithelial cells infected with E7 protein</td>
<td>DMEM, 10% FBS, 2mM L-Glutamine, G418 0.5mcg/ml</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human breast cancer (p53 defective)</td>
<td>RPMI, 10% FBS, 2mM L-glutamine, PSN*</td>
</tr>
<tr>
<td>HTC</td>
<td>Rat hepatoma cells</td>
<td>DMEM, 10%FBS, 2mM L-glut.</td>
</tr>
</tbody>
</table>

*PSN - 50units penicillin, 50ng Streptomycin, 0.1mg neomycin per ml

### Table 2.9 Buffers and solutions used for cell culture

<table>
<thead>
<tr>
<th>name</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1xPBS</td>
<td>140mM NaCl, 3mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄, pH 7.4</td>
</tr>
<tr>
<td>4% para-formaldehyde</td>
<td>4 g of para-formaldehyde in 100 ml of 100mM Na-phosphate buffer</td>
</tr>
<tr>
<td>Antifading solution</td>
<td>2% pyrogallate in 95% glycerol</td>
</tr>
<tr>
<td>0.25% Trypsin solution</td>
<td>0.25% (w/v) trypsin, 1mM EDTA in PBS</td>
</tr>
<tr>
<td>HeLa lysis buffer</td>
<td>50mM Tris, pH7.4, 200mM NaCl, 1% glycerol, 0.5% Triton X-100, 1mM EDTA, 1mM DTT , all protease and phosphatase inhibitors as listed in the table 2.7a</td>
</tr>
<tr>
<td>Name</td>
<td>Protein expressed</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>pDsRed-C1</td>
<td>Red Fluorescent Protein (RFP)</td>
</tr>
<tr>
<td>pRFPccdB</td>
<td>RFP</td>
</tr>
<tr>
<td>pRFP-Unp</td>
<td>RFP-mUnp</td>
</tr>
<tr>
<td>pRFP-Usp15</td>
<td>RFP-mUSP15</td>
</tr>
<tr>
<td>pRFP-UnpNL51Mut</td>
<td>RFP-mUnpNL51Mut</td>
</tr>
<tr>
<td>pRFP-UnpNL2Mut</td>
<td>RFP-mUnpNL2Mut</td>
</tr>
<tr>
<td>pRFP-UnpNL3Mut</td>
<td>RFP-mUnpNL3Mut</td>
</tr>
<tr>
<td>pRFP-UnpL459Gmut</td>
<td>RFP-mUnpL459Gmut</td>
</tr>
<tr>
<td>pRFP-UnpCR2Mut</td>
<td>RFP-mUnpC461Gmut</td>
</tr>
<tr>
<td>1.4Rev-GFP</td>
<td>RevNESmut-GFP</td>
</tr>
<tr>
<td>1.4/RevNES</td>
<td>1.4Rev-RevNES-GFP</td>
</tr>
<tr>
<td>1.4/UnpNES1</td>
<td>1.4Rev-mUnpNES1-GFP</td>
</tr>
<tr>
<td>1.4/UnpNES2</td>
<td>1.4Rev-mUnpNES2-GFP</td>
</tr>
<tr>
<td>Table 2.10 (continued)</td>
<td><strong>Plasmids for bacterial expression</strong></td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td><strong>pPR2all</strong></td>
<td><strong>β-galactosidase</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> Tac-promoter driven; lacZ was inserted using BamHI/HindIII sites. The plasmid is for β-galactosidase fusions production.</td>
</tr>
<tr>
<td><strong>UNshort/pPR2</strong></td>
<td><strong>UnpNLSshort-β-galactosidase</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> UnpNLSshort double-stranded oligo is cloned into pPR2all using Xmal site</td>
</tr>
<tr>
<td><strong>UNlong/pPR2</strong></td>
<td><strong>UnpNLSlong-β-galactosidase</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> UnpNLSlong PCR-product is cloned into pPR2all using Xmal site</td>
</tr>
<tr>
<td><strong>pDEST17</strong></td>
<td><strong>6xHis-</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> A GATEWAY Destination vector, for expression of proteins as 6xHis fusions, T7 promoter driven</td>
</tr>
<tr>
<td><strong>pDest17-Unp</strong></td>
<td><strong>6xHis- mUnp (full length)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> mUnp full length in pDest17</td>
</tr>
<tr>
<td><strong>pDest17-Usp15</strong></td>
<td><strong>6xHis-mUsp15 (full length)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> mUsp15 full length in pDest17</td>
</tr>
<tr>
<td><strong>UnpL457G-D17</strong></td>
<td><strong>6xHis- mUnpL457Gmut</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> mUnpL459Gmut full length in pDest17</td>
</tr>
<tr>
<td><strong>pGEX-4T</strong></td>
<td><strong>GST</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> Pharmacia Biotech Taq-promoter driven vector, contains internal LaqIa gene</td>
</tr>
<tr>
<td><strong>GST-Unp(var)</strong></td>
<td><strong>Unp(var. region)-GST</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> mUnp gene region, corresponding to a.a 539-789 (variable region) cloned into pGEX-4T using EcoRI/SalI sites</td>
</tr>
<tr>
<td><strong>GST-Usp15(var)</strong></td>
<td><strong>Usp15 (var.region)-GST</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ampR</strong> mUsp15 gene region, corresponding to a.a 526-797 (variable region) cloned into pGEX-4T using EcoRI/SalI sites</td>
</tr>
<tr>
<td><strong>pDONR201</strong></td>
<td><strong>The entry vector for a GATEWAY system, not an expression vector</strong></td>
</tr>
<tr>
<td></td>
<td><strong>kanR</strong> Used for introducing full-length Unp and Usp15 into the GATEWAY system</td>
</tr>
</tbody>
</table>
Table 2.11 Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GATEWAY primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AttB1-UnpFW</td>
<td>5'-gggg aca agt ttg tac aaa gca ggc ttg ggc gaa ggc ggg ggg ggc agg-3'*</td>
<td>To clone full-length mUnp gene into pDONR201 vector, to create the mUnp Entry clone</td>
</tr>
<tr>
<td>AttB2-UnpREV</td>
<td>5'-gggg ac cac ttg gta caa gaa gca ggc tgg gta gag ggc aca tca gtt ggt gtc c-3'*</td>
<td></td>
</tr>
<tr>
<td>AttB1-Usp15FW</td>
<td>5'-gggg aca agt ttg tac aaa gca ggc ttg ggc gaa ggc gga ggc ggc ggg agg-3'*</td>
<td>To clone full-length mUsp15 gene into pDONR201 vector, to create the mUsp15 Entry clone</td>
</tr>
<tr>
<td>AttB2-Usp15REV</td>
<td>5'-gggg ac cac ttg gta caa gaa ggc tgg gtt gca ctt tcg tta att agt gtg c-3'*</td>
<td></td>
</tr>
<tr>
<td>* In normal: the attB sites, in bold: the gene-specific nucleotides,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| <strong>PCR primers:</strong>     |                                               |                                                                             |
| AntbFW               | 5'-cag aat tct aca atc acm gat tyc ac agg-3' | The primers are for cloning of the variable regions of Unp and Usp15 into pGEX-4T, to express proteins for antibody generation. Suit both mUnp and mUsp15. |
| AntbBACKWARD         | 5'-cct gtc gac ggt ggt gaa gag ctc ctc agg-3' |                                                                             |
| hUnpEx3FW            | 5'-atc tct gac gtt cgt ccg tgg gtt cct cct acc agg-3' | To PCR and sequence the exon 3 of endogenous Unp in HeLa and MPF cells |
| hUnpEx3REV           | 5'-ttc tct gac gtt cgt ccg tgg gtt cct cct acc agg-3' |                                                                             |
| hUnpEx4FW            | 5'-ttc tct gac gtt cgt ccg tgg gtt cct cct acc agg-3' | To PCR and sequence the exon 4 of endogenous Unp in HeLa and MPF cells |
| hUnpEx4REV           | Caa tgg tgt ctc ctc ctc cct acc agg-3' |                                                                             |
| LongNLSR (mUnp, FW)  | 5'-ttc ccg ggg aca gtt aga ccc ga cca agg-3' | To PCR the mUnp &quot;Long NLS&quot; and clone it into pPR2all for expression of UnpNLS-long-β-gal fusion protein |
| LongNLSL (mUnp, REV) | 5'-ttc ccg ggg tca tgc tgc ccc caa cca agg-3' |                                                                             |</p>
<table>
<thead>
<tr>
<th>Primer name</th>
<th>sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutagenesis primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L459G-UnpFW</td>
<td>5'-cct ttt caa atc gac cgg tgt ttg ccc aga atg tg-3'</td>
<td>To create a pRb-binding mutant by mutating L459 to G in CR2 site of mUnp. The primers introduce an extra AegI site.</td>
</tr>
<tr>
<td>L459G-UnpREV</td>
<td>5'-cac att ctg ggc aaa cac cgg tct att tga aaa gg-3'</td>
<td>To create a pRb-binding mutant by mutating C461 to G in CR2 site of mUnp. The primers introduce an extra Apal site.</td>
</tr>
<tr>
<td>UnpCR2mutFW</td>
<td>5'-c aaa tgg act tgt cgg cca gaa tgt gc-3'</td>
<td></td>
</tr>
<tr>
<td>UnpCR2mutREV</td>
<td>5'-gc cga ttc tgg ggc ccc caa aga att cga ttt g-3'</td>
<td></td>
</tr>
<tr>
<td>UnpNLS1mutFW</td>
<td>5'-gaa ccc cct aaa gaa ttc ggc tta cct cza ggc c-3'</td>
<td>To create a putative NLS1 mutant by mutating 414-KK-415 to 414-NS-415 of mUnp. The primers introduce an extra EcoRI site</td>
</tr>
<tr>
<td>UnpNLS1mutREV</td>
<td>5'-ggg ctc cag tta agg cga att ctt tac ggg ctt c-3'</td>
<td></td>
</tr>
<tr>
<td>UnpNLS2mutFW</td>
<td>5'-gcc cgg ctc cgt cga cgg acc cgc tgc tct tta cct tca g-3'</td>
<td>To create a putative NLS2 mutant by mutating 703-KR-704 to 703-QL-704 of mUnp. The primers introduce an extra PvuII site</td>
</tr>
<tr>
<td>UnpNLS2mutREV</td>
<td>5'-ctg aac tga aag acc cgc tgg tgc ctc ggc 'gg c-3'</td>
<td></td>
</tr>
<tr>
<td>UnpNLS3mutFW</td>
<td>5'-cag agg cgc ctc cga cga cag cgg cag tag c-3'</td>
<td>To create a putative NLS3 mutant by mutating 770-KK-771 to 770 NS 771 of mUnp. The primers introduce an extra EcoRI site</td>
</tr>
<tr>
<td>UnpNLS3mutREV</td>
<td>5'-gct act cgg ccc tgg gaa ttc ttc cgc cgc tgg ctc g-3'</td>
<td></td>
</tr>
<tr>
<td>UnpLL140142AAFW</td>
<td>5'-gta ttt gct gga ggc gaa ggc ctg tga gaa cag-3'</td>
<td>To create the NES2 mutant in mUnp-RFP by mutating two C-terminal L to A of NES2</td>
</tr>
<tr>
<td>UnpLL140142AAREV</td>
<td>5'-ctg ttc tca cag cgg tcc gcc ccc acc cca atc t-3'</td>
<td></td>
</tr>
<tr>
<td>UnpNES1KF (FW)</td>
<td>5'- tgg tcc cag cgc gta ctt cga ata aat t-3'</td>
<td>To create a Kpnl site inside mUnp NES1 to make a truncated mUnp(ΔNES1)-RFP gene fusion</td>
</tr>
<tr>
<td>UnpNES1KR (REV)</td>
<td>5'-aat tta ttc cag gta ccc ggt gcc acc a-3'</td>
<td></td>
</tr>
<tr>
<td>UnpNES2KF (FW)</td>
<td>5'-ctg aag ctc tgt ggt acc agt gac ccc acc-3'</td>
<td>To create a Kpnl site inside mUnp NES2 to make a truncated mUnp(ΔNES1+2)-RFP gene fusion</td>
</tr>
<tr>
<td>UnpNES2KR (REV)</td>
<td>5'-ggt ggg gtc act ggt acc aca gag ctt cag03'</td>
<td></td>
</tr>
<tr>
<td><strong>Sequencing primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NLS1mutSEQ</td>
<td>5'-cag atg gg tgt gca agg-3'</td>
<td>To sequence the NLS1 mutants of mUnp</td>
</tr>
<tr>
<td>NLS2,3mutSEQ</td>
<td>5'-ca a ggt ctc tag gag tag c-3'</td>
<td>To sequence the NLS2 or 3 mutants of mUnp</td>
</tr>
<tr>
<td>Primers to create double stranded oligos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td><strong>SmlNLSR (mUnp, FW)</strong></td>
<td>5'-ccg gga acc tga gca tgt cac agc aga aga aga gag cgg cgg tac-3'</td>
<td></td>
</tr>
<tr>
<td><strong>SmlNLSL (mUnp, REV)</strong></td>
<td>5'-ccg ggt acc ggc ttc ttc ttc ttc ggc tgt gac atg ctc agg tgc ttc-3'</td>
<td></td>
</tr>
<tr>
<td><strong>FWNES1</strong></td>
<td>5'-gat ccc gag cag cac tta atc gat gag ctg gag atg ctg gta cca ggc gaa cca-3'</td>
<td></td>
</tr>
<tr>
<td><strong>REVNES1</strong></td>
<td>5'-gat ccc ggt tgt ctc gtc ctc gca cca cca cat agt cca gct cat cga tta agt gct cct tgc-3'</td>
<td></td>
</tr>
<tr>
<td><strong>FWNES2</strong></td>
<td>5'-gat ccc gac tgc aaa gbg gaa gbg tgt tgt tgt ctg gag ctg aac atc ttc tgt gag aac cca-3'</td>
<td></td>
</tr>
<tr>
<td><strong>REVNES2</strong></td>
<td>5'-gat ccc ggt tgt tct cac aga gct cta gct cca gca aat aca ctt cca ctt tgc agt ggc-3'</td>
<td></td>
</tr>
<tr>
<td><strong>UnpNES2mut/1.4RevF</strong></td>
<td>5'-gat ccc gag cag ctc gtc ctc gtt ctt tgt tgt ctg gag ggc cgc tgt gag aac cca-3'</td>
<td></td>
</tr>
<tr>
<td><strong>UnpNES2mut/1.4RevR</strong></td>
<td>5'-gat ccc ggt tgt ctc cag ctt cca gca aat aca ctt cca ctt tgc agt ggc-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.11 (continued)**

<table>
<thead>
<tr>
<th>Primers</th>
<th>5'-act ccc gtt ctg gat aat gt-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPR2SEQ1</td>
<td>5'-ttc ttc gtt gga aca aac g-3'</td>
</tr>
<tr>
<td>RFP3'SEQ</td>
<td>5'-gat cca cca ggc cct gaa gc-3'</td>
</tr>
</tbody>
</table>

To sequence the inserts cloned into pPR2all plasmids. The primers anneal up- and downstream of the insert, respectively.

The primer is for sequencing of 5'-end of products, cloned into pDsRED-C1 (RFP vector).

These primers annealed to each other are for cloning into pPR2all plasmid for expression of mUnpNLS-short-β-gal fusion protein.

These primers annealed to each are for cloning into Rev(1.4)-GFP reporter plasmid for expression of Rev(1.4)- mUnp NES1-GFP in mammalian cells.

These primers annealed to each are for cloning into reporter plasmid for expression of Rev(1.4)- mUnp NES2-GFP in mammalian cells.

These primers annealed to each create a mUnp NES2 mutant, where two C-term Leu are changed to Ala. For cloning into Rev(1.4)-GFP reporter plasmid;
Chapter 3

IDENTIFICATION OF FUNCTIONAL NUCLEAR LOCALISATION AND NUCLEAR EXPORT SEQUENCES IN UNP
3.1 Introduction

As mentioned in the Introduction (Section §1.4), Unp belongs to a large family of ubiquitin-specific proteases (UBPs), whose function is to cleave ubiquitin from a wide variety of proteolytic substrates and from precursor molecules of ubiquitin (Section §1.3). Interestingly, murine Unp displays oncogenic properties when overexpressed in nude mice (Gupta et al., 1994). It has also recently been shown that Unp interacts with all three members of the pRb family of tumour suppressor proteins (Blanchette et al., 2001; DeSalle et al., 2001). There is, however, a discrepancy in the literature concerning its subcellular localisation. Although Unp was originally described as a nuclear protein, and a putative Nuclear Localisation Sequence (pNLS) was identified within it (Gupta et al., 1994; Gupta et al., 1993), another report described it as a mainly cytoplasmic protein (Frederick et al., 1998).

In this chapter, a rigorous analysis was carried to determine the subcellular localisation of Unp. This information will be essential in order to understand the mechanism of Unp-mediated oncogenic transformation including the functional significance of Unp interaction with members of the Rb family of proteins.

3.2 Aims

The specific aims of this chapter were to determine:
1. The subcellular localisation of RFP-Unp in HeLa cells.
2. Whether Unp has a functional NLS(s)
3. Whether Unp also possesses a functional NES(s).
4. The mechanism of NLS-mediated nuclear import of Unp.
3.3 Results

3.3.1 Generation of expression construct for RFP-Unp

In order to investigate the subcellular localisation of Unp, a recombinant fusion of Unp with the red fluorescent protein (RFP) from *Discosoma* species, DsRed (Matz et al., 1999) was generated. DsRed subsequently will be referred to as RFP. The RFP protein has several advantages over other known fluorescent tags:

1. RFP is less sensitive to pH fluctuations compared to Green Fluorescent Protein (GFP) (Matz et al., 1999).

2. Like GFP, RFP is resistant to photobleaching. Unlike GFP, the bright red fluorescence of RFP is outside the range of most background autofluorescence, and produces a strong signal-to-noise ratio (Living Colors, CLONTECHniques, 1999).

3. RFP is spread more evenly throughout the nucleus and the cytoplasm and does not localise in discrete dots or foci like Enhanced Cyan Fluorescent Protein (ECFP) (Mizuno et al., 2001).

To create RFP-Unp, the GATEWAY cloning technique (Invitrogen) was used as described in Section §2.3.11 and illustrated by Figure 2.1. The pCG51 plasmid containing full-length mUnp cDNA was amplified with primers AttB1-UnpFW and AttB1-UnpREV (Table 2.11). The PCR product, full-length Unp flanked by attB sites, was recombined with plasmid pDONR201 to create the Unp-Entry vector, which was recombined with pRFP-ccdB destination vector to produce the expression clone, pRFP-Unp. The pRFP-ccdB destination vector (see Table 2.10) was produced by Melanie Johnson-Saliba (Nuclear Signalling laboratory, JCSMR, ANU). The Unp insert was sequenced with various Unp-specific primers to ensure that it did not contain any mutations.

3.3.2 Expression and subcellular localisation of the RFP-Unp (wt) fusion in transformed HeLa cells

HeLa cells were chosen for the expression of RFP-Unp because the level of the endogenous Unp, as detected by Western blotting, was low (see Figure 3.1).
Figure 3.1 Level of endogenous Unp in cell lines as determined by Western blotting
Total cell lysate was prepared from HeLa cells (lane 2), HTC cells (lane 3), or HepG2 cells (lane 4), and an aliquot (130 µg total protein) (see Section §2.6.3) was electrophoresed in a 10% SDS-PA gel. Recombinant 6xHis-Unp purified from bacterial cells (lane 1) (Section § 2.4.1.1) was used as a positive control. Proteins were transferred to a PVDF membrane and probed with commercial anti-Unp antibody (Zymed) followed by an HRP-conjugated anti-rabbit antibody and ECL detection. The position of the 97 kDa molecular mass marker is shown on the left.

Figure 3.2 Expression of RFP-Unp in HeLa cells detected by Western blotting
Total cell lysate from HeLa cells expressing RFP alone (lane 1) or RFP-Unp (lane 2) was prepared 48 hours post-transfection (see Section §2.6.3), and an aliquot (90 µg total protein) was electrophoresed in a 10% SDS-PA gel. Proteins were transferred to the PVDF membrane and probed with a polyclonal anti-Unp antibody raised in the course of this study (see Section 4.3.3) followed by an HRP-conjugated anti-rabbit antibody and ECL detection. Positions of molecular mass markers are shown on the left.
HeLa cells were analysed for the expression of RFP-Unp or RFP alone 48 hours after transfection. Western blot analysis showed that RFP-Unp was expressed as a full-length protein. Importantly, no degradation products were observed (see Figure 3.2).

Expression of RFP-Unp was analysed at the single cell level by confocal laser scanning microscopy (CLSM). The initial experiment revealed a disparate expression pattern for RFP-Unp. Thus RFP-Unp was observed to accumulate in either the nucleus or cytoplasm, with a small percentage of cells where RFP-Unp was spread evenly throughout both of these cellular compartments (Figure 3.3, panel A).

In contrast, the cells expressing RFP alone exhibited fluorescence throughout the cell, with no pronounced nuclear or cytoplasmic staining (see Figure 3.3, panel B).

These results show that RFP-Unp can clearly be localised in the nuclei of HeLa cells. Since RFP-Unp has a molecular weight of 138 kDa (28 kDa RFP + 110kDa Unp), this implies that the protein is actively imported into the nucleus, through the function of an active NLS within Unp.

The examination of the amino acid sequence of Unp revealed that Unp contains several putative NLSs (pNLS). Additionally, at least two putative NESs (pNES) were also identified. Figure 3.4 shows the amino acid sequence of Unp, with the putative NLSs and NESs highlighted. pNLS3 has already been mentioned in the introduction to this chapter as a putative NLS described by Gupta et al (Gupta et al., 1993). To ascertain whether any of these pNLSs can serve as functional NLSs, a number of point mutations within the putative NLSs were introduced as indicated below.

3.3.3 Mutagenesis studies revealed that pNLS3 is a functional NLS

To investigate whether any of the pNLSs were functional, mutagenesis of each putative NLS signal within the pRFP-Unp plasmid was performed as described in Section §2.3.10. The UnpNLS1mutFW/REV primers (Table 2.11) were used to mutate nucleotides encoding the amino acids K\(^{414}\)R\(^{415}\) to N\(^{414}\)S\(^{415}\) within putative Unp NLS1, and the UnpNLS2mutFW/REV primers (Table 2.11) were utilised to create mutation K\(^{703}\)R\(^{704}\) to Q\(^{703}\)L\(^{704}\) within pNLS2. Finally, UnpNLS3mutFW/REV primers (Table 2.11) were used to mutate K\(^{770}\)K\(^{771}\) to N\(^{770}\)S\(^{771}\) of pNLS3. Figure 3.5 illustrates these substitutions.

The choice of residues for mutagenesis was based both on generation of diagnostic restriction sites and previously published mutations of other NLSs.
HeLa cells were transfected with plasmids expressing RFP-Unp (panel A) and RFP alone (panel B) and observed by confocal microscopy 48 hours after transfection.
The mUnp sequence is shown in the single letter amino acid code with putative NLSs (pNLSs) are highlighted in red, putative NESs (pNESs) are in green.

Wild type pNLSs are shown on the left and mutated pNLSs on the right, with the mutated amino acid residues are in blue.
Restriction digest and sequencing across the mutated region with primers NLS1mutSEQ and NLS2,3mutSEQ (Table 2.11) confirmed the presence of the desired mutations.

The RFP-UnpNLS1 mutant did not show subcellular localisation markedly different from RFP-Unp wt in transfected HeLa cells (see Table 3.1 and Figure 3.6). Image analysis indicated no significant difference ($p = 0.487$, see Section §2.7 for statistical analysis details) between the two constructs in terms of the nuclear to cytoplasmic ratio, Fn/c. This result demonstrates that the pNLS1 does not act as a functional NLS, or that it is totally redundant with another NLS in Unp.

Unexpectedly, the RFP-UnpNLS2 mutant showed significantly ($p < 0.001$) enhanced nuclear localisation compared to the wild type protein (Table 3.1, and Figure 3.6). Clearly, pNLS2 is dispensable for Unp nuclear localisation.

Finally, the mutation of pNLS3 caused a significant change in Unp localisation showing predominantly cytoplasmic localisation of the RFP-UnpNLS3 mutant (see representative cell images in Figure 3.7). In 60.4% of cells the mutant protein was localised in the cytoplasm, compared to 40% in case of the wt protein. The Fn/c average ratio calculated for cells expressing the mutant was also significantly lower with a value of 1.09 for NLS3 mutant compared to 1.39 for wild type (see Table 3.1 and Figure 3.6). The Fn/c ratios of cells expressing RFP-Unp wt and RFP-Unp NLS3 mutant were highly significantly different ($p$ value less than 0.0002). This result clearly indicates that the pNLS3 is required for Unp nuclear localisation.

### 3.3.4 A putative cdk2 phosphorylation site in Unp?

The RFP-Unp-pNLS2 mutant produced an unexpected result, accumulating more strongly in the nucleus than the wild type. The amino acid sequence of this putative NLS, $^{699}\text{QPRHKR}^{704}$, does not precisely resemble any other known NLSs, which are usually enriched in arginine and lysine residues. Histidine is also a positively charged amino acid, and therefore, it was considered that $^{699}\text{QPRHKR}^{704}$ sequence may be a putative NLS. Close examination of this region of Unp revealed that the amino acid sequence, $^{701}\text{RHKRL}^{705}$, resembles a cyclinA/E-cdk2 binding motif (the consensus motif is $Z(R/K)XL$, where $Z$ and $X$ are usually basic) (Adams et al., 1999).

To examine whether Unp and cyclin A or E interact, co-immunoprecipitation experiments from total HeLa cell extracts were performed (see Section §2.6.3 for
Table 3.1 Subcellular localisation of RFP-Unp wt, RFP-Unp-pNLS1, RFP-Unp-pNLS2, and RFP-Unp-pNLS3 mutants in HeLa cells

<table>
<thead>
<tr>
<th>protein</th>
<th>Number of cells analysed</th>
<th>% of cells</th>
<th>Fn/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFP-Unp wt</td>
<td>Total</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>N&gt;C</td>
<td>46</td>
<td>51.1</td>
</tr>
<tr>
<td></td>
<td>C&gt;N</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>N=C</td>
<td>8</td>
<td>8.9</td>
</tr>
<tr>
<td>RFP-Unp NLS1mut</td>
<td>Total</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>N&gt;C</td>
<td>30</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>C&gt;N</td>
<td>16</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>N=C</td>
<td>9</td>
<td>16.3</td>
</tr>
<tr>
<td>RFP-Unp NLS2 mut</td>
<td>Total</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>N&gt;C</td>
<td>73</td>
<td>74.5</td>
</tr>
<tr>
<td></td>
<td>C&gt;N</td>
<td>19</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>N=C</td>
<td>6</td>
<td>6.1</td>
</tr>
<tr>
<td>RFP-Unp NLS3 mut</td>
<td>Total</td>
<td>96</td>
<td>100</td>
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<tr>
<td></td>
<td>N&gt;C</td>
<td>29</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>C&gt;N</td>
<td>58</td>
<td>60.4</td>
</tr>
<tr>
<td></td>
<td>N=C</td>
<td>9</td>
<td>9.4</td>
</tr>
</tbody>
</table>

HeLa cells were transfected with the plasmids expressing RFP-Unp (wt), RFP-Unp-NLS1, RFP-Unp-NLS2, or RFP-Unp-NLS3 mutants and observed by confocal microscopy 48 hours after transfection. Images were analysed for fluorescence in different cellular compartments (nucleus and cytoplasm) using the NIH image software (see Section 2.6.8) and the ratio of nuclear to cytoplasmic fluorescence (Fn/c was determined as described in Section 2.6.8. The images were termed "N>C" if Fn/c > 1.1, "C>N" if Fn/c < 0.9, and "N=C" if 0.9<Fn/c < 1.1. The mean Fn/c and standard error (SE) were calculated for each localisation category.
Figure 3.6 Subcellular localisation of RFP-Unp wt, RFP-Unp-pNLS1, RFP-Unp-pNLS2, and RFP-Unp-pNLS3 mutants expressed in HeLa cells.

The results from Table 3.1 were plotted as a histogram of the percentage of cells falling into each localisation category shown in the colour key, for each protein indicated below the columns.

Figure 3.7 Representative images of HeLa cells expressing RFP-Unp wt or the RFP-Unp-pNLS3 mutant.

HeLa cells were transfected with RFP-Unp (panel A) or RFP-Unp-NLS3 (panel B) and CLSM images were taken 48 hours after transfection.
Unp was immunoprecipitated using anti-Unp antibody (see Sections § 4.3.1 and §4.3.3 for antibody description). Western blotting was then performed using anti-Cyclin A or E antibodies to detect if cyclin A or E were co-immunoprecipitated with Unp. Total cell lysate was also probed with anti-Cyclin A or E antibodies as a control for cyclin expression. Cyclin A but not cyclin E was detected in the total cell lysate. It was not possible, however, to detect whether cyclin A co-immunoprecipitated with Unp since the secondary antibodies reacted with the IgG heavy chain, which has the same molecular weight as cyclin A (data not shown). In order to avoid detection of IgG heavy chain, the antibodies against Unp would have to be bound covalently to Protein A-sepharose beads, so that they would not elute from the beads. However, this would have required large amounts of antibodies (2 mg), which were not available at that time. Therefore, further work is required to determine whether amino acids within the pNLS2 are a cyclinA/E-cdk2 binding site.

Next, the ability of CyclinA-cdk2 complex to phosphorylate Unp was tested in vitro. The experiment was performed by Dr. Boris Sarcevic (Garvan Institute of Medical Research, Sydney, Australia) using bacterially expressed 6xHis-Unp (full-length) produced in this study and GST-pRb(773-928) (as a positive control) in the presence of [γ32P]ATP as described in Section §2.4.3. Figure 3.8 shows that 6xHis-Unp was phosphorylated by cyclinA/cdk2 (lane 3), while incubation of 6xHis-Unp in the reaction mix in the absence of CyclinA-cdk2 did not result in Unp phosphorylation (lane 2). GST-Cyclin A was autophosphorylated (lane 1). Heavy phosphorylation of GST-pRb(773-928) was expected (lane 4) because firstly, the region 773-928 of pRb contains 7 CyclinA-cdk2 phosphorylation sites, and secondly, 5 µg of GST-pRb(773-928) (unlike 2 µg for Unp) was used for the reaction.

Taken together, these results raise the possibility that Unp localisation and nuclear transport may be regulated by cdk phosphorylation in a cell-cycle specific manner.

**3.3.5 Importin α and β ELISA binding assay**

The finding that the NLS3 is functional prompted an attempt to elucidate the mechanism of NLS3-mediated nuclear import of Unp. The first step of NLS-mediated nuclear protein import involves NLS recognition by importins (see Section §1.6.3 and
Figure 3.8 In vitro phosphorylation of Unp by Cyclin A/cdk-2.

Purified GST-cyclinA/cdk2 was incubated with [$\gamma^{32}$P]ATP either alone (lane 1) or in the presence of 2µg of 6xHis-Unp (Lane 3) or 5 µg of GST-pRb(773-928) (lane 4). As a control, 2µg of 6xHis-Unp was incubated with [$\gamma^{32}$P]ATP in the absence of GST-cyclinA/cdk2 (lane 2). (The phosphorylation reaction is described in detail in Section §2.4.3). Following phosphorylation, the proteins were separated on a 10% SDS-PAGE and visualised by autoradiography. The red arrow indicates phosphorylated 6xHis-Unp. The green arrow indicates an autophosphorylated GST-cyclin A. The Blue arrow indicates phosphorylated GST-pRb(773-928). Lane 4 also indicates the presence of multiple pRb phosphorylation states (above the blue arrow) as well as phosphorylated degradation products (below the blue arrow). This experiment was performed by Dr. Boris Sarcevic, Garvan Institute of Medical Research, Sydney, Australia.
references therein). The amino acid sequence of NLS3 is similar to the basic NLSs, such as that of T-ag, recognised by the importin α/β1 heterodimer. However, certain basic NLSs are recognised by importin β1 alone (Lam et al., 1999). To test the possibility of Unp recognition by importins, an ELISA-based binding assay was performed with two Unp constructs: Unp NLS3 fused to β-galactosidase (Unp-NLS3-β-gal), and the full-length 6-histidine-tagged Unp (6xHis-Unp). The first construct was used to test NLS3 recognition by importins in isolation, and the second one was used to test importin binding in the context of the whole Unp protein. Both Impα and Impβ were expressed as GST-fusion proteins.

In order to create the Unp-NLS3-β-gal fusion protein, short double-stranded oligonucleotides encoding Unp pNLS3 were introduced into XmaI sites of pPR2all vector (see Table 2.10 for vector description). The resulting plasmid was sequenced with primers pPR2SEQ1/2 and the integrity of the plasmid was confirmed. The fusion protein was purified as described in Section §2.4.1.3. The details of 6xHis-Unp cloning and purification are described in Sections §4.3.3 and §2.4.1.3. Both fusion proteins were assessed for importin α/β1 heterodimer binding, as well as Impα and Impβ1 monomer binding, as described in Section §2.5.4. A peptide containing the NLS of SV40 Large T-antigen (T-ag) fused to β-Galactosidase was used as a positive control, whilst β-galactosidase alone was used as a negative control.

The results (see Figure 3.9 and Table 3.2) imply that both NLS3-containing proteins have a higher affinity for Impα/β1 heterodimer than for Impα or β alone (Fig.3.9 A, B). Interestingly, the affinity of Impα/β for both fusion proteins was similar (Kd_{6xHis-Unp} = 54 nM and Kd_{Unp-NLS3-β-gal} = 45 nM). Unp-NLS3-β-gal is also recognised well by Impα alone (Kd = 46.5 nM). However, full-length Unp binds Impα very weakly (Kd = 346 nM). Both proteins bind Impβ weakly (Kd_{6xHis-Unp} = 107 nM and Kd_{Unp-NLS3-β-gal} = 153 nM) (see Table 3.2). The maximum level of Impα binding to Unp-NLS3 was half that of Impα/β consistent with the fact that both Impα and Impβ contain GST moieties (Fig. 3.9 B). Comparison of importin binding to the T-ag NLS and NLS3 of Unp revealed that T-ag binds Impα/β1 with much higher affinity than the Unp NLS3 (Kd_{T-ag-NLS} = 4.4 nM). Overall, the results of ELISA-binding assay indicate that binding of importin α1/β1 heterodimer to NLS3 is the likely mechanism of nuclear import of Unp.
**Figure 3.9 ELISA binding assay**

The binding affinities of 6xHis-Unp fusion protein, termed Unp, (panel A), UnpNLS3-β-gal fusion protein (panel B), and T-ag-NLS-β-gal fusion protein as a positive control (panel C) for importin α/β, importin α, or importin β were measured using ELISA-based binding assay. Fusion proteins were coated on microtiter plates and hybridised with increasing amount of importins as described in Section §2.5.4. Curves were fitted for the function $B(x) = B_{\text{max}}(1-e^{-kB})$, where $x$ is the concentration of importins. The apparent dissociation constant, $K_d$, represents the concentration of importin required for half maximum binding (see Table 3.2).
Table 3.2 Importin binding parameters of Unp and the Unp-NLS3-β-Gal fusion protein peptides as measured using an ELISA-based binding assay

<table>
<thead>
<tr>
<th>Protein</th>
<th>Importin binding parameters&lt;sup&gt;x&lt;/sup&gt;</th>
<th>Importin binding parameters&lt;sup&gt;x&lt;/sup&gt;</th>
<th>Importin binding parameters&lt;sup&gt;x&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mImpα</td>
<td>mImpβ</td>
<td>mImpα/β</td>
</tr>
<tr>
<td></td>
<td>Kd (nM)</td>
<td>Bmax (% α/β)</td>
<td>Kd (nM)</td>
</tr>
<tr>
<td>His&lt;sub&gt;e&lt;/sub&gt;-Unp</td>
<td>346 ± 121 (1)</td>
<td>57 ± 27</td>
<td>107 ± 59 (1)</td>
</tr>
<tr>
<td>Unp-NLS3-β-Gal</td>
<td>55 ± 5 (3)</td>
<td>48 ± 21</td>
<td>153 ± 78 (2)</td>
</tr>
<tr>
<td>T-ag-NLS-β-Gal</td>
<td>27 ± 10 (3)</td>
<td>34 ± 9</td>
<td>ND (2)</td>
</tr>
</tbody>
</table>

<sup>x</sup>Importin binding parameters were determined as described in Section 2.5.4 from experimental data fitted as shown in Figure 3.9. Results for the apparent dissociation constant (Kd) and maximal level of binding (expressed relative to the binding by importin α/β). Results represent the mean ± SE (n in parentheses); where n = 1, the SE is determined from the curve fit. ND, not able to be determined due to low binding.
3.3.6 Unp subcellular localisation responds to Leptomycin B treatment; identification of putative NESs

The experiments described above demonstrated that Unp contains a functional NLS. In addition to that, amino acid sequence analysis revealed that Unp may possess two Nuclear Export Signals (Fig. 3.4). NESs are usually rich in leucine (L) and/or isoleucine (I) residues, which are critically spaced. In some cases, valine and methionine residues may substitute for L or I. The consensus sequence for NESs, such as that of Rev is \( L/I/M/VX_{(3,4)}L/I/M/VX_{(2,3)}L/I/VX/L/I/V \) (where X is any amino acid) (Jans et al., 1998). Figure 3.4 illustrates the Unp amino acid sequence with two putative NESs highlighted. The presence of a NLS and a functional NES could explain the controversy concerning Unp localisation. Whereby, Unp could be a protein that shuttles in signal-dependent fashion between nuclear and cytoplasmic compartments.

To test the functionality of putative Unp NESs, Leptomycin B (LMB) treatment of HeLa cells expressing RFP-Unp was performed. LMB is an antifungal agent, which inhibits export of proteins containing NES motifs mediated by CRM1 export receptor (see Section §1.6.3.2 for more details and references). When cells expressing an NES-containing protein are treated with LMB, the protein accumulates in the nucleus because LMB prevents the NES/CRM1 interaction and subsequent nuclear export. Therefore, if Unp contains a functional NES recognised by CRM1, LMB treatment should lead to increased accumulation of RFP-Unp in the nucleus.

To assess the optimal concentration of Leptomycin B in HeLa cells the reporter construct Rev(1.4)-GFP (see Table 2.10 for plasmid description) was used. 24 hours post-transfection with Rev(1.4)-GFP plasmid, LMB was added to the cells at various concentrations (1, 2, 4, 6, or 8 ng/ml) for 4 hours. The range of concentrations was based on other LMB experiments described elsewhere (Henderson and Eleftheriou, 2000; Henderson and Percipalle, 1997). The minimal concentration that inhibited the export of Rev(1.4)-GFP in HeLa cells was established to be 4-6 ng/ml (see Figure 3.10). The incubation of cells expressing Rev(1.4)-GFP for 8 hours produced identical results. The concentration of 4 ng/ml and incubation for 8 hours was used for further experiments.

HeLa cells expressing RFP fusions were treated with LMB in the following way: the growth media was removed 45-48 hours post-transfection; half of the samples
Figure 3.10 Subcellular localisation of Rev(1.4)-RevNES-GFP construct in HeLa in the absence and presence of LMB

HeLa cells were transfected with Rev(1.4)-RevNES-GFP construct, and 24 hours post-transfection cells received 0, 2, 4, 6, or 8 ng/ml of LMB. 4 hours after LMB addition, images were analysed using confocal microscopy (see Section 2.6.7) and scored as having either nuclear/nucleolar, cytoplasmic, or (nucl.+ cytoplasmic) localisation. The optimal LMB concentration was 4 ng/ml. Panel A: the histogram represents the percentage of cells having Rev(1.4)-RevNES-GFP localised in the nucleus, cytoplasm, or nucleus and cytoplasm (see colour key) before LMB treatment (left) and after 4 hour treatment with 4 ng/ml LMB (right). “N” indicates a number of cells scored. Representative cell images are shown of Rev(1.4)-RevNES-GFP localisation in the nucleus and the nucleolus as well as in the cytoplasm in HeLa cells non-treated with LMB (panel B) and treated with 4 ng/ml LMB for 4 hours (panel C).
received fresh media containing LMB at concentration of 4 ng/ml, while the other half received fresh LMB-free media; all samples were then incubated for a further 8 hours.

The LMB-treated cells expressing RFP-Unp exhibited increased nuclear fluorescence compared to the non-treated samples. RFP-Unp localised in the nucleus of 85.5% of LMB-treated cells, while only 49% of non-treated cells had RFP-Unp in the nucleus. The Fn/c for LMB-treated samples was 3.1, significantly higher (p < 0.0001) than for untreated cells (Fn/c of 1.63) (Table 3.3 and Figure 3.11). The Fn/c ratios of cells from two samples were statistically compared by the Student’s T-test (see Section §2.7). LMB had no effect on the cells expressing RFP only (Table 3.4 and Fig. 3.12). Similar results were obtained in two identical experiments (data not shown).

This result clearly indicates that subcellular localisation of RFP-Unp is responsive to LMB treatment, which shows that Unp is actively exported from the nucleus through a functional NES(s) recognised by CRM1.

3.3.7 Cloning of pNES1 and pNES2 into 1.4Rev-GFP reporter plasmid

In order to assess the functionality putative NESs within Unp, an in vivo export assay was used. The assay is based on the ability of a functional NES to export the reporter construct, Rev(1.4)-GFP, from the nucleus. The reporter construct consists of Rev protein fused to GFP, and was a kind gift of Dr. Beric Henderson, Westmead Cancer Institute, Sydney. Rev is HIV-1 regulatory protein, which is able to shuttle between the nucleus and the cytoplasm in the host cell in order to export viral mRNA. A strong NLS and NES of Rev define its shuttling properties. The reporter construct contains a GFP fusion of modified Rev protein, where the NES of Rev is mutated (non-active), and therefore the mutant, Rev(1.4)-GFP, accumulates in the nucleoli of transfected cells. The details of the cloning of the reporter construct are described elsewhere (Henderson and Eleftheriou, 2000; Headerson and Percipalle, 1997). When a functional NES is introduced into the pRev(1.4)-GFP reporter plasmid, the nuclear export of Rev can be restored.

To create Rev(1.4)-UnpNES1-GFP and Rev(1.4)-UnpNES2-GFP constructs, double-stranded oligonucleotides, encoding both Unp NESs, were inserted into BamH1/AgeI digested Rev(1.4)-GFP plasmid. The inserts were generated by annealing
Table 3.3 Subcellular localisation of RFP-Unp protein expressed in HeLa cells in the presence and the absence of LMB.

<table>
<thead>
<tr>
<th>protein</th>
<th>Number of cells analysed</th>
<th>% of cells</th>
<th>Fn/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>1.63±0.13</td>
</tr>
<tr>
<td>N&gt;C</td>
<td>49</td>
<td><strong>49</strong></td>
<td>2.58±0.17</td>
</tr>
<tr>
<td>C&gt;N</td>
<td>42</td>
<td>42</td>
<td>0.66±0.02</td>
</tr>
<tr>
<td>N=C</td>
<td>9</td>
<td>9</td>
<td>1.0±0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>protein</th>
<th>Number of cells analysed</th>
<th>% of cells</th>
<th>Fn/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>62</td>
<td>100</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>N&gt;C</td>
<td>53</td>
<td><strong>85.5</strong></td>
<td>3.48±0.3</td>
</tr>
<tr>
<td>C&gt;N</td>
<td>6</td>
<td>9.7</td>
<td>0.75±0.06</td>
</tr>
<tr>
<td>N=C</td>
<td>3</td>
<td>4.8</td>
<td>1.03±0.02</td>
</tr>
</tbody>
</table>

HeLa cells were transfected with the RFP-Unp construct, and 48 hours post-transfection half of the cells received 4ng/ml of LMB, while the other half received LMB-free media. 8 hours after LMB addition, images were analysed for fluorescence in different cellular compartments (nucleus and cytoplasm) using NIH image software (see Section 2.6.8) and the ratio of nuclear to cytoplasmic fluorescence (Fn/c) was determined as described in Section 2.6.8. The images were termed “N>C” if Fn/c > 1.1, “C>N” if Fn/c < 0.9, and “N=C” if 0.9< Fn/c < 1.1. The mean Fn/c and standard error (SE) were calculated for each localisation category.

Figure 3.11 Subcellular localisation of RFP-Unp expressed in HeLa cells changes upon LMB treatment. The results from Table 3.3 were plotted as a histogram of the percentage of cells falling into each localisation shown in the colour key, for the proteins indicated below the columns (top panel). The lower panels show representative images of RFP-Unp localisation in the absence (left panel) and presence (right panel) of 4 ng/ml of LMB.
Table 3.4 Subcellular localisation of RFP protein expressed in HeLa cells in the presence and the absence of LMB.

<table>
<thead>
<tr>
<th>protein</th>
<th>Number of cells analysed</th>
<th>% of cells</th>
<th>Fn/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFP No LMB</td>
<td>Total 32</td>
<td>100</td>
<td>1.03±0.02</td>
</tr>
<tr>
<td></td>
<td>N&gt;C 5</td>
<td>15.6</td>
<td>1.28±0.05</td>
</tr>
<tr>
<td></td>
<td>C&gt;N 5</td>
<td>15.6</td>
<td>0.84±0.01</td>
</tr>
<tr>
<td></td>
<td>N=C 22</td>
<td>68.8</td>
<td>1.02±0.01</td>
</tr>
<tr>
<td>RFP LMB added</td>
<td>Total 62</td>
<td>100</td>
<td>1.07±0.03</td>
</tr>
<tr>
<td></td>
<td>N&gt;C 53</td>
<td>26.3</td>
<td>1.26±0.04</td>
</tr>
<tr>
<td></td>
<td>C&gt;N 6</td>
<td>5.3</td>
<td>0.72±0.07</td>
</tr>
<tr>
<td></td>
<td>N=C 3</td>
<td>68.4</td>
<td>1.02±0.01</td>
</tr>
</tbody>
</table>

Transfections and analysis were as per Table 3.3.

Figure 3.12 Subcellular localisation of RFP expressed in HeLa cells does not change upon LMB treatment. The results from Table 3.4 were plotted as a histogram of the percentage of cells falling into each localisation shown in the colour key, for the protein indicated below the columns (top panel). The lower panels show representative images of RFP localisation in the absence (left panel) or presence (right panel) of 4 ng/ml of LMB.
single-stranded oligos, FWNES1 and RevNES1; and FWNES2 and RevNES2, (see Table 2.11) to each other as described in Section §2.3.7. The presence of inserts within the Rev(1.4)-GFP plasmid was confirmed by sequencing in both directions through the insert with primers flanking the Rev protein (CMV promoter and GFP gene).

Figure 3.13 illustrates the proteins encoded by the reporter construct containing the Unp pNES1 and pNES2, the positive (Rev(1.4)-RevNES-GFP), and the negative (Rev(1.4)-GFP) control constructs respectively.

3.3.8 Unp pNES2 is a functional NES

The subcellular localisation of all four GFP-fusion proteins in HeLa cells was compared. As expected, Rev(1.4)-GFP fusion was mainly localised in the nucleoli (89% of cells), with faint cytoplasmic staining observed in 11% of cells (Figure 3.14). As expected, the Rev(1.4)-wtRevNES-GFP fusion was localised exclusively in the cytoplasm of the majority of cells (80%), and throughout the cell in the rest of cells (Figure 3.14). Rev(1.4)-pNES1-GFP fusion localised in almost identical fashion to the negative control (Figure 3.14), establishing that the pNES1 of Unp is not able to export the fusion protein out of the nucleus in the presence of the Rev NLS. On the other hand, the Rev(1.4)-pNES2-GFP fusion revealed a strikingly different subcellular localisation. Even though all cells still had fluorescence in the nucleus, more than 72% of cells had strong cytoplasmic fluorescence as well (Figure 3.14). Therefore, it can be concluded that Unp contains a functional NES (pNES2), which is weaker than the Rev NES but is able to facilitate nuclear export of the reporter Rev(1.4)-GFP protein. The other putative NES, pNES1, failed to export the reporter from the nucleus under similar conditions.

3.3.9 Actinomycin D treatment of HeLa cells expressing Rev(1.4)-pNES1-GFP and Rev(1.4)-pNES2-GFP

Actinomycin D (Act D) is a transcriptional inhibitor that can also prevent nucleolar association and nuclear import of Rev protein by an undefined mechanism (Rodriguez and Henderson, 2000). Act D treatment of cells expressing Rev-derivatives provides an excellent tool to detect weak NESs that are normally not able to overcome Rev NLS-mediated nuclear import. As shown above, nuclear export activity of Rev
Figure 3.13 Schematic representation of Rev(1.4)-GFP reporter based constructs.
A: Rev(1.4)-GFP reporter.
B and C: the Unp pNES1 and Unp pNES2 respectively, cloned in the reporter construct (see Section 3.3.7 for details).
D: WT RevNES cloned into Rev(1.4)-GFP reporter.
The location of the NLS and mutated NES in Rev are indicated.
Figure 3.14 Unp NES2 is able to export the reporter construct Rev(1.4)-GFP from the nucleus of HeLa cells.

HeLa cells were transfected with Rev(1.4)-GFP, Rev(1.4)-NES1-GFP, Rev(1.4)-NES2-GFP, or Rev(1.4)-RevNES-GFP constructs and were imaged 24 hours post-transfection. Images were analysed and scored for subcellular localisation as described in legend to Fig. 3.10. Representative images are shown in the lower panel of Rev(1.4)-GFP, Rev(1.4)-NES1-GFP, Rev(1.4)-NES2-GFP, or Rev(1.4)-RevNES-GFP localisation in HeLa cells 24 hours post-transfection. The arrow indicates that Unp NES2 has pronounced nucleocytoplasmic localisation.
fusion facilitated by Unp pNES1 was not detected, indicating that pNES1 is too weak to override the strong Rev NLS. Therefore, expression of Rev(1.4)-pNES1-GFP in HeLa cells was performed in the presence of Act D, in order to block Rev NLS-associated nuclear import. The Rev(1.4)-pNES2-GFP construct was used as a positive control. To ensure that cytoplasmic GFP fusion proteins were products of nuclear export and not newly synthesised proteins, the cells were co-treated with cycloheximide (CHX), an inhibitor of protein translation.

Act D (5 µg/ml final concentration) was added to cells expressing GFP-fusions 24 hours post-transfection for duration of 3 to 4 hours. CHX (15 µg/ml final concentration) was added at the same time either alone (negative control) or together with Act D. The results showed that cytoplasmic localisation of Rev(1.4)-pNES1-GFP fusion was increased approximately 2-fold upon Act D treatment (56.2% in the presence of Act D and 26% in the absence of Act D). A control, Rev(1.4)-pNES2-GFP, was localised in the cytoplasm of 79% of non-treated cells and in 98% of Act D-treated cells. The experiment was repeated two more times, but Rev(1.4)-pNES1-GFP did not respond consistently to Act D treatment with an enhanced cytoplasmic localisation. On one occasion it was increased from 10% (non-treated cells) to 16% (Act D added), and another time the cytoplasmic localisation was decreased upon Act D treatment from 15% to 7.5% (data not shown). Therefore, it was concluded that Unp pNES1 is indeed very weak.

### 3.3.10 Unp NES 2 is partially sensitive to LMB treatment

As described earlier (Section §3.3.6), RFP-Unp fusion protein accumulates in the nucleus of HeLa cells upon LMB treatment. However, approximately 15% of cells retain RFP-Unp in the cytoplasm despite LMB treatment, implying that CRM1-mediated export is not the only mechanism of Unp transport out of the nucleus, which may be the reason why LMB does not completely inhibit RFP-Unp export. Identification of a functional NES (NES2) in Unp made it possible to explore whether nuclear export of the reporter construct facilitated by Unp NES2 could be completely inhibited by LMB treatment. Therefore, HeLa cells expressing Rev(1.4)-pNES2-GFP construct, with Rev(1.4)-pNES1-GFP and Rev(1.4)-wtRevNES-GFP as negative and positive controls
respectively, were treated with LMB. As expected, Rev(1.4)-wtRevNES-GFP localised to the nuclei of LMB treated cells (79%), confirming that LMB is active. Rev(1.4)-pNES1- GFP localisation did not change substantially upon LMB treatment (13% and 9% nuclear localisation in the absence or presence of LMB treatment). In contrast, Rev(1.4)-pNES2-GFP fusion was detected in the cytoplasm of 45% of cells compared to 21% for the positive control Rev(1.4)-RevNES- GFP (see Figure 3.15). This result correlates with the finding that LMB does not completely inhibit RFP-Unp export, and provides additional evidence that CRM1-mediated export may not be the only pathway mediating Unp nuclear export.

3.3.11 Attempts to express truncated ΔNES1- and Δ(NES1+NES2)-RFP-Unp fusion proteins, and an NES2 RFP-Unp mutant

The Rev(1.4)-GFP-based in vivo nuclear export assay revealed that Unp NES2 is able to partially export Rev(1.4)-GFP from the nucleus, which implies that NES2 represents an active export signal within Unp. To determine whether this NES is the only functional export signal in Unp, two truncated versions of RFP-Unp construct were generated where NES1 or NES1 plus NES2 were removed (Figure 3.16). The truncated fusions were designated as ΔNES1-RFP-Unp and Δ(NES1+NES2)-RFP-Unp and their integrity confirmed by DNA sequencing, therefore successful expression of the truncated fusion proteins was expected. However, following several transfections of HeLa cells with ΔNES1- and Δ(NES1+NES2)-RFP-Unp fusion proteins, no RFP fluorescence was detected. The positive control, RFP-Unp fusion, was successfully expressed at the same time under identical conditions. Western blot analysis of extracts from HeLa cells expressing truncated ΔNES1-RFP-Unp and Δ(NES1+NES2)-RFP-Unp fusions using a Unp antibody revealed a band of approximately 85 kDa in both lysates instead of the expected 130 and125 kDa proteins respectively, and a very weak band of approximately 125 kDa in the Δ(NES1+NES2)-RFP-Unp lysate (Figure 3.17). The 85 kDa species were absent from cells expressing RFP-Unp (wt) protein and presumably represent a degradation product of the proteins with N-terminal truncations. Therefore, it was not possible to use these constructs for functional studies.
Figure 3.15 Unp NES2 is only partially sensitive to LMB treatment.
HeLa cells were transfected with Rev(1.4)-NES1-GFP, Rev(1.4)-NES2-GFP, or Rev(1.4)-RevNES-GFP constructs. 24 hours post-transfection, half of the samples were treated with 4ng/ml of LMB for a period of 8 hours. Images were analysed and scored for subcellular localisation as described in the legend to Fig. 3.10.
Figure 3.16 Schematic representation of truncated ΔNES1 and Δ NES(1+2) RFP-Unp constructs
Proteins with their predicted molecular mass are indicated on the left. The location of pNES1 and pNES2 are indicated by the pink bars. The KpnI site in the polylinker is in blue, while KpnI sites introduced by site-directed mutagenesis to allow deletion of fragments are in green.

Figure 3.17 Expression of RFP-Unp ΔNES1 and ΔNES(1+2) fusion proteins in HeLa cells as analysed by Western blotting
Total cell lysates from HeLa cell transfected with RFP-Unp (lane 1), RFP-Unp ΔNES(1+2) (lane 2), or RFP-Unp ΔNES1 (lane 3) were prepared 48 hours after transfection. 100 µg aliquots were resolved by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane and probed with polyclonal anti-Unp antibody (see Sections §2.5.3 and §4.3.3) followed by an HRP-conjugated anti-rabbit antibody and ECL. * indicates anti-Unp antibody-reactive ~85kDa proteins, presumably degradation products of RFP-Unp ΔNES(1+2) and Unp ΔNES1 (see text for details).
Next, two point mutations within NES2 of Unp were made. The C-terminal leucine residues of NES2 within RFP-Unp plasmid (\^{133}VEVYLLELK\^{142}) were mutated to alanines (\^{133}VEVYLLEAK\^{142}) by site-directed mutagenesis as described in Section §2.3.10 to generate plasmid RFP-UnpNES2mut. Sequence analysis confirmed that mutations were successfully introduced. However, despite numerous attempts, no RFP fluorescence was detected in HeLa cells transfected with RFP-Unp NES2mut. Two more rounds of LKL\^{142} -> AKA\^{142} mutagenesis and subsequent HeLa cells transfections with newly mutated RFP-Unp gave the same result. Therefore, it was concluded that mutation of two Leu residues probably results in protein instability and degradation, similar to that of truncation mutants described above.

### 3.3.12 The NLS3 mutation reduces RFP-Unp sensitivity to LMB

Finally, LMB treatment of HeLa cells transfected with the RFP-UnpNLS3 mutant was performed. It was hypothesised that the NLS3 mutant would be less sensitive to LMB than the wild type. The rationale underlying this experiment was that, if the NLS3 is functional, the mutant protein lacking the NLS3 would not accumulate in the nucleus of LMB-treated cells to the same extent as the wt protein, where the NLS3 is present. Thus, it was expected that the difference between RFP-Unp wt and the NLS3 mutant localisation in cells treated with LMB ((RFP-Unpwt)+LMB vs (RFP-Unp-NLS3m)+LMB) would be more striking than in non-treated cells (RFP-Unpwt vs RFP-UnpNLS3m), representing additional evidence for NLS3’s role in nuclear targeting.

Table 3.5 and Figure 3.18 summarise data obtained from this experiment. As expected, wt RFP-Unp accumulated in the nuclei of most LMB-treated HeLa cells (81%), while only a small portion of cells had cytoplasmic staining (10.6%). The NLS3 mutant, on the other hand, localised in the nuclei of 59.5% of LMB-treated cells and 31% of cells had RFP-Unp NLS3 mutant localised in the cytoplasm. The most striking difference, however, was in the Fn/c values where a significant difference (\(p = 0.0006\)) was observed between the values for wild type (3.06) and NLS3 mutant (1.8). This data strongly supports the previous results (Section §3.3.3) and confirms that NLS3 is a functional nuclear import signal for Unp.
Table 3.5 Subcellular localisation of RFP-Unp wt and RFP-Unp-pNLS3 mutant in LMB-treated HeLa cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of cells analysed</th>
<th>% of cells</th>
<th>F(n/c)mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFP-Unp wt plus LMB</td>
<td>47</td>
<td>100</td>
<td>3.06±0.3</td>
</tr>
<tr>
<td>N&gt;C</td>
<td>38</td>
<td>81</td>
<td>3.57±0.32</td>
</tr>
<tr>
<td>C&gt;N</td>
<td>5</td>
<td>10.6</td>
<td>0.79±0.03</td>
</tr>
<tr>
<td>N=C</td>
<td>4</td>
<td>8.4</td>
<td>1.05±0.03</td>
</tr>
<tr>
<td>RFP-Unp NLS3 mutant plus LMB</td>
<td>42</td>
<td>100</td>
<td>1.8±0.17</td>
</tr>
<tr>
<td>N&gt;C</td>
<td>25</td>
<td>59.5</td>
<td>2.5±0.18</td>
</tr>
<tr>
<td>C&gt;N</td>
<td>13</td>
<td>31</td>
<td>0.7±0.05</td>
</tr>
<tr>
<td>N=C</td>
<td>4</td>
<td>9.5</td>
<td>0.97±0.02</td>
</tr>
</tbody>
</table>

HeLa cells were transfected with RFP-Unp (wt) or RFP-UnpNLS3mut constructs, and 48 hours post-transfection samples received 4ng/ml of LMB. 8 hours after LMB addition, images were analysed as per Table 3.1

Figure 3.18 Subcellular localisation of RFP-Unp and RFp-UnpNLS3mut expressed in LMB-treated HeLa cells. The results from Table 3.5 were plotted as a histogram and represent the percentage of cells falling into each localisation indicated in the colour key, for the RFP-Unp protein indicated below the columns.
3.4 Discussion

3.4.1 Subcellular localisation of Unp

At the onset of this study there was controversy in the literature about the subcellular localisation of Unp. Initially, Unp was discovered by Gupta et al. (Gupta et al., 1993) and described as a nuclear protein, ubiquitously expressed in all tissues tested. The name, Unp, Ubiquitous Nuclear Protein is based on these characteristics. However, another report described Unp as a cytoplasmic protein (Frederick et al., 1998). In order to understand Unp function(s), it was important to clarify its subcellular localisation. Therefore, one of the aims of this study was to address this issue.

To study the localisation of Unp, it was tagged with RFP and expressed in HeLa cells. HeLa cells were chosen because the endogenous concentration of Unp was low. The expression of the RFP-Unp fusion in HeLa cells revealed that RFP-Unp can localise in the nucleus as well as in the cytoplasm. While these results do not support observations of Gupta and colleagues (Gupta et al., 1994), who reported that Unp is mainly nuclear, neither do they agree with the results obtained by Frederick and coworkers (Frederick et al., 1998), who showed that Unp is mainly cytoplasmic.

Different results obtained by three independent laboratories may appear controversial, but in fact are consistent with our data indicating that Unp is able to shuttle between the nucleus and cytoplasm. Factors such as cell origin, stage of the cell cycle, or level of expression can promote a shift in the localisation of a shuttling protein between the nucleus and the cytoplasm. For example, the tumour suppressor protein p53 is excluded from the nucleus in diverse neoplasms (Moll et al., 1995; Moll et al., 1992) and in embryonic stem cells (Aladjem et al., 1998). The cyclin B1-cdc2 complex translocates to the nucleus during prophase (Toyoshima-Morimoto et al., 2001). Dostie and colleagues (Dostie et al., 2000) have shown that subcellular localisation of overexpressed eukaryotic translation initiation factor 4E (eIF4E) depends on the amount of expression. When a low amount of DNA was used for transfection, eIF4E localised predominantly in the cytoplasm, while transfections with larger amounts of DNA resulted in accumulation in the nucleus.
Therefore, localisation of a shuttling protein, such as Unp, may alter between different cell types under different conditions. Chapter 4 of this thesis explores the localisation of endogenous Unp in variety of cell lines and shows that it indeed changes accordingly to cell type. The results of Gupta et al and Frederick et al are consistent with this conclusion. Thus endogenous Unp protein in a mouse fibroblast cell line, NIH3T3, was mainly nuclear (Gupta et al., 1994), while in human lung fibroblasts, IMR90, the overexpressed human orthologue of mUnp was mainly cytoplasmic but was also detected in a small portion of nuclei (Frederick et al., 1998).

3.4.2 Unp contains a functional NLS

The sequence analysis of mUnp revealed the presence of three putative NLSs within the Unp protein. Point mutations were introduced into each of these pNLSs, one at a time, and mutants were expressed in HeLa cells as RFP-Unp fusion proteins.

The pNLS1, consisting of amino acids 413KKP416 did not appear to be functional, because the RFP-Unp-pNLS1 mutant had similar subcellular localisation to RFP-Unp wt.

Most interestingly, mutation of pNLS2 resulted in increased rather than reduced nuclear accumulation of RFP-fusion protein. The pNLS2 contains a Z(R/K)XL cyclin A/E-binding motif, which has been found in proteins such as E2F1, p21, p107 (Adams et al., 1996; Chen et al., 1996; Zhu et al., 1995), and pRb (Adams et al., 1999). Cyclin binding to the protein via this motif promotes its phosphorylation by the cyclin dependent kinase, cdk2. Even though it was not possible to show that Unp binds to cyclin A or E, Unp was phosphorylated by the CyclinA/cdk2 complex in vitro. The cdk2 phosphorylation site is a serine/threonine-proline (S/T-P). It has been shown that Ser residues of Unp are phosphorylated in vivo (D. Gray, personal communications). Unp contains three putative cdk2 phosphorylated Ser sites (250SP251, 294SP295, and 642SP463) so it appears quite likely that Unp is phosphorylated by cdk2 in vivo.

Notably, it is well known that phosphorylation can promote or inhibit nuclear transport (see Section §1.6.5 and references therein). Therefore, it is possible that the level of cdk2-mediated phosphorylation may control Unp subcellular localisation. It is attractive to postulate that mutation of the cyclin binding site (701RHKRL705) which, in
turn, would inhibit/reduce phosphorylation of Unp, may caused increased retention of Unp in the nucleus.

Finally, mutation of the third putative NLS, pNLS3 (76QPQKKKK772), caused a significant shift in localisation of RFP-Unp to the cytoplasm. Moreover, LMB treatment of cells expressing the mutant protein versus wild type showed that the NLS3 mutant was not able to accumulate in the nucleus to the same extent, which further supports the idea that pNLS3 is a functional NLS. However, the RFP-Unp-NLS3 mutant protein was still localised in the nucleus of more than 30% of cells not treated with LMB and in 60% of cells treated with LMB. This suggests that either the export mechanism(s) is stronger, or that the NLS3-mediated nuclear import is not the only means of Unp entry to the nucleus. Several models of alternative Unp import to the nucleus can be suggested. First, Unp interaction with any member of the pRb family could serve as a piggyback mechanism (see Section §4.3.8), such that Unp binds to pocket proteins in order to be transported to the nucleus. Second, Unp might contain additional NLSs, which may not resemble currently identified NLSs. Third, as mentioned above, modifications such as phosphorylation may also contribute to changes in subcellular localisation as well as the cell cycle phase. It is also possible that Unp entering the nucleus via different mechanisms may have different roles.

### 3.4.3 Unp NLS3 is recognised by Importin α/β

The ELISA binding assay showed that full-length Unp binds to importin α/β1 heterodimer rather than to importin α or β1 alone. The UnpNLS3-β-gal fusion binds to the Impα/β heterodimer with a similar affinity as full-length Unp. However, in contrast to full-length Unp, UnpNLS3-β-gal also has a high affinity to Impα alone. This could be explained by the fact that NLS3 within the UnpNLS3-β-gal construct was removed from the context of a full-length Unp.

The binding affinity of UnpNLS3-β-gal fusion protein to Impα/β1 was much lower than that of T-ag, but it was similar to that of other nuclear localised cellular proteins. Thus the NLS of pRb is recognised by Impα/β with a Kd of 45nM (Efthymiadis et al., 1997), whilst the NLS of Drosophila Melanogaster transcription factor Dorsal is recognised by Impα/β with affinity of 26 nM. Therefore, it can be surely
concluded that UnpNLS3 is specifically recognised by the heterodimer Impα/β. It is, however, worth remembering that the binding affinity between Unp and importins was tested using bacterially expressed fusion proteins. The proteins expressed in bacteria do not undergo post-translational modifications, such as phosphorylation, normally occurring in mammalian cells. Therefore, if Unp is phosphorylated near the NLS3 the real binding affinity to importins would be different from the ones registered by the ELISA assay (see Section §1.6.5 and references therein).

In addition, there is a formal possibility that other types of importin β (see Section §1.6.3), other than importin β1 tested here, facilitate UnpNLS3-mediated nuclear transport, or that other isoforms of importin α are involved in recognition of Unp NLS.

3.4.4 Unp is actively exported from the nucleus

RFP-Unp expressed in HeLa cells was sensitive to LMB. The fact that RFP-Unp accumulates in the nuclei of LMB-treated cells leads to two significant conclusions. The first is that RFP-Unp is actively imported into the nucleus, as for a protein of approximately 140kDa, passive diffusion through the NPC is impossible. The second is that RFP-Unp is actively exported from the nucleus and this export is CRM1-dependent.

Although the majority of LMB-treated cells had strong nuclear accumulation of RFP-Unp, there was always a small proportion of cells where RFP-Unp was predominantly cytoplasmic. This can be explained by the fact that Unp nuclear import may be cell cycle dependent. It takes 22 hours for a HeLa cell to pass through one cell cycle. Therefore, given that HeLa cells were treated with LMB for only 8 hours, not all cells may have been in the appropriate stage of the cell cycle to import RFP-Unp into the nucleus. LMB treatment for longer than 16 hours is not applicable, because it is toxic to mammalian cells. However, 16 hours also do not cover the whole cell cycle of HeLa cells. Therefore, it did not prove possible to examine localisation of RFP-Unp in HeLa cells treated with LMB for the period of a whole cell cycle.

Alternatively, CRM1 may not be the only receptor to export Unp from the nucleus. There are a number of export receptors which are not sensitive to LMB treatment, including calreticulin which can recognise the same type of NES as the CRM1 receptor (Holaska et al., 2001). The fact that export of Rev(1.4)-GFP fusion protein facilitated by
Unp NES2 was not completely inhibited by LMB supports the idea of dual alternative export pathways for Unp, probably through NES2 (see below).

Close examination of the amino acid sequence of Unp revealed that it contains two NES-like motifs in its N-terminus that may explain its sensitivity to LMB. Both pNESs were tested for their ability to export a reporter construct, Rev(1.4)-GFP, from the nucleus. The pNES2 showed the ability to do so, whereas pNES1 did not. However, in the presence of actinomycin D, an inhibitor of Rev NLS-mediated nuclear import, pNES1 showed weak export activity in one such experiment. Therefore, the role of pNES1 in Unp export cannot be formally excluded.

NES2, on the other hand, appeared to be active even in the absence of Act D, although pNES2-mediated export was rather weak by comparison with that mediated by the NES of Rev (see Section §3.3.8 and Figure 3.14). It is worth noting, however, that the NES2 tested here was competing against a strong Rev NLS. Interestingly, analysis of the literature showed that functional NESs of several other cellular proteins had a comparably weak export activity when tested in experiments using the same reporter construct (Henderson and Eleftheriou, 2000). Thus Unp NES2 is in fact stronger than the NESs of the tumour suppressor protein p53, oncogene hdm2 or PML, transcription factors AP2 and TFIIIA, or the transcription inhibitor protein IκB-α (Henderson and Eleftheriou, 2000). Therefore, Unp NES2 is definitely a functional export signal.

Unfortunately, it was not possible to test whether pNES2 is the only functional NES within Unp because the truncated ΔNES1-RFP-Unp, Δ(NES1+NES2)-RFP-Unp fusion proteins, as well as the RFP-UnpNES2mut were present at very low levels in HeLa cells, presumably due to rapid degradation. It was assumed that truncation of N-terminal NES-containing region of Unp, changed the protein native folding, rendering the protein accessible to degradation. Interestingly, the degradation products of truncated RFP-Unp, detected by Unp antibodies, had the same molecular weight (80-90 kDa) as a degradation product commonly seen when recombinant Unp was produced in bacteria (Gilchrist and Baker, 2000). The N-terminus of Unp is rich in leucine, isoleucine residues and other hydrophobic residues that could be heavily involved in forming hydrophobic interactions and controlling overall Unp protein folding. Since NESs are also Leu/Ile-rich motifs, it is possible that the residues that form NES, may alternatively be involved in stabilising the Unp protein secondary or tertiary structure. Hence, when
the N-terminus was deleted, the Unp protein did not fold properly and subsequently degraded. Even the RFP-Unp NES2mut fusion, with only two mutated Leu residues, was not expressed to a detectable level in HeLa cells.

Since truncation of NES1 or NES1+NES2, and site-directed mutation of NES2 failed to produce Unp proteins that could be analysed, other experiments to test whether Unp possesses additional NESs can be suggested. Thus, there is a formal possibility that the amino acids surrounding pNES1 and 2, which were not tested here, are involved in the export of Unp. Chestukhin and co-workers have shown recently that the p130, the Rb family member, contains a Leu-rich NES-like motif which was not sufficient for nuclear export of a GFP reporter. However, a larger fragment of p130 (79 aa, containing the NES-like motif within it) was able to export the GFP reporter to the cytoplasm (Chestukhin et al., 2002). It is also plausible that NES1 and NES2 work cooperatively, as they are spaced by only 32 amino acids and could represent a similar Leu-rich motif as found in p130, mentioned above. Therefore, the region containing both NES1 and NES2 sequences could be cloned into Rev(1.4)-GFP construct to test its export ability.

Finally, there are several other Leu-rich motifs in Unp, which do not exactly resemble the Rev-like NESs, but could be tested for the export ability in the Rev-GFP reporter assay.

Taken together, the results of this chapter describe, for the first time, Unp as a protein whose transport into and out of the nucleus is actively regulated. Unp contains a functional NLS (NLS3), which is recognised by the nuclear import receptor Impα/β. Disruption of this NLS causes Unp to localise in the cytoplasm. Unp also contains a functional NES as shown by the ability of this NES to export a Rev(1.4)-GFP reporter construct from the nucleus. This chapter also demonstrated that the subcellular localisation of Unp can be altered by LMB treatment. Taken together, these findings show that Unp is a shuttling protein, which implies that this characteristic is likely to be important for its function as a ubiquitin specific protease. The next chapter explores whether the endogenous Unp has the same characteristics as the overexpressed fusion protein.
Chapter 4

SUBCELLULAR LOCALISATION OF THE ENDOGENOUS UNP AND THE INVOLVEMENT OF THE RB FAMILY OF PROTEINS IN UNP LOCALISATION
4.1 Introduction

The results described in the previous chapter established that Unp is able to shuttle between nucleus and cytoplasm, since it contains a functional NLS and an active NES. In these experiments, Unp fused to a fluorescent tag, RFP, was overexpressed in HeLa cells. However, given that the transport properties of an overexpressed protein may not be the same as the endogenous protein, the major aim of this chapter was to investigate whether endogenous Unp would function as a shuttling protein.

Another very interesting aspect of Unp function is its interaction with the pRb family of proteins. As discussed in the Introduction chapter (see Section §1.4.5) Unp interacts with all three pRb family members, pRb, p130 and p107. Here, it was also investigated whether this interaction influences Unp localisation.

4.2 Aims

The specific aims pursued in this chapter were:

1. To generate antibodies that can distinguish between Unp and its close homologue Usp15.
2. To determine the subcellular localisation of endogenous Unp in several cell lines.
3. To examine sensitivity of endogenous Unp to LMB treatment.
4. To create a pRb-binding mutant of Unp and assay its subcellular localisation.
5. To compare the subcellular localisation of endogenous Unp in Rb family defective and Rb family “normal” cell lines.
4.3 Results

4.3.1 Plasmid constructs for bacterial expression of Unp and Usp15 variable region as GST-fusion proteins

Immunohistochemical techniques are the only tool for detection and analysis of endogenous protein localisation. To perform immunohistochemical experiments, antibodies against the protein(s) of interest are required. Previously, at least three different laboratories have described antibodies raised against Unp ((DeSalle et al., 2001; Frederick et al., 1998; Gupta et al., 1994). However, these antibodies were generated before the existence of the close Unp homologue, Usp15, was reported (Baker et al., 1999). Usp15 shares 61% identity with Unp, with the N-terminal region 69% identical to Unp and the C-terminal region being 66% identical. The only regions which display a significant difference between the two proteins, are the so-called variable regions of approximately 230 amino acids (the proteins share 33% identity within this region) (see Fig. 4.1).

The first attempt to raise an anti-Unp antibody was performed by Gupta et al (Gupta et al., 1994). They used a sequence near the C-terminus of Unp, \(932 \text{LGSFPGSGGVKLS}^{945}\) as an antigen. Even though this antigen sequence does not align well with Usp15, the specificity of this antibody was still questionable as it reacted with a 180 kDa protein in NIH3T3 cell lysates instead of the expected 110 kDa (predicted molecular mass of Unp). Frederick and co-workers (Frederick et al., 1998) raised a peptide antibody against a region of Unp almost identical to that of Usp15 (Unp: \(\text{CHFSKADTIATIEK}\); Usp15: \(\text{RRFSKADTIDTIEK}\), where the identical residues are underlined). Therefore, it is quite likely that both proteins were being detected in their localisation studies. The commercially available anti-Unp antibody (Zymed, USA) (DeSalle et al., 2001), was raised against a C-terminal Unp peptide. The antibody was thus tested to see if it cross-reacted with Usp15. The Zymed antibody indeed detected protein of the expected size in bacterial lysates expressing either Unp or Usp15, clearly indicating cross-reactivity (see Fig. 4.2). Therefore, at the time of the beginning of this study, no reliable antibody to detect endogenous Unp or Usp15 was available.
**Figure 4.1 mUnp and mUsp15 protein sequence comparison.**

A: A diagram showing the conserved and the variable regions of mUnp (aa 537-793) and mUsp15 (aa 547-781). Variable regions coloured red and blue, respectively.

B: Sequence alignment of mUsp15 and mUnp. The variable regions of both proteins are highlighted by colour, mUnp is red, mUsp15 is blue. Primers to amplify the variable regions were designed from the DNA sequence encoding the conserved residues in pink.
Figure 4.2 Cross-reactivity of the commercial anti-Unp antibody (Zymed) with Usp15.

Total cell lysates from bacteria expressing Usp15 (lane 1), Unp (lane 2) or an empty pKK plasmid (lane 3) were electrophoresed in a 10% SDS-PAGE gel. Proteins were transferred to the PVDF membrane and probed with a commercially available anti-Unp antibody from Zymed (1:1000 dilution), followed by an HRP-conjugated anti-rabbit antibody (diluted 1:4000) and ECL detection (see Section §2.5.3). Positions of molecular mass marker are shown on the left (lane M).
To generate specific antibodies for Unp and Usp15, the variable regions of Unp (amino acids 539-786) and mouse Usp15 (amino acids 526-797) were chosen (see Fig. 4.1). Unp or Usp15-coding DNA was amplified with primers AntbFW and AntbBACKWARD (see Table 2.11 for sequence and primer description). PCR products generated with these primers contained an EcoRI site at the 5’-end and a Sall site at the 3’-end. The PCR products and pGEX-4T-1 plasmid (Table 2.10) were digested with EcoRI and Sall enzymes, and purified products were ligated. The final products were designated as GST-Unp(var) and GST-Usp15(var). DNA sequencing confirmed that the inserts were present and no reading frame shifts occurred (cloning, PCR and sequencing procedures are described in Section §2.3 of this thesis).

4.3.2 Optimisation of GST-Unp(var) and GST-Usp15(var) protein expression and purification

It had previously been observed that Unp is difficult to express in bacterial cells due to degradation problems (Gilchrist and Baker, 2000). Moreover, it was expected that the expression of only the variable region of the proteins (Unp as well as Usp15) may be even more challenging, since they may not fold properly and be more susceptible to aggregation and/or degradation. Because of these difficulties, a major aim of this work was to produce a sufficient amount of recombinant protein for rabbit immunisations. In order to minimise Unp and Usp15 protein degradation in bacteria, a protease-deficient E.coli strain, BL21(DE3) was used.

4.3.2.1 Small-scale protein expression

A first attempt to express GST-Unp(var) and GST-Usp15(var) was performed using a small scale preparation of bacterial cells (5 ml) induced with 1mM IPTG (see Section §2.4.1.1 for details). Total cell lysates (samples before and after induction) were analysed by SDS-PAGE and Coomassie-blue staining. Figure 4.3 shows that a high level of expression was obtained for both fusion proteins after induction with IPTG.

This success led to an attempt to purify both fusion proteins utilising affinity chromatography using GSH agarose beads. Following IPTG-induced expression of these recombinant proteins the cells were lysed by ultrasonication and the soluble proteins were bound to GSH agarose beads. SDS-PAGE analysis showed that a surprisingly low quantity of both proteins was present in eluates (Fig.4.4). This result
suggested that most of the recombinant proteins were insoluble and/or as predicted above were highly susceptible to proteases.

To investigate the possibility that the recombinant proteins were insoluble, a third round of small-scale recombinant protein expression was performed. This time the total protein lysate; the soluble fraction/supernatant after ultrasonication; and the insoluble fraction/pellet after ultrasonication were analysed. The bacterial cells for all fractions were first induced with IPTG. Figure 4.5 shows that both recombinant proteins, GST-Unp(var) and GST-Usp15(var), were detected in the “insoluble” fraction. Therefore, the recombinant proteins were mainly insoluble.

In order to increase the quantity of soluble recombinant proteins it was attempted to solubilise the precipitated GST-Unp(var) and GST-Usp15(var).

4.3.2.2 Solubilisation studies

Recombinant proteins expressed in bacteria often precipitate because they are expressed at a high concentration due to a strong promoter encoded by a multi-copy plasmid. Due to poor folding, such proteins form aggregates and precipitate, forming so-called inclusion bodies. High concentrations of urea (8 M) or guanidine hydrochloride (6 M) are widely used to solubilise the inclusion bodies. The proteins solubilised this way do not maintain their natural conformation. If the protein is expressed as a 6xHis-fusion it can be bound to Ni\(^{2+}\)-beads in the presence of denaturants. However, when the GST-tag is used, the solubilised proteins have to be refolded to allow the GST portion to bind to the GSH beads. There are several refolding procedures, some of which involve dialysis from high to low concentration of denaturant in a step-wise manner. Others, like drop-refolding, allow protein to refold when the protein in a high-denaturant buffer is added drop-wise to a low denaturant solution, and the rapid change of solution molarity allows the protein to refold without precipitation.

To estimate the minimal concentration of Urea required for solubilisation of GST-Unp(var) and GST-Unp(var), PBS buffer containing 0.5, 1, 2, 4, 6, and 8 M urea was used. Figure 4.6 indicates that complete solubilisation of precipitated proteins was achieved at 8 M urea. The proteins solubilised in 8 M Urea were dialysed step-wise against 6M, 4M, 2M, 0M urea in order to remove denaturant and allow re-folding. The proteins were mixed with GSH agarose beads but SDS-PAGE analysis revealed that the
Figure 4.3 Small-scale expression of GST fusions of Unp and Usp15 variable regions.

Total *E. coli* cell lysates expressing GST-Unp(var) or GST-Usp15(var) were electrophoresed in a 10% SDS-PAGE gel and stained in 0.5% Coomassie blue solution to visualise proteins. The presence (+) or absence(-) of 1mM IPTG is indicated above the lanes. The red and blue arrows indicate expression of GST-Unp(var) and GST-Usp15(var) recombinant proteins, respectively. Positions of molecular mass markers are shown on the left (lane M).

Figure 4.4 Small-scale expression and purification of GST fusions of Unp and Usp15 variable regions.

Soluble fractions of total *E. coli* cell lysates expressing GST-Unp(var) (lanes 1) and GST-Usp15(var) (lane 2), affinity purified and eluted recombinant proteins GST-Unp(var) (lanes 3) and GST-Usp15(var) (lane 4), and GSH beads with bound GST-Unp(var) (lanes 5), or GST-Usp15(var) (lane 6) were analysed by 10% SDS-PAGE, followed by protein visualisation by 0.5% Coomassie blue staining. The red and blue arrows indicates full-length GST-Usp15(var) and GST-Unp(var) proteins, respectively. Positions of molecular mass markers are shown on the left (lane M).
Figure 4.5 GST-Unp(var) and GST-Usp15(var) are largely insoluble.
Total cell lysate (1), insoluble fraction (2), and soluble fraction (3) of bacteria expressing GST-Unp(left) or GST-Usp15 (right) variable regions were electrophoresed in a 10% SDS-PAGE gel and stained with 0.5% Coomassie blue solution to visualise the proteins (see text for details). The arrow indicates the GST-Unp(var) and GST-Usp15(var) recombinant proteins. Positions of the molecular mass markers are shown on the right (lane M).

Figure 4.6 Concentration dependency of solubilisation of GST-Unp(var) fusion protein with urea
Insoluble (1) and soluble (2) fractions of bacteri cell lysates expressing GST-Unp(var) were analysed by 10%SDS-PAGE after solubilisation with different concentrations of urea (urea concentrations are indicated above each sample). The proteins were visualised by staining the gel with 0.5% Coomassie blue solution. The arrow indicates the GST-Unp(var) recombinant protein. Positions of the molecular mass markers are shown on the right (lane M).
solubilised proteins did not bind to the glutathione beads. The drop-refolding procedure was not successful either, since the solubilised proteins also did not bind to the GSH beads (see Fig. 4.7).

Strong denaturants such as 8 M urea can sometimes be replaced with milder reagents (e.g., detergents, like Triton X-100). Accordingly, the inclusion bodies formed by GST-Unp(var) and GST-Unp(var) recombinant proteins, were also tested for solubilisation by Triton X-100 added to the PBS buffer at concentrations of 0.5% and 2%. In addition, a commercially available bacterial protein extraction reagent, B-Per (Pierce) was used to solubilise the inclusion bodies. However, none of these methods succeeded in solubilising the precipitated proteins (see Fig. 4.8).

4.3.2.3 Attempts to increase the quantity of soluble proteins by improving the expression conditions

Because all the attempts to solubilise the recombinant proteins in a form still able to bind GSH agarose failed, the next approach was to optimise the expression conditions by minimising protein insolubility during expression.

Protein insolubility can be minimised if bacteria are grown at low temperatures, and the recombinant protein expression is induced with lower concentrations of IPTG for a shorter period of time. Therefore, the incubation temperature (37°C or 25°C), length of IPTG induction (30 minutes, 1, 2, or 3 hours), and final IPTG concentration (0.1, 0.05, or 0.01 mM) were varied. The induced bacterial cells were harvested; equal amounts of pelleted bacterial cells were resuspended in PBS and lysed by ultrasonication, and only soluble fractions were analysed by SDS-PAGE. Figure 4.9 (A-D) illustrates the results of these experiments. As can be seen from Figure 4.9 (B), the best conditions, which yielded the highest concentrations of soluble proteins were:

1. Incubation at 25°C;
2. IPTG induction at 0.01 mM final concentration
3. Induction for a period of 3 hours.

These optimal conditions were used for a large-scale (10L bacterial culture) expression of both recombinant proteins and subsequent purification using GSH beads (Figure 4.10). The details of large-scale expression are described in Section §2.4.1.2. The proteins illustrated on Figure 4.10 were used for rabbit immunisation.
Figure 4.7 Solubilised GST-Unp(var) does not bind to GSH beads. Lysates containing GST-Unp(var) solubilised with 8 M urea were either dialysed (I, left lanes) or subject to drop-refolding (II, right lanes) (see Section 4xxx). Following incubation with GSH agarose beads, aliquots of unbound protein from cell lysate expressing GST-Unp (1), protein eluted from beads (2), and beads boiled after elution (3) were analysed by 10% SDS-PAGE, followed by protein visualisation with 0.5% Coomassie-blue solution. The positions of the molecular mass marker are shown in the centre (lane M).

Figure 4.8 Attempts to solubilise GST-Unp(var) with detergents. (1): insoluble fraction (2): soluble fraction of E. coli cells expressing GST-Unp(var) analysed by 10% SDS-PAGE and staining with 0.5% Coomassie blue after solubilisation with: I: 0.5% Triton-X100; II: 2% Triton X100; III: B-PER extraction reagent (Pierce). The arrow indicates GST-Unp(var). Positions of the molecular mass markers are shown on the right side of each gel panel (lane M).
Figure 4.9 Optimisation of conditions for expression of GST-Unp(var) and GST-Usp15(var).

The expression of GST-Unp(var) and GST-Usp15(var) was induced by IPTG at various concentrations: (lane 1) 0.1 mM IPTG, (lane 2) 0.05 mM IPTG, and (lane 3) 0.01 mM IPTG for the periods of time and temperatures as indicated above each gel image. The cells were spun down, equal amounts of cell pellets were resuspended in PBS and solubilised by sonication and centrifuged. Soluble fractions of total *E. coli* cell lysates expressing GST-Unp(var) and GST-Usp15(var) fusion proteins were analysed by 10% SDS-PAGE and staining with 0.5% Coomassie blue. The red arrow indicates the GST-Usp15(var) recombinant protein and the blue arrow indicates GST-Unp(var) recombinant protein. Positions of the molecular mass markers are shown on the left side of each gel panel (lane M).
Figure 4.10 Large-scale expression of Unp and Usp15 variable regions as GST-fusion proteins.

The GST-Unp(var) and GST-Usp15(var) recombinant proteins were expressed in 10 L bacterial culture at 25°C and induced with 0.01mM IPTG for 3 hours. Following cell lysis and centrifugation, the GST-fusions in soluble fraction were bound to GSH-agarose and eluates analysed by 10% SDS-PAGE. Proteins were visualised by 0.5% Coomassie blue staining solution.

The red and blue arrows indicate GST-Usp15(var) and GST-Unp(var) respectively, the black arrow indicates the free GST protein.
4.3.3 Rabbit immunisation, crude sera testing, and affinity purification of the antibodies

New Zealand female rabbits were immunised with purified GST-Unp(var) and GST-Usp15(var) recombinant proteins as described below:

- **Primary injection (day 1)**: 150µg of protein mixed with Freund's Complete Adjuvant in a ratio 1:1
- **First booster (day 8)**: 150 µg of protein mixed with Freund's Incomplete Adjuvant in a ratio 1:1
- **Second Booster (day 15)**: 150 µg of protein mixed with Freund's Incomplete Adjuvant in a ratio 1:1
- **First Bleeding (day 22)**: 5 ml of blood collected
- **Second Bleeding (day 29)**: 10 mls of blood collected

Serum was separated from collected blood and tested for its specificity by Western blotting. Crude bacterial lysates expressing non-tagged full-length Unp, or Usp15, or an empty plasmid, prepared as described in Section §2.4.1.1, were probed with Unp and Usp15-specific serum, diluted 1 in 1000. As shown in Figure 4.11, Unp-specific serum recognised full-length Unp but not Usp15. The Usp15-specific serum also reacted only with Usp15 but not with Unp. Both sera cross-reacted weakly with some bacterial proteins. Therefore, it was concluded that specific antibodies for Unp and Usp15 were successfully raised.

In order to use these antibodies for immunostaining of mammalian cells, they need to be purified from crude serum. Since the use of GST-tagged variable regions of Unp and Usp15 for antibody affinity purification would result in co-purification of GST-specific antibodies, 6xHis-tagged Unp (full-length) and Usp15 (full-length) constructs had to be generated.

These constructs were made utilising the GATEWAY system, which is described in detail in Section §2.3.11 of this work. Figure 2.1 provides a schematic diagram describing the GATEWAY cloning procedure. Unp and Usp15 coding DNA were amplified with AttBl/2-UnpFW/Rev and AttB1/2-Usp15FW/Rev primers (see Table 2.11 for primer sequences) to obtain Unp or Usp15 full-length cDNA respectively, suitable for GATEWAY cloning. Then entry clones were created by recombination of
**Figure 4.11 The Unp and Usp15 specific crude serum do not cross-react.**

Total cell lysates from bacteria expressing Usp15 (lane 1), Unp (lane 2), or an empty pKK plasmid (lane 3) (see Section 2.4.1.1) were electrophoresed in a 10% SDS-PA gel. Proteins were transferred to a PVDF membrane and duplicate membranes were probed with Unp-specific crude serum (Panel 1) or with Usp15-specific crude serum (Panel 2), followed by an HRP-conjugated anti-rabbit antibody and ECL detection. Positions of molecular mass marker are shown on the left.

**Figure 4.12 SDS-PAGE analysis of 6xHis-Unp and 6xHis-Usp15 recombinant protein expression.**

Affinity purified 6xHis-Unp (lane 1) and 6xHis-Usp15 (lanes 2) recombinant proteins were loaded on a 10% SDS-PA gel. Electrophoresed proteins were detected by staining with Coomassie blue. The arrows indicate full-length 6xHis-Unp/Usp15. Positions of molecular weight marker are shown.

**Figure 4.13 Western blot analysis with the Unp and Usp15 affinity purified antibodies.**

Affinity purified 6xHis-Unp (lanes 1 and 4) and 6xHis-Usp15 (lanes 2 and 3) recombinant proteins were loaded on a 10% SDS-PA gel. Electrophoresed proteins were transferred to a PVDF membrane and probed with affinity-purified anti-Unp antibody (left panel) or with affinity-purified anti-Usp15 antibody (right panel), as indicated under the panels, followed by an HRP-conjugated anti-rabbit antibody and ECL detection. Positions of the 97 kDa molecular mass marker is shown on the left.
In addition, the Unp antibody did not detect non-specific bands in total HeLa cell extracts (e.g., Fig. 4.19). The antibody also failed to detect endogenous Unp in HeLa cells due either to insufficient protein level, or that the antibody does not detect denatured protein.
attB-flanked PCR product and pDONR201 vector in the BP reaction (see Section §2.3.11), resulting in the creation of an Entry clone. The Entry clone was then recombined with pDEST-17 plasmid (Table 2.10) in the LR reaction, to create final products, pDEST17-Unp or pDEST17-Usp15, expression clones encoding 6xHis tagged fusions of full-length Unp or Usp15. DNA sequencing confirmed plasmid integrity. The 6xHis-Unp and 6xHis-Usp15 proteins were then expressed and purified under conditions, determined for GST-Unp(var) and GST-Usp15(var) (see Figure 4.12 and Section §2.4.1.3). The 6xHis-Unp and 6xHis-Usp15 proteins were then used to affinity purify the anti- Unp and Usp15 antibodies from crude sera as described in Section §2.5.2. The affinity-purified anti-sera were then used for Western blot analysis against recombinant Unp and Usp15 proteins. Figure 4.13 shows that the antibodies recognise their cognate proteins and do not cross react. These antibodies also detected recombinant human Unp or Usp15 (data not shown).

§ 4.3.4 Subcellular localisation of endogenous Unp and Usp15 in HeLa and NIH3T3 cells is mutually exclusive

As mentioned earlier, Unp and Usp15 are very close homologues, which are approximately 61% identical. This similarity is also reflected in the fact that both are ubiquitin specific proteases, and can bind pRb (Usp15 also binds pRb, p130 and p107; C Angelats, R. Baker, personal communication). Therefore, this raises the interesting question of why higher eukaryotic cells possess two such similar enzymes?

Most interestingly, immunostaining of HeLa cells (cervical cancer) revealed that endogenous Unp and Usp15 proteins have mutually exclusive subcellular localisation. While the anti-Unp antibody stained only the nucleus, excluding the nucleolus, the anti-Usp15 antibody stained primarily the cytoplasm and the nucleolus, but not the nucleus (see Figure 4.14). These significant results show that although Unp and Usp15 may perform similar enzymatic functions, they appear to carry out their roles in different cellular compartments.

These results were confirmed in the NIH3T3 mouse fibroblast cell line (see Figure 4.15) although a greater heterogeneity of staining was observed. Unp was found mainly in the nuclei of 23% of cells, exclusively in the cytoplasm of 27% of cells, and throughout the cell in 50% of cells. Usp15, in contrast, did not show such heterogeneity,
Figure 4.14 Unp and Usp15 localise in different subcellular compartments in HeLa cells.

HeLa cells were fixed with 4% paraformaldehyde, stained with anti-Unp antibody (top panel) or with anti-Usp15 antibody (bottom panel) diluted 1:100 as described in the Chapter 2.6.6. Following several washes with PBS, cells were stained with anti-rabbit FITC-conjugated secondary antibody (Jackson Laboratories, Co), mounted on microscope slides and analysed by confocal microscopy.
Figure 4.15 Unp and Usp15 have different localisation in NIH3T3 cells. NIH3T3 cells were fixed, stained, and imaged as described in the legend for Figure 4.14.
being never seen in the nucleus, but rather localised near the plasma membrane, and enriched near some membranes but not others (see Figure 4.15).

To eliminate the possibility of non-specific reaction of the antibodies with unrelated proteins, the antibodies were blocked with Unp or Usp15 proteins prior to adding them to the HeLa cells. The results of this experiment showed no staining at all (not shown), demonstrating the specificity of the antibodies and lack of cross-reactivity with other proteins in the fixed cell preparations.

4.3.5 Endogenous Unp has different subcellular localisation in different cell lines

The finding that Unp steady-state localisation in HeLa and NIH3T3 cell lines is different clearly supports the observation described in Chapter 3 that Unp is a shuttling protein. It also implies that Unp localisation and shuttling properties may be regulated differently in different cell lines. To explore this further, the localisation of endogenous Unp in a range of cell lines was analysed.

Immunostaining of other available cell lines was therefore performed: HepG2 (human hepatocellular carcinoma); Saos-2, (human osteosarcoma); and MDA-MB-231 (human breast cancer). The results of immunostaining revealed a whole range of endogenous Unp subcellular localisations. Thus, Unp was entirely cytoplasmic in HepG2 cells, while in Saos-2 cells Unp was exclusively in the nucleus. Staining of MDA-MB-231 cells showed that Unp was more nuclear in 51.5% of cells, exclusively cytoplasmic in 27.5% of cells, and throughout the cell in 21% of cells. As mentioned above, NIH3T3 cells had yet another ratio of nuclear to cytoplasmic localisation for Unp. Here 23% of cells had more nuclear Unp localisation, 27% had Unp localisation shifted to the cytoplasm and 50% of cells had even distribution of Unp in the nucleus and in the cytoplasm. Figure 4.16 summarises the results of immunostaining of all cell lines.

4.3.6 Endogenous Unp is sensitive to Leptomycin B treatment

It is worth noting that all of the above cell lines are transformed, and it was therefore of interest to investigate the subcellular localisation of endogenous Unp protein in a non-transformed cell line. Mouse primary embryonic fibroblasts (MPEFs)
Figure 4.16 Subcellular localisation of endogenous Unp in a variety of cell lines.

Cells were fixed and stained using anti-Unp antibodies as described in the legend to Figure 4.14. Cells were visualised by confocal microscopy and scored by eye for fluorescence being mainly nuclear (N>C), mixed nuclear-cytoplasmic (N=C), or mainly cytoplasmic(C>N), and these percentages plotted in the histogram above the name of each cell line. Representative images are shown below each cell line name.
This experiment was repeated once with a single 8-hour LMB time point and similar nuclear accumulation was observed.
were chosen. MPEF cells were obtained from 13-day old mouse embryos, and were a kind gift from Dr Klaus Matthaei (JCSMR, ANU). Immunostaining of endogenous Unp protein revealed that it was mainly cytoplasmic in MPEF cells (67% of cells). Only 14% of cells had nuclear localisation of Unp and 19% of cells had Unp-specific staining throughout the cell (see Figure 4.17). As shown above, endogenous Unp was also mainly cytoplasmic in HepG2 and NIH3T3 cells. However, since HepG2 and NIH3T3 are transformed cells, the nuclear transport machinery may be altered in these cell types. Therefore, to prove that endogenous Unp is a shuttling protein, MPEF cells were used for Leptomycin B (LMB) treatment. LMB (4 ng/ml) was applied to MPEF cells for 0, 2, 4, 8, and 16 hours. The subcellular localisation of Unp in different samples was then compared. As expected, Unp gradually accumulated in the nucleus from 14% (0 hours) to 33% (16 hours) of cells in response to LMB treatment (see Figure 4.18). Therefore, it can be concluded that endogenous Unp in MPEF cells is a shuttling protein.

This result also raised the possibility that the shuttling properties of Unp are dramatically altered in HeLa and Saos-2 cells as well as in HepG2 cells, since Unp in these cells is exclusively nuclear (HeLa, Saos-2) or exclusively cytoplasmic (HepG2 cells).

4.3.7 Creation and characterisation of pRb-binding mutants of Unp

Several observations suggest that Unp may have several mechanisms for nuclear-cytoplasmic transport. Firstly, mutation of Unp NLS3 did not cause an exclusive cytoplasmic localisation of RFP-Unp (Section §3.3.3) indicating that Unp can enter the nucleus via a different mechanism. Secondly, Unp export was not completely inhibited by LMB, suggesting that Unp can exit the nucleus via a CRM1-independent mechanism (See Sections §3.3.6 and §4.3.6).

One of the alternative ways Unp may be transported into or out of the nucleus, is via a piggy-back mechanism mediated by a shuttling protein (Dostie et al., 2000; Turpin et al., 1999). Two laboratories have shown recently that Unp interacts in vivo with pRb, and in vitro with p130, and p107 (Blanchette et al., 2001; DeSalle et al., 2001). Unp contains two conserved regions, CR1 and CR2, comprised of consensus sequences LHE and LXCXE, respectively (where X is any aa). These regions are present in a number of
Figure 4.17 Subcellular localisation of endogenous Unp in Mouse Primary Embryonic Fibroblasts (MPEF).
MPEF cells were stained, fixed, visualised by CLSM and analysed visually as described in the legend to Figure 4.16.

Figure 4.18 Endogenous Unp in MPEF cells accumulates in the nucleus upon Leptomycin B treatment.
MPEF cells were grown on cover slips until they reached 70-80% confluency, and were then treated with 4 ng/ml of LMB for 0, 2, 8, and 16 hours. The cells were fixed in 4% paraformaldehyde and stained with anti-Unp antibodies and scored visually as described in the legend to Figure 4.16.
cellular and viral proteins and are known to be the interaction sites for pocket proteins (Section §1.5.1 and Buyse et al., 1995; Fattaey et al., 1993; Whyte et al., 1989). Interestingly, Unp interaction with different pRb family members is mediated by different regions of Unp. For example, Unp-pRb interaction is disrupted by mutation of the Unp LXCXE motif alone (Blanchette et al., 2001), whereas disruption of Unp-p130 interaction requires mutations of both LXCXE and LHE motifs (ibid). On the other hand, interaction of Unp with p107 is not disrupted even when both CR motifs are mutated (ibid).

In order to investigate whether Unp interaction with pRb can influence Unp subcellular localisation, the 459LXCXE463 motif in RFP-Unp was mutated. The C461 residue was chosen for substitution by G, because Blanchette et al have shown that this mutation disrupts Unp association with pRb in vitro (Blanchette et al., 2001).

Site-directed mutagenesis with primers UnpCR2mutFW/Rev (see table 2.11) was used to introduce a C461G mutation within RFP-Unp. The mutation was confirmed by DNA sequencing and the mutated construct was designated as RFP-UnpC461Gmut. HeLa cells were transfected with the RFP-UnpC461Gmut and RFP-Unp wt as a positive control (details of transfection procedure are described in Section §2.6.2). Both populations of cells were analysed under the confocal microscope 48 hours post-transfection. Surprisingly, in contrast to the positive control, no fluorescence was observed in cells transfected with RFP-UnpC461Gmut. In order to investigate why the mutant protein was not expressed, several attempts to improve expression of RFP-UnpC461Gmut were made. Plasmid DNA encoding RFP-UnpC461Gmut was purified using Wizard PureFection kit (Promega) in order to obtain better quality DNA, different concentrations of DNA and the Cytofectene transfection reagent were used to optimise transfection efficiency. Finally, other transfection reagents, Fugene and Lipofectamine, were used for transfections. None of the above changes overcame the lack of expression of the Unp mutant. The lack of expression was confirmed by Western analysis, which revealed a very weak band of the expected size (Figure 4.19). The basis of the lack of expression of this mutant is discussed below (see Section §4.4.3).

Given that mutation of C461 resulted in lack of protein expression, an alternative mutant was derived.
**Figure 4.19** Western blot demonstrating very poor expression of RFP-UnpC461Gmutant.

HeLa cell expressing RFP alone (lane 1), RFP-Unpwt (lane 2), and RFP-UnpC461G mutant (lane 3) were lysed as described in Section §2.6.3 48 hours after transfection. The total cell lysates were electrophoresed in a 10% SDS-PA gel. Western blotting was performed as described in the legend to Figure 4.2, using the Unp antibody raised during this study.

The experiment was performed by R. Baker.

**Figure 4.20** The RFP-UnpL459Gmutant interacts with GST-pRb as demonstrated by GST-pull down assay.

HeLa cell expressing RFP alone (lane 1), RFP-Unpwt (lane 2), and RFP-UnpL459G mutant (lane 3) were lysed 48 hours after transfection as described in §2.6.3. The lysates expressing RFP alone (lane 4), RFP-Unpwt (lane 5), and RFP-UnpL459G mutant (lane 6) were incubated with bacterially expressed GST-pRb bound to GSH beads. The total cell lysates and proteins, which bound to GST-Rb were electrophoresed in 10% SDS-PA gel. Western blotting was performed as described in the legend to Figure 4.2, using the Unp antibody raised during this study.

The experiment was performed by R. Baker.
It has been reported that that mutation of the leucine residue in the LXCXE conserved region disrupts adenovirus E1A protein binding to pRb (Corbeil and Branton, 1994). Based on this finding, L\textsuperscript{459} was mutated to G in RFP-Unp, which resulted in the RFP-UnpL459G mutant. HeLa cells transfected with the RFP-UnpL459G mutant clearly expressed the mutated protein (Figure 4.20, lane 3). A GST-pull down was performed to assess the L459G mutant - Rb binding, but the pull-down experiment between bacterially expressed GST-pRb and HeLa cell lysates expressing RFP-UnpL459Gmut or RFP-Unp wt showed that the mutant could still bind GST-pRb (Figure 4.20). Therefore, the RFP-UnpL459G mutant was not useful for Unp localisation studies. Given that both attempts to create a pRb-binding mutant of Unp failed, a different approach needed to be developed. The next section presents such an attempt.

### 4.3.8 Involvement of the pRb family of proteins in endogenous Unp localisation

In order to address the question whether pRb or other pocket proteins are involved in determining Unp subcellular localisation, the latter was analysed in Rb family “defective” cell lines.

Analysis of the literature describing Saos-2 and HeLa cells revealed several interesting facts. Saos-2 cells, where Unp is found only in the nucleus (Section §4.3.5), are pRb-defective (Goodrich et al., 1991). More specifically, pRb in Saos-2 cells is truncated at its C-terminus. This defective pRb protein lacks its NLS and therefore is localised only in the cytoplasm. As a consequence of this, the truncated form of pRb is not functional (Shew et al., 1990). Interestingly, both mRNA and the protein levels of p130 and p107 are also significantly depleted in Saos-2 cells (Jiang et al., 2000).

In addition, the HeLa cell line, where Unp was also localised only in the nucleus, expresses HPV18 virus E6 and E7 proteins. Notably, the presence of E7 herpes virus proteins prevents pRb dephosphorylation, thus keeping it hyperphosphorylated and therefore inactive (Goodwin and DiMaio, 2000). More intriguingly, E7 enhances p130 and p107 degradation by the proteasome. Therefore, levels of p130 and p107 proteins in HeLa cells are also dramatically depleted (ibid). Taken together, Saos-2 and HeLa cells
have similar Rb family status: the pRb protein is inactive, and the proteins levels of p130 and the p107 are depleted.

As mentioned before, Unp interacts with pRb, p107 and p130. It was thus hypothesised that the events triggered by pRb inactivation in both cell lines may have resulted in Unp localisation in the nucleus. To examine this possibility, a modified Saos-2 cell line, SR-40, was obtained. In these cells the pRb phenotype has been rescued through stable expression of a wild type Rb cDNA (SR-40 cell line was a kind gift from David Jans, JCSMR, ANU). However, immunostaining of SR-40 cells (see Section §2.6.6 for details) revealed no changes in endogenous Unp localisation compared to Saos-2 cells (Figure 4.21 A, B). As a control for pRb expression, both cell lines (Saos-2 and SR-40) were stained with anti-pRb antibodies (Pharminingen) and this confirmed that pRb is cytoplasmic in Saos-2 cells and expressed throughout the cell in SR-40 cells with some nuclear accumulation (Figure 4.21 C, D). Since introducing wild type pRb did not change Unp subcellular localisation, it was concluded that pRb is not involved in the regulation of Unp localisation in these cells.

However, it is plausible that other Rb family proteins, p130 and p107, may play a role in the regulation of Unp subcellular localisation. To investigate this, NIH3T3 cells infected with HPV16 E7 protein were used. E7 protein contains CR1 and CR2 Rb-binding motifs and has high affinity for all pRb family proteins. When E7 is present in the cell, it binds strongly to all pRb family members through its LXCXE motif, displacing any other Rb-interacting proteins, predominantly Unp, which bind the Rb family of proteins through the same LXCXE motifs. Another important feature of E7 is that it downregulates p130 and p107 proteins level by targeting them for destruction by the proteasome. It was predicted that if p130 and p107 were involved in Unp shuttling or anchoring in the cytoplasm, Unp localisation in the presence of E7 protein would be more nuclear.

Therefore, Unp localisation was analysed in NIH3T3 cells stably transformed with plasmids expressing three different proteins: first, HPV16 E7 (wild type) protein (these cells are designated as NIH3T3/E7). Second, N-terminally deleted HPV16 E7 protein where the deletion removes the LXCXE binding motif, which is situated in the N-terminus of E7 protein (cells designated as NIH3T3/ΔE7). Third, an empty plasmid, as a
Unp in Saos-2 cells (pRb-)

Unp in SR-40 cells (pRb+)

pRb in Saos-2 cells (pRb-)

pRb in SR-40 cells (pRb+)

Figure 4.21 Unp localisation in Saos-2 cells does not change upon introducing an active pRb.
Saos-2 (Panel A) and SR-40 (Panel B) cells were stained with anti-Unp primary antibodies followed by anti-rabbit FITC-conjugated secondary antibodies.
To visualise pRb localisation, Saos-2 (Panel C) and SR-40 (Panel D) were stained with mouse anti-pRb antibody (Pharmingen) followed by anti-mouse Texas Red-conjugated antibody (Jackson Laboratory Co) (see Section §2.6.6). Cells were then fixed on microscope slides and analysed by confocal microscopy. Representative images are shown.
negative control (cells designated as NIH3T3). These cell lines were a kind gift from Dr. Grant MacArthur, Peter Macallum Cancer Institute, Melbourne, Australia.

The results of immunostaining revealed that Unp was indeed significantly more nuclear in NIH3T3/E7 cells (47%) compared to NIH3T3 cells (23%) (p=0.0006) (Figure 4.22). Unp nuclear localisation in NIH3T3/ΔE7 cells was not significantly different than in NIH3T3 cells (p=0.18) (see Figure 4.22).

This experiment provides support to the hypothesis that Unp localisation in the cell may be altered by E7-mediated downregulation of p130 and p107 proteins. Therefore the p130 and/or p107 proteins may play a role in the determination of Unp localisation, perhaps through a piggyback mechanism.
Figure 4.22 Localisation of Unp in NIH3T3 cells expressing E7 or E7 Rb-binding mutant.

The NIH3T3 cells were stably transfected with an empty vector (NIH3T3/contr), or with wild type E7 protein (NIH3T3/E7), or with N-terminal-truncated E7 protein (NIH3T3/dE7) as indicated below the columns. Cells were fixed, stained, and imaged as described in the legend to Figure 4.16. The subcellular localisation of endogenous Unp in these cells was analysed by confocal microscopy by scoring cells with pronounced nuclear, or cytoplasmic or nuc+ cyt staining with Unp antibodies and the percentages plotted in the histogram. The numbers of cells scored is indicated under each set of columns.
4.4 Discussion.

4.4.1 Endogenous Unp is a shuttling protein

The main aim of the study described in this chapter was to determine whether endogenous Unp, like the exogenously expressed RFP-Unp protein fusion, is a shuttling protein (see Chapter 3). The antibodies raised against Unp provided the essential tool needed to carry out this aim. They were proven to recognise only their cognate proteins and not to cross react. Moreover, they helped to determine subcellular localisation of endogenous Unp and its sensitivity to LMB (Sections §4.3.3-4.3.6).

Thus, immunostaining of a variety of cell lines showed that endogenous Unp has a different localisation in different cell lines. Unp localisation varied from being totally cytoplasmic (HepG2 cells) to totally nuclear (HeLa and Saos-2 cells). This indicates that Unp’s nucleocytoplasmic transport properties may be differently regulated in these cells. Most importantly, experiments with MPEF cells showed that endogenous Unp is sensitive to LMB. This finding provides direct evidence that endogenous Unp, like the exogenous RFP-Unp, also shuttles between the nucleus and cytoplasm.

Interestingly, even though MPEF cells were treated with LMB for 16 hours, only 33% of cells showed Unp accumulation in the nucleus. One cell cycle round of MPEF cells, which is shorter than that of HeLa cells, is estimated to be 16-20 hours. The precise estimation of a cell cycle length of MPEF is complicated given that the procedure utilised for generation of these cells results in purification of a mixture of different cell types, the majority of which are fibroblasts. Since treatment of MPEF cells with LMB was for 16 hours, this would probably cover most of phases of a cell cycle. Therefore, it can be concluded that Unp partial accumulation in the nucleus is either due to an alternative export mechanism additional to CRM1-mediated export or because the CRM1 receptor in MPEF cells was not completely inhibited by the amount of LMB used for the experiment.

Notably the overexpressed RFP-Unp fusion protein had a mixed cytoplasmic/nuclear distribution in HeLa cells, whereas endogenous Unp was exclusively nuclear. This may indicate that the nuclear import machinery was overwhelmed by the level of RFP-Unp and could not import it all in the nucleus, and/or the nuclear retention mechanism for Unp was overwhelmed by RFP-Unp levels.
4.4.2 Analysis of subcellular localisation of endogenous Unp in different cell lines

The results of immunostaining of different immortalised cell lines showed that Unp has a diverse range of subcellular localisations (Section §4.3.5). This finding demonstrates that Unp shuttling properties are controlled differently in different cell lines. In addition, given that most of the immortalised cell lines used are well described, they could be a useful tool to clarify what factors control and/or alter nuclear transport of Unp in these cells.

Unp is totally cytoplasmic in HepG2 cells. These cells are derived from a human hepatocellular carcinoma. A search of literature revealed no reports of the pRb status of HepG2 cells. However, different research groups have shown that HepG2 cells have altered expression levels and activity of various protein kinases. Thus, compared to normal hepatocytes, expression and activation of serine/threonine protein kinase B (PKB) is upregulated in HepG2 cells (Syed et al., 2002). Another serine/threonine kinase, hSGK1, is also expressed at higher levels in HepG2 cells compared to non-transformed hepatocytes (Fillon et al., 2002). Levels of expression of protein kinase C (PKC) isoforms are also altered in HepG2 cells (Ducher et al., 1995). Based on these findings, it would be interesting to investigate whether an alteration in the phosphorylation status of Unp can explain its cytoplasmic localisation.

MDA-MB-231 breast cancer cells, where p53 is inactivated, have very similar Unp subcellular localisation to MPEF cells (see Section §4.3.6). MPEF cells are not immortalised and not transformed, and have a wild type p53 pathway. Accordingly, it is possible to suggest that Unp subcellular localisation is not influenced by p53-dependent signalling.

In HeLa and Saos-2 cells, where Unp is totally nuclear, the function of Rb proteins is disrupted. This raises the possibility that Rb proteins may have a role in Unp subcellular localisation.

4.4.3 Unp localisation and Rb family of proteins

Several attempts were made during this study to characterise the link between Unp localisation and its interaction with Rb proteins. The attempts to create Rb-binding mutants of Unp by mutating the conserved amino acids within LXCXE region were not
successful in regards to Unp localisation. However, these results are very interesting and contradictory to previously published findings.

Firstly, the mutation of C461 resulted in an almost complete lack of protein expression. Recent preliminary experiments performed in this laboratory suggest that C461, together with the other three Cys residues in Unp (C464, C708, and C801), may be involved in the formation of a Zinc finger, a structure common for most of Ubps (R. Baker, E. Sutcliffe, personal communication). Therefore, mutation of this residue may have prevented folding of Unp and/or the subsequent tetramerisation of RFP, which is required for fluorescence. The Western blot analysis showed a very low level of full-length proteins (Figure 4.19) implying that most of the protein was degraded. These preliminary results also put into question the conclusion of Blanchette et al that C461 is important for interaction with pRb based on in vitro experiments (Blanchette et al., 2001). They reported that mutation of UnpC461 prevented interaction with pRb. However, this experiment used bacterially expressed GST-UnpC461G, which may not be subject to degradation to the same extent as in mammalian cells. The lack of interaction with pRb may be, therefore, an indirect effect of the lack of proper folding. However, DeSalle et al observed that an untagged Unp protein with LXCXE mutated to AXRXH was expressed to the same level as a wild type in U2OS cells (DeSalle et al., 2001). While different expression constructs and cell lines were used, it is unclear why different protein stabilities were observed.

Secondly, even though it has been shown that all three conserved residues in LXCXE motif are involved in interaction with Rb protein (Lee et al., 1998), the results of this work suggested that these residues are not equally important for binding to Rb, at least with respect to Unp. Thus the pull-down assay (Figure 4.20) showed that mutation of the conserved L residue of Unp’s LXCXE motif did not disrupt its interaction with GST-pRb. Similar results were obtained by DeSalle et al who demonstrated that mutation of a third conserved residue of LXCXE motif, E, results only in partial loss of interaction with pRb (DeSalle et al., 2001).

The experiments with endogenous Unp showed that pRb is unlikely to be involved in Unp subcellular localisation, since introduction of a full-length functional pRb into Saos-2 cells did not change Unp localisation. However, expression of E7 protein of a high-risk HPV16 in NIH3T3 cells targets p130 and p107 for proteasome-
mediated degradation and prevents pocket proteins interaction with other LXCXE proteins, changed Unp localisation. Therefore, it is possible that Unp interaction with p130 or p107 provides a piggyback mechanism for Unp facilitating transport out of the nucleus. Of relevance in this context is the fact that p130 also shuttles between nucleus and cytoplasm, and its nuclear export is not LMB-sensitive (Chestukhin et al., 2002). Therefore, it is possible that Unp partial insensitivity to LMB relates to its interaction with p130 with may contribute to Unp exit from the nucleus.

A third Rb family member, p107, is found in the cytoplasm and the nucleus. It is likely that p107 can shuttle, because it is able to import E2F4 factor into the nucleus (see Section §1.5.4 for references), and it contains a Leu-rich region, almost identical to nuclear export signal of p130 (Chestukhin et al., 2002). Therefore, it is plausible that p107 may also serve as a transporter for Unp nuclear-cytoplasmic transport.

Clearly, these results are preliminary and more detailed studies are required. For example, subcellular localisation of endogenous or recombinant Unp could be examined in cells lacking pocket proteins (ie primary cells from knock out pRb/-/-, or p130/-/-, or p107/-/- mouse embryos) or in cells where pocket proteins are overexpressed.

Finally, there are other possible explanations for Unp exclusive nuclear localisation in HeLa and Saos-2 cells. It can be suggested that Unp accumulation in the nucleus is due to mutations or truncations of the Unp gene, which may result in a production of an NES-truncated or mutated protein leading to disruption of protein export to the cytoplasm. There is evidence supporting this idea: the gene that encodes human Unp is positioned on chromosome 3, locus 3p21.31 (Gray et al., 1995). It was shown recently that this locus of chromosome 3 is a subject to loss of heterozygosity (LOH) and other deletions and aberrations in many cancer types including cervical cancer ((Braga et al., 2002) and references therein).

### 4.4.4 Usp15 subcellular localisation may provide a clue to its function.

During this study antibodies against two closely related ubiquitin-specific proteases, Unp and Usp15, were generated. These antibodies specifically recognise their cognate proteins and do not cross-react. The immunostaining of HeLa and NIH3T3 cell lines with these antibodies showed that Unp and Usp15 localise in different
compartments of the cell. This is the first demonstration that these two proteins may have different roles, perhaps deubiquitinating different substrates in separate cellular compartments.

Usp15 function in vivo has not been rigorously studied. In vitro experiments with recombinant Usp15 showed that it can cleave linear fusions of ubiquitin with β-gal and GST, as well as the ubiquitin-proline bond, a property previously unique to Unp (Baker et al., 1999). It has also been recently shown that Usp15 can interact with all Rb family members (C. Angelats, R. Baker, personal communications). Clearly, detailed studies are required to determine cellular substrates of Usp15.

Based on immunostaining results obtained during this study, several suggestions about the possible functions of Usp15 can be made. Firstly, it was shown that Usp15 is localised in the nucleolus of HeLa cells, while Unp was never detected there. The nucleolus is a site for transcription of ribosomal RNA, which later is exported from the nucleus to the cytoplasm as a part of the ribonucleoprotein particle (RNP) (Olson et al., 2000). It is well established that some proteins within RNP are degraded by the proteasome (Iervolino et al., 2002). Therefore, it is possible that Usp15 may be involved in the export of ribosomal RNA, and/or stabilisation of proteins within RNPs.

In NIH3T3 cells (as well as MDA-MB-231 cells, data not shown), Usp15 was localised near cellular membranes. This intriguing funding suggests that Usp15 may interact with membrane-associated proteins or be involved in endocytosis. It has been established that ubiquitination and deubiquitination are involved in control of protein trafficking into endosomes and subsequent degradation (see Section §1.2.3 for more information and references). Clearly, these observations warrant further studies.

The experiments described in this chapter also provide some clues about mechanisms of Usp15 nuclear-cytoplasmic transport. The fact that Usp15 was found in the nucleoli of HeLa cells suggests that it is actively imported into the nucleus, which implies that it contains an NLS. Park et al have shown that the almost identical rat orthologue of Usp15, Ubp109, contains a functional NLS (Park et al., 2000). This NLS is also present in Usp15, 755PDLKKR760.

In addition to nucleolar localisation in HeLa cells, Usp15 localises in the cytoplasm of HeLa and NIH3T3 cells near the cellular membrane. This suggests that Usp15 may possess an NES(s) in order to be exported into the cytoplasm. Analysis of
the amino acid sequence revealed that Usp15 contains two Rev-like putative NESs, One of them is identical to Unp NES2. Another has strong similarity to Unp NES1, but contains additional L and I residues, which implies that it could be a stronger export signal than either Unp NES1 or NES2:

\[
\begin{align*}
\text{98} & \quad \text{LIDELDYILL}^{107} \quad \text{mUsp15 pNES} \\
\text{102} & \quad \text{LIDELDYVLL}^{111} \quad \text{mUnp pNES1} \\
\text{129} & \quad \text{VEVYLTELKL}^{138} \quad \text{mUsp15 pNES} \\
\text{133} & \quad \text{VEVYLLEEKL}^{141} \quad \text{mUnp pNES2}
\end{align*}
\]

Considering the subcellular localisation of Usp15 in HeLa and NIH3T3 cells, and the fact that Usp15 contains putative NLS and NES(s), it can be hypothesised that Usp15 may, like Unp, be a shuttling protein. This observation is supported by experiments performed by Park et al, who showed that endogenous Ubp109 (rat orthologue of Usp15) localises in both the nucleus and cytoplasm of L6 cells, while exogenous myc-tagged Ubp109 is found only in the nucleus of NIH3T3 cells (Park et al., 2000). Therefore like Unp, the different localisation of Ubp109 in different cell types implies that it is may also be a shuttling protein.
Chapter 5

GENERAL DISCUSSION
5.1 Unp subcellular localisation and the mechanism of Unp nuclear transport

At the onset of this study the subcellular localisation of Unp was unclear. Gupta et al identified Unp and later showed, by subcellular fractionation of NIH3T3 cells, that endogenous Unp localised primarily in the nucleus (Gupta et al., 1994; Gupta et al., 1993). Antibodies raised against a C-terminal peptide of Unp (LGSFPGSDGGVKL946) were used for this study. However, the antibodies reacted only with a 180-kDa protein, which caused some concern, since the molecular weight of Unp is approximately 110 kDa.

Another group (Frederick et al., 1998) showed that endogenous Unp was observed mainly in the cytoplasmic fraction of HeLa cells after subcellular fractionation, and exogenously expressed c-myc-Unp was mainly localised in the cytoplasm of IMP90 cells. Given that the antibody used to detect endogenous Unp in HeLa cells was raised against a sequence similar to that of Usp15, it is possible that both proteins were detected in their study, again putting the results into question.

The present study showed that Unp contains a functional NLS (NLS3, QPQKKKK772) since most of the protein accumulated in the cytoplasm when NLS3 was mutated within the context of an RFP-Unp fusion protein (Section §3.3.3). Importinα/β appeared to be able to recognise this sequence specifically (Section §3.3.5). Unp was also shown to contain an active NES (NES2, VEVYLLELKL142). A peptide encoding the NES was able to export a Rev(1.4)-GFP reporter contract from the nucleus (Section §3.3.8). Consistent with this, the study also demonstrated that Unp subcellular localisation is sensitive to Leptomycin B treatment, indicating that the CRM1 receptor is involved in Unp nuclear export (Section §3.3.6). Therefore, these data strongly suggest that Unp is a shuttling protein containing the necessary targeting signals for nuclear import and export, which are recognised by distinct members of the importin family of nuclear import (Section 3.3.5) and export receptors (Section §3.3.6).

The ability of Unp to shuttle between the nucleus and the cytoplasm in a signal-dependent manner is presumably the basis of its disparate location observed in different cell types both mentioned above, and presented in this study (Section 4.3.5).
5.2 Differences in subcellular localisation of Unp and Usp15

Another achievement of this work was the generation of specific antibodies against Unp and Usp15 proteins. These essential tools enabled a definitive determination of the subcellular localisation of endogenous Unp and Usp15 proteins for the first time.

As mentioned above, other researchers had previously generated antibodies for Unp detection before this study was commenced. However, these antibodies were either unreliable as they detected incorrectly-sized protein (Gupta et al., 1994), or were expected to be cross-reactive with the close Unp homologue, Usp15 (commercial anti-Unp antibody from Zymed, USA; anti-Unp antibody generated by Frederick et al., 1998).

The antibodies raised here were shown to recognise only their cognate proteins. Immunostaining with anti-Unp antibodies revealed that the localisation of endogenous Unp is different depending on the cell line, and that endogenous Unp is sensitive to LMB (Sections, §4.3.5, 4.3.6). The knowledge obtained in this set of experiments is consistent with the data generated using the transfection constructs for Unp, supporting the idea that Unp is a shuttling protein.

Given that Unp and Usp15 have high sequence identity, common biochemical characteristics, and that both contain Rb-binding domains, it is intriguing why the cell requires both of these proteins. Immunostaining experiments using specific anti-Unp and Usp15 antibodies demonstrated that Unp and Usp15 localise to different subcellular compartments, being essentially mutually exclusive (Section §4.3.4). Unp was exclusively nuclear, but was excluded from the nucleolus in HeLa cells, whereas Usp15 was detected only in the nucleolus and in the cytoplasm. Results for NIH3T3 cells confirmed the fact that Usp15 was not present in the nucleus. Intriguingly, Usp15 was also observed near the cellular membrane and on the cytoplasmic periphery in NIH3T3 cells (Section §4.3.4). Clearly, these two homologues enzymes are targeted to very different subcellular locations, implying quite different cellular roles relating to particular substrates in the different cellular sites.
5.3 Subcellular localisation of other Deubiquitinating enzymes

The study presented here was focussed on the nuclear transport properties of the Unp protein in an attempt to gain new insights into its possible function. Unp belongs to a large family of deubiquitinating enzymes, or DUBs. DUB function, in general, is to cleave ubiquitin off proteins and ubiquitin precursor molecules (reviewed in Baker, 2000; Wilkinson, 2000). Such an activity can enhance or inhibit proteasome-mediated proteolysis. Despite the diversity of DUB enzymes, not much is known about their precise function, substrate specificity, and the functional significance of each member of DUB family including Unp. It is clear that the determination of subcellular localisation of a protein can assist in understanding the function of a protein.

The human DUB, USP7 (also known as HAUSP), was first described as being exclusively nuclear associated with PML nuclear bodies (Everett et al., 1997). Subsequently, the tumour suppressor protein p53 was identified as a cellular partner of USP7. Moreover, it was shown that p53 is a substrate for USP7 (Li et al., 2002a). Therefore, it is now clear that the exclusive nuclear localisation of USP7 is essential for the stabilisation of p53 in the nucleus. p53 is degraded in the absence of USP7, once it is exported into the cytoplasm (Freedman and Levine, 1998; Geyer et al., 2000).

Determination of the subcellular localisation of a mouse DUB, Fam, enabled the elucidation of a new substrate, β-catenin, for this DUB. Fam was first shown to stabilise the Ras-effector, AF-6, and to colocalise with it at cell-cell contact sites (Taya et al., 1998), (Kanai-Azuma et al., 2000). Moreover, it is well known that one of the key proteins in establishing cell-cell-adhesion is β-catenin (Adams and Nelson, 1998). It has also been established that β-catenin is degraded via the ubiquitin-proteasome pathway (Aberle et al., 1997). Examination of relationships between Fam and β-catenin has shown that the two proteins interact in vivo, and Fam regulates the degradation of β-catenin (Taya et al., 1999).

These two examples show how elucidating the subcellular localisation of a DUB can provide a clue to its function. There are very few such examples in the current literature, and no thorough or systematic study of cellular localisation of a DUB has
been performed. Subcellular localisation, substrates, and interacting partners of other selected DUBs are summarised in Table 1.1 and Section §1.3.

Importantly in this context, this study has established that Unp is a nuclear-cytoplasmic shuttling protein. A number of shuttling proteins have been described previously, but Unp is the first DUB reported to have this ability. Even though the enzymatic substrates of Unp are unknown, and the reasons for Unp shuttling are still obscure, it seems likely that the shuttling of Unp is essential for performing its function (see below).

5.4 Unp and other shuttling proteins

Recent intensive studies have shown that shuttling proteins are key factors in relaying information between the two major cellular compartments, the nucleus and the cytoplasm (reviewed in Gama-Carvalho and Carmo-Fonseca, 2001). The list of nuclear-cytoplasmic shuttling proteins includes transport receptors and adaptors, numerous transcription factors, cell cycle regulators, RNA-binding proteins, and steroid hormone receptors.

Shuttling between the nucleus and cytoplasm is important because it provides a control mechanism to ensure that the specific function of a protein is carried out in the correct subcellular compartment at the required time. This is exemplified by the shuttling of E2F transcription factors. E2F1-3 each contain intrinsic NLSs, and are accordingly localised mainly in the nucleus. E2F4, in contrast, does not possess an NLS but imported into the nucleus by p107, p130, or DP2 proteins during the G0-early G1 phase (see Cartwright and Helin, 2000). It relocates back to the cytoplasm in mid-G1 phase. This simple sequestration of E2F4 back into the cytoplasm is sufficient to inactivate its function. Attachment of an NLS to E2F4 results in relocation of the protein into the nucleus and disruption of its shuttling. E2F4 stays in the nucleus and drives the cells into an S-phase, thereby altering the cell cycle (Muller et al., 1997).

Another example of the importance of shuttling is the regulation of function of a protein called Adenomatous Polyposis Coli (APC). APC primarily localises in the cytoplasm, but nuclear localisation of APC has been observed in several human tumour cells (see Henderson, 2000). The role of APC is to bind to β-catenin (an oncoprotein and transcriptional activator) in the nucleus and transport it back to the cytoplasm for
proteasomal degradation (*ibid*). Preventing APC shuttling by mutation of its two strong NESs leads to accumulation of APC in the nucleus, resulting in cellular transformation because β-catenin can no longer be degraded (*ibid*).

Shuttling of cyclinB/cdc2 complex between the nucleus and cytoplasm is important for the normal progress of the cell cycle. Lui and co-workers showed that this shuttling can be disrupted by overexpression of the Myt 1 protein kinase, which phosphorylates cyclinB/cdc2 and thus negatively regulates its activity. Phosphorylation of the cyclinB/cdc2 complex prevents its shuttling and delays the entry of the cell into the G2 phase of the cell cycle (Liu et al., 1999).

These three examples not only illustrate the importance of shuttling in the regulation of fundamental cellular processes but also show how the disruption of shuttling can lead to cell transformation.

The purpose for Unp shuttling is not known. However, several models explaining the significance of Unp shuttling can be proposed. It is possible that Unp has a substrate “X” localised in the nucleus. This substrate may be stabilised at a particular stage of the cell cycle and be degraded at a different stage. Unp located in the nucleus would result in the stabilisation of protein “X”. On the other hand, export of Unp out of the nucleus, at the appropriate stage of the cell cycle would allow the subsequent degradation of protein “X” in the nucleus (Figure 5.1 A). This hypothesis could also apply to the stabilisation of a cytoplasmic protein “Y”. In this case, Unp retention in the cytoplasm would protect “Y” from degradation. Translocation of Unp into the nucleus would then allow the degradation of protein Y (Figure 5.1 A).

It also cannot be excluded that Unp has substrates in both the nucleus and cytoplasm, which require stabilisation at different stages of the cell cycle. In this case Unp would shuttle between the two compartments in order to prevent degradation of such substrates (Figure 5.1 B).

A third hypothetical scenario is that Unp shuttles because it is associated with another shuttling protein, which needs to be stabilised whether it is in the nucleus or cytoplasm (Figure 5.1 C).

Therefore, disruption of Unp shuttling in all of these hypothetical examples, would cause alteration of Unp’s target stability, which may result in deregulation of certain cellular pathways and in tumourigenic transformations.
Figure 5.1 Models for the significance of Unp shuttling.

A: Unp stabilises either protein “X” in the nucleus, or protein “Y” in the cytoplasm. Protein “X” or “Y” degrades when Unp leaves the cellular compartment.

B: Unp stabilises several different substrates, which are localised in the nucleus (A) or the cytoplasm (B) by shuttling between the two compartments.

C: Unp shuttles together with another shuttling protein in order to prevent its degradation.

Key:
- Multi-ubiquitin chain
- X, Y, A, B, Hypothetical and Z Unp substrates.
Chapter 5  General Discussion

5.5 The functional significance of the interaction between Unp and Rb proteins

Understanding the biological function of Unp will require determination of the physiological significance of its interaction with the pocket proteins. It is known that Unp interacts with the pocket proteins (Blanchette et al., 2001; DeSalle et al., 2001). However, it is not known whether shuttling of Unp can be influenced by its interaction with the Rb family, or whether potential Unp substrate(s) are Rb-interacting proteins or involved in Rb-pathways. It is also unclear whether oncogenic properties of Unp are related to its interaction with Rb family members.

Unp is probably not involved in the stabilisation of pocket proteins since it has been shown that overexpression of Unp does not increase the level of pRb (M. Pagano, personal communications). Therefore, it can be assumed that Unp interaction with members of the Rb family has a different function. Indeed, this thesis showed that Unp nucleocytoplasmic transport is altered in pRb/p130/p107 deficient HeLa and Saos-2 cell lines, whereby Unp is localised exclusively in the nucleus. However, introduction of functional pRb into Saos-2 cells did not change Unp localisation, which implies that pRb itself is not involved in Unp nuclear transport (Section §4.3.8). On the other hand, sequestration of p130 and p107 proteins in NIH3T3 cells by viral E7 protein changed Unp localisation, rendering it more nuclear (Section §4.3.8). Given that p130 and p107 interact with Unp in vivo, it is possible that these two proteins are involved in Unp trafficking to the cytoplasm or retention of Unp in the cytoplasm (see Figure 5.2). Clearly, more experiments are required to prove this, since E7 protein modifies many other cellular pathways regulating cell cycle progression, cell differentiation and transcription (reviewed in McMurray et al., 2001).

5.6 A model explaining the oncogenic properties of Unp

Given that Unp is a deubiquitinating enzyme, it is likely that the interaction between Unp and Rb proteins serves to link Unp and its enzymatic substrate(s). Pocket proteins interact with numerous other proteins. One family of proteins which regulate Rb activity, are the cyclins. Blanchette et al. (2001) have evidence that Unp does not interact with cyclin D, which is involved in keeping pRb in an active,
Figure 5.2 A model showing how Unp-p130/107 interaction may influence Unp subcellular localization.

A: p130/p107-Unp interaction may serve to export Unp out of the nucleus, or to anchor Unp in the cytoplasm. B: expression of E7 protein in cells results in proteasome-mediated degradation of p130/p107 or in displacing Unp form p130/p107. As a result, Unp is not exported from the nucleus or not retained in the cytoplasm and accumulates in the nucleus.
hypophosphorylated state. On the other hand, results of this thesis suggest that Unp localisation may be regulated by cyclinsA/E-cdk2 phosphorylation. Unp contains a putative cyclin A/E binding site (pNLS2) and mutation of this site changes Unp cellular localisation. In addition, a cyclin A-cdk2 complex is able to phosphorylate Unp in vitro (Sections §3.3.3 and §3.3.4). Therefore, Unp subcellular localisation might be influenced by cyclinA/E-cdk2 phosphorylation.

On the other hand, since cyclins A and E are degraded by the proteasome in a cell cycle-dependent manner (Sudakin et al., 1995; Singer et al., 1999), it is attractive to propose that cyclin A or E may be a substrate for Unp deubiquitination. Therefore, a complex interplay between enzymatic activities of Unp and cyclin A/E may exist (see Figure 5.3). If Unp stabilises cyclin A/E, this would result in maintaining pRb in the hyperphosphorylated inactive state, and subsequent transformation. Although highly speculative, this model can explain the oncogenic properties of Unp.

Interestingly, levels of cyclin A and E are elevated in a variety of different tumours (reviewed in (Yamb et al., 2002). This elevation leads to a prolonged activity of both cyclins, which together with other cell cycle proteins abrogate the Rb/E2F pathway (Halaban, 1999).

5.7 Future directions

The main achievement of the work described in this thesis was the demonstration that Unp is a shuttling protein. However, as mentioned above, reasons for its shuttling are unknown. In order to resolve this issue, Unp enzymatic substrates need to be identified. Yeast two-hybrid screen and subsequent in vitro pull-down and in vivo co-immunoprecipitation experiments may be applied to identify Unp interacting proteins. Immunoprecipitation with specific antibodies and subsequent Western blotting with anti-Ubiquitin antibodies as well as inhibition of proteasome activity would test whether these proteins are ubiquitinated and degraded by the proteasome. Given that not all ubiquitinated proteins are targeted for proteasome degradation, a role for Unp in reversing monoubiquitination of some proteins (eg histones) should also be investigated. The selected proteins can be further tested by pulse-chase assays, whether they are stabilised by Unp. Subcellular localisation, shuttling properties, and the
Figure 5.3 A model explaining the oncogenic properties of Unp
Unp/cyclin A/E interaction may result in the phosphorylation of Unp and the stabilisation of cyclin A/E. Phosphorylated Unp leaves the nucleus, and stabilised cyclin A/E -cdk2 complex hyperphosphorylates pRb, rendering it inactive.
stability of putative Unp substrates may then be further tested in mammalian cells in the presence and absence of overexpressed Unp (CLSM, Western blot analysis etc).

Another question that needs to be answered is the significance of the interaction between Unp and the Rb family of proteins. The experiments have indicated that Unp subcellular localisation may depend on p130 or p107 pocket proteins. To investigate this further, Unp localisation following co-expression of Unp and pocket proteins in mammalian cells may be examined. Analysis of the subcellular localisation of endogenous Unp in cells overexpressing one or several pocket proteins may be performed, as well as examining Unp localisation in cells lacking one or combination of pocket proteins.

Unp oncogenic properties also need more detailed investigation. Firstly, it needs to be established whether Unp and cyclin A/E interact in vivo (two-hybrid screen, co-immuno precipitation with larger amounts of antibodies for covalent linking to Sepharose A, co-immunostaining of cells). Pulse-chase assays and/or co-expression of Unp and cyclins A or E would determine whether Unp is able to stabilise either of these cyclins.

Secondly, tumour formation in nude mouse and colony formation assays with a range of different Unp mutants (eg NLS or NES, cyclin A/E-binding site, Rb-binding site, active Cys mutants) can be performed to investigate whether alterations of Unp enzymatic activity, shuttling properties, and Rb/cyclin(s) binding, play a role in Unp-mediated tumourigenesis.

In addition to the key experiments suggested above, Unp nucleocytoplasmic transport properties still require further clarification. A two-hybrid screen may also identify whether Unp interacts with nuclear transport receptors other than Impα/β and CRM1, or if there are any proteins, that are involved in piggyback nucleocytoplasmic transport of Unp. Experiments involving generation of a series of truncated Unp fusion proteins and analysis of their subcellular localisation would help to elucidate other regions of Unp involved in the nuclear import or export.

Unp subcellular localisation during different cell cycle phases also needs to be resolved. Cell synchronisation either by serum starvation or by applying reagents such
as hydroxyurea, mimosine or nocodazole and subsequent analysis of Unp localisation (either overexpressed or endogenous) in these cells will clarify if Unp localisation is cell cycle dependent.

Finally, given that Usp15 contains putative NLS, NESs, and pRb family interaction motifs, their functionality should be explored, using analogous approaches to those used in this thesis, and those suggested above. It is still to be established whether Usp15 is an oncogene. This should be addressed using colony formation, nude mouse, or transgenic mouse models.

5.8 Concluding remarks

The work presented here successfully achieved several goals. The subcellular localisation and some aspects of mechanisms of nuclear-cytoplasmic trafficking of Unp were elucidated. Antibodies specific to Unp and its homologue, Usp15, were raised for the first time, and novel data was generated addressing the important question of why the cell possesses two very similar deubiquitinating enzymes, Unp and Usp15.

Finally, the research carried out in this thesis provided a new insight in the role of the deubiquitinating enzyme Unp in the cell. The nucleocytoplasmic shuttling properties of Unp characterised rigorously here for the first time are presumably fundamental for its function.
References
6. REFERENCES


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